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L-carnitine supplementation during vitrification or warming of *in vivo*-produced ovine embryos does not affect embryonic survival rates, but alters *CrAT* and *PRDX1* expression

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ABSTRACT

L-carnitine is an antioxidant and β-oxidation stimulator substance commonly used to improve metabolic performance of oocytes and embryos in *in vitro* systems. However, few studies have evaluated its beneficial effects in embryos produced *in vivo*. This study aimed to evaluate the effect of L-carnitine supplementation into vitrification or warming solutions on the post-warming character of day 6–7 *in vivo*-produced ovine embryos. L-carnitine (3.72 mM) was added to vitrification (Experiment 1) or warming solutions (Experiment 2). In experiments 1 and 2, the embryos were vitrified using straw and cryo-tip protocols, respectively. *In vitro* culture (IVC) of warmed embryos was performed for 72 h in order to evaluate survival rates, reactive oxygen species (ROS) levels, total cell number (TCN), number of apoptotic cells, apoptotic index evaluation, and gene expression analysis of carnitine palmitoyltransferase I and 2 (*CPT1* and *CPT2*), carnitine O-acetyltransferase *(CrAT),* and peroxiredoxin-1 (*PRDX1*). In experiment 1, survival rate, ROS levels after 24 h of IVC, total cell number at 24 h and 72 h, apoptotic cells and apoptotic index at 72 h of IVC were similar in embryos vitrified in medium supplemented with LC or not. Gene expression analysis showed no differences in *CPT1* and *CPT2* mRNA relative abundance in embryos of both experiments compared to fresh embryos (FE); however, CrAT was downregulated ($p < 0.05$) in C1, and PRDX1 was downregulated ($p < 0.05$) in both the control (C1) and L-carnitine (LC1) groups, compared to FE. Moreover, *CrAT* and *PRDX1* were upregulated ($p < 0.05$) in C2, and *CrAT* was downregulated ($p < 0.05$) in LC2, in relation to FE. Although the short-term LC supplementation at 3.72 mM did not improve survival, and quality parameters of *in vivo*-produced ovine embryos, it could affect their quality at a molecular level. In conclusion, further investigations with different concentrations of LC and tools are needed for improvement of the efficiency of these strategies.

1. Introduction

Gamete and embryo cryopreservation are essential strategies to accelerate genetic improvement of livestock. Cryopreservation is widely used in human artificial reproduction techniques, animal reproduction for improvement of breeding programs, and preservation of genetic diversity of endangered species [1]. However, cellular cryodamage in reproductive cells is common unless a well-tailored cryopreservation protocol has been developed. Damage includes cytoskeletal fractures and alteration of distribution pattern and mitochondrial activity [2], delay in the resumption of DNA and protein synthesis, and changes in levels of gene expression [3,4]. In some instances, these problems can be reduced by using vitrification procedures that minimize the exposure time to cryoprotectants and ice crystal formation [5,6]. However, changes in the intracellular redox status are difficult to avoid and

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have been found to influence the survival ability of cells. Oxidative stress during cryopreservation may be generated through different mechanisms, including osmotic stress and increased oxidative metabolism [7]. Microarray analysis of gene expression alteration induced by cryopreservation, have revealed that eukaryotic cells express different genes at various time points after warming in the categories of energy metabolism, oxidative stress scavengers, cell rescue systems, including heat shock proteins, and gene products localized in organelles [8]. These results are taken as evidence that cryopreservation induces damage to organelles and activates rescue systems associated with energy generation and subsequent oxidative stress [8,9]. Mitochondrial injuries are responsible for decreased oxidative metabolism [10], as observed in frozen/thawed two-cell mouse embryos, especially after slow cooling. Since reactive oxygen species (ROS) are known to induce breaks in the DNA chain, the presence of DNA damage in cryopreserved cells represents a further indication of oxidative stress during cryopreservation [10,11]. Thus, oxidative stress generation is considered a main cause of structural and molecular damage during sperm cryopreservation.

Storey [12] reported that high antioxidant defenses after warming can defend cells against oxidative damage. The addition of vitamin E to the cryopreservation solution improved sperm motility, but did not prevent DNA fragmentation [13]. Two-cell stage cryopreserved mouse embryos to showed high levels of intracellular hydrogen peroxide, which is known to have deleterious effects on embryo development [14]. These effects can be prevented by addition of the antioxidant ascorbate to the cryopreservation solutions [10].

L-carnitine (LC) is a quaternary amine, naturally synthesized from the methionine and lysine, that performs significant functions in intermediary metabolism in most mammalian tissues [15,16]. It is mainly synthesized in the liver and is present especially in muscle and liver tissues of all animals. In living cells, it is required to transport fatty acids from cytosol to mitochondria during lipid breakdown to generate metabolic energy [17]. Carnitine acyltransferases are enzymes that participate in the intermediary metabolism of fatty acids [18]. They are responsible for modulating acetyl-coenzyme A (CoA) and acyl-CoA pools in several cell compartments and contributing to cellular energetic homeostasis [18]. Carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2) are located in the outer and inner mitochondrial membranes. They transfer of acyl-fatty esters between CoA and carnitine, allowing their transport across the mitochondrial membranes and the continuity of the β-oxidation pathway [19]. Carnitine O-acetyltransferase (CrAT) is a key enzyme in the intermediary metabolism in mitochondria, peroxisomes, and endoplasmic reticulum, modulating intracellular ratios of the acetyl-CoA/CoA and acyl-CoA/CoA through the reversible transfer of acetyl- and acyl-groups between CoA and carnitine, which are important for energy production and regulation of cellular activities [18]. In addition to its contribution to cellular homeostasis through proper functioning of mitochondrial metabolism, carnitine acts as an antioxidant that neutralizes free radicals, especially superoxide anions and protects cells against oxidative damage-induced apoptosis [20]. In response to high levels of ROS, the cell increases the expression of peroxiredoxin 1 (PRDX1), which plays an important role as an antioxidant, reducing levels of hydrogen peroxide and alkyl hydroperoxides [21]. Positive effects of LC on *in vitro* culture of ovine [22], bovine [23], swine [24] and murine embryos [11] have previously been reported. However, no study has assessed the role of LC on *in vivo*-produced embryos. Therefore, the present study used a sheep model to evaluate the effect of LC supplementation into vitrification (Experiment 1) or post-warming (Experiment 2) dilution/wash solutions, on cryopreservation outcomes, such as the embryonic survival, apoptotic index, free radical levels, and expression profile of the genes involved with LC metabolism and oxidative stress.

2. Material and methods

This experiment was conducted under the approval of the Animal Care and Usage Committee, of the Universidade Federal Fluminense (protocol # 699/2015), Brazil, and the ethical principles of the Sociedade Brasileira de Experimentação Animal were followed.

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.2. Experiment 1: Effect of L-carnitine addition into vitrification solution on the post-thawing character

To evaluate the effect of LC addition to vitrification solution on post-thawing character of i*n vivo*-produced day 6–7 ovine embryos, 154 embryos were uniformly allocated into Control (C1, n: 73) or L-carnitine (LC1; n: 81) groups. Embryos in the LC1 and C1 groups were vitrified with solutions with or without 3.72 mM (0.06 mg/mL) LC (Sigma C0158 inner salt), respectively [25]. After warming, all embryos were *in vitro* cultured (IVC) for 72 h. Re-expansion rate analysis was performed at 24, 48, and 72 h, and hatching rates were calculated at 72 h after warming. After 24 h of IVC, samples of re-expanded embryos were harvested. From these, some embryos were frozen at −80 °C for gene expression analysis, and the rest underwent staining for ROS and total cell number evaluations. The remaining embryos were maintained on culture for survival rate, total cell number and apoptosis evaluation at 72 h of IVC.

2.2.1. In vivo embryo production

In Experiment 1, 25 Santa Ines ewes were submitted to three sessions of superovulation [4] and were naturally mated with males of the same breed. Animals were submitted to general anesthesia to collect embryos by laparotomy, as previously described [26]. Embryos were recovered between days 6 and 7 after natural mating. After collection, embryos were morphologically evaluated under a stereomicroscope (Nikon, Tokyo, Japan) and classified according to their developmental stage and quality, following the standard international criteria [28]. Embryos ranged from morulae to hatched blastocysts, and those classified as grades I and II, were uniformly distributed within the experimental groups. A sample of fresh embryos (FE) was directly snap frozen on dry ice at −80 °C to form a physiological control group for gene expression analysis.

2.2.2. Vitrification/warming of embryos

Vitrification was performed according to Guignot et al. [29], with minor modifications. Glycerol (G) and ethylene glycol (EG) were used in three vitrification solutions: solution 1 (10% G), solution 2 (10% G and 20% EG); and solution 3 (25% G, 25% EG and 1 M sucrose). Embryos of the same developmental stage were vitrified together in the same French straw. Briefly, pairs of embryos underwent two washes in OCM solution (Ovum Culture Medium, 20% newborn calf serum (NBCS) in phosphate-buffered saline (PBS), then transferred through solution 1 for 5 min, solution 2 for 5 min and solution 3 for 30 s. The straw was pre-prepared with a 5-cm column of 0.85 M galactose in OCM, a column of air, a 0.5-cm column of solution 3, and a second 0.5-cm airgap. After exposure to solution 3, embryos were immediately aspirated in a 1 cm column into the straw, followed by a 0.5 cm air column and a final column of 0.85 M galactose solution. The straw was immediately immersed

in liquid nitrogen (N_2). All straws were stored in a liquid N2 tank. For warming, the straws were withdrawn one by one, kept in air for 5 s and for 15 s in a water bath at 22 °C. The full contents of each straw was deposited on an empty plate, mixed immediately and kept at room temperature for 5 min. The embryos were aspirated in a minimum of solution, transferred to OCM for 5 min at room temperature, and then washed in OCM at 39 °C in the plate.

2.2.3. IVC and survival evaluation

Warmed embryos were transferred to 50 μL of CR2 medium for IVC at 38.5 °C, 5% CO_2, 5% O_2 and maximum humidity. CR2 medium is composed of CR2 stock (108 mM sodium chloride (NaCl), 3 mM potassium chloride (KCl) 26 mM bicarbonate, 10 mM glycine, 1 mM alanine, 1 mM glutamine and 4.3 mM lactate hemi calcium in Milli-Q water) supplemented with 0.36 mM sodium pyruvate, 50 mg/ml streptomycin/penicillin, 0.01 g bovine serum albumin (BSA), 100 μL Modified Eagle's Medium (MEM), 200 μL β-mercaptoethanol (BME), and 2.5% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Survival rates were evaluated with morphologic criteria, on the basis of the integrity of the embryo membranes and the zona pellucida (with the exception of hatched blastocysts), and re-expansion of the blastocoel. The embryos were evaluated after 24, 48, and 72 h. The hatching rate was observed at 72 h of IVC.

2.2.4. ROS levels and total cell number evaluation at 24 h of IVC

In Experiment 1, the evaluation of total cell number (TCN) and ROS levels were performed by Hoechst 33342 and CellROX Green staining (both Molecular Probes, Eugene, OR, USA), respectively. ROS levels were assessed at 24 h to evaluate the antioxidant effect of LC in early re-expanded embryos and to correlate its level with gene expression analysis of PRDX1, also evaluated at 24 h. Viable embryos at 24 h of IVC were washed in PBS plus 0.1% polyvinyl alcohol (PVA) and culture in PBS-PVA medium containing CellROX Green (10 mM) and Hoechst 33342 (2.5 μM) for 30 min in the dark. Afterward, embryos were submitted to three washes in PBS-PVA, fixed for 30 min in 4% paraformaldehyde (PFA) and then washed in PBS-PVA. Embryos were mounted on glass slides and covered with coverslips and evaluated within 24 h under a fluorescence microscope (Olympus BX53, Tokyo, Japan), with an excitation/emission wavelength 350/461 for Hoechst and 485/520 for CellROX Green. Images were captured by an Olympus DP73 camera coupled to the same microscope. TCN was manually assessed by counting the blastomers stained with Hoechst (in blue), while the ROS evaluation was performed by measuring the green fluorescence intensity (CellROX Green) using ImageJ software.

2.2.5. Apoptosis evaluation and TCN at 72 h of IVC

In Experiment 1, viable embryos at 72 h of IVC were evaluated for TCN by Hoechst 33342 staining and apoptosis by the caspase-3 immunofluorescence staining protocol. Cell counting was performed manually, and the calculation of the apoptotic index was performed using the equation [apoptotic index = (apoptotic cells/total cell number) * 100]. For this, embryos were fixed in 4% PFA and stored at 10 °C. Embryos underwent membrane permeabilization with 0.5% Triton in PBS for 30 min in a 4-well plate, followed by three washes of 10 min each in 0.2% Tween solution in PBS. Blocking of non-specific sites for 1 h in 3% BSA solution in PBS was followed by overnight incubation (12 h) with anti-caspase 3 (rabbit) primary antibody (Sigma C8487, 1:20 in PBS) at 10 °C. Subsequently, embryos underwent three washes (10 min/each) in 0.2% Tween solution in PBS, followed by new blocking of non-specific sites for 1 h. Following incubation with secondary antibody Alexa Fluor 555 (goat anti-rabbit IgG; 1: 400 in PBS) (Molecular Probes, Eugene, OR, USA) in PBS was carried out for 2 h in darkness. After, embryos underwent three washes, they were protected from the light in 0.2% Tween solution in PBS for 10 min each, and in the last wash, Hoechst 33342 (1:50) was added to the washing solution. Embryos were mounted on slides, covered by coverslips, and evaluated under a fluorescence microscope (Nikon Eclipse TS100, Tokyo, Japan) with the excitation/emission wavelength of 555/565 for Alexa Fluor 555. Images were captured by a DCM510 digital camera (Alltion, Guangxi, China) coupled to the same microscope.

2.2.6. Analysis of CPT1, CPT2, CrAT, and PRDX1 expression

In both experiments, total RNA extraction was performed with 3 pools of 5 re-expanded embryos per/group using an RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and treated with DNase. Complementary DNA (cDNA) was synthesized using the Superscript III First-strand Supermix kit (Invitrogen, Carlsbad, CA, USA) and a random primer hexamer. Quantification of cDNA from each pool per group was performed using 1 μL of the sample and an ND-100 spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The relative quantification was performed in triplicate using the real-time polymerase chain reaction (PCR) technique (ABI Prism1 7300, Applied Biosystem, Foster City, CA, USA) and reactions using the Power SYBR Green PCR Master Mix (Applied Biosystem) mix, 400 ng of cDNA, nuclease-free water, and the specific primers for each reaction. PCR reactions consisted of the following steps: denaturation of the cDNA at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s for ribbon opening, annealing temperature of each specific primer for 30 s, and synthesis of the new ribbon at 60 °C for 30 s. As negative controls, reactions were prepared with the cDNA omitted during the amplification reaction. After each PCR run, an analysis of the Melting curve was performed to confirm the generation of a single specific product. The sequences of the primers used and the size of the amplified fragments for all the transcripts are shown in Table 1. The efficiency of the primers for each reaction was determined by the LinRegPCR® software and considered in the calculation of the relative quantification. The analysis of quantitative PCR results was performed using REST® software, and the results were expressed relative to a calibrator. For evaluation of expression, a calibrator group with FE embryos in the blastocyst stage was used. Thus, the data obtained were expressed as N times relative to the calibrator group.

2.3. Experiment 2: Effect of L-carnitine addition into warming solution on the post-thawing character

To evaluate the effect of the addition of L-carnitine into the warming solution of *in vivo*-produced day 6–7 ovine embryos, vitrified embryos without LC were thawed in a solution supplemented with 3.72 mM (0.6 mg/mL) LC (LC2) or solutions without LC supplementation (C2). Afterward, 67 embryos were IVC for 72 h. Survival rate analysis was performed at 24, 48, and 72 h, and hatching rates were calculated at 72 h IVC. A sample of live embryos was removed from IVC after 24 h and frozen at −80 °C for gene expression analysis. The remaining embryos were kept on IVC for survival evaluation at 72 h.

2.3.1. In vivo embryo production

In Experiment 2, 26 Santa Ines ewes were subjected to hormonal treatment with medroxyprogesterone acetate, cloprostenol, equine chorionic gonadotropin, and lecirelin [4] and were mated with males of the same breed. Embryos were recovered transcervically under local anesthesia [27]. The time of recovery, classification, and distribution within experimental groups were performed as Experiment 1.

Table 1
Sequence of specific primers used for the gene expression analysis of *in vivo*-produced ovine embryos submitted to supplementation with L-carnitine in the vitrification or rewarming solution.

Product	3′-5′ Primer Sequence	Melting T°	Product Size	GeneBank number/Reference
CPT1	F CGACTGGTGGGAGGAATACA R TGCGTCTGTAAAGCAGGATG	60 °C	155 bp	Sharma et al. [7]
CPT2	F TTGTGCCTTCCTTCCTGTCT R GAGGTGTCTGGCCTTGTCAA	60 °C	149 bp	Sharma et al. [7]
CrAT	F GTTCAGCAGGACCAAGAAGC R TGCAGTGACGAGTTCCAGGAC	60 °C	150 bp	Sharma et al. [7]
PRDX1	F CAAAGCAACAGCTGTTATGC R GAGAATCCACAGAAGCACC	60 °C	197 bp	Sengodan et al. [21]
ACTB	F GGAAATGCTGCGTGACATTAAG R TGTGTTGGCGTAAGGTCTTTG	60 °C	273 bp	Sharma et al. [7]

Abbreviations: *CPT1*: carnitine palmitoyltransferase 1; *CPT2*: carnitine palmitoyltransferase 2; *PRDX1*: peroxiredoxin 1; *CrAT*: carnitine O-acetyltransferase; *ACTB*: Homo sapiens actin beta.

2.3.2. Experiment 2 - vitrification/warming of embryos

Embryos were vitrified and warmed according to Gibbons et al. [30], with minor modifications. Embryos were kept for 20 min at room temperature in the base medium (MB), consisting of PBS supplemented with 20% FBS. For vitrification, embryos were exposed to three different vitrification solutions: solution 1b (MB + 10% G) for 5 min, solution 2b (MB + 10% G and 10% EG) for 5 min, and solution 3b (MB + 25% G and 25% EG) for 30 s at room temperature. During the 30 s in solution 3b, the embryos were aspirated in 1 μL of this solution into 10 μL plastic tips, which were decoupled from the micropipette and immersed in liquid N2. The embryo-containing tips were individually stored in previously labeled cryotubes in a liquid N2 tank. For warming, the tips were removed from the cryotubes and directly dipped in warming solution 1 (W1) composed of MB plus 12.5% G, 12.5% EG and 0.5 M sucrose for 5 min at room temperature. Then, the embryos were recovered and transferred to W2 (OCM plus 0.5 M sucrose) for 5 min, then transferred to W3 (0.25 M sucrose in OCM) for a further 5 min and finally washed in OCM at 39 °C. In the LC2 group, W1, W2, and W3 solutions were all supplemented with 3.72 mM (0.6 mg/mL) LC.

2.3.3. In vitro culture and survival evaluation

As performed in Experiment 1, warmed embryos were transferred to 50 μL of CR2 medium for IVC at 38.5 °C, 5% CO_2, 5% O_2, and maximum humidity. The same criteria and time point of evaluation used in Experiment 1 were also applied in Experiment 2.

2.4. Statistical analysis

Averages of re-expansion and hatching rates were compared by the chi-squared test. Means of TCN, apoptotic cells, apoptotic index, and fluorescence intensity were compared by the Student's t-test. Statistical analyses were performed using GraphPad Prism version 5.6 software, with a significance level of 5% ($p < 0.05$).

3. Results

3.1. Experiment 1: Effect of addition of L-carnitine into vitrification solutions on the post-thawing character

The results of the survival analysis are presented in Table 2. The recovery rates after warming were 77.8% (63 recovered embryos/81 vitrified) for LC1 and 58.9% (43/73) for C1. Despite increases in re-expansion rates associated with LC addition into vitrification solution by 9, 15, and 13% at 24, 48, and 72 h, respectively, this difference was not significant ($p > 0.05$).

3.1.1. TCN and levels of ROS at 24 h after warming

The representative images of C1 and LC1 blastocysts stained with Hoechst and CellROX Green are shown in Fig. 1. Twenty-two embryos were stained for this analysis. Blastocysts vitrified in the presence of L-carnitine showed similar intracellular ROS levels ($p = 0.09$) to blastocysts vitrified without supplementation (890.0 ± 844.9 vs. 858.2 ± 683.7, arbitrary fluorescence units; Fig. 2). Regarding the TCN after 24 h of IVC, the means for C1 and LC1 were respectively, 89 ± 22 and 82.2 ± 28 cells, with no difference between groups ($p > 0.05$) (Fig. 3).

3.1.2. Apoptosis and TCN at 72 h after warming

Four viable re-expanded embryos per group were evaluated for TCN and apoptosis at 72 h. No difference ($p > 0.05$) was observed in the TCN between C1 (86 ± 19.9) and LC1 (68 ± 25.2) ($p > 0.05$) (Fig. 3). The number of apoptotic cells (Fig. 4) and apoptotic index (Fig. 5) were 3.7 ± 1.4 and 4.37% for C1 and 4.5 ± 4.7 and 5.23% for LC1, making them statistically similar ($p > 0.05$).

3.1.3. Expression of CPT1, CPT2, CrAT, and PRDX1 in embryos vitrified with or without LC supplementation

At 24 h of IVC, the relative abundances of *CPT1* and *CPT2* transcripts showed no difference between C1 and LC1 embryos or in comparison with the FE group. However, *CrAT* was downregulated ($p < 0.05$) in C1 embryos, whereas in the LC1 embryos there was no difference ($p > 0.05$) compared to the FE group. *PRDX1* mRNA were downregulated ($p < 0.05$) in vitrified (C1 and LC1 groups) embryos compared to FE (Fig. 6).

3.2. Experiment 2: Effect of the addition of L-carnitine into warming solutions on the post-thawing character

Survival results of Experiment 2 are presented in Table 3. Similarly to Experiment 1, adding L-carnitine to the warming solution increased the blastocoel re-expansion rates by 14, 14, and 16% at 24, 48, and 72 h, respectively, although no difference

Table 2
Effect of adding L-carnitine[a] to the vitrification solutions on the post warming survival and hatching rates of *in vivo*-produced ovine embryos.

Groups	Re-expansion rates			Hatching rate[b] % (n)
	24 h % (n)	48 h % (n)	72 h % (n)	
C1	41 (17/41)	53 (17/32)	68 (22/32)	9 (3/32)
LC1	50 (20/40)	68 (15/22)	81 (18/22)	13 (3/22)

[a] 3.72 mM.
[b] Evaluation at 72 h IVC.

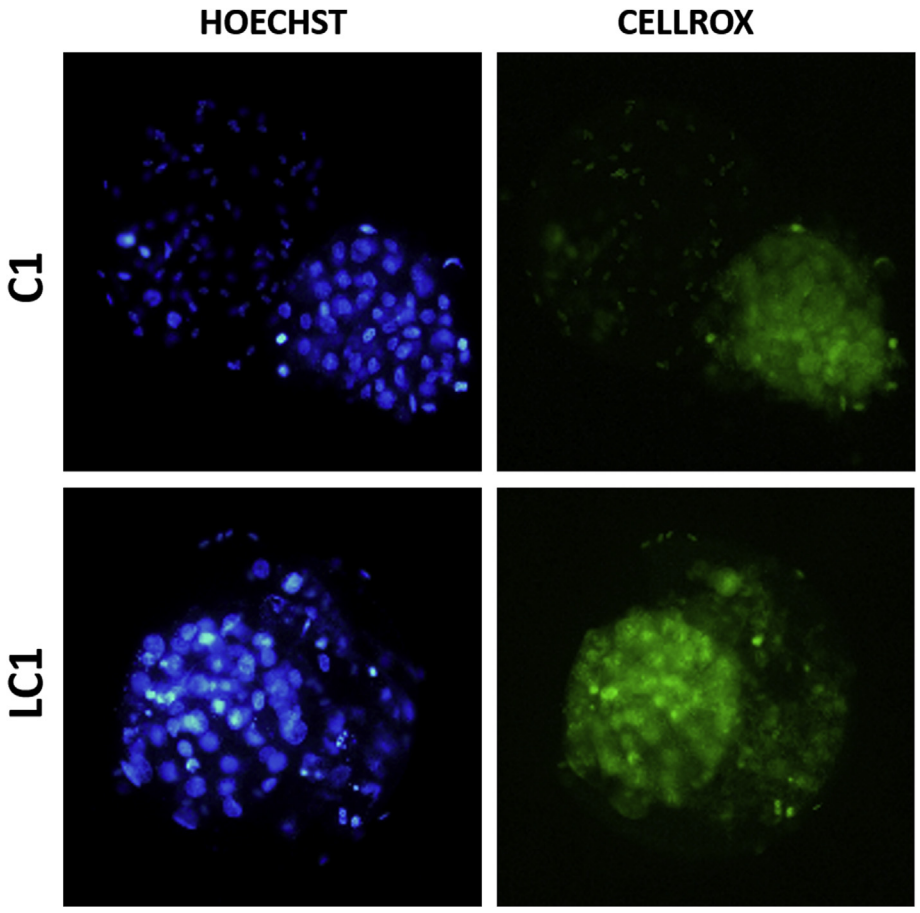

Fig. 1. Representative images of *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine stained for total cell number (in blue) and harmful reactive oxygen species (ROS, in green) at 24 h of *in vitro* culture after warming. Original magnification, x 20. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

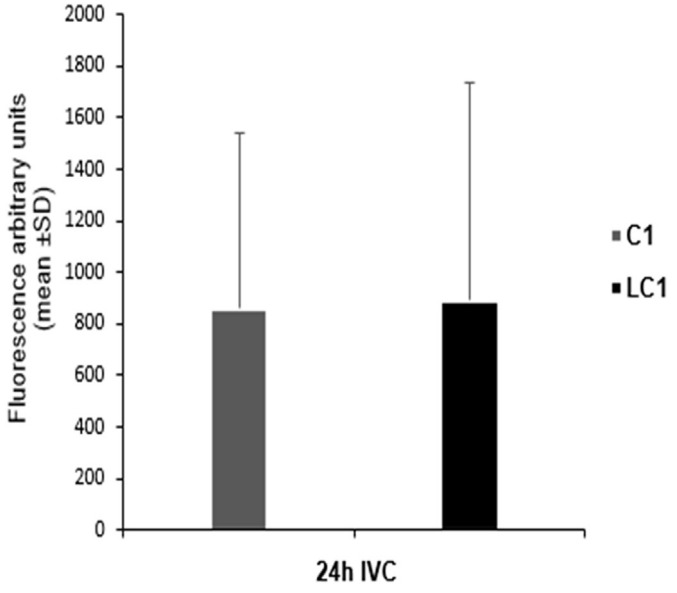

Fig. 2. Intracellular reactive oxygen species (ROS) levels of *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine, evaluated by fluorescence intensity emitted after staining with CellROX Green at 24 h of *in vitro* culture after warming ($p > 0.05$).

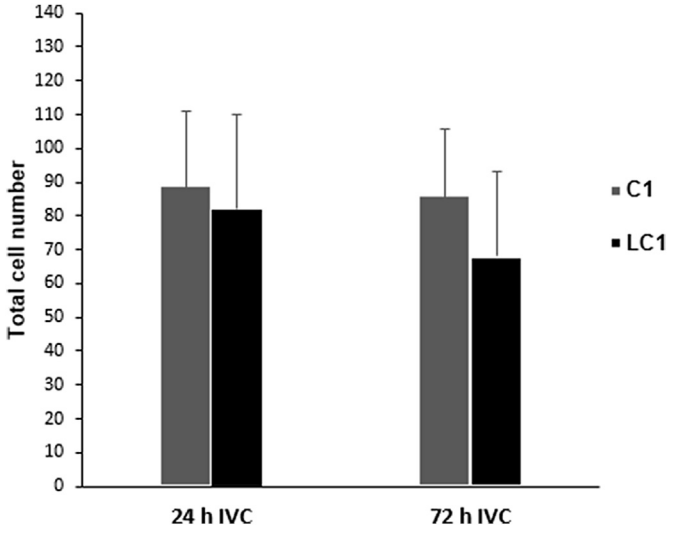

Fig. 3. –Total cell number (TCN; n) at 24 and 72 h of IVC after warming of *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine, evaluated by Hoechst 33342 ($p > 0.05$).

($p > 0.05$) was observed between the LC2 and C2 groups. Hatching rates were 11% higher at 72 h with LC supplementation, but this difference was not statistically significant ($p > 0.05$).

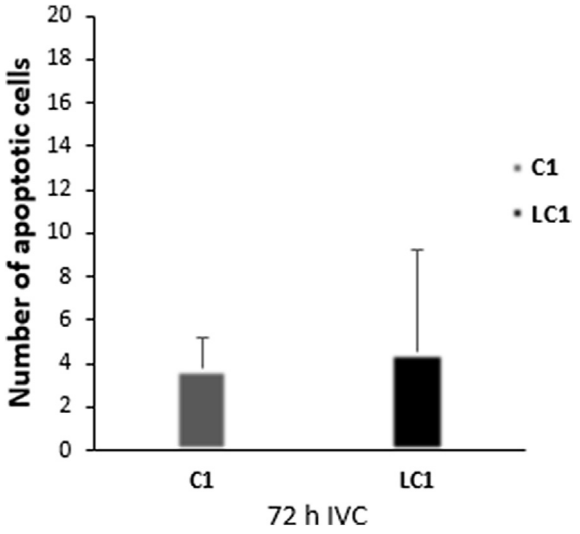

Fig. 4. Number of apoptotic cells (n) at 72 h of IVC after warming of *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine, evaluated by the caspase-3 immunofluorescence technique ($p > 0.05$).

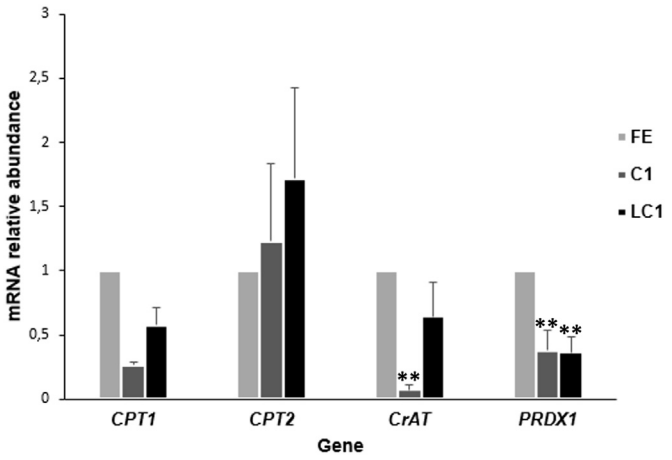

Fig. 6. Relative expression of *CPT1*, *CPT2*, *CrAT*, and *PRDX1* in *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine, at 24 h of *in vitro* culture after warming. FE: fresh embryos. (*) upregulated; (**) downregulated ($p < 0.05$). (Based in 3 pools of 5 re-expanded embryos sampled at 24 h after warming).

3.2.1. *Expression of CPT1, CPT2, CrAT, and PRDX1 in embryos warmed with or without LC supplementation*

No difference ($p > 0.05$) was observed in the relative abundance of *CPT1* and *CPT2* mRNA in vitrified (C2 and LC2) embryos compared to FE. However, *CrAT* expression was upregulated ($p < 0.05$) in the C2 group and downregulated ($p < 0.05$) in the LC2 group compared to FE. No difference ($p > 0.05$) was observed in *PRDX1* expression between LC2 and FE embryos; however, the abundance of these transcripts was upregulated in C2 compared to FE (Fig. 7).

4. Discussion

L-carnitine is an essential metabolite for energy production and has the potential to neutralize the embryotoxic effects of H_2O_2 in culture medium. Oxidative stress during cryopreservation generated through different mechanisms is associated with several deleterious cryopreservation effects. However, our results showed that treatment of *in vivo*-produced ovine embryos with L-carnitine (3.72 mM) during vitrification or warming did not or slightly affect the embryo survival rate, ROS intracellular level, apoptosis index, and levels of mRNA expression of *CPT1* and *CPT2*. Baldoceda et al. reported that the beneficial effect of L-carnitine supplementation during IVC on embryonic survival after vitrification is due to alteration in the amount of intracytoplasmic lipids [31]. Excessive formation of lipid droplets has also been associated with variations in mitochondrial function, which likely affects lipid metabolism [32,33]. This could be an explanation for our results, since *in vivo*-produced embryos have a lower lipid content than *in vitro* ones. Several studies have related CPT1 and CPT2 [34,35] to mitochondrial adenosine triphosphate (ATP) production during oxidative phosphorylation in eukaryotic cells. However, we observed that L-carnitine supplementation does not affect the abundance of these transcripts, which supports the hypothesis that the effect observed in the present study is due to a low amount of lipids in *in vivo*-produced ovine embryos.

Interestingly, the time of LC supplementation in the vitrification protocol affected the expression of *CrAT* (Experiment 1 and 2), although caution is necessary while comparing the results of both experiments, due to the different vitrification protocols. In comparison to fresh embryos, *CrAT* expression was similar or downregulated when LC was added to vitrification or warming medium, respectively. These data suggest a beneficial effect of LC during the initial exposure to the cryoprotectant, possibly protecting molecules against the deleterious effects of free radicals. CrAT is an essential enzyme for mitochondrial homeostasis, regulating the

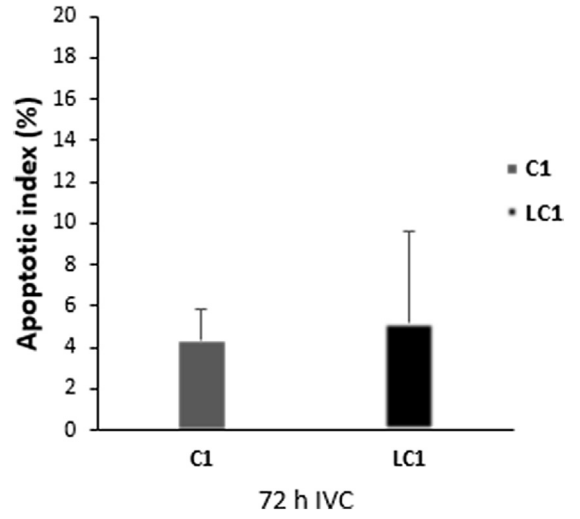

Fig. 5. Apoptotic index (%) at 72 h of IVC after warming of *in vivo*-produced ovine embryos vitrified in the absence (C1) or presence (LC1) of 3.72 mM L-carnitine ($p > 0.05$).

Table 3
Effect of adding L-carnitine[a] to the post vitrification warming and dilution solutions on the subsequent survival and hatching rates of *in vivo*-produced ovine embryos.

Groups	Re-expansion rates			Hatching rate[b] % (n)
	24 h % (n)	48 h % (n)	72 h % (n)	
C2	27 (9/33)	33 (11/33)	48 (16/33)	6 (2/33)
LC2	41 (14/34)	47 (16/34)	64 (22/34)	17 (6/34)

Evaluation at 24 h included the sample of embryos used for gene expression analysis.
[a] 3.72 mM.
[b] Evaluation at 72 h IVC.

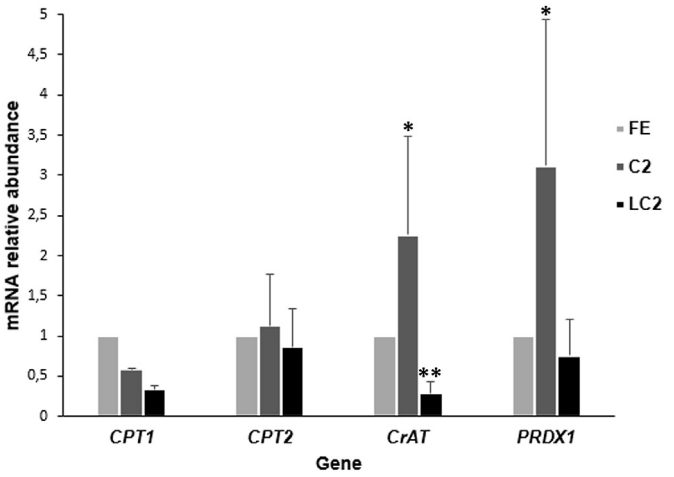

Fig. 7. Relative expression of *CPT1*, *CPT2*, *CrAT*, and *PRDX1* in vitrified *in vivo*-produced ovine embryos, warmed in the absence (C2) or presence (LC2) of 3.72 mM L-carnitine, after 24 h of *in vitro* culture. FE: fresh embryos. (*) upregulated; (**) downregulated ($p < 0.05$). (Based in 3 pools of 5 re-expanded embryos sampled at 24 h after warming).

acylCoA and CoA pools, whose imbalances result in blockage of energetic pathways and impairment of oxidative metabolism and ATP production in the cell [20,36]. Changes in *CrAT* expression and a high acylcarnitine/free carnitine ratio are associated with mitochondrial dysfunction [37]. Low levels of LC increase the levels of acylCoA and reduces free CoA into the mitochondria, impairs the activity of dehydrogenases and, consequently, mitochondrial metabolism of carbohydrates and amino acids [38].

Contradictory results (between the two experiments) on *CrAT* expression were observed in embryos vitrified and thawed without LC supplementation in comparison to the fresh control. As demonstrated by several studies, cryopreservation reduces embryonic viability, possibly due to intracellular changes during cryopreservation, which can lead to a disorganized response. According to Lesse [39], embryos with less viability would present greater metabolic alterations, greater amino acid turnover, and lower levels of antioxidant enzymes under stressful conditions. Again approving the previous hypnosis further experiment is required to investigate the effect time of LC addition on the expression of CrAT using the same vitrification protocol.

LC supplementation into vitrification medium neither affected ROS levels in ovine embryos 24 h after rewarming, nor the number of apoptotic cells and the apoptotic index at 72 h of IVC. These results can be attributed to the high quality of the embryos and the culture under low tension of O_2. The beneficial effects of antioxidants are best observed under high O_2 culture conditions [40,41]. It is important to highlight that in the present study, we used a 5% O_2 atmosphere during IVC, aiming to mimic the uterine conditions [42] which may have minimized the effects of LC in ROS levels. Corroborating these results, in Experiment 1 the expression of *PRDX1* was similar for the vitrified embryos in the presence or absence of L-carnitine; however, both were downregulated relative to FE. PRDX1 acts as an ROS scavenger, as well as a modulator of survival and cell death pathways [21]. Cells constantly subjected to oxidative stress, such as cancer cells have high expression of members of the PRDX family, in response to increased levels of ROS [43,44]. Both vitrified and fresh embryos are cultured in the same conditions; therefore, differences in *PRDX1* expression should be referred to vitrification procedures.

Apoptosis evaluation was performed by caspase-3, a member of aspartate-specific cysteine protease enzyme family called caspases, which are important in intracellular apoptotic signaling that occurs in response to oxidative stress [45,46]. DNA fragmentation is the major consequence of caspase-3 activation by high levels of ROS [45]. In cryopreserved 8-cell murine embryos, a reduction in apoptosis and an increase in survival rates was observed after 4 h of IVC in LC treatment supplemented with 0.3 mg/mL, half the dosage used in the present study, whereas the 2-fold dosage produced a less noticeable increase of embryo survival and quality [11]. Moawad et al. [25] observed that L-carnitine supplementation at 3.72 mM during vitrification of germinal vesicle mouse oocytes improved pre-implantantion development following maturation and fertilization, although the same dosage has also been used in IVM before IVF, extending the supplementation period, and therefore, differing from the supplementation strategy used in our study. Such differences may explain the divergent results in embryo developmental capacity between Moawad's study and ours. The cryotolerance of the oocytes is completely different from the blastocysts, in addition to species differences.

Due to the difference in developmental dynamics of embryos in the morula and blastocyst stage, in the present study, embryos from both stages were cultured together, in the same drop (by virtue of n). Normally, the re-expansion of the blastocyst occurs within the first 24 h of culture after vitrification, while in embryos at the morula stage, the expansion takes place after this period (48–72 h). Based on this, we believe that the increased number of re-expanded embryos observed at 48 h and 72 h of culture is due to the embryos in the morula stage.

5. Conclusion

The results obtained here demonstrate that the dosage of 3.72 mM of LC was efficient in promoting some cellular responses in the level of gene expression related to energy homeostasis and antioxidant cell defense. However, such effect did not result in extra benefits in embryo quality parameters. Although not significant, LC supplementation was able to enhance embryo survival and hatching rates by approximately 10–15%, an increase that may be significant when applied to large-scale *in vivo* embryo production systems. Finally, to improve the efficiency of this strategy, further investigations need to be carried out with different concentrations of LC and tools.

Conflict of interest statement

The authors report that there are no conflicts of interest among authors and between authors and other people, institutions, or organizations.

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