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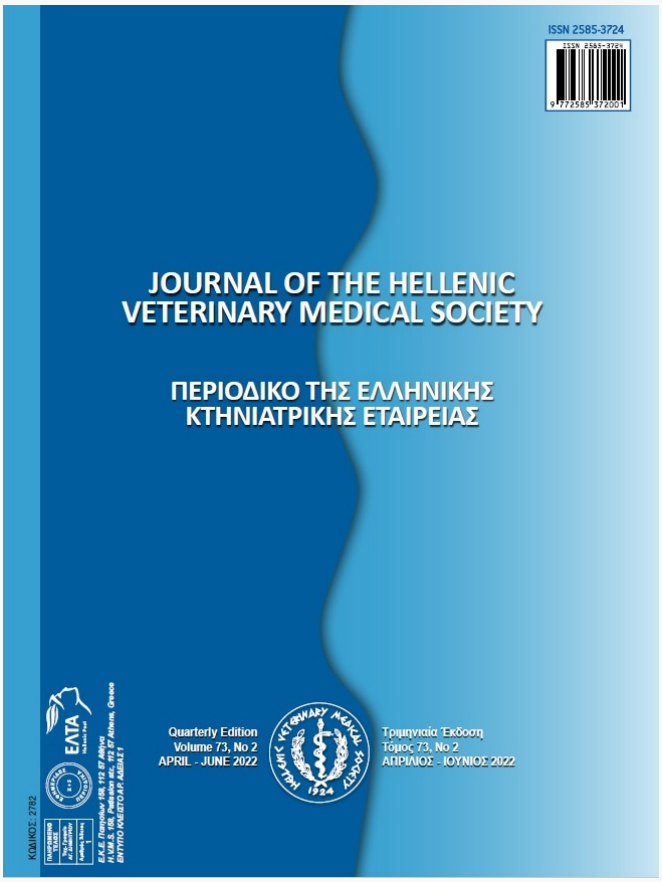

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Research article
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Effects of L-carnitine and different cryodevices on the vitrification and developmental competence of *invitro* fertilized buffalo oocytes

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ABSTRACT: The cryopreservation of immature oocytes from buffalo provides a continuous non-seasonal source of female gametes. The current study compared different cryodevices and L-carnitine (LC) supplementation to the vitrification medium on the viability and developmental competenceofbuffalo oocytes. The immature oocytes were vitrified in TCM119 medium supplemented with 0.0, 0.3, 0.6 or 1.2 mg/ml of LC in either a straw (SD), open pulled-straw (OPS), or solid surface device (SSD). Vitrification in a straw resulted in higher rates of recovery (52.7 %)and viability (85.5 %) of oocytes in medium containing 0.6 mg/ml LC ($P<0.05$). Use of OPS resulted in greater recovery (50.2 %) and viability rates (79.7%)in medium containing 0.3 mg/mlLC ($P<0.05$). There were greater recovery (54.1%) and viability (83.3%)rates for medium containing 0.6 mg/ml LC and SSD ($P<0.05$).Abnormalities of the cytoplasm (all cryodevices) and zona pellucida (SD) were higher when medium contained 1.2 mg/ml LC ($P<0.05$) was used.Furthermore, the maturation rates of oocytes were the greatest when using OPS and 0.3 mg LC (46.9 %, $P<0.05$). The rates of fertilization (60.5%), cleavage (27.9 %)and development of fertilized oocytes to blastocysts (16.3 %)were the greatest when using OPSand vitrification medium with 0.3 mg LC ($P<0.05$). In conclusion,the proper LC concentration differs at different cryodevices.The rates of recovery and viability were the greatest for medium with 0.6 mg/ml LC in either SD or SSD. However, when using OPS the best results were recorded when the medium contained 0.3 mg/ml LC.

Keywords: L Carnitine, Buffalo Oocytes, Straw, Open Pulled-Straw, Solid Surface Device

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INTRODUCTION

Buffalos have a prominent role in the economy in many developing countries including Egypt. They have many advantages in comparison to cattle such as resistance to many diseases, the ability to utilize low quality roughages, and the production of high-quality milk (Choudhary et al., 2016).

Cryopreservation is used to enhance the handling of gametes from buffalo for both research and commercial purposes (Attanasio et al., 2010). Moreover, cryopreservation of oocytes collected from slaughtered animals of high genetic value offers a chance to overcome the loss of valuable germplasm and to enhance genetic improvement of this species (Wani et al., 2004; Moawad et al., 2019).

There are two main techniques to cryopreserve gametes and embryos: controlled slow freezing (Gautam et al., 2008) and vitrification (Yamada et al., 2007; Busardò et al., 2016). The controlled slow freezing method for cryopreservation of oocytes and embryos frequently has disadvantages due to osmotic shock and intracellular ice crystallization which may severely damage the cells (Ledda et al., 2007).

Vitrification is a valuable method for cryopreservation as cells are exposed to high concentrations of cryoprotectants and ultra-rapid cooling ratesprevent formation of icecrystals (Bus et al., 2019; Chang et al., 2019; Gutnisky et al., 2019). Furthermore, vitrification is a simple and fast technique of cryopreservation that does not require expensive equipment (Scholz and Navas 2014; Zolini et al., 2019; Gutnisky et al., 2019).

The high lipid content in buffalo oocytes makes them sensitive to oxidative damage (Liebermann 2002; Asgari et al., 2012; Schiewe et al., 2017). Moreover, vitrification of oocytes decreases the antioxidant glutathione while increases amounts of hydrogen peroxide (H_2O_2) (Kelly et al., 2006; Schiewe et al., 2017). Therefore, the increase in the amount of intracellular lipids in oocytes reduces their rate of survival and embryo quality after cryopreservation (Romek et al., 2009). As physical changes to lipids leads to cryoinjury, large intracellular lipid droplets resolve into small lipid droplets that alter the morphology of oocytes during cryopreservation (Romek et al., 2009).

The mechanical removal of lipid droplets (Nagashima et al., 1995; Bartolac et al., 2018) or the decreasing of their abundance by using chemical agents (Men et al., 2006) are promising approaches to improve the tolerance of oocytes to cryopreservation. L-carnitine (LC) enhances lipid metabolism in cells of animals and is very important in the transport of fatty acids from the cytosol to the mitochondria to undergo beta-oxidation (Men et al., 2006). Supplementation of embryo culture medium with L-carnitine reduces lipid content in bovine embryos and increases tolerance of bovine oocytes to cryopreservation (Chankitisakul et al., 2013). L-carnitine also has antioxidant activity that protects cells from DNA damage (Jiang et al., 2012; Lahneche et al., 2019). The double role of LC as an antioxidant and an essential component of lipid metabolism makes it a promising agent for improving oocyte cryopreservation efficacy and subsequent embryonic development (El-Shalofy et al., 2017; Moawad et al., 2019).

The present study evaluated the effects of different concentrations of L-carnitine (LC) in vitrification medium and different cryo-devices on the efficacy of oocyte vitrification and warming by evaluating effects on rates of recovery,viability, morphological abnormalities, and developmental competence.

MATERIAL AND METHODS

The present study was carried out in the Theriogenology Department, Faculty of Veterinary Medicine, Mansoura University, Egypt, in collaboration with the Department of Artificial Insemination and Embryo Transfer, Animal Reproduction Research Institute (ARRI), Al-Haram, Giza between January and December 2017. The chemicals used in this study were purchased from Sigma Chemical Co (St. Louis, MO, USA). All experimental procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, 35516 Mansoura, Egypt.

Recovery and classification of immature oocytes

Ovaries from apparently normal reproductive organs were collected from buffalo heifers and adults of unknown breeding history within 30 min following slaughter and evisceration in the EL-sharkawy abattoir as described previously (Mostagir et al., 2019). Briefly, the ovaries were kept in a thermos flask containing warm normal saline during transport to the laboratory within 2-3 h after slaughter. In the laboratory, ovaries were washed in fresh, sterile physiological saline to further remove any contaminants and were dried with sterile paper towels.Follicles (2 to 8 mm) were aspirated by the use of an 18-gauge needle attached to a

10 ml syringe. The oocytes were pooled in a 15 ml falcon tube and allowed 10-15 min to settle to the bottom of the tube. Then, about 5ml of the medium into which the oocytes settled was aspirated and placed into a sterile 100 mm polystyrene petri dish. The cumulus-oocyte complexes (COCs) were recovered and selected by using a stereomicroscope (Nikon, Tokyo, Japan).The collected COCs were transfered into another dish containing fresh pre-warmed washing medium. The oocytes were then classified into one of three grades based on their morphological appearance as previously described by Sadeesh et al., (2016).

Grade aoocyteshad more than 4 layers of cumulus cells and homogenous cytoplasm. Grade boocytes hadcomplete or partial (2-4) layers of cumulus cells and homogenous cytoplasm. Grade c oocytes were denuded with non-homogeneous cytoplasm. Non-spherical, vacuolated, or fragmented oocytes were classified as degenerated. Good quality COCs were those having homogenous cytoplasm and dense compact cumulus cells. Grades a and b oocytes were used in this study. The effects of LC supplementation in vitrification medium and different cryodevices on developmental competence of the immature buffalo ooctes were checked in three replicates (Figure 1).

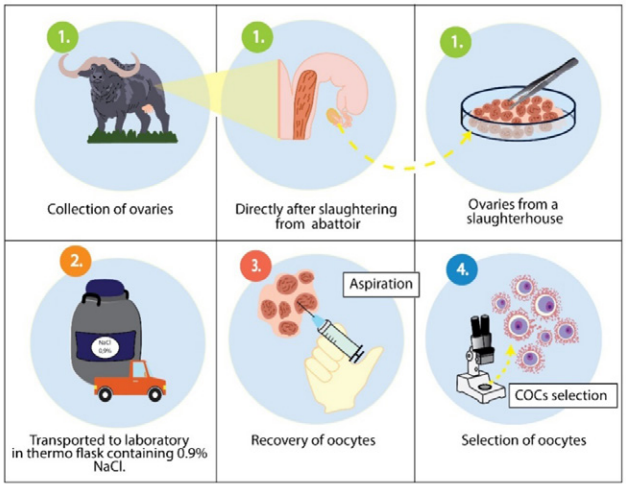

Figure 1: recovery and classification of immature buffalo oocytes

Vitrification of immature oocytes

Equilibration and vitrification of selected oocytes were done as described by El-Shalofy et al., (2017). In brief, equilibration and vitrification solutions were prepared using TCM 119 medium supplemented with 10% fetal calf serum (FCS) as the basic medium. Five COCs were placed in 50µl drops of basic medium (TCM119) for 1 min and then transferred to an equilibrated solution [100µl drops of TCM 119containing 10% EG (etylene glucol) and 10% dimethylsulfoxide (DMSO)] for 10 min. Then equilibrated COCs were transferred to the vitrification solution (100µl drops of TCM 119 containing 20%EG and 20% DMSO), as well as L-carnitine (LC) at 0.0, 0.3, 0.6 or 1.2 mg/ml for 1 min (Phongnimitr et al., 2013). The cryo-devices used for the vitrification process were: i) Straws (SD): five to ten oocytes were immediately loaded in 0.25-ml mini-straws in the middle column of the vitrifying solution separated by air bubbles and sealed with polyvinyl alcohol powder. The straws were precooled by keeping them in liquid nitrogen vapor at a height of about 5 cm from the surface of the liquid nitrogen level (LN_2) to avoid cracking of straws for 1 min, following which they were dipped vertically in LN_2. ii) Open pulled straws (OPS): Oocytes were immediately loaded in open pulled straw (OPS) as described previously (Vajta et al., 1998a; Attanasio et al., 2010) with slight modifications. Briefly, 0.25 ml semen straws were modified by removing the cotton plug and heating them over a hot plate to approximately 100ºC for 10-15 seconds and then stretching them manually until the inner diameter was approximately one-half the original diameter. The straws were cooled in the air for 10 sec before being cut in the middle using a razor plate. Subsequently, oocytes of each group were loaded in OPSs by capillary action and pre-cooled by keeping them in liquid nitrogen vapor at a height of about 5 cm from the surface of the LN_2 for 1 min, followed by dipping vertically into the LN_2 tank. iii) Solid surface device (SSD) for cryopreservation was as described by Gupta et al., (2007). In brief, oocytes from a group of 5 COCs with vitrification solution were aspirated into a glass pipette and the solution containing the COCs were put into a dry surface of a hollow metal cube pre-cooled by immersion in LN_2. The pellets of pre-cooled oocytes were placed into cryotubes and immersed into liquid nitrogen.

Warming

Straw: after a period of storage, the vitrified oocytes in straws were thawed in a water bath at 37°C for 1 min before the content of each straw was expelled into a tissue culture dish containing 1, 0.5, 0.25 M sucrose solutions, respectively and held for 3 min in each concentration of sucrose for stepwise rehydration of oocytes. Finally, the COCs were incubated in basic medium (TCM119) for 3min at room temperature.

Open pulled straw: the loaded ends were sub-

merged into 1ml sucrose for 3 min at 37°C; then moved to decreasing concentrations of sucrose solution as described for straws and finally to the TCM 119 for 3min at room temperature.

Solid surface device: the vitrified droplets were warmed by putting them into 1ml of sucrose for 3min at 37°C and then in decreasing concentrations of sucrose as noted previously.

Evaluation of vitrified/warmed immature oocytes quality

Oocyte recovery rate and morphological assessment

The recovery rate was defined as the number of oocytes counted after the end of rehydration compared to the total number of oocytes vitrified (Moawad et al., 2019). Vitrified /warmed oocytes were examined under a stereomicroscope for evaluation of their morphological characteristics as reported by Sharma et al., (2010). The oocytes with a spherical and symmetrical shape and no signs of lyses/degeneration were considered normal, whereas oocytes with ruptured zona pellucida, fragmented cytoplasm, or degenerative signs were classified as abnormal and discarded (Figure 2)

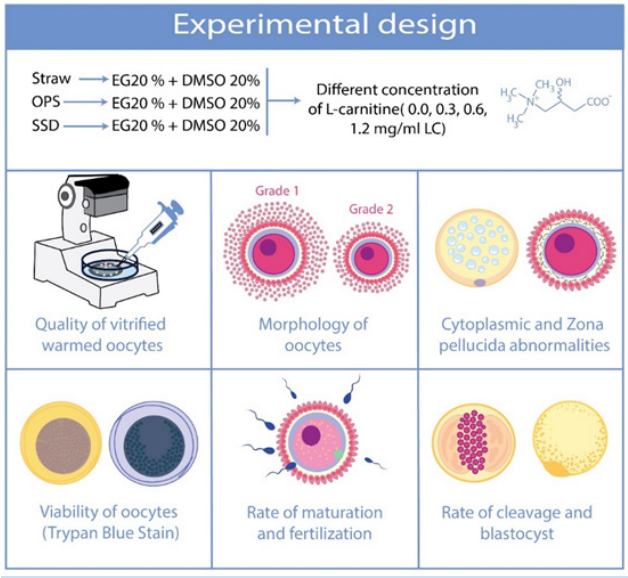

Figure 2: Experimental design of the present study (first panel, different treatments). Evaluation of vitrified/warmed immature oocytes quality (second panel, left); oocytes morphology (second panel, middle), cytoplasmic and zona pellucida abnormalities (second panel, right); viability of oocytes (third panel, left); the maturation and fertilization rates (third panel, middle), and cleavage and blastocyst (third panel, right)

Evaluation of oocytes' viability

The trypan blue exclusion test was used for the evaluation of oocytes' viability.Trypan blue stain is a useful and quick method to assess the initial quality and viability of oocytes, because it provides an assessment of cell membrane integrity, as those cells with damaged or non-intact cell membranes permit the passage of the trypan blue toward the nucleus of the oocyte (Fig. 2). Trypan blue solutions (0.05%) were prepared by dissolving trypan blue in phosphate buffer saline (PBS; pH = 7.0). Oocyte staining was performed at room temperature for 2 min (Mostagir et al., 2019; Moawad et al., 2019).Oocytes (n= 806) were categorized based on the degree of dye exclusion. Unstained oocytes were classified as live and fully stained oocytes were considered dead (Moawad et al., 2019).

In vitro maturation and fertilization

Oocyte maturation:The vitrified-warmed good quality COCs (homogenous oocyte cytoplasm and dense compact cumulus cells, n= 173) were rinsed three times in maturation medium and 10- 15 oocytes were transferred to a 100 µl drop of maturation medium under mineral oil and placed in an incubator for 20-22 hat 38.5°C and an atmosphere of 5% CO_2 in the air with maximum humidity.

Assessment of maturation rate: Maturation rates were determined under a stereomicroscope by assessing the expansion of cumulus cells. Oocytes with complete or moderate cumulus expansion and oocytes with slight or no cumulus cell expansion, but with the first polar body extruded into the perivitelline space, were considered mature (Fig. 2, Mostagir et al., 2019).

Semen preparation and oocyte fertilization: First, the fertilization dish was prepared by adding 50 µl drops of modified Tyrode's medium (TALP) into a sterile disposable petri dish and then covered with mineral oil and incubated at 38.5°C with an atmosphere of 5% CO_2 in air with maximum humidity. Mature oocytes were subjected to repeated gentle pipetting to remove surrounding cumulus cell to allow easy penetration of the sperm and washed three times in TALP medium before adding to the fertilization drops (10 oocytes/drop).

Spermatozoa were capacitated in vitro in the TALP medium. Three straws of frozen semen with known concentrations of sperm (2×10^6 sperm/ml) were

thawed for 1 min in a 37°C water bath. Immediately after thawing, the most motile spermatozoa were separated in sperm-TALP medium containing 6 mg/ml BSA for 1 hby the swim-up technique (Mostagir et al., 2019). Then, the uppermost layer of the medium containing the most motile spermatozoa were collected using a plastic Pasteur pipette and transferred into small test tubes containing 3 ml of SP-TALP medium. The freezing medium was washed out by centrifugation at 2,000 rounds per minute (rpm) for 5 min to form sperm pellets. The supernatant was discarded and 1 ml of TALP added and mixed with the pellet before being centrifuged again and the supernatant was discarded again to completely remove the freezing medium. Next, 1 ml TALP medium containing 10 mg/ml heparin (for in vitro capacitation of sperm) was added, mixed with the sperm pellet and incubated at 38.5°C in an atmosphere of 5 % of CO_2in air with maximum humidity for 10 min before being evaluated for motility. If motility was acceptable (60-80%), 20 µl of semen was co-incubated with each fertilization drop for 18 h.

Invitro culture and embryo development

The fertilization and embryo culture was carried out as described previously (Chung et al., 2007; Räty et al., 2011). The culture dish was prepared by adding 50µl drops of culture medium (synthetic oviduct fluid - SOF) into a sterile disposable petri dish, covered with mineral oil and incubated at 38.5°C in an atmosphere of 5% CO_2 in air with maximum humidity. The oocytes were freed from loosely bound spermatozoa and any remaining attached cumulus cells by gentle pipetting. The culture medium was replaced every 48 h with fresh medium until day 7 post-incubation to prevent accumulation of ammonium resulting from amino acid degradation and removal of oocytes that had not cleaved. During the culture period, the gentle shaking of the culture dish was done to allow a uniform environment among fertilized oocytes.The percentage of cleaved oocytes was recorded 48h post-insemination and those that developed to the blastocyst stage were recorded at 5 and 7-days post-insemination, respectively.

Assessment of fertilization and embryonic development

The assessment of fertilization and embryonic development in the present study was carried out as described previously (Chung et al., 2007; Räty et al., 2011). The presumptive zygotes (n= 79) were denuded from surrounding cumulus cells and mounted on slides with coverslips at 18 h post-insemination. Subsequently, they fixed in acetic acid/ethanol (1:3) solution for at least 24 h. The presumptive zygotes were stained with 1% orcein dissolved in 45% acetic acid solution and examined for evidence of fertilization based on the presence of decondensed sperm heads or male pronuclei with their accompanying sperm tails in the cytoplasm. Oocytes with two pronuclei and a clear second polar body were considered to have been fertilized normally. The percentage of cleaved oocytes was assessed at 48 h post-insemination (Fig. 2; Chung et al., 2007).

Statistical analysis

The normality of quantitative parameters was assessed using normal probability plots and the Kolmogorov-Smirnov test generated with the UNIVARIATE procedure of SAS (Elmetwally, 2012; Gohar et al., 2018; Elmetwally et al., 2018). The experimental data are expressed as means ± SEM (standard error of the mean). To determine the effect of cryodevices as will as the different concentrations of L-carnintine on the vitrification and developmental competence of *in vitro* fertilized buffalo oocytes, a one-way analysis of variance (ANOVA) was used followed by Duncan's multiple comparison test. The rates of recovery, viability, abnormalities of cytoplasm and abnormlities of zona pellucida are expressed as percentages. Statistical analyses were done using SAS® (version 9.2, SAS Institute, Cary, NC, USA). Differences were considered to be significant at $P \leq 0.05$.

RESULTS

The recovery rate, the vibility rate and certain abonormalities rates (zona pellucida and cytoplasmic) of buffalo oocytes vitrified by using different cryodevices and different L-carnitine treatments

The recovery rate, the rate of normal and abnormal (zona pellucida and cytoplasmic abnormalities) buffalo oocytes after vitrification in straws (SD), open pulled straws (OPS) and solid surface devices (SSD) with different concentrations of L-carnitine (0.0, 0.3, 0.6 and 1.2 mg/ml) are presented in Table 1. The highest recovery ratewas recorded when 0.6 mg LCwas used at straw and solid surface devicegroups and when 0.3 mg/ml LC was used at OPS group.

The greatest viability rates of oocytes were observed when using 0.6 mg/ml LC at SD and SSD groups and 0.3 mg LC at OPS group.Viability rates

Table 1: Recovery and Viability of vitrified buffalo oocytes, as well as abnormalities in their cytoplasm and zona pellucida following different treatments with L carnitine and various cryodevices

LC concentration (mg/ml)	SD (straw)	OPS (open pulled straw)	SSD (solid surface device)
oocytes recovery rate % (n/total n)			
controls	35.2 (61/173)$^{b, \beta}$	31.4 (52/166)$^{b, \beta}$	40.8 (53/130)$^{b, \alpha}$
0.3	43.7 (86/197)$^{ab, \beta}$	50.2 (74/147)$^{a, \alpha}$	48.6 (82/169)$^{ba, \alpha}$
0.6	52.7 (95/181)$^{a,\alpha}$	39.7 (51/128)$^{b,\beta}$	54.1 (99/183)$^{a, \alpha}$
1.2	31.6 (48/152)$^{b, \alpha}$	26.8 (42/155)$^{bc, \beta}$	32.1 (63/196)$^{c, \alpha}$
Morphologically normal oocytes % (n/total n)			
controls	71.1 (43/61)$^{b, \alpha}$	57.6 (30/52)$^{b, \beta}$	62.3 (33/53)$^{c, \beta}$
0.3	70.1 (72/86)$^{b, \beta}$	79.7 (59/74)$^{a, \alpha}$	77.8 (64/82)$^{b, \alpha}$
0.6	85.5 (81/95)$^{a, \alpha}$	61.1 (31/51)$^{b, \beta}$	83.3 (84/99)$^{a, \alpha}$
1.2	70.8 (34/48)$^{b, \alpha}$	41.1 (17/42)$^{c, \beta}$	66.9 (42/63)$^{c, \alpha}$
Zona Pellucida abnormalities % (n/total n)			
Controls	27.6 (48/173)$^{ba, \alpha}$	25.8 (43/166)$^{a, \alpha}$	23.9 (31/130)$^{a, \beta}$
0.3	24 (47/197)$^{b, \alpha}$	19.1 (28/147)$^{b, \beta}$	20.7 (35/169)$^{b, \alpha}$
0.6	21.5 (39/181)^{bc}	18.8 (24/128)^{b}	18 (33/183)^{b}
1.2	29.8 (45/152)$^{a, \alpha}$	25.2 (39/155)$^{a, \alpha}$	22.4 (44/196)$^{a, \beta}$
Cytoplasmic abnormalities % (n/total n)			
controls	34 (59/173)^{a}	30.7 (51/166)^{c}	36.2 (47/130)^{b}
0.3	32.2 (64/197)^{ab}	30.7 (45/147)^{c}	30.8 (52/169)^{c}
0.6	25.8 (47/181)$^{b, \beta}$	41.3 (53/128)$^{b, \alpha}$	27.9 (51/183)$^{c, \beta}$
1.2	38.6 (59/152)$^{a, \gamma}$	84.2 (75/155)$^{a, \alpha}$	45.4 (89/196)$^{a, \beta}$

^{a,b,c} significant differences in the same column, $^{\alpha,\beta,\gamma}$ significant differences in the same row

were also high when 0.3 mg/ml was used at SSD groups. The viability rates were similar when using 0.3 and 1.2 mg/ml L-carnitine when compared with control for SD group. The percentage of zona pellucida abnormalities for oocytes after vitrification and warming at all cryo-device groups was higher in1.2 mg/ml LC concentration and in controls. The highest percentage of cytoplasmic abnormalities was recorded when 1.2 mg/ml LC was used at all cryo-devices groups. The cytoplasmic abnormalities were the highest (84.2%) at OPS when 1.2 mg/ml LC was used (Table 1).Furthermore, the rates of cytoplasmic abnormalities were also high in controls at SD and SSD.

Effects of LC on maturation, fertilization, cleavage, and blastocyst rates of vitrified warmed buffalo oocytes using different cryodevices

The maturation rate of oocytes was greater when using 0.3 mg/ml LC at OPS group (46.9%, OPS, $P<0.05$) than control at the same cryo-device group (30.4%). However, there was no other significant effect of LC concentration or cryo-device on maturation rate.It is important to notice that other groups had also increased maturation rates (0.6 mg/ml LC at SD group: 37.1% and 0.6 mg/ml LC at SSD group:41.9 %). The greatest fertilization rates ($P<0.05$)was observed when 0.3 mg/ml LCand OPS was used (60.5%) compared to controls of the same group (38.2%), to 0.6 mg/ml LC at SD group (31.7%) and at SSD group (48.7%). The cleavage rate was the greatest ($P<0.05$) for both 0.3 mg/ml LCat OPS group (27.9%) and 0.6 mg/ml LC at the SSD group (30.8%) compared to controls (14.45%; $P<0.05$) and 0.6 mg/ml at SSD group (17.1%).

The rate of blastocyst development was the greatest in 0.3 mg/mlLC at OPS group (16.3%) compared to control group (3.7%: $P<0.05$) and 0.6 mg/ml in SD group (7.3%).Good blastocyst rate was also recorded when 0.6 mg/ml LC was used at SSD group (12.82%) compared to controls.

DISCUSSION

Cryopreservation of certain mammalian embryos is now a common procedure;however, there are substantial differences of the efficiency of this method depending on the stage, species, and origin of cells produced either in vivo or in vitro. Factors associated with variations in those differences include the number of intracellular lipid droplets and the different microtubular structures, chilling injury, and volume/surface ratio, all of which play a crucial role in penetration of the cryoprotectant (Vajta et al., 1998b).

The recovery rate of buffalo oocytes after vitri-

fication and warming by using straws and the solid surface device was increased in medium with 0.6 mg/ml LC compared with the absence of LC and 0.3 or 1.2 mg/ml LC; whereas the recovery rate of oocytes when using OPS was greater in 0.3 mg/ml LC group compared with control, 0.6 and 1.2 mg/ml LC. The significant effect of LC could be due to the amelioration of detrimental effects of vitrification on mitochondrial function, which causes ATP loss and reduces the competence of vitrified buffalo oocytes (Zhao et al., 2011b; Zhao et al., 2011a). LC supplementation improves ATP production in mouse cells (Zare et al., 2017; Zolini et al., 2019). The metabolism of lipids in rats and bovine cells is promoted by L-carnitine which is considered a beta-oxidation factor via fatty acids transport from the cytosol to the mitochondria (Kerner and Hoppel 2000; Chankitisakul et al., 2013). Cryoprotection devices play a crucial role in the success of *in vitro* fertilization procedures. Additionally, the extent of ice crystals contributes to the success of cryobiology processes. In the present study, we found a significant increase in viability rate of oocytes when using 0.6 mg/ml (SD, SSD) or 0.3 mg/ml (OPS) LC compared with control and 1.2 mg/ml LC concentrations. As concern OPS, lower concentration of LC was enough because of the minimum surface area in OPS, which reduces the volume of vitrification solution and allows direct contact between oocytes and liquid nitrogen compared to those parameters when using SD (Vajta et al., 1998b; Attanasio et al., 2010), because the surface area of straw and solid surface device are large; thus, this theoretically needs more L-carnitine concentration to have a potential antioxidant effect. Smaller drop size and a rapid cooling rate improve vitrification success (Arav and Zeron 1997; Arav and Natan 2019). Previous studies on the viability rate of oocytes in buffalo (Sharma et al., 2010; Choudhary et al., 2016), pigs (Cuello et al., 2010), and mice (Zhou et al., 2007) indicated better results when OPS was used.

Oocytes cryopreservation-induced cryodamage causes morphological and functional damage to oocytes and results in poor development. Oocyte cryopreservation is frequently associated with alterations of the zona pellucida and cytoplasm of oocytes after freezing and thawing (Huang et al., 2008; Son et al., 2019).In the present study, we found a higher rate of zona pellucida and cytoplasmic abnormalities to be associated with the highest concentration of LC (1.2 mg) and all cryodevices used. The concentration of 0.6 mg/ml L carnitine showed the lowest rate of zona pellucida abnormalities when using SD, OPSor SSD; furthermore the concentrations of 0.3 and 0.6 mg/ml when using OPS were also related to low rate of zona pellucida abnormalities, probably because the diameter of OPS is smaller than SD and SSD. Accordingly, the L-carnitine concentrations could inversely related to the surface area. At the same time, the higher zona pellucida abnormatities when using 1.2 mg/ml L-carnitine, may be due to reduced lipid density that is unfavourable for the development competence of oocytes. Consistent with this result, Sprícigo et al., (2017) reported that lower concentrations of LC were associated with a significant improvement of vitrification of immature bovine oocytes. Similarly, excessive concentrations of LC may reduce lipid density that is unfavorable to *in vitro* development of bovine embryos (Sutton-McDowall et al., 2012). Therefore, in the current study, we investigated various concentrations of LC to determine the effects on zona pellucida abnormalities and found that 1.2 mg/ml LC was detrimental when compared to 0.3 and 0.6 mg/ml L-carnitine.

In the present study, the use of low doses of L-carnitine improved recovery and survival rates of oocytes and minimized abnormalities in zona pellucida and cytoplasm compared to the absence of LC. However, there is a different threshold that LC affects positively oocyte quality according to the cryo-deviceused. L-carnitine stimulates lipid metabolism in animal cells because of its role in the transport of fatty acids from the cytosol to the mitochondria for beta-oxidation (Kerner and Hoppel, 2000). Consequently, L-carnitine improves ATP production in animal cells (Vanella et al., 2000)**.** Similar results were obtained after adding LC to the maturation medium before vitrification of in vitro-matured calf oocytes (Sprícigo et al., 2017).

The success of oocytes vitrification is determined by the developmental competence including *invitro* maturation and fertilization rates,as well as clevage andblastocysts formation rates. Results of the present study demonstrated that the maturation rate of oocytes was significantly increased when 0.3 mg/ml LC and OPS were used. This may be attributed to the damage of certain cytoplasmic components when no LC added in vitrification medium and the maturation rate and developmental competence of vitrified oocytes was decreased compared to low doses of LC. Ruppert-Lingham et al., (2003) reported that vitrification of germinal viscule oocytes causes damage to the

surrounding cumulus cells, which consequently disrupts their communication with enclosed oocytesthat is very important for the successful completion of *in-vitro* maturation and subsequent embryonic development (Mostagir et al., 2019). Thus, supplementation of the basic vitrification medium with LC may avoid that damage (El-Shalofy et al., 2017). The oxidative activity of mitochondria is decreased mildly by vitrification, but increased by adding LC to vitrification medium in mice (Moawad et al., 2019). Furthemore, it is important to notice that according to our results, there is different threshold of LC for different cryo-devices.

There are several reports about lower maturation rates of immature oocytes following cryopreservation by using basic media alone in human (Khalili et al., 2017; Hatırnaz et al., 2018; Son et al., 2019), mouse (Yeo et al., 2008), cow (Isachenko et al., 2001) and ewe (Peng et al., 2013) oocytes. In the present study, the use of OPS to vitrify immature buffalo oocytes improved the maturation rate after warming, perhaps because the tip of the OPS has a small diameter and thin wall to enhance cryopreservation efficiency. Oocytes held in OPS with a very small volume of vitrification solution undergo a faster rate of cooling and warming (a theoretical rate of 20, 000°C/min) than those in conventional straws (2,500°C/min) (Vajta et al., 1998b). Moreover, oocytes in vitrification medium (1-2 μl) in OPS are warmed directly during dilution,quickly expelled (within 1 sec) and immediately diluted. This reduces exposure to inappropriate temperatures. OPS decreases chilling injury, and toxic and osmotic damage of oocytes (Vajta et al., 1998b).

CONCLUSION

In conclusion, it seems that different LC concentrations are possitivelly affect oocyte quality after vitrification at different cryodevices. In the present study, the vitrification of buffalo oocytes could be improved by using OPS and 0.3 mg LC in combination with EG and DMSO as cryoprotectants. Further research is needed to understand the mechanism by which different LC concentrations act at different cryopreservation procedures.

CONFLICT OF INTEREST

None to declare.

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