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Would L-carnitine improve the survival and viability of vitrified/warmed in vitro bovine blastocysts?

A L-carnitina melhora a sobrevivência e a viabilidade de blastocistos bovinos vitrificados e aquecidos?

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ABSTRACT: The experiment was conducted to verify the effects of delipidation using L-carnitine on the development and survival of *in vitro*-produced bovine embryos, cryopreserved by vitrification, using the evaluation of the intracytoplasmic content of lipids, cellular apoptosis, mitochondrial potential, the rates of re-expansion and hatching post-warming. The embryos were cultured without the addition of L-carnitine (Control), in the presence of L-carnitine (0.6 mg/mL) added to the culture medium of D1 to D7 (L-Ivc), during the culture before vitrification and post-warming re-culture (L-IvcR) and during *in vitro* maturation, *in vitro* culture before vitrification and post-warming re-culture (L-IvmIvcR). The supplementation with L-carnitine did not change the cleavage rate in D3 ($p > 0.05$) but increased the production of blastocysts in D7 by 20.7% and the proportion of grade I embryos by 30.1% in LIvc ($p<0.05$), as well as the re-expansion rate (2-hour) ($p<0.05$), but the hatching rates and the number of degenerate structures at the 24 and 48-hour post-warming did not differ between treatments ($p>0.05$). The treatment with L-carnitine was effective in reducing cell apoptosis, intracytoplasmic content of lipids, and mitochondrial potential of blastocysts ($p<0.001$). In conclusion, media supplementation with 0.6 mg/mL of L-carnitine during *in vitro* embryo production improved vitrified/warmed bovine blastocysts' survival and viability, reducing cell apoptosis and intracellular lipids' amount and positively impacting mitochondrial activity potential. Under the presented conditions, this supplementation can be used only in the culture medium since no additional benefit was verified by adding this L-carnitine concentration during oocyte maturation, blastocyst culture, or post-warming culture.

Keywords: L-carnitine; *in vitro* embryo; bovine; embryo vitrification.

RESUMO: O experimento foi realizado para verificar os efeitos da deslipidação usando L-carnitina no desenvolvimento e sobrevivência de embriões bovinos produzidos *in vitro*, vitrificados, usando a avaliação intracitoplasmática de lipídios, apoptose celular e potencial mitocondrial, taxas de reexpansão e eclosão pós-aquecimento. Os embriões foram cultivados sem L-carnitina (Controle), com L-carnitina (0,6 mg/mL) no meio de cultura (D1-D7, L-Ivc), durante a cultura antes da vitrificação e recultura pós-aquecimento (L-IvcR) e durante a maturação e cultura *in vitro* antes da vitrificação e recultura pós-aquecimento (L-IvmIvcR). A suplementação com L-carnitina não alterou a taxa de clivagem em D3 ($p >0,05$), mas aumentou a produção de blastocistos (D7) em 20,7% e a proporção de embriões de grau I em 30,1% (LIvc, $p<0,05$), e a taxa de reexpansão (2 horas) ($p<0,05$); as taxas de eclosão e o número de estruturas degeneradas 24-48 horas pós-aquecimento não diferiram ($p>0,05$). O tratamento com L-carnitina foi eficaz na redução de apoptose celular, conteúdo intracitoplasmático de lipídios e potencial mitocondrial dos blastocistos ($p<0,001$). Em conclusão, a suplementação do meio com 0,6 mg/mL de L-carnitina durante a produção de embriões *in vitro* melhorou a sobrevivência e a viabilidade dos blastocistos bovinos vitrificados/aquecidos, reduzindo a apoptose celular e a quantidade de lipídios intracelulares e impactando positivamente o potencial de atividade mitocondrial. Nas condições apresentadas, essa suplementação pode ser usada apenas no meio de cultura, uma vez que nenhum benefício adicional foi verificado com a adição dessa concentração de L-carnitina durante a maturação dos oócitos, a cultura de blastocistos ou a cultura pós-aquecimento.

Palavras-chave: L-carnitina; embrião *in vitro*; bovino; vitrificação de embriões

INTRODUCTION

The need to speed up genetic improvement in dairy and beef cattle has contributed to developing biotechnologies such as *in vitro* embryo production (IVP) programs. The main objective of IVP is to obtain viable embryos from females of high genetic value and those no longer capable of producing offspring using conventional techniques. *In vitro*-produced embryos are more sensitive to cryopreservation than *in vivo* embryos. The successful cryopreservation of mammalian oocytes and embryos offers opportunities for the preservation of germplasm and the transportation of genetics within the country and internationally (Viana *et al.*, 2018), which is the key to further expanding the PIV embryo industry.

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It is becoming increasingly clear that mitochondrial activity and adenosine triphosphate (ATP) levels play an essential role in determining the quality and competence of the oocyte (Nagano *et al*., 2006), and influence the metabolism and developmental capacity of the pre-implantation embryo. In mammalian oocytes and embryos, fatty acids are stored in cells mainly as triacylglycerol (TAG) within lipid droplets surrounded by specialized proteins (Fair *et al*., 2001), intracellular lipids serve as an important energy source for ATP production in mitochondria via β-oxidation (Sturmey *et al*., 2009). The β-oxidation metabolic pathway is expressed in *in vivo* and *in vitro* embryos (Dunning *et al*., 2010). The use of endogenous lipids as an energy source by bovine oocytes is a determining factor during maturation (Cetica *et al*., 2002), and also for embryo quality (Sturmey *et al*., 2009), as they can generate abundant ATP even with low levels of extracellular substrates. The cytoplasm of mammalian oocytes and embryos is naturally rich in lipids, as is bovine follicular fluid, which has abundant levels of triglycerides and free fatty acids (Dunning *et al*., 2011). However, oocytes and embryos cultured in the presence of fetal serum have a greater amount of intracellular lipid when compared to those produced *in vivo* or in serum-free systems (Sudano *et al*., 2012).

The *in vitro* culture environment can modulate gene expression in mammalian cells and embryos (Zhou *et al*., 2008) and influence the transcriptome of blastocysts, particularly lipid metabolism, and the response to oxidative stress, altering development rates and sensitivity to cryopreservation (Held-Hoelker *et al*., 2017; Zhao *et al*., 2024). The inverse relationship between embryo quality and lipid content has been demonstrated by the accumulation of lipid content within embryos, with the location of these droplets compromising the development and quality of embryos produced *in vitro* vs. *in vivo*. The cytoplasm's lipid content is related to cryoinjury in oocytes and blastocysts. In addition to the accumulation of intracellular lipids, differences have been found in the lipid profile of PIV blastocyst membranes, directly altering the physicochemical properties of the cell membrane, including fluidity, permeability, and thermal phase behavior (Sudano *et al*., 2012). *In vitro*-originated embryos are more sensitive to cryopreservation than *in vivo* embryos, and the pregnancy and birth rates of vitrified embryos are lower than those of fresh embryos (Dunning; Robker, 2017), with the highest percentage of loss occurring before 45 days of gestation (Alexopoulos *et al*., 2008). The success of cryopreservation depends greatly on the quality of the blastocysts (Carrillo-González; Maldonado-Estrada, 2020; Carrillo-González *et al*., 2021), and embryos classified as grade I or II show slight ultrastructural changes after cryopreservation (Dalcin *et al*., 2013). This difference in the development potential of oocytes and embryos produced in vitro and in vivo may be due to the absence of cofactors to facilitate the β-oxidation metabolic pathway (Sutton-McDowall *et al*., 2012) such as L-carnitine, forskolin, and others (Li *et al*., 2021; Vasková *et al*., 2023).

L-carnitine (LC) (3-hydroxy-4-N-trimethylamoniobutyrate) is a small water-soluble molecule that plays an important role in fat metabolism. LC is an enhancer of lipid metabolism in animal cells. Its main function is facilitating free fatty acid transport from the cytosol to the mitochondria, supplying beta-oxidation, and generating adenosine triphosphate (ATP) (Li *et al*., 2021; Darzi; Zandi; Ghaedrahmati, 2025).

This experiment was based on the hypothesis that L-carnitine would increase the survival rates of vitrified/warmed *in vitro*-produced bovine embryos, aiming to establish a specific protocol for preparing IVP bovine embryos for cryopreservation.

MATERIAL AND METHODS

This experiment was approved by the Ethical Committee in Animal Experimentation of the Federal University of Goiás (Protocol 021/15).

Media composition

Maturation medium (IVM) - TCM 199 medium with Earle's salts and L-glutamine (Gibco®, Invitrogen Co, Grand Island, NY, USA) added with 10% fetal bovine serum (v/v), 0.2 mM pyruvate, 5 mg/mL luteinizing hormone (Lutropin-V®, Bioniche Co, Belleville, ON, Canada), 1 mg/mL follicle-stimulating hormone (Folltropin®, Bioniche Co., Belleville, ON, Canada), 75 µg/mL amikacin and 1mM cystamine.

Fertilization medium (IVF) - TALP-FERT supplemented with 6 mg/mL bovine serum albumin (BSA) - free of fatty acids, 0.2 mM pyruvate, 30 µg/mL heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine, and 75 µg/mL amikacin.

Capacitation medium (CAP) - Tyrode's HEPES buffered medium, supplemented with 0.2 mM pyruvate and 75 µg/mL amikacin. Culture medium (CIV) - SOFaa 80 supplemented with 2.7 mM myoinositol, 0.2 mM pyruvate, 2.5% fetal bovine serum (v/v), 5 mg/mL fatty acid-free BSA, 75 ug/mL amikacin.

L-carnitine solution - maturation/cultivation medium plus 0.6mg/mL L-carnitine (Sigma-Aldrich® Merck KGaA, Darmstadt, Germany), prepared on the day of use.

Vitrification solutions - Maintenance solution (MS) - HEPES-buffered TCM-199 medium (GIBCO® BRL, Invitrogen) supplemented with 20% fetal bovine serum (Cultilab, Campinas, Brazil). Vitrification solution 1 (VS1) - SM solution supplemented with 7.5% ethylene glycol (Sigma Co., St. Louis, MO, USA) and 7.5% dimethyl sulfoxide (DMSO, Sigma Co., St. Louis, MO, USA).

Vitrification solution 2 (SV2) - SM solution added with 15% ethylene glycol, 15% DMSO, and 0.5 M sucrose (Sigma Co., St. Louis, MO, USA).

Heating solutions -Maintenance Solution (SM) - HEPES-buffered TCM-199 medium (GIBCO® BRL, Invitrogen) supplemented with 20% fetal bovine serum (Cultilab, Campinas, Brazil). Devitrification Solution 1 (DV1) - SM solution supplemented with 1.0 M sucrose (Sigma Co., St. Louis, MO, USA). Devitrification Solution 2 (DV2) - SM solution supplemented with 0.5 M sucrose (Sigma Co., St. Louis, MO, USA).

Experimental design

After selection, 2,100 COCs from 10 aspiration sessions were randomly assigned to treatments and subjected to in vitro maturation. For the treatments, LC (0.6 mg/mL) was added to maturation (Ivm), culture (Ivc), and embryo post-warming culture (PW), in four treatments: no LC (Control), L-Ivc, L-IvcR, and L-IvmIvcR media. On D7 the blastocysts classified as Grade 1 and 2 were vitrified using the Cryotop method; 700 embryos were vitrified and rewarmed. Of the total, 124 embryos were used for cell apoptosis analysis, 116 for intracytoplasmic lipid analysis and measurement, 120 for membrane potential, and 284 for re-expansion and hatching evaluation. L-carnitine effects were evaluated on the production rates, cell death, lipid quantification, mitochondrial activity, re-expansion, and hatching rates in vitrified blastocysts.

In vitro Embryo Production

For *in vitro* production, all the steps were performed under the following incubation conditions- 38.8°C, 5% CO2, and saturated relative humidity without condensation, in the same incubator. The epifluorescence microscope Olympus BX43 (Tokyo, Japan) was used for fluorescence evaluations.

Cumulus-oocyte complex (COCs) from Bos indicus x Bos Taurus were aspirated from ovaries collected at a slaughterhouse and processed for in vitro embryo production as described elsewhere (Marques *et al.*, 2021). Briefly, after selection, COCs Grade 1 were maturated in 200 µL drops (for 30-35 oocytes) of Maturation Medium (IVM) for 22-24 hrs (38.8°C, 5% CO2 and saturated relative humidity without condensation), then transferred to 200 µL drops of Fertilization medium (IVF) and inseminated with conventional commercial semen from a Holstein bull proved for IVP previously prepared trough the Percoll 45-90% gradient and diluted to a final concentration of 1.0 x 106 viable sperm/ml (Parrish; Krogenaes; Susko-Parrish, 1995). After 18 hrs, presumable zygotes were partially denuded by successive pipetting and cultivated in 200 µL of culture medium (CIV) for 7 days. On D3 (72 hpi) cleavage rate was assessed and 50% of IVC was replaced. Morphological quality and blastocyst rate were evaluated in D7 (Bó; Mapletoft, 2013).

Vitrification, warming, and re-culture of embryos

On D7, expanded blastocysts were cryopreserved using the Cryotop vitrification method (Kuwayama, 2007) on a warmer plate at 36°C, using Ingá-med® rods (Maringá-PR, Brazil) and stored in liquid nitrogen. Briefly, blastocysts were placed in a drop of 70 µL of SM medium and then passed through three drops of 70 µL of SV1 medium, each for three minutes. Subsequently, they were washed in three drops of 50 µL of SV2 medium, positioned on the rod with a minimum solution (±0.2µL) for 40 to 90 seconds, and immediately immersed in liquid nitrogen. One week later, vitrified embryos were warmed (39°C) and immersed in 2 µL of DV1 medium, transferred to 200 µL drop of DV1 (1 Min) and 200 µL of DV2 (3 Min). Immediately afterward, embryos were washed in two drops of 200 µL of SM solution for 5 Min.

Mitochondrial Activity Analysis and Total number of cells

The mitochondrial membrane potential of the blastocysts was assessed 24 hours after warming (Santos et al., 2014). After incubation of embryos with 0.5 µM C24H24Cl2N2O (MitoTracker Orange CMTMRos®, Life Technologies Corporation, Carlsbad, CA, USA) for 30 min at 38.5^{0}C in PBS/PVA and fixed in 3.7% paraformaldehyde for 24 hs at 4°C, blastocysts were exposed to 10µg/mL-1 solution of Hoechst 33342 (Invitrogen Corp., Carlsbad, CA) for 10 min, protected from light, washed in PBS/PVA, placed on glass slides with ProLong®Gold (Molecular Probes, Eugene, OR), covered with a coverslip and examined under the epifluorescence microscope(wavelength filter of 550 nm for MitoTracker Orange and 365 nm for Hoechst 33342).

Evaluation of intracellular lipid content

The intracellular lipid droplets of the embryos were labeled with the specific lipid dye Nile Red (NR) (Invitrogen™ Molecular Probes™, Eugene, OR) based on the method of Genicot *et al.* (2005). Briefly, 1 mg/mL-1 of NR stock solution (Molecular Probes) was prepared by dilution in dimethyl sulfoxide (DMSO) and stored at room temperature in the dark. The embryos were fixed in 4% paraformaldehyde for 24 hours, washed in PBS-PVP three times (10 min each), and stained for three hours in 500 µL of working solution (10µg/mL-1 of NR stock solution dissolved in physiological saline (0.9% NaCl) with 1mg/mL-1 of PVP). The blastocysts were washed three times in PBS-PVP and then incubated in a 10µg/mL-1 solution of Hoechst 33342 (Invitrogen Corp., Carlsbad, CA) for 10 minutes, protected from light, washed three times in PBS-PVP and the slide was mounted. ProLong®Gold (Molecular Probes, Eugene, OR) was used to mount the slides and coverslips for observation under an epifluorescence microscope. The photo analysis procedures for Nile Red were the same as those for the assessment of mitochondrial potential. The relative fluorescence intensity of the structures was considered to be directly proportional to the amount of intracellular lipids.

For mitochondrial potential and lipid content, the images were saved in TIFF format using a camera attached (Olympus SC30, Tokyo, Japan) to the microscope, and the exposure time and light intensity were standardized. The fluorescence intensity and the total cell count of the embryos were calculated individually using Image J software (version 1.46; National Institutes of Health, Bethesda, MD). The fluorescence intensity (average pixel intensity) per area was corrected by removing the background, the corrected value was divided by the number of cells to obtain the average pixel intensity per cell, normalized to the Control, and set to a numerical value of 1.0. The structures' relative fluorescence intensity was directly proportional to mitochondrial activity and lipids content.

Apoptotic cells evaluations

The TUNEL assay (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) was used to assess cell apoptosis according manufacturer´s instructions (In Situ Cell Death Detection kit (Roche, Mannheim, Germany). After staining, blastocysts were mounted using ProLong®Gold (Molecular Probes, Eugene, OR) and observed under an epifluorescence microscope. The nuclei of all cells were stained in blue (365 nm), and the nuclei of TUNEL-positive cells, i.e. those with fragmented DNA, were visualized in green (450 nm). The blastocysts were evaluated individually and all the cells marked on both filters were counted to measure the total number of cells (TCN) and the number of apoptotic cells (ACN).

Statistical Analysis

Statistical analyses were performed using the program R version 3.0.2 (2018) at a 5% significance level. Data that did not show a normal distribution using the Shapiro-Wilk test were compared using the non-parametric Kruskal-Wallis test (mitochondrial activity and quantification of intracellular lipids). The number of cleaved embryos, blastocysts, embryonic quality, the average total number of cells, and the number of apoptotic cells between treatments were analyzed using the generalized linear model with a binomial distribution, testing contrasts between pairs of treatments. Quantitative data were presented as means and qualitative data as percentages.

RESULTS AND DISCUSSIONS

This study was designed to test the best time to add L-carnitine in the routine procedures for *in vitro* production to enhance the survival and viability of vitrified blastocysts post-warming. The results highlighted the positive effect of adding 0.6 mg/mL of L-carnitine on the quality and cryotolerance of bovine blastocysts produced *in vitro*.

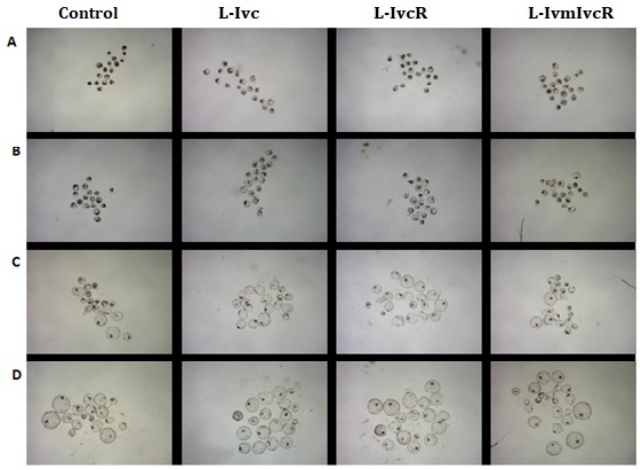
LC supplementation did not alter the cleavage rate at D3 ($p>0.05$), but increased the production of blastocysts at D7 in L-Ivc and grade I embryos in L-IvcR ($p<0.05$) compared to the Control (Table 1).

The re-expansion rate (2-hour) was higher in the L-Ivc, and the hatching rate at 24 hours did not differ between treatments, nor did the number of degenerates ($p>0.05$), but hatching at 48 hours was higher in L-Ivc ($p<0.05$) compared to the Control (Table 2).

In the evaluation of apoptotic cells, TNC did not differ ($p>0.05$) and ACN was lower in the L-IvcR and LMivCivR groups when compared to L-Ivc and Control ($P <0.001$; Table 3).

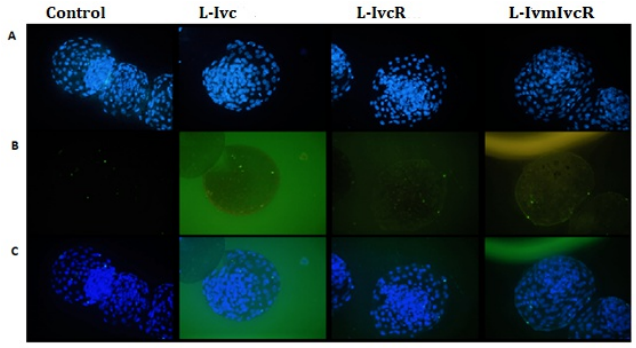
The kinetics of development of embryos vitrified by the Cryotop method warmed and re-cultivated, evaluated at 2-hour, and hatching at 24 and 48-hour post-warming are exposed in Figure 1. Figure 2 shows images illustrating the total and apoptotic cell count after the treatments used following warming and reculturing of vitrified embryos.

Figure 1 – Kinetics of embryos vitrified, warmed, and post-warming culture for 48 hrs. A- Zero-hour; B- 2-Hour; C – Hatched, 24-Hour; D – Hatched, 48-hour.



Source: Author's collection.

Figure 2 – Post-warming culture for 48 hrs. A- Zero-hour; B- 2-Hour; C – Hatched, 24-Hour; D – Hatched, 48-hour. Cell apoptosis, TUNEL Assay. 365 nm wavelength filter for Hoechst 33342 (A), 450 nm wavelength filter for TUNEL positive (B), and merge (C).



Source: Author's collection.

Table 1 – *In vitro* production rate of bovine blastocysts, without L-carnitine (Control) and cultured in the presence of LC in the IVC (L-Ivc and L-IvcR), in the IVM + IVC (LMivCivR).

Treatments	Cleavage (%)	Blastocysts D7	
		Total (n, %)	Grade I Blastocysts (n, %)
Control	89.2 (266/298)[a]	34.9 (104/298)[b]	17.4 (52/298)[b]
L-Ivc	90.4 (276/305)[a]	48.9 (149/305)[a]	24.9 (76/305)[ab]
L-IvcR	89.1 (271/304)[a]	44.1 (134/304)[ab]	27.6 (84/304)[a]
L-IvmIvcR	90.6 (270/298)[a]	39.9 (119/298)[ab]	21.8 (65/298)[ab]

[ab]Different letters in the column show difference ($p<0.05$ using the generalized linear model with binomial distribution).

Table 2 – Re-expansion rate (2 hrs) and hatching (24 and 48 hrs) of expanded blastocysts produced in the absence of L-carnitine (Control), L-carnitine added to the IVC (L-Ivc), to the IVC and the re-cultivation media after warming (L-IvvR), and L-carnitine added to the IVM, IVC and + the re-cultivation media after warming (LIvmIvcR).

Treatments	Re-expansion(%) 2-hour	Hatching (%)		
		24-hour	48-hour	Unviable
Control	67.6 (46/68)[b]	47.1 (32/68) [a]	72.1 (49/68)[b]	4.4 (3/68) [a]
L-Ivc	87.5 (63/72)[a]	50.0 (36/72) [a]	90.3 (65/72)[a]	1.4 (1/72) [a]
L-IvcR	78.4 (58/74)[ab]	52.7 (39/74) [a]	86.5 (64/74)[ab]	1.4(1/74) [a]
L-IvmIvcR	75.7 (53/70)[ab]	45.7 (32/70) [a]	77.1 (54/70)[ab]	4.3 (3/70) [a]

[ab]Different letters in the column show difference ($p<0.05$ using the generalized linear model with binomial distribution).

Table 3 – Cell apoptosis by the TUNEL assay of expanded blastocysts produced in the absence of L-carnitine (Control), L-carnitine added to the IVC (L-Ivc), to the IVC and the re-cultivation media after warming (L-IvcPW), and L-carnitine added to the IVM, IVC and + the re-cultivation media after warming (LIvmIvcR).

Treatments	N	TNC	ACN
Control	31	165,3[a]	6,5[a]
L-Ivc	31	168,5[a]	5,7[a]
L-IvcR	31	175,1[a]	3,2[b]
L-IvmIvcR	31	179,5[a]	3,6[b]

TNC- total Number of Cells, ACN- Apoptotic Cells Number. abDifferent letters in the column show difference ($p<0,001$).

Adding L-carnitine improved the competence of in vitro matured oocytes and increased the number of oocytes progressing to the Metaphase II (MII) stage, especially those with low development potential (Knitlova *et al*., 2017; Carrilo-González *et al*., 2021). Despite studies showing its positive effect on oocyte maturation, LC did not improve embryo production from prepubertal heifer oocytes, vitrified or not (Spricigo *et al*., 2017). In the present study, no improvement in cleavage rates was observed (Table 1), which corroborates previous results in bovine embryos (Held-Hoelker *et al*., 2017).

Regarding the number of blastocysts on D7 (Table 1), there was a positive effect of LC, when added to the IVC medium, as well as grade I embryos ($p<0.05$). Improved embryo production was associated with LC due to the increase in the β-oxidation pathway, providing a better supply of ATP to the embryos (Dunning *et al*., 2010, Li *et al*., 2021), and its positive effect was evident when added to oocytes with low developmental potential (Knitlova *et al*., 2017). The action of LC, either by stimulating the production of antioxidants or activating the β-oxidative pathway, increases mitochondrial activity and ATP production, protecting cells against apoptosis.

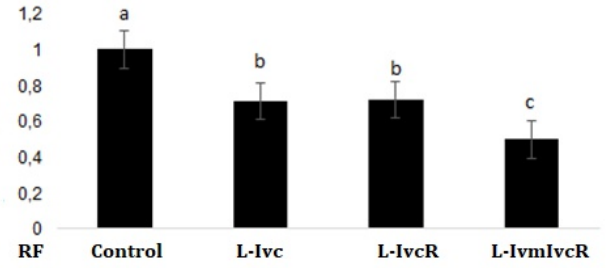
Positive results were obtained after warming the blastocysts in the re-expansion (2-hour) and hatching (48-hour) (Table 2, Figure 1) in the treatments with LC, and a significant improvement of hatching in the embryos previously cultured during 7 days in the presence of LC, in comparison with Control (90.3% vs 72.1%; $p<0.05$). Hatching is a demanding process in which the blastocyst escapes through the zona pellucida (ZP) before implantation. The thickness of the ZP is an important indicator for the successful hatching and implantation of transferred embryos (Balakier *et al*., 2012). In vitro culture of mammalian embryos changes the characteristics of the ZP; PIV embryos have a thicker ZP, especially the inner layer, and the cryopreservation process also alters the ZP leading to its hardening. The zona pellucida of embryos treated with LC is significantly thinner (Khanmohammdi *et al*., 2016). Vitrification leads to embryo injuries, and cryoprotectants play a crucial role in minimizing these negative effects (Souza *et al*., 2018), but to evaluate the survival and viability of cryopreserved blastocysts, the post-warming culture is necessary to verify their developmental kinetics (Hwang *et al*., 2020). As shown in Table 2, the addition of LC only in the ICV medium was sufficient to improve blastocysts survival and viability, suggesting that the extra manipulation required to add LC in the IVM and/or in the IVC medium used during the post-warming culture would be not necessary. Blastocysts produced in the presence of LC and cryopreserved by the slow freezing method improved the cryotolerance, evaluated by blastocysts' expansion and hatching ability (Zolini *et al*., 2019).

The total number of cells (TNC) in blastocysts and the number of cells in the inner cell mass (ICM) indicates the embryo quality, and is crucial for successful implantation (Lee *et al*., 2015). Our study showed no increase in the TNC (Table 3), corroborating previous research which found no difference in TNC and ICM, or in the ICM/trophectoderm ratio in cattle (Knitlova *et al*., 2017). However, the significant decrease in apoptotic cells in LC-treated and vitrified embryos (Table 3, $p<0.001$) is an important result, since the quality of a blastocyst predicts its ability to implant and its future development, and apoptosis that occurs during this stage can be detrimental because of the damage in the embryo homeostasis (Brad *et al*., 2007).

This reduction in the number of apoptotic cells and the greater potential for re-expansion and hatching in vitrified blastocysts treated with LC may be

associated with the production of antioxidants and the reduction in lipid content. There is an inverse relationship between embryo quality and lipid content; the accumulation of lipids within embryos and the location of these droplets compromise the development and quality of embryos produced *in vitro* (vs. *in vivo*) (Sudano *et al*., 2012, Annes *et al.*, 2019). In addition to the accumulation of intracellular lipids, there are differences in the lipid profile of IVP blastocyst membranes, which can lead to alterations in membrane fluidity (Sudano *et al.*, 2012). The LC-treated blastocysts, independent of the medium or time of supplementation, showed significantly lower amounts of intracellular lipid content (Figure 3, p<0.001).

Figure 3 – Quantification of intracellular lipids by fluorescence using Nile Red post-warming. The analyses were carried out in triplicate (n = 116). (p<0.001, Kruskal-Wallis test). Y-axis: mean relative fluorescence. Control was set to 1.00.

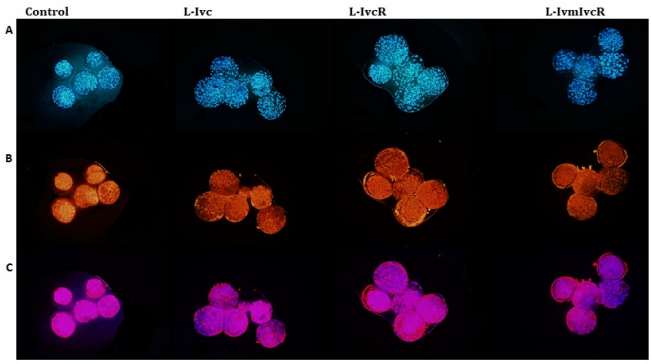


Source: Author's collection.

This decrease in lipid content is associated with activating the β-oxidation pathway. Using endogenous lipids as an energy source by bovine oocytes is a determining factor during maturation and for embryo quality, as these lipids can generate an abundance of ATP even with low levels of extracellular substrates (Li *et al.*, 2021). In this context, the addition of LC in both, IVM and IVC seems to be a good alternative to reduce lipid content and enhance blastocysts quality.

In Figure 4 is the lipid droplet staining with Nile Red. Images are representative of D7 blastocysts 24 hours post-warming.

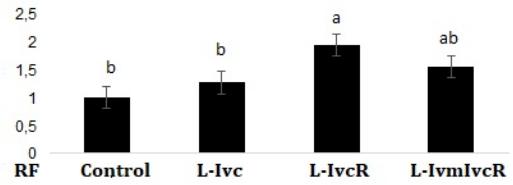
Figure 4 – Representative images of the intracellular lipid content of the blastocysts 24 hours post-warming using Nile Red and Hoechst 33342. 365 nm wavelength filter for Hoechst 33342 (A), 550 nm wavelength filter for Nile Red (B), and merge (C).



Source: Author's collection.

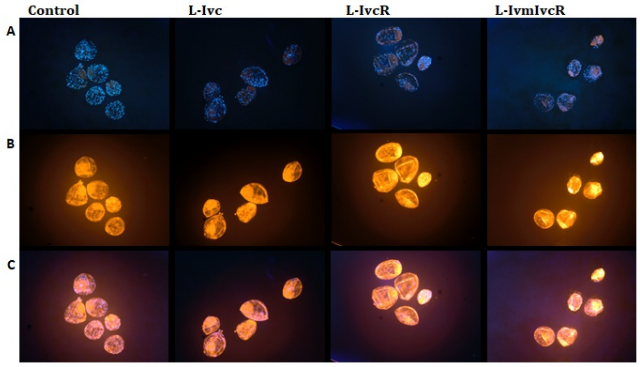
The mitochondrial membrane potential data assessed by fluorescence are shown in Figures 5 and 6. The mitochondrial membrane potential was significantly higher in the post-warming L-IvcR blastocysts (p<0.001) than in Control and L-Ivc.

Figure 5 – Quantification of mitochondrial membrane potential (MMP) after 24 hours post-warming blastocysts. The analyses were carried out in triplicate (n= 120). (p< 0.001 by Kruskal-Wallis test). Y-axis: Relative fluorescence, Control was set to 1.00.



Source: Author's collection.

Figure 6 – Representative images of the mitochondrial membrane potential of post-warming blastocysts after 24 hours using MitoTracker Orange CMTMRos® and Hoechst 33342. 365 nm for Hoechst 33342 (A), 550 nm wavelength filter for MitoTracker Orange (B), and merge (C).



Source: Author's collection.

Mitochondrial organization and ATP levels play an essential role in determining the oocyte's quality and competence, influencing the preimplantation embryo's metabolism and developmental capacity (Van Blerkom, 2011), and play a crucial role in ensuring their efficient performance in energy production. The translocation of mitochondria to the central regions is a natural process during oocyte *in vivo* maturation the redistribution of active mitochondria is related to the high ATP production required during this phase (Yildrim; Seli, 2024). Studies suggest that the protective effect of LC occurs preferentially around mitochondria (Miyamoto *et al*., 2010); LC can stabilize mitochondrial membranes and increase the energy supply to the organelle, protecting the cell from apoptosis (Yildirim; Seli, 2024) Cryopreservation causes damage to cells such as cytoskeletal fractures (ultrastructural changes resulting from disorganization of actin filaments), changes in the distribution of organelles, including areas without cytoplasmic organelles (Golgi complex located far from the nucleus), changes in the pattern of mitochondrial distribution and activity, the presence of large vesicles and a decrease in inter-cellular junctions (Dalcin *et al.*,

2013), a delay in the resumption of protein synthesis, changes in gene expression levels and DNA damage, indicating additional oxidative stress during cryopreservation. The protective effects of LC may be by decreasing lipid droplets around mitochondria and/or by improving the energy metabolism necessary for cell survival under oxidative stress (Aksu *et al*., 2012). So, the increased mitochondrial potential in the blastocysts exposed to LC during culture (L-Ivc), culture post-warming (L-IvcR), and during maturation, culture, and post-warming (L-IvmIvcR) is compatible with the decreased intracellular lipid content in the same groups of blastocysts. In both, fresh and vitrified-warmed bovine embryos, there was a time-dependent effect of culture conditions on the embryo quality regarding hatching rates, cell loss, and oxidative stress recovery, allowing the embryos to restore their developmental competence (Silva *et al*., 2021).

CONCLUSIONS

In conclusion supplementations with 0.6 mg/mL of L-carnitine during *in vitro* embryo production improved vitrified/warmed bovine blastocysts' survival and viability, reducing cell apoptosis and intracellular lipids' amount and positively impacting mitochondrial activity potential. Under the presented conditions, this supplementation can be used only in the culture medium since no additional benefit was verified by adding this L-carnitine concentration during oocyte maturation, blastocyst culture, or post-warming culture.

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