Livestock Science 232 (2020) 103933

Contents lists available at ScienceDirect

Livestock Science

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

Effects of L-carnitine on embryo development of vitrified swamp buffalo oocytes following in vitro fertilization

Yuanyuan Liang[a,b], Ton Yoisungnern[a], Yanni Huang[b], Rangsun Parnpai[a,*]

[a] Embryo Technology and Stem Cell Research Center, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

[b] Department of Reproductive Medicine, Liuzhou General Hospital, Guangxi 545006, China

ARTICLE INFO

Keywords:
Buffalo
oocytes
Vitrification
L-carnitine

ABSTRACT

The aim of this study was to evaluate the effect of L-carnitine (LC) supplemented in vitro maturation (IVM) medium on the cryotolerance and development of buffalo oocytes. Mitochondrial activity, reactive oxygen species (ROS) content, and lipid droplet distribution of vitrified-warmed buffalo oocytes were also observed. Oocytes were matured in IVM medium supplemented with 0, 0.3, 0.6, and 1.2 mg/mL LC and some of them were vitrified by the Cryotop® method. The highest survival, morula, and blastocyst rates were obtained with 0.6 mg/mL LC, irrespective of whether oocytes were fresh or vitrified-warmed. The density of active mitochondria in vitrified oocytes was significantly higher with 0.6 mg/mL LC than without LC treatment. In contrast, H_2O_2 levels and lipid droplet content were significantly lower following LC treatment than without it. In conclusion, supplementation with 0.6 mg/mL LC during IVM improves the buffalo oocytes' survival rate and subsequent embryo development after in vitro fertilization. This may be linked to improved mitochondrial activity, enhanced β-oxidation, and reduced ROS levels.

1. Introduction

Oocytes' cryopreservation offers potential applications in scientific research, stock raising, endangered species conservation, and the preservation of women's gametes before chemotherapy or radiation therapy (Andrabi and Maxwell, 2007). Chilling injury, osmotic stress, cryoprotectance (CPA) toxicity and ice crystallization are the major damaging factor during cryopreservation (Mazur et al., 1972). A large size, low volume-to-surface ratio, high lipid contents, and the nature of the plasma membrane are associated with the limited success of oocytes' cryopreservation (Leibo, 1980). To date, vitrification has received more attention than slow freezing as a cryopreservation protocol because it causes less damage to the oocytes (Kuleshova and Lopata, 2002). Buffalo oocytes' cryopreservation is of great value in preserving female gametes, especially in developing countries. However, the large number of lipid vacuoles inside buffalo oocytes makes the oocyte sensitive to oxidative damage and chilling injuries (Boni et al., 1992; Mondadori et al., 2010). Mitochondria, Golgi complex, endoplasmic reticulum, and lipid are clustered in the buffalo oocytes' cortical region (Mondadori et al., 2010) which is also the first location to undergo freezing damage. Lipid droplet removal has been reported to improve oocytes' and embryos' cryotolerance (Ogawa et al., 2010; Nagashima et al., 1995).

Lipids are essential for generating ATP via oxidative phosphorylation (OXPHOS) in the mitochondria. Although OXPHOS is a vital part of metabolism, it produces reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, which lead to lipid peroxidation as well as the propagation of free radicals, apoptosis, and mitochondrial dysfunction (Yin et al., 2015).

L-carnitine (LC) is a small water-soluble molecule with an important role in normal physiology. It was first isolated from bovine muscle in 1905 by two Russian scientists (Gulewitch and Krimberg, 1905), and only the L-isomer was found to be bioactive (Binienda and Virmani, 2003). LC serves as an intra-mitochondrial vehicle for the transfer of long-chain fatty acids inside mitochondria for β-oxidation, the transport of short- and medium-chain acyl groups from the peroxisome to mitochondria, the regulation of intracellular acyl-CoA/free CoA ratio, and the removal of toxic acyl residues from mitochondria (Melegh et al., 1993; Vaz et al., 2002). Moreover, it protects the cell from DNA damage, minimizes insult to the oocytes' cytoskeleton, and acts as an antioxidant to prevent embryo apoptosis (Mansour et al., 2009). LC promotes lipid metabolism as well as ATP production in animal cell (Kerner J, Hoppel, 2000) and has been reported to act as a potent antioxidant with very few side effects (Dunning and

* Corresponding author.
E-mail address: rangsun@g.sut.ac.th (R. Parnpai).

https://doi.org/10.1016/j.livsci.2020.103933
Received 27 June 2019; Received in revised form 5 December 2019; Accepted 10 January 2020
Available online 11 January 2020
1871-1413/

Robker, 2012). Given these positive characteristics, scientists are now interested in the role of LC in freezing resistance in farm animals.

Over the last two decades, a number of studies have shown the positive influence of LC supplementation on female fertility. LC added in vitro matruration (IVM) medium was shown to decrease lipid droplet and ROS levels, as well as increase mitochondrial activity in porcine oocytes (Somfai et al., 2011). We previously reported that 0.3 mg/mL LC added to maturation medium improved the nuclear maturation rate of swamp buffalo oocytes (Phongnimitr et al., 2013a). In particular, LC supplementation in maturation, fertilization, and culture media for bovine oocytes revealed that 0.6 mg/mL in maturation medium led to the highest embryo development (Phongnimitr et al., 2013b). Supplementation of IVM medium with LC was found to maintain similar embryo developmental competence in vitrified-warmed IVM bovine oocytes as in non-vitrified ones (Chankitisakul et al., 2013). Moawad et al. (2014) showed that LC supplementation (0.6 mg/mL) in vitrification/warming and IVM medium increased the proportions of immature mouse oocytes with normal MII spindles. LC improved cytoplasmic and nuclear maturation of immature mouse oocytes by reducing ROS levels in the maturation medium (Zare et al., 2015). Manzano et al. (2015) reported improved embryo developmental potential, higher blastocyst formation rate, increased total cell count and activity when bovine oocytes were matured in medium supplemented with 0.1–0.5 mg/mL LC. Recently, LC supplemented during maturation was shown to improve the development of bovine embryos from meiotically less competent oocytes and accelerate blastocyst formation (Knitlova et al., 2017). At the same time, a combination of 0.6 mg/mL LC during maturation and 0.23 μg/mL Resveratrol prior to vitrification was shown to decrease spindle damage and affect gene expression of vitrified bovine oocytes (Sprícigo et al., 2017). More recently, acetyl-L-carnitine (ALC, an ester of L-carnitine) supplementation during IVM was suggested to improve buffalo oocyte quality after vitrification by enhancing mitochondrial function and altering the phospholipid composition of vitrified oocyte membranes (Xu et al., 2018). ALC affects mitochondrial function, regulates oocyte-derived paracrine factors, and promotes the production of steroid hormones, thus increasing the quality of matured oocytes and embryo development (Xu et al., 2019).

Based on the above-mentioned benefits of LC addition to IVM medium, the present study sought to examine the effect of LC supplementation on fertilization and embryo developmental competence, cryotolerance during maturation, mitochondrial activity, ROS levels, and lipid droplet variation in buffalo oocytes.

2. Materials and methods

2.1. Oocyte collection and IVM

Buffalo ovaries were obtained from a slaughterhouse and transported within 4 h to the laboratory in physiological saline solution (0.9% NaCl) at room temperature. Cumulus-oocyte complexes (COCs) were collected from follicles 2–8 mm in diameter and washed in Dulbecco's phosphate-buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrrolidone (PVP). Each of 20 COCs was randomly cultured in 100 μL IVM medium supplemented with at 0 (control), 0.3 (0.3 group), 0.6 (0.6 group), and 1.2 (1.2 group) mg/mL LC at 38.5 °C under a humidified atmosphere of 5% CO_2 for 23 h. The IVM medium was TCM199 supplemented with 10% fetal bovine serum (FBS), 0.02 IU/mL follicle stimulating hormone (FSH, Antrin®, Denka Phamaceutical, Japan), 50 IU/mL human chorionic gonadotropin (Chorulon®; Intervet Inc., Rushmore, MN, USA) and 1 μg/mL 17-β-estradiol.

2.2. Oocyte vitrification and warming

Vitrification was performed as previously described by Liang et al. (2011) and was combined with the Cryotop® method (Kitazato Supply Co., Tokyo, Japan).

After IVM, COCs were partially denuded by gentle pipetting with a pulled-pipette using 0.1% (w/v) hyaluronidase. A group of five oocytes was placed in base medium (BM: TCM199-Hepes + 20% FBS) supplemented with 10% (v/v) dimethylsulfoxide (MERCK, Darmstadt, Germany) and 10% (v/v) ethylene glycol (EG) for 1 min, and then exposed to vitrification solution (BM supplemented with 20% (v/v) DMSO, 20% (v/v) EG, and 0.5 M sucrose) at 22–24 °C for 30 s. Oocytes were loaded onto the end tip of Cryotop® in a small volume of vitrification solution and plugged into liquid nitrogen (LN_2) immediately. The warming of vitrified-oocytes proceeded by direct immersion into 3 mL of 0.5 M sucrose in BM at 38.5 °C for 5 min, followed by transfer into BM without sucrose for 5 min. Finally, the vitrified-warmed oocytes were kept in BM under humidified atmosphere of 5% CO_2 at 38.5 °C for 1 h before being used.

2.3. Evaluation of oocyte viability

After warming, oocytes were counted to calculate the recovery rate and live/dead status using fluorescein diacetate (FDA) staining as described previously (Mohr and Trounson 1980). Briefly, oocytes were treated with 2.5 μg/mL FDA in phosphate buffered saline (PBS) supplemented with 5 mg/mL BSA at 38.5 °C for 2 min in dark room, washed three times in PBS supplemented with 5 mg/mL bovine serum albumin (BSA). The oocytes were evaluated under a fluorescent microscope (IX71, Olympus, Tokyo, Japan) with UV irradiation and a U-MWIB3 filter with an excitation wavelength of 460–495 nm and emission of 510 nm. Oocytes displaying a bright green fluorescence were considered alive and used in subsequent experiments.

2.4. Preparation of Bovine oviduct epithelial cells (BOECs)

Bovine oviducts were collected along with buffalo ovaries from the local slaughterhouse. BOECs were mechanically isolated from the oviducts according to the method by Parnpai et al. (1999). Oviducts were cut, separated from the surrounding connective tissue, and rinsed with PBS without calcium or magnesium. The oviduct was gently squeezed in a stripping motion with forceps along the ampulla and isthmus to obtain BOECs. These were centrifuged at 300 × *g* for 5 min, washed two times in TCM-199-Hepes supplemented with 10% FBS, and then washed twice in TCM-199 supplemented with 10% FBS. The cells were cultured for 48 h in 5% CO_2 in air at 38.5 °C to form aggregates of BOECs. Finally, BOEC aggregates were selected and washed three times in embryo culture medium.

2.5. In vitro fertilization (IVF) and in vitro embryo culture (IVC)

The frozen fertile bull sperms were thawed in air for 10 s and placed in 38 °C water bath for 30 s. Thawed sperms were placed in the bottom of 5 mL snap tube containing 2 mL of Tyrode's albumin lactate pyruvate (TALP) medium and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 30 min to allow the sperm to swim up. The top 1.5 mL of medium were collected and centrifuged at 400 x *g* for 5 min. The pellets were re-suspended in TALP medium and washed twice at 400 x *g* for 5 min. Groups of 10–15 COCs were placed in 100 μL droplets of TALP medium, and incubated with fertile-thawed sperms at a concentration of 3 × 10^6 cells/mL at 38.5 °C under humidified atmosphere of 5% CO_2 in air for 12 h. The embryos (20 embryos/100 μL) were cultured in modified synthetic oviduct fluid supplemented with amino acids (mSOFaa) medium (Parnpai et al., 1999) supplemented with 3 mg/mL fatty acid-free BSA at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 for two days. Only 8-cells stage embryos were co-cultured with BOECs in mSOFaa medium supplemented with 0.25 mg/mL glucose in a humidified atmosphere of 5% CO_2 in air for two days. After that, the embryos were cultured in mSOFaa medium supplemented with 0.5 mg/mL glucose for an additional three days (seven days in total).

2.6. Evaluation of mitrochondrial activity in vitrified oocytes

All evaluated vitrified-warmed buffalo oocytes were denuded before incubated with 200 nM of Mito Tracker Red CMXRos (MTR) dye in PBS containing 3% PVP for 30 min. After staining, the oocytes were fixed in 2% paraformaldehyde in PBS for 20 min. Subsequently, oocytes were incubated in PBS supplemented with 10 μg/mL H33342 for 5 min, and then fixed carefully on glass slide at dark room. Oocytes in 0 mg/mL and 0.6 mg/mL L-carnitine treatment groups were processed simultaneously using the same batches of working. Only the oocytes with signs of second metaphase plate with the first polar body (confirmed by H33342) were included in our analysis. For mitrochondrial evaluation, sample were observed at 200 × magnification using A1 laser scanning confocal microscope (Nikon, Tokyo, Japan) equipped with helium-neon laser (543 nm). The mitochondria distribution was measure in the equatorial plane, at where the largest diameter of each oocyte was taken using the same setting. The abundance of active mitochondria in the equatorial section of the oocyte was evaluated by measuring pixel numbers from inverted greyscale images using the NIH ImageJ (ver. 1.48) software. Relative mitochondria density was expressed as the mean intensity of MTR-positive pixels in the total area of the oocyte equatorial section surface.

2.7. Measurement of intracellular H_2O_2 levels in vitrified oocytes

The intracellular levels of H_2O_2 in MII-stage vitrified-warmed oocytes were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes, Eugene, OR, USA) as previously described (Nasr-Esfahani et al., 1990). Denuded oocytes with first polar bodies were selected for measurement. Oocytes were incubated in TCM-199 medium supplemented with 10 mM DCHFDA and 3 mg/mL PVP for 15 min. Next, oocytes were washed twice in the above medium without DCHFDA and carefully mounted on a glass slide with a coverslip. The slide was evaluated under a fluorescent microscope (IX71; Olympus) with UV irradiation and a U-MWIB3 filter with an excitation wavelength of 480 nm and emission of 510 nm. Digitalization of fluorescence intensity to evaluate intra-oocyte H_2O_2 levels was performed in ImageJ. Relative fluorescence intensity units (FIU) were calculated from the mean total fluorescence, with the control group set as 100%. The H_2O_2 level in MII-stage oocytes matured in vitro with or without 0.6 mg/mL LC was compared.

2.8. Measurement of intracellular lipid content in vitrified oocytes

Vitrified-warmed buffalo oocytes were denuded from cumulus cells and fixed with 4% paraformaldehyde for 48 h. After that, oocytes were washed in PBS three times (10 min each) and stained overnight in 500 μL of a 10 μg/mL Nile Red (Molecular Probes) solution in 0.9% NaCl with 1 mg/mL PVP (Genicot et al., 2005). The oocytes were then washed twice in PBS, mounted with 10 μg/mL H33342 on glass slides, and examined immediately at room temperature in a dark room. The distribution of lipid droplets was investigated only in MII-stage oocytes (confirmed by H33342 staining) by the same laser scanning confocal microscope described above. An equatorial section image at the largest diameter of each oocyte was taken at the same settings for each sample. Lipid content was evaluated using ImageJ. Relative lipid content was expressed as the mean intensity of Nile Red-positive pixels in the total area of the oocyte equatorial section. The lipid content in MII-stage oocytes matured in vitro with or without 0.6 mg/mL LC was compared.

2.9. Experimental design

In experiment 1, the effect of different concentrations of LC (0, 0.3, 0.6, 1.2 mg/mL) added to IVM medium on embryo development after IVF was evaluated. In experiment 2, the effect of different concentrations of LC (0, 0.3, 0.6, 1.2 mg/mL) added to IVM medium on the survival rate of vitrified-warmed oocytes and subsequent embryo development following IVF was evaluated. In experiment 3, mitochondrial activity of vitrified-warmed buffalo oocytes matured in vitro with 0 and 0.6 mg/mL LC was compared (based on the results of experiment 1 and 2). In experiment 4, intracellular H_2O_2 levels in vitrified-warmed buffalo oocytes matured in vitro with 0 and 0.6 mg/mL LC were compared. In experiment 5, intracellular lipid content in vitrified-warmed buffalo oocytes matured in vitro with 0 and 0.6 mg/mL LC was compared.

2.10. Statistical analysis

Each experiment was replicated at least four times. Data for survival, and embryo development were arcsine-transformed and analyzed by analysis of variance (ANOVA) using statistical analysis software (SAS Institute Inc., Cary, NC, USA). *P*-values <0.05 were considered statistically significant. Mitochondrial fluorescence intensity, H_2O_2 levels, and lipid content were analyzed using one-way ANOVA followed by Tukey's post-hoc test (StatSoft Inc., Tulsa, OK, USA). *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Effect of LC supplementation on embryo development

As shown in Table 1, when the oocytes were subjected to IVF and *cultured* in vitro, no difference in cleavage rates was observed between LC groups and the control group. Similarly, there was no significant difference between groups regarding the number of embryos developing to the 8-cell stage. The highest rates of embryos developing to the morula and blastocyst stages were obtained with 0.6 mg/mL LC, while the other LC groups gave similar results as the control group.

3.2. Effect of LC supplementation on survival of vitrified-warmed buffalo oocytes and subsequent embryo development after IVF

As shown in Table 2, survival of vitrified groups was over 96%, and did not differ from fresh control group (100%). Cleavage and morula rates were not significantly different among vitrified groups, but they were significantly lower than those of fresh oocytes. The ability of embryos development to develop to the blastocyst stage was similar in vitrified oocytes given either 0 or 0.3 mg/mL LC, and significantly lower than with 0.6 or 1.2 mg/mL LC, which were again similar to each other. The developmental ability of embryos was significantly lower in all vitrified groups than in the fresh control group in terms of cleavage and morula or blastocyst formation rates. There was no significant difference in terms of survival, 8 cell, morula and blastocysts yield after IVM in a medium supplemented with 0.6 mg/mL and 1.2 mg/mL. Base on the low dosage and economical, it is safer to choose 0.6 mg/mL LC in IVM medium for subsequent experiments.

Table 1
Effects of L-carnitine addition to IVM medium on embryo developmental competence of buffalo oocytes after IVF.

Groups	No. COCs	Cleaved (%)	No. (%) embryo developed to		
			8-Cell	Morula	Blastocyst
0 mg/mL	144	95 (66)	51 (35)	26 (18)^{b}	22 (15)^{b}
0.3 mg/mL	142	100 (70)	57 (40)	29 (20)^{ab}	24 (17)^{b}
0.6 m/mL	144	108 (75)	62 (43)	39 (27)^{a}	32 (22)^{a}
1.2 mg/mL	145	108 (74)	59 (41)	34 (23)^{ab}	30 (21)^{a}

*9 replicates were performed. Different superscripts within a column indicate significant differences ($P < 0.05$, ANOVA).

Table 2
Effects of L-carnitine addition to IVM medium on survival rate of vitrified oocytes and subsequent embryo development after IVF*.

Groups	No. COCs	Survived (%)	No. IVF	Cleaved (%)	No. (%) embryo developed to		
					8 Cell	Morula	Blastocyst
0 mg/mL	119	114 (96)	114	63 (55)^{b}	29 (25)^{b}	7 (6)^{b}	4 (4)^{c}
0.3 mg/mL	121	117 (97)	117	66 (56)^{b}	31 (26)^{b}	8 (7)^{b}	5 (4)^{c}
0.6 mg/mL	120	116 (97)	116	72 (62)^{b}	36 (31)^{ab}	12 (10)^{b}	9 (8)^{b}
1.2 mg/mL	121	116 (96)	116	70 (60)^{b}	35 (30)^{ab}	12 (10)^{b}	9 (8)^{b}
Fresh control	124	124 (100)	124	91 (73)^{a}	53 (43)^{a}	30 (24)^{a}	24 (19)^{a}

* Different superscripts within a column indicate significant differences ($P < 0.05$, ANOVA).

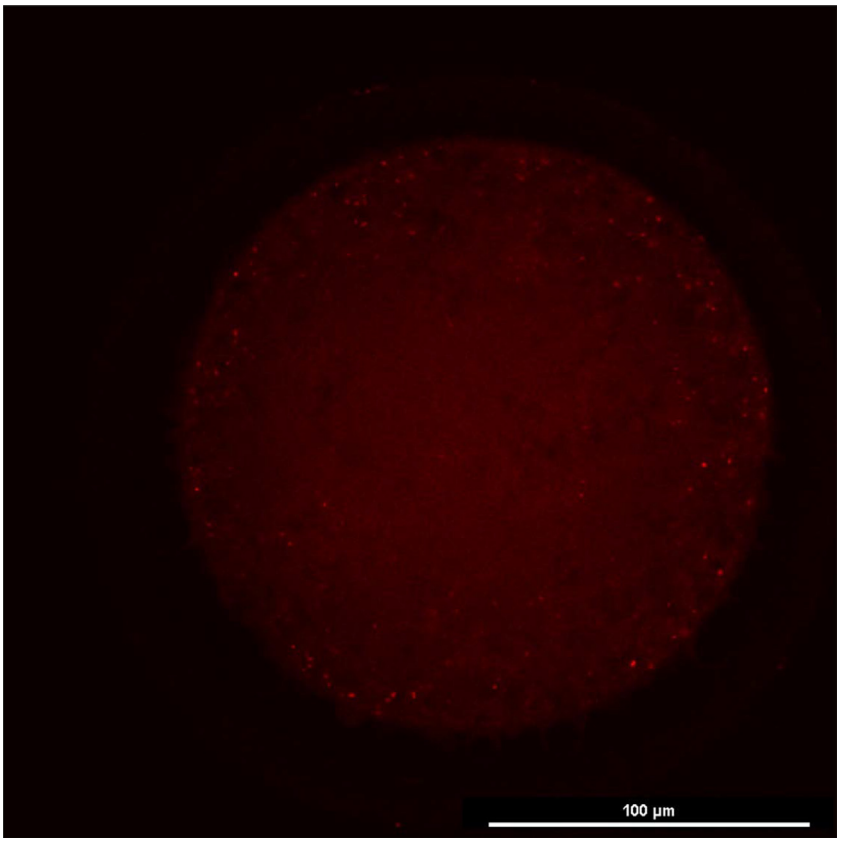

Fig. 1. Distribution of mitrochondria in MII stage oocytes matured in the absence of L-carnitine (0 mg/mL).

3.3. Evaluation of mitrochondrial activity in vitrified oocytes

Based on the results of experiments 1 and 2, 0.6 mg/mL LC was used to investigate the effect of LC on mitochondrial activity. A high density of active mitochondria was found both in the peripheral areas and the center of MII-stage vitrified-warmed buffalo oocytes treated with LC compared with non-LC-treated oocytes (Figs. 1 and 2, respectively). When the equatorial sections of oocytes were subjected to digital image analysis, a significantly higher density of MTR-labeled pixels was measured in LC-treated oocytes compared with non-LC-treated oocytes (Fig. 3).

3.4. Evaluation of intracellular H_2O_2 levels in vitrified oocytes

As shown in Figs. 4 and 5, at the end of the IVM culture, vitrified-warmed oocytes matured with 0.6 mg/mL LC exhibited lower DCHFD fluorescence intensity than vitrified-warmed oocytes matured without LC. Fig. 6 shows H_2O_2 levels expressed as FIU and given as mean ± standard error of the mean.

3.5. Evaluation of intracellular lipid content in vitrified oocytes

As shown in Figs. 7 and 8, at the end of IVM culture, most of the vitrified-warmed oocytes matured in the presence of 0.6 mg/mL LC presented fewer lipid droplets than vitrified-warmed oocytes matured without LC. Digital image analysis of equatorial sections revealed significantly less Nile Red-positive staining in the LC group compared with the non-LC group ($P < 0.05$) (Fig. 9).

4. Discussion

In this study, we investigated the effect of LC supplementation on cryotolerance of buffalo oocytes during in vitro maturation. Additionally, we assessed the effect of LC on mitochondrial activity, H_2O_2 levels, and lipid droplet content in vitrified-warmed oocytes. The present findings indicate that LC supplementation in IVM medium clearly enhanced the development of embryos derived from vitrified oocytes. Levocarnitine or LC is a biologically active stereoisomer of 3-carboxy-2-hydroxy-N, N, N-trimethyl-1-propanaminium. It exists as a highly polar, small zwitterion (an ion that while electrically neutral, carries both a positive and a negative electrical charge in different parts

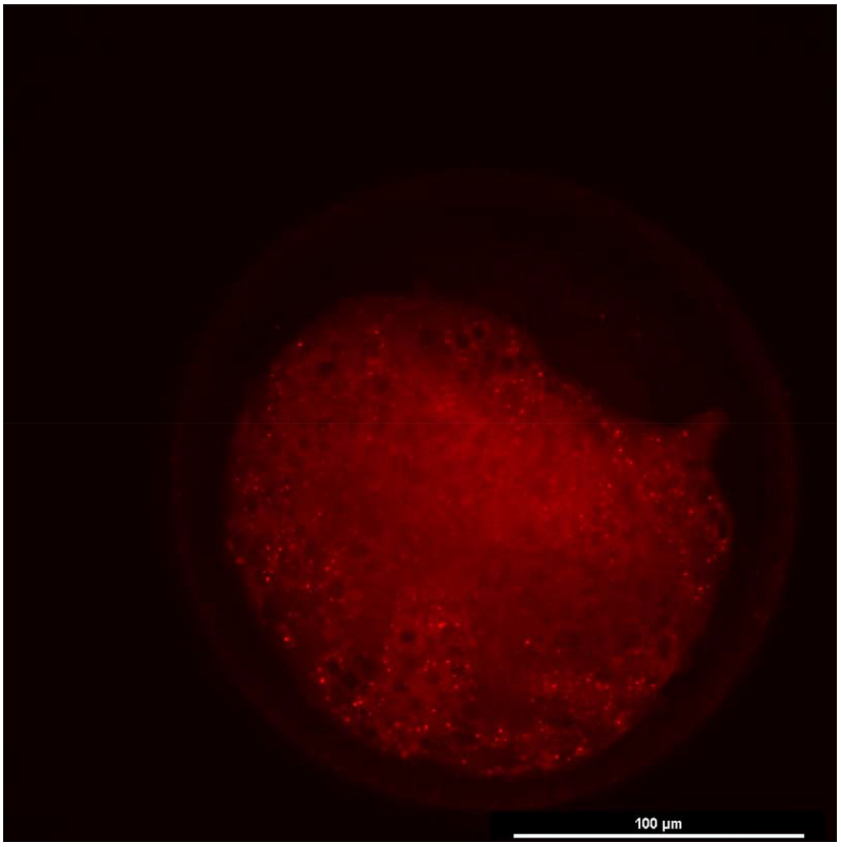

Fig. 2. Distribution of mitrochondria in MII stage oocytes matured in the present of L-carnitine (0.6 mg/mL).

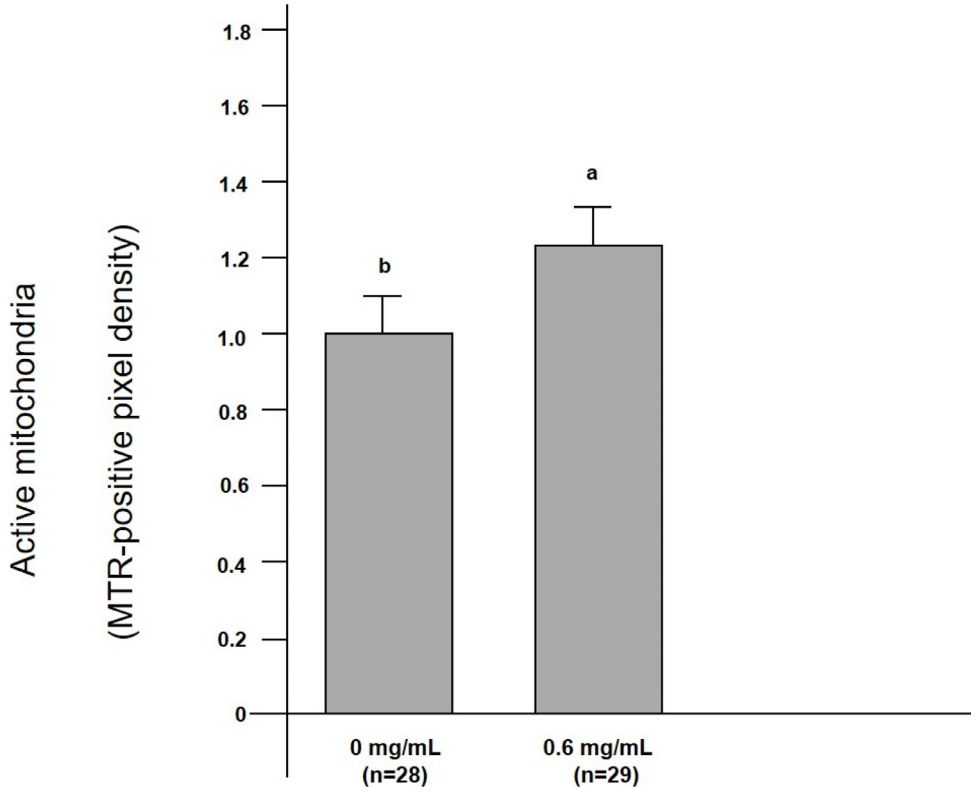

Fig. 3. Density of mitrochondria in MII stage oocytes matured in the absence (0 mg/ml) or presence (0.6 mg/mL) of L-carnitine.

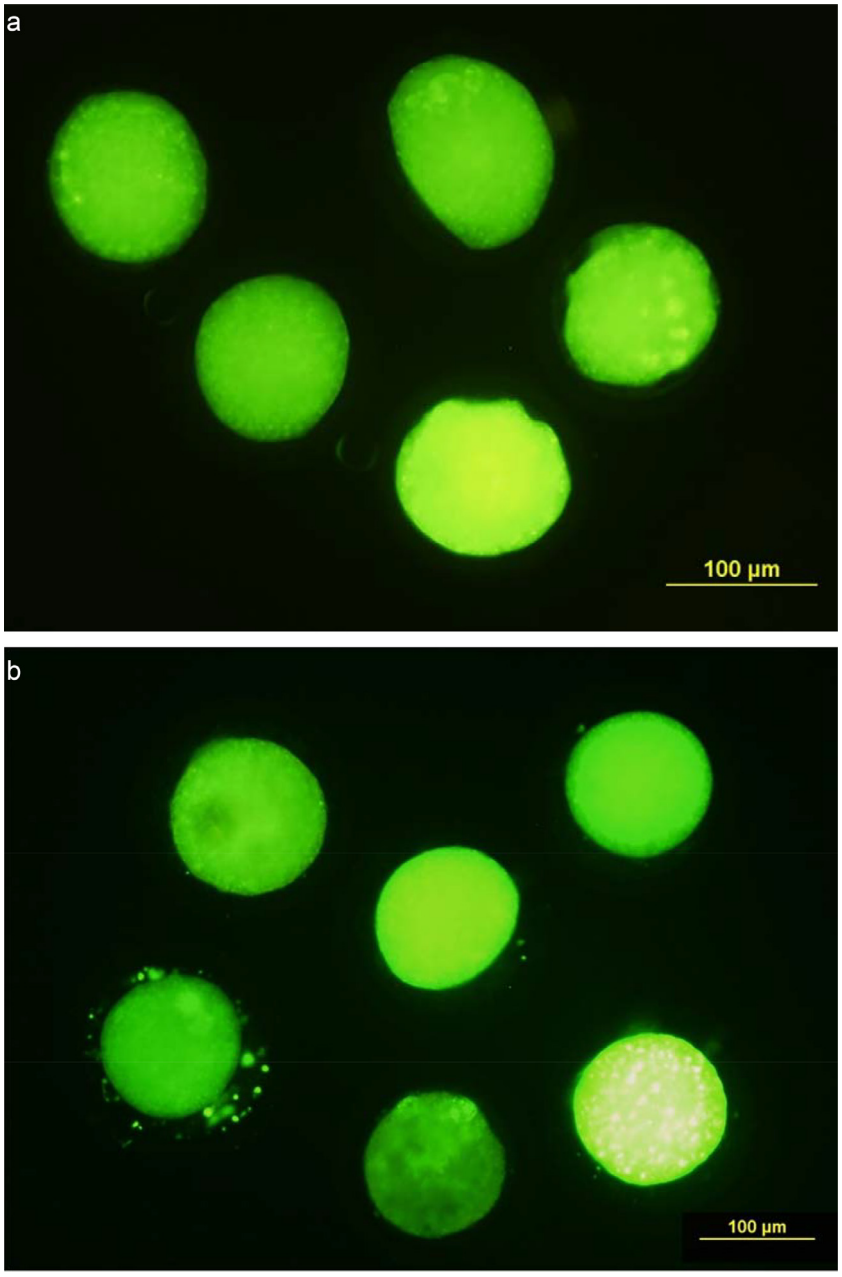

Fig. 4. a, b. Intracellular H_2O_2 levels in MII stage IVM buffalo oocytes matured in the absence (0 mg/mL) of L-carnitine.

of the molecule, as seen in certain amino acids and protein) (Binienda and Virmani, 2003). LC has been reported to act as a potent antioxidant with very few side effects (Dunning and Robker, 2012). This has sparked an interest in its role providing freezing resistance in farm animals. Compared to other animals, buffalo ooplasm is characterized by a large number of lipid droplets (Boni et al., 1992; Mondadori et al., 2010). This high lipid content is directly correlated with the oocytes' sensitivity to oxidative damage and freezing injury.

The observed beneficial effect of LC on oocyte maturation and embryo development may result from lipid utilization via β-oxidation to generate the ATP necessary for meiosis and cytoplasmic maturation (Ferguson and Leese, 2006). Similarly, Phongnimitr et al. (2013a) reported that LC supplementation during bovine oocytes' IVM improved nuclear maturation and embryo development. Sprícigo et al. (2017) found that addition of 0.6 mg/mL LC to the IVM medium of prepubertal bovine oocytes restored the expression of certain genes. Meiotically less competent bovine oocytes were shown to significantly accelerate blastocyst formation from meiotically more competent oocytes when 0.5 mg/mL LC was supplemented during IVM (Knitlova et al., 2017). When porcine oocytes were treated with 0.5 mg/mL LC during IVM, the rate of blastocyst formation was significantly higher, but it decreased when 2 mg/mL LC was used (Wu et al., 2011). A similar decrease in blastocyst development was found in murine oocytes with 1.25, 2.5, and 5 mg/mL LC (Abdelrazik et al., 2009). In buffalos, 0.5 mg/mL ALC supplementation in IVM was reported to decrease oocytes' freezing injury and improve blastocyst formation from 3.4 to 7.95% (Xu et al., 2018). Present findings demonstrate that supplementation with 0.6 and 1.2 mg/mL LC gives similar results as those reported previously. Although blastocyst formation was the same with 0.6 and 1.2 mg/mL LC, it's safer and economical to choose low dose group under the same biological potency. Even safe drugs can have harmful effects when the dosage is increased. Given the comparatively low cytotoxicity of 0.6 mg/mL LC, this dose was regarded as optimal for subsequent experiments. Considering that LC acts as an antioxidant by reducing the

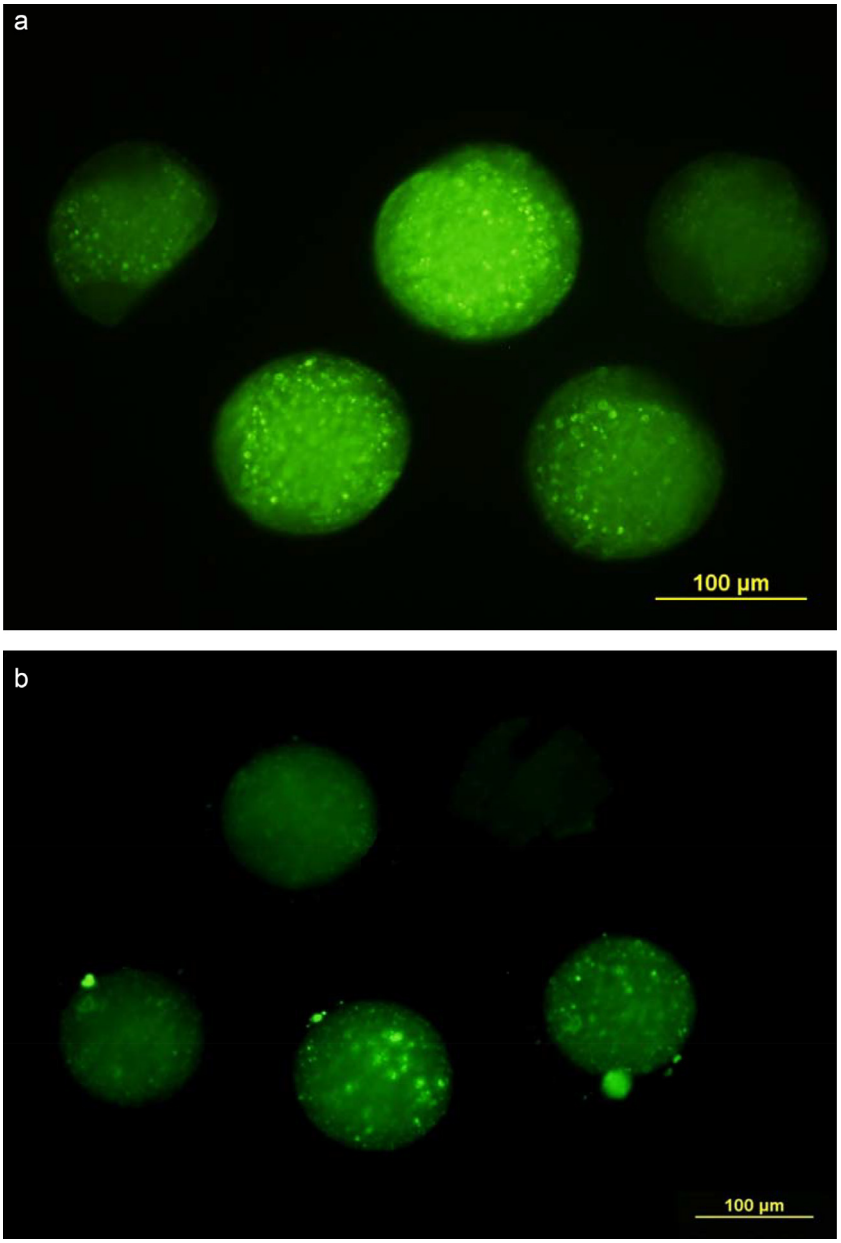

Fig. 5. a, b. Intracellular H_2O_2 levels in MII stage IVM buffalo oocytes matured in the presence (0.6 mg/mL) of L-carnitine.

level of ROS during IVM (Somfai et al., 2011; Wu et al., 2011), we propose that LC supplementation may improve embryonic development after IVF of buffalo oocytes due to: (1) increased endogenous lipid catabolism via β-oxidation to generate the ATP necessary for oocyte metabolism; (2) its beneficial effect in protecting cells from apoptosis; or (3) its antioxidant activity during maturation.

In the present study, only oocytes that survived the warming stage were subjected to IVF and subsequent in vitro culturing until blastocyst stage. In vitrified oocytes, LC supplementation in IVM medium had no positive effect on survival and cleavage rates, but improved further embryo development. Our result was similar to those observed by Chian et al. (2004), Morató et al. (2008), Sripunya et al. (2010), and Phongnimitr et al. (2013b), who also used the Cryotop® method to vitrify bovine oocytes. A lower embryo developmental ability is inevitable after vitrification and warming. Vitrification causes the fractionation of the zona pellucida, disruption of gap junctions between cumulus cells and oocytes, disappearance of microvilli on the oolemma surface (Wu et al., 2006), and abnormality in spindle and chromosome structure (Morató et al., 2008). Supplementation with LC during oocyte maturation was reported to significantly increase β-oxidation, and improve both fertilization and blastocyst development (Dunning et al., 2010; 2011). Ferguson and Leese (2006) indicated that endogenous lipid metabolism played an important role during oocyte maturation and, potentially, during subsequent embryonic development. Furthermore, incubation of oocytes and embryos with LC significantly reduced oocyte cytoskeleton damage and decreased the level of embryo apoptosis (Mansour et al., 2009).

Our results suggest that LC helps buffalo oocytes withstand the freezing damage caused by impaired lipid metabolism during IVM. In cattle, LC significantly increased the density of active mitochondria in oocytes in the central areas (Yamada et al., 2006). Somfai et al. (2011) revealed that mitochondrial translocation induced by LC was associated with a significant reduction of intracellular lipid content. Similarly, ALC improved vitrified buffalo oocytes' quality by decreasing phospholipid content (Xu et al., 2018). The greater density and distribution of active mitochondria in the presence of LC is likely related to faster nuclear

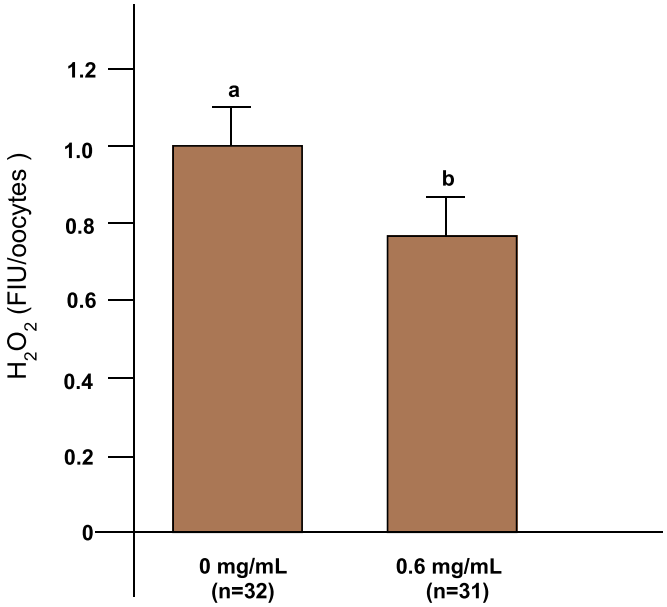

Fig. 6. H_2O_2 levels expressed by fluorescence intensity unit (FIU) in MII stage oocytes matured in the absence (0 mg/mL) or presence (0.6 mg/mL) of L-carnitine.

maturation of oocytes, in line with earlier findings correlating the proximity of active mitochondria with chromatin in porcine oocytes (Sun et al., 2001). Redistribution of active mitochondria was found to correlate with elevated ATP production during MI–MII transition, suggesting the importance of mitochondrial ATP for chromosome segregation in mouse oocytes (Yu et al., 2010).

At the end of the IVM culture, a lower level of intracellular H_2O_2, as expressed by mean FIU per oocyte, was detected in oocytes matured in the presence of LC. H_2O_2 plays important roles in host defense and oxidative biosynthetic reactions. H_2O_2 can be produced intracellularly from a variety of sources, though mostly superoxide anions (O^{2-}) formed from the partial reduction of oxygen. When H_2O_2 levels are low, the cells' antioxidant enzymes provide protection from oxidative damage. In contrast, higher levels of H_2O_2 initiate a signaling cascade, which may result in the inactivation of antioxidant enzymes and the consequent inability to quench H_2O_2 (Veal et al., 2007). Our results show that LC-treated oocytes were associated with reduced intracellular H_2O_2 levels, indicating an antioxidant effect of LC. Somfai et al. (2011) suggested a dual effect of LC on both lipid metabolism and ROS levels in porcine oocytes. Reduced ROS levels in LC-treated oocytes indicate that improved MII and cleavage rates may be related to the effect of LC on the oocytes' redox status. Alternatively, the beneficial effect of LC on meiotic progression and development may be attributed to enhanced metabolism, as suggested by a previous report, whereby LC improved mouse oocyte development in the absence of compounds (e.g., glucose) that promoted ROS formation (Dunning et al., 2010). Here, the greater developmental competence of vitrified buffalo oocytes indicates enhanced β-oxidation of fatty acids, as manifested by the concomitant lower intensity of lipid droplets following LC treatment.

In conclusion, we report that supplementation with LC during IVM augments buffalo oocytes' cryotolerance and mitochondrial activity, while reducing H_2O_2 levels and lipid content. More detailed in vitro assessments are required to determine the influence of such brief LC treatment on sperm function and blastocyst quality.

CRediT authorship contribution statement

Yuanyuan Liang: Conceptualization, Methodology, Investigation, Writing - review & editing. **Ton Yoisungnern:** Formal analysis, Writing - original draft. **Yanni Huang:** Project administration, Supervision,

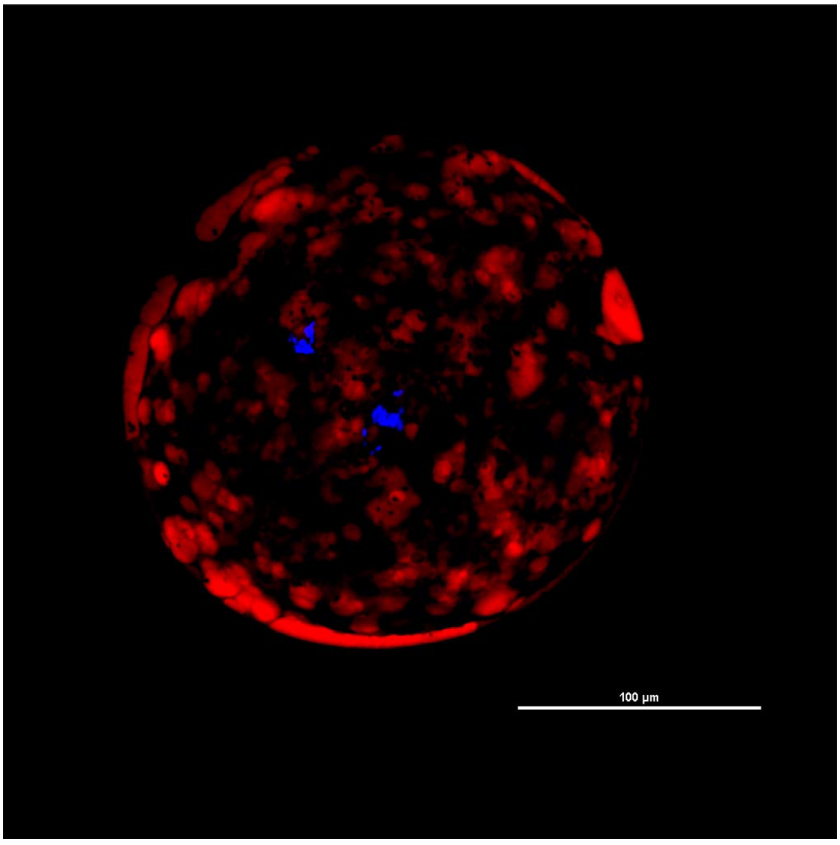

Fig. 7. Distribution of lipid droplets in MII stage IVM oocytes matured in the absence (0 mg/mL) of L-carnitine.

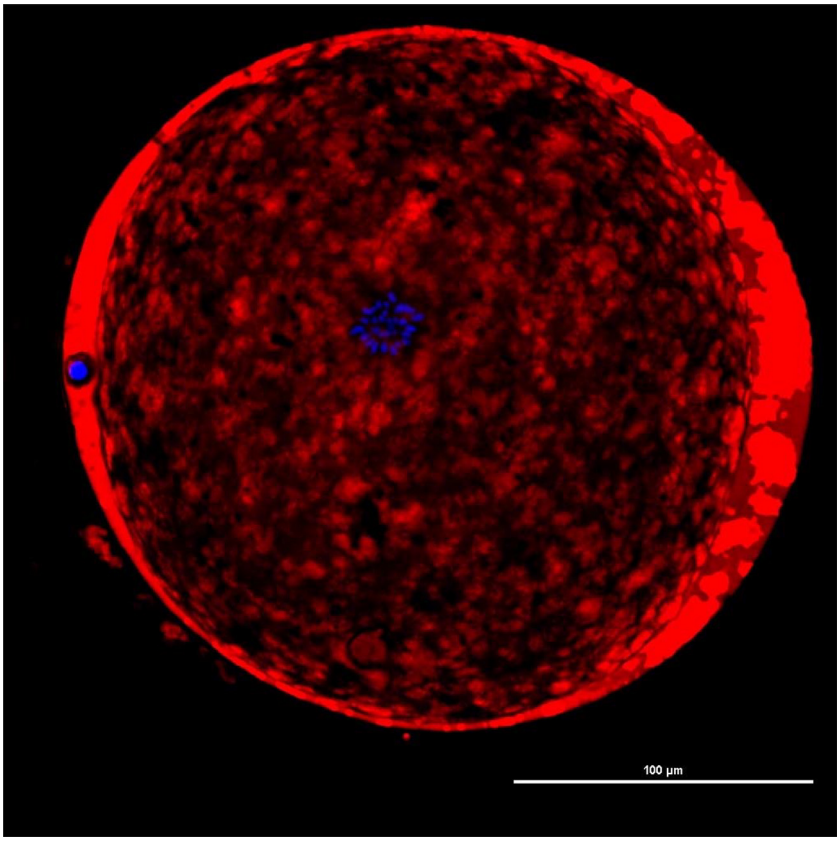

Fig. 8. Distribution of lipid droplets in MII stage IVM oocytes matured in the presence (0.6 mg/mL) of L-carnitine.

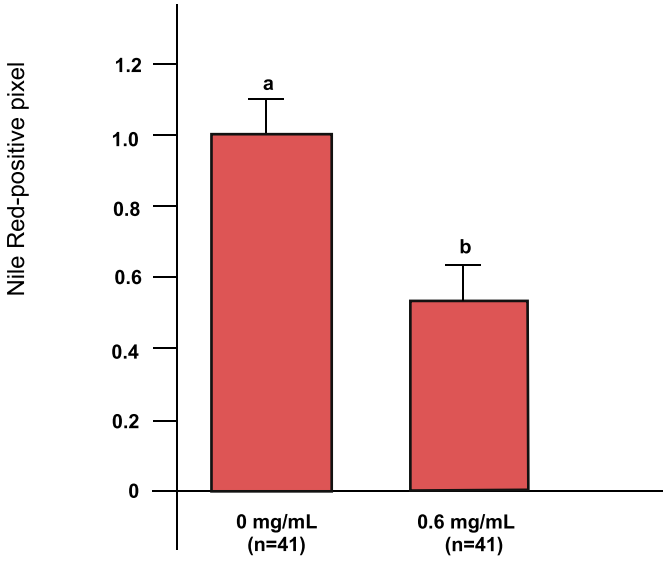

Fig. 9. Density of lipid droplets in MII stage IVM oocytes matured in the absence (0 mg/ml) and presence (0.6 mg/mL) of L-carnitine.

Visualization. **Rangsun Parnpai:** Resources, Funding acquisition, Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

None.

Acknowledgements

This research was supported by the Thailand Research Fund (BRG5080026) and Suranaree University of Technology. Ton Yoisungnern was supported by a SUT post-graduate fellowship. We would like to thank Editage (www.editage.com) for English language editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2020.103933.

References

Abdelrazik, H., Sharma, R., Mahfouz, R., Agarwal, A., 2009. L-carnitine decrease DNA damage and improves the in vitro blastocyst development rate in mouse embryos. Fertil. Steril. 91, 589–596.

Andrabi, S.M.H., Maxwell, W.M.C., 2007. A review on reproduction biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci. 99, 223–243.

Binienda, Z., Virmani, A., 2003. The mitochondriotropic effects of L-carnitine and its esters in the central nervous system. Curr. Med. Chem. – Cent. Nervous Syst. Agents 3, 275–278.

Boni, R., Santella, L., Dale, B., Roviello, S., Di Palo, R., Barbieri, V., 1992. Maturazione in vitro di oociti buffalini: indagine ultrastrutturale. Acta. Med. Vet. 38, 153–161.

Chankitisakul, V., Somfai, T., Inaba, Y., Techakumphu, M., Nagai, T., 2013. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes. Theriogenology 79, 590–598.

Chian, R.C., Kuwayama, M., Tan, L., Tan, J., Kato, O., Nagai, T., 2004. High survival rate of bovine oocytes matured in vitro following vitrification. J. Reprod. Dev. 50, 685–696.

Dunning, K.R., Cashman, K., Russell, D.L., Thompson, J.G., Norman, R.J., Robker, R.L., 2010. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol. Reprod. 83, 909–918.

Dunning, K.R., Akison, L.K., Russell, D.L., Norman, R.J., Robker, R.L., 2011. Increase beta-oxidation and improved oocyte developmental competence in response to L-carnitine during ovarian in vitro follicle development in mice. Biol. Reprod. 85, 548–555.

Dunning, K.R., Robker, R.L., 2012. Promoting lipid utilization with L-carnitine to improve oocyte quality. Anim. Reprod. Sci. 134, 69–75.
Ferguson, E.M., Leese, H.J., 2006. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol. Reprod. Dev. 73, 1195–1201.
Genicot, G., Leroy, J.L.M.R., Van Soom, A., Donnay, I., 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology 63, 1181–1194.
Gulewitch, V.S., Krimberg, R., 1905. Zur kenntnis der extraktivstoffe der muskeln. II. MItteilung: über das carnitin. Zeits für Physiol. Chem. 45, 326–330.
Kerner, J., Hoppel, C., 2000. Fatty acid import into mitochondrial. Biochim. Biophys. Acta 1486, 1–17.
Knitlova, D., Hulinska, P., Jeseta, M., Hanzalova, K., Kempisty, B., Machatkova, M., 2017. Supplementation of L-carnitine during in vitro maturation improves embryo development from less competent bovine oocytes. Theriogenology 102, 16–22.
Kuleshova, L., Lopata, A., 2002. Vitrification can be more favourable than slow cooling. Fertil. Steril. 78, 449–454.
Liang, Y.Y., Phermthai, T., Nagai, T., Somfai, T., Parnpai, R., 2011. In vitro development of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. Theriogenology 75, 1652–1660.
Leibo, S.P., 1980. Water permeability and its activation energy of fertilized and unfertilized mouse ova. J. Membr. Biol. 53, 179–188.
Mansour, G., Abdelrazik, H., Sharma, R.K., Radwan, E., Falcone, T., Agarwal, A., 2009. L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. Fertil. Steril. 91, 2079–2086.
Manzano, P.C.V.J., Ocampo, M.B., Ocampo, L.C., Maylem, E.R.S., Lazaro, J.V., 2015. Improved bovine blastocyst developmental potential by L-carnitine supplementation. Int. J. Sci. Res. Knowl. 3, 21–29.
Mazur, P., Leibo, S.P., Chu, E.H., 1972. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. Exp. Cell. Res. 71, 345–355.
Melegh, B., Sumegi, B., Sherry, A.D., 1993. Preferential elimination of pivalate with supplemental carnitine via formation of pivaloylcarnitine in man. Xenobiotica 23, 1255–1261.
Moawad, A.R., Xu, B., Tan, S.L., Taketo, T., 2014. L-carnitine supplementation during vitrification of mouse germinal vesicle stage-oocytes and their subsequent in vitro maturation improves meiotic spindle configuration and mitochondrial distribution in metaphase II oocytes. Hum. Reprod. 29, 2256–2268.
Mohr, L.R., Trounson, A.O., 1980. The use of Fluorescien diacetate to assess embryo viability in the mouse. J. Reprod. Dev. 58, 189–196.
Mondadori, R.G., Santin, T.R., Fidelis, A.A., Name, K.P., da Silva, J.S., Rumpf, R., Báo, S.N., 2010. Ultrastructure of in vitro oocyte maturation in buffalo (*Bubalus bubalis*). Zygote 18, 309–314.
Morató, R., Izquierdo, D., Paramio, M.T., Mogas, T., 2008. Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosome configuration and embryo development. Cryobiology 57, 137–141.
Nagashima, H., Kashiwazaki, N., Ashman, R.J., Grupen, C.G., Nottle, M.B., 1995. Cryopreservation of porcine embryos. Nature 374, 416.
Nasr-Esfahani, M.H., Aitken, J.R., Johnson, M.H., 1990. Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. Development 109, 501–507.
Ogawa, B., Ueno, S., Nakayama, N., Matsunari, H., Nakano, K., Fujiwara, T., Ikezawa, Y., Nagashima, H., 2010. Developmental ability of porcine in vitro matured oocytes at the meiosis II stage after vitrification. J. Reprod. Dev. 56, 356–361.
Parnpai, R., Tasripoo, K., Kamonpatana, M., 1999. Development of cloned swamp buffalo embryos derived from fetal fibroblasts: comparison in vitro cultured with or without buffalo and cattle oviductal epithelial cells. Buffalo J. 15, 371–384.
Phongnimitr, T., Liang, Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetampinich, C., Parnpai, R., 2013a. Effects of L-carnitine supplemented in maturation medium on the maturation rate of swamp buffalo oocytes. Buffalo Bull. 32, 613–616.
Phongnimitr, T., Liang, Y.Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetampinich, C., Parnpai, R., 2013b. Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. Anim. Sci. J. 84, 719–725.
Somfai, T., Kaneda, M., Akagi, S., Watanabe, S., Haraguchi, S., Mizutani, E., Dang-Nguyen, T.Q., Geshi, M., Kikuchi, K., Nagai, T., 2011. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. Reprod. Fertil. Dev. 23, 912–920.
Sprícigo, J.F., Morató, R., Arcarons, N., Yeste, M., Dode, M.A., López-Bejar, M., Mogas, T., 2017. Assessment of the effect of adding L-carnitine and/or resveratrol to maturation medium before vitrification on in vitro-matured calf oocytes. Theriogenology 89, 47–57.
Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K., Parnpai, R., 2010. A comparison of cryotop and solid surface vitrification methods for the cryopreservation of in vitro matured bovine oocytes. J. Reprod. Dev. 56, 176–181.
Sun, Q.Y., Wu, G.M., Lai, L., Park, K.W., Cabot, R., Cheong, H.T., Day, B.N., Prather, R.S., Schatten, H., 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction 122, 155–163.
Vaz, F.M., Melegh, B., Bene, J., Cuebas, D., Gage, D.A., Bootsma, A., Vreken, P., van Gennip, A.H., Bieber, L.L., Wanders, R.J., 2002. Analysis of carnitine biosynthesis metabolites in urine by HPLC-electrospray tandem mass spectrometry. Clin. Chem. 48, 826–834.
Veal, E.A., Day, A.M., Morgan, B.A., 2007. Hydrogen peroxide sensing and signaling. Mol. Cell. 26, 1–14.
Wu, C., Rui, R., Dai, J., Zhang, C., Ju, S., Xie, B., Lu, X., Zheng, X., 2006. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol. Reprod. Dev. 73, 1454–1462.
Wu, G.Q., Jia, B.Y., Li, J.J., Fu, X.W., Zhou, G.B., Hou, Y.P., Zhu, S.E., 2011. L-carnitine enhances oocyte maturation and develpoment of parthenogenetic embryos in pig. Theriogenology 76, 785–793.
Xu, H.Y., Yang, X.G., Lu, S.S., Liang, X.W., Lu, Y.Q., Zhang, M., Lu, K.H., 2018. Treatment with acetyl-L-carnitine during in vitro maturation of buffalo oocytes improves oocyte quality and subsequent embryonic development. Theriogenology 118, 80–89.
Xu, H.Y., Geng, S.S., Li, T.T., Fu, Q., Lu, S.S., Liang, X.W., Lu, Y.Q., Zhang, M., Yang, X.G., Lu, K.H., 2019. Maturation of buffalo oocytes in vitro with acetyl-L-carnitine improves cryotolerance due to changes in mitochondrial function and the membrane lipid profile. Reprod. Fertil. Dev. 31, 386–394.
Yamada, T., Imai, H., Yamada, M., 2006. Beneficial effects of acetyl- L-carnitine treatment during IVM on post-fertilization development of bovine oocytes in vitro. Eprod. Fertil. Dev. 18, 280–281.
Yin, F., Sancheti, H., Liu, Z., Cadenas, E., 2015. Mitochondrial function in ageing: coordination with signalling and transcriptional pathways. J. Physiol. https://doi.org/10.1113/JP270541.
Yu, Y., Dumollard, R., Rossbach, A., Lai, F.A., Swann, K., 2010. Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. J. Cell. Physiol. 224, 672–680.
Zare, Z., Masteri Farahani, R., Salehi, M., Piryaei, A., Ghaffari Novin, M., Fadaei Fathabadi, F., Mohammadi, M., Dehghani-Mohammadabadi, M., 2015. Effect of L-carnitine supplementation on maturation and early embryo development of immature mouse oocytes selected by brilliant cresyle blue staining. J. Assist. Reprod. Genet. 32, 635–643.