Original Article

The effect of L-carnitine on Oocyte Mitochondrial Activity after Cryopreservation

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Abstract

Background: Mitochondria are cellular organelles required for energy production, vital to reproduction, especially oocyte maturation and fertilization. It has been seen that oocyte cryopreservation (OC) can cause mitochondria damage, aggregation of lipid droplets near mitochondria and endoplasmic reticulum, and cryoinjury. In recent studies use of antioxidants such as L- carnitine can increase the number of active mitochondria and decrease intracellular ROS levels. The present study aimed to determine the beneficial effect of L –carnitine on oocyte mitochondrial activity after vitrification.

Materials and Methods: In the present experimental study, 6-8 weeks of female NMRI mice were taken from the Royan Institute of Iran and stimulated with 7.5 IU Pregnant Mare Serum Gonadotrophin (PMSG) and 10 IU of human chorionic gonadotropin (HCG) after 48 hours was injected. After stimulation, oocytes were collected, and MII oocytes were selected. A two-step vitrification procedure was done, and 0.6mg/ml of L –carnitine was added to both ES and VS mediums. After two weeks, oocyte thawing was performed, intracellular GSH level was also measured mitochondrial membrane potential was measured. Captured images were analyzed by J software (Version 1.40; and obtained data were analyzed using SPSS Ver.20.

Results: Average difference in intracytoplasmic GSH level in the study group was significantly higher than the control group ($P<0.001$). So, L –carnitine could successfully increase the oocyte intracytoplasmic GSH level. Also, it has been seen that the LC supplement could successfully grow oocyte mitochondrial function and subsequent mitochondrial membrane potentials($P<0.001$).

Conclusion: Adding LC to the cryopreservation media could increase mitochondrial activity, GSH level, and mitochondrial membrane potentials. Adding LC to the cryopreservation could enjoy the beneficial effect of L –carnitine on oocyte mitochondrial activity after vitrification and minimize mitochondrial damage and boost oocyte quality which can lead to successful fertilization and embryo growth.

Keywords: L-carnitine, Cryopreservation, Oocyte, Mitochondria

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Introduction

Mitochondria are cellular organelles known as the cell's powerhouse and have an essential role in cell metabolism, cell differentiation, calcium homeostasis, fatty acid oxidation, and apoptosis[1]. Also, this double-membrane-bounvital to reproduction science oocyte mitochondria are responsible for oocyte maturation,

regulation of calcium oscillation during fertilization, and production of ATP during preimplantation. Therefore, oocyte mitochondrial dysfunction can adversely impair reproductive processes[2, 3]. Besides, nowadays, advances in Assisted Reproductive Technique (ART), especially in cryopreservation, and improvements in vitrification technology, oocyte cryopreservation (OC) had organelle is d od dramatically. Although oocyte cryopreservation enjoys clinical benefits of achieving the desired pregnancy rate and could be the best option for those encountering fertility-threatening situations such as therapy for cancer or rheumatologic disease, premature ovarian insufficiency, or need for ovarian surgery as a measure to preserve fertility, recent studies show that oocyte freezing can cause morphological changes, functional damage, aggregation of lipid droplet near to mitochondria and endoplasmic reticulum and cryoinjury[4, 5]. Moreover, irreversible damage to oocyte cortical mitochondria, J-aggregate formation elimination, and mitochondrial membrane potential changes have been associated with oocyte cryopreservation[5]. Therefore, doing considerable research to boost oocyte freezing media and maybe use antioxidants that can minimize the production of Reactive Oxygen Species (ROS) could reduce damage to mitochondria. One of the antioxidants that can be used for this purpose is L –carnitine science. Previous studies have observed that this antioxidant can increase the number of active mitochondria, decrease intracellular ROS levels, and improve in vitro embryo development[6, 7]. Hence, using L –carnitine besides cryopreservation techniques may eliminate and reduce the destructive effect of freezing on oocyte mitochondria. The present study aimed to determine the beneficial effect of L –carnitine on oocyte mitochondrial activity after vitrification.

Methods

The Ethics Committee approved this experimental study at Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1394.334), Tehran, Iran.

Animal Care and oocyte collection: For oocyte collection, 6-8 weeks female NMRI mice were taken from the Royan Institute of Iran and treated with favorable and consistent conditions. After adapting the mice to laboratory conditions, the research was started. To stimulate superovulation, 7.5 IU of pregnant mare serum gonadotrophin (PMSG) was administered intraperitoneally, and 10 IU of human chorionic gonadotropin (HCG) after 48 hours was injected. After 14 hours, mice were sacrificed, and Cumulus complex was obtained from the oviductal ampulla in sterile condition and placed in HTCM media containing 5mg/ml Streptomycin, 6mg Penicillin, and 10% FBS. Then Cumulus cells were immediately removed from the oocyte by putting them in media containing hyaluronidase and, after this, by mouth pipetting. Metaphase II (MII) oocytes were selected, and those with normal morphology, regular contours, and light coloration were included in the study. Until vitrification time, selected oocytes were kept in K+ modified simplex optimized medium (KSOM) at 37˚C with 5% CO2. Totally, 28 oocytes were obtained and randomly divided into two groups study and control.

Vitrification of mouse oocytes: For oocyte vitrification, a two-step vitrification procedure was utilized using KITAZATO Vitrification KIT (Kitazato Biopharmaceuticals, Japan). Also, as carrier and vitrification, the crypto (Kitazato) was used. So, based on kit protocol and using a procedure reported by Kuwayama, the vitrification was performed[8]. First of all, equilibration solution (ES) consisting of 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) dimethylsulfoxide (DMSO) was used, and oocytes in the vitrification group were pretreated for 9 minutes by ES Media. Then vitrification solution (VS) consisting of 15 % (v/v) EG, 15 % (v/v) DMSO, and 0.5 M sucrose was used to survey the antioxidant effect of L –carnitine on mitochondrial membrane potential; according to a previous study, 0.6 mg/ml of L –carnitine[7] was added to both ES and VS mediums. Oocytes were washed in VS medium at least three times for 60 seconds. With minimal VS concentration (<1 µL), 4 to 6 oocytes were transferred to crypto and immediately immersed in liquid nitrogen.

Thawing of oocytes: After two weeks of keeping the oocyte in liquid nitrogen, the thawing process of the vitrified oocyte was performed. For this purpose, four-step dilution procedure (Kitazato Biopharmaceuticals, Japan) were used. First, the protective cap was removed, and a warming solution (0.5 M sucrose at

37˚C) was used for 1 minute that crypto was containing the oocytes and immersed. In the second step, diluent solutions were used, and oocytes were placed in 0.25 M sucrose for 3 minutes and 0.125 M sucrose for 5 minutes. In the third step, the oocytes for 1 minute were immersed in sucrose-free washing media. Finally, after the warming procedure, Oocytes were transferred into KSOM medium and then incubated at 37˚C with 5% CO2 for at least 1 hour. Eventually, oocytes were prepared for intracellular measurement of GSH Levels and evaluation of mitochondrial membrane potential.

Intracellular Measurement of GSH Levels: To survey intracellular measurement of GSH levels after warming, oocytes were washed in PBS/PVA droplets (1mg PVA/1mlPVS) several times. Then oocytes were immersed in PBS/PVA droplets containing ten μM H2DCFDA or ten μM Cell Tracker Blue in the dark for 30 minutes at 37°C with 5% CO2. Again oocytes were washed in PBS/PVA droplets and were placed in 20 μL αMEM drops under mineral oil. Then the intracellular measurement of the GSH level of MII oocyte was investigated with blue color under a fluorescence microscope with UV filters (485nm filters are green fluorescence, and 590nm are represented in red). The images were recorded in TIFF format, and the data of each oocyte was quantified with Image J software (Version 1.40; National Institutes of Health, Bethesda, MD). The tests were repeated three times, and all the steps were done in the dark.

Evaluation of Mitochondrial Membrane Potential: To obtain oocyte mitochondrial membrane potential, tetrachloro-1,1′,3,3′- tetraethylbenzamideazolyl-carbocyanine iodide (JC-1, Invitrogen) was used. For this purpose, 10 μg JC-1 dye was dissolved in 1 ml DMSO and kept at a temperature of 20°C. Then One microlitre of the above solution was added to 100 microliters of TCM medium, and droplets were performed for staining. In the next step, all oocytes were placed in droplets containing color for 10 minutes in a 37°C incubator and 5% CO2. Afterward, a fluorescence microscope with UV filters (485nm filters are green fluorescence and 590nm are represented in red) was surveyed, and images were captured in TIFF format. Captured images were analyzed by J software (Version 1.40; National Institutes of Health, Bethesda, MD). Finally, the red-green fluorescence ratio was then evaluated in the images. When the mitochondrial membrane potential is high, JC-1 monomers accumulate and form aggregates called J-aggregates that are detected by red fluorescence. In contrast, when the potential of the mitochondria membrane is low, JC-1 is a monomer that is seen with green fluorescence. In each group, 13 oocytes were examined in three replicates. All the staining steps were done in the dark.

Statistical analysis: Statistical analysis was done using SPSS Ver.20 (SPSS, Chicago, IL, USA). First, the normal distribution of obtained data was measured using the Kolmogorov Smirnov nonparametric test. Given the result of this test distribution of green color the intensity was (P=0.95), the distribution of red color intensity was (p=0.88), and the distribution of GSH Level was (P=0.53), following a normal distribution. Therefore, to analyze mentioned data T-test was used. The mean and standard deviation of the intensity of the green and red colors and GSH levels in both groups (With and without an encounter with L –carnitine) are shown in Table 1.

Results

GSH Levels in the mouse oocytes cytoplasm: As shown in the figure1 average difference in intracytoplasmic GSH level in the study group was significantly higher than in the control group (P<0.001). This data indicates that L –carnitine could successfully increase oocyte intracytoplasmic GSH levels in the present study and, therefore, high intracellular GSH levels after adding L –carnitine to vitrification media.

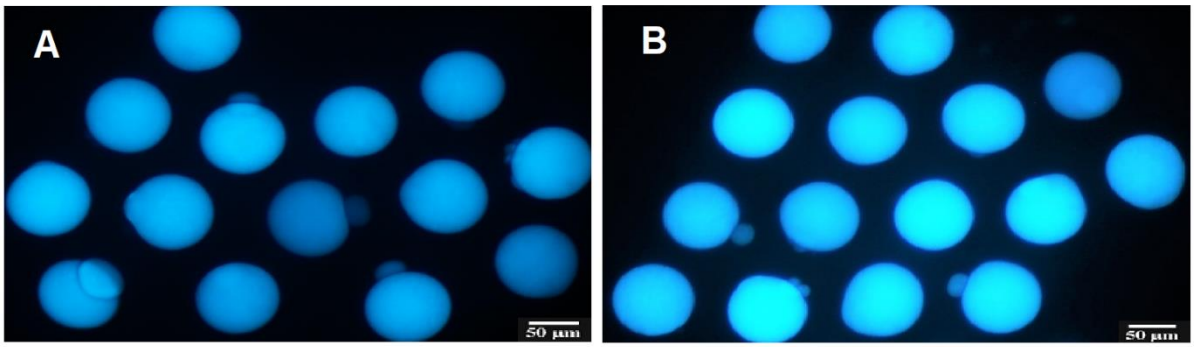

Figure 1. Representative images in MII oocytes from control (a) and lcarnitine-treated (b) groups, stained with Cell Tracker Blue to detect GSH as blue fluorescent. In the treated group compared with the control, level of GSH levels had significant increase. Scale bar = 50 µm.

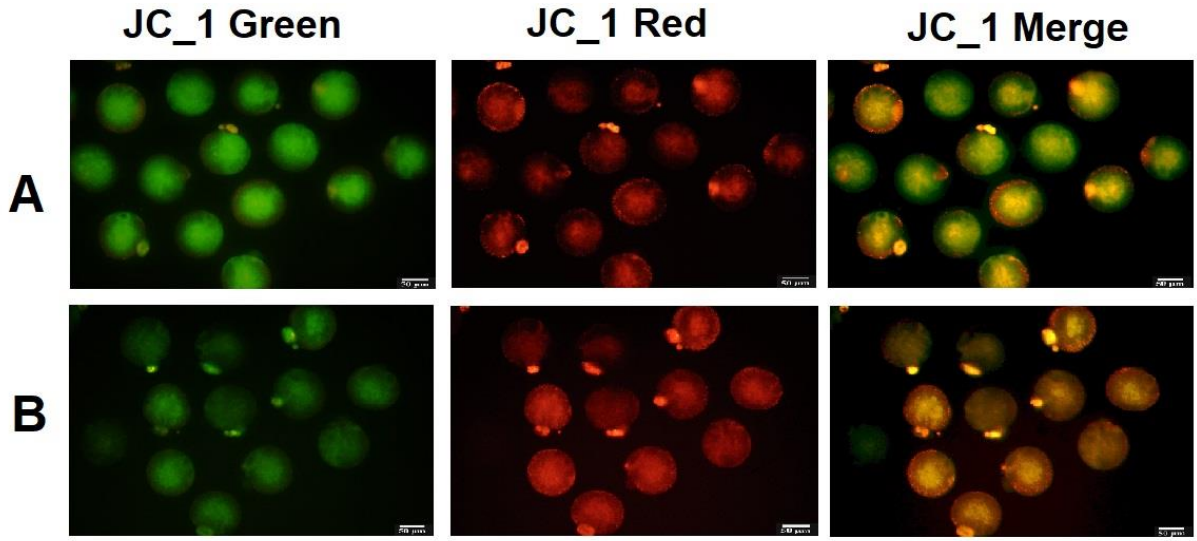

Figure 2. JC-1 Representative images in MII oocytes from control (a) and l_carnitine-treated (b) groups. ΔΨm was significantly increased in lcarnitine-treated group compared to control group Scale bar = 50 µm.

Mitochondrial Membrane Potential: Studies have shown that LC increases the mitochondrial function in different cells; for further investigation of the role of LC on oocyte development, mitochondrial membrane potentials were measured using 5, 5 ', 6, 6'-tetrachloro-1, 1', 3, 3 '(tetra ethyl benzamide azolyl carbocyanine iodide) JC-1 Fluorescence images were stored in TIFF format, and then the intensity of the fluorescence pixels of each oocyte was analyzed using Image J software and then the red: green fluorescence ratio was evaluated in the images. In the present study, the mean and standard deviation of red to green intensity in the case group was (1.88±0.59) and in the control group was (0.75±0.24), which was 15.07 % higher in the case group. Thus, the LC supplement could successfully increase oocyte mitochondrial function and subsequent mitochondrial membrane potentials ($P<0.001$) (Figure 2).

Discussion

The present study obtained satisfactory results from L-carnitine's effect on oocyte mitochondrial activity. After cryopreservation, L-carnitine could improve mouse oocytes' GSH level and mitochondria membrane potential. Today, oocyte cryopreservation is a rapidly advancing, breakthrough technique that enables women to postpone pregnancy to a later date due to cancer, aging, gonadotrophic drugs, and treatment with assisted reproductive technologies. Also, the overall pregnancy rate achieved by vitrified oocytes is not satisfactory[9, 10]. Besides, in several studies, it has been reported that

oocyte mitochondrial activity could be negatively affected by cryopreservation [5, 11-13]. Maybe such studies lead researchers to look for a substance that can reduce vitrification's harmful effects on oocyte mitochondrial activity. In a survey by Adel R., Moawad et al. found that adding L-Carnitine supplement to vitrification and warming solution can significantly increase peripherally concentrated mitochondrial distribution[9]. Also, in another experimental study by Moawad AR et al., L-Carnitine supplement was added to vitrification and thawing of mouse GV-oocytes, IVM, and embryo culture, and they found that LC supplementation during vitrification is sufficient for preimplantation development from the GV-oocytes that otherwise have lower developmental competence in culture [7]. In addition, Sprícigo et al. 2016 added L-Carnitine to the maturation medium before vitrification, and they found that LC supplementation could not have a beneficial effect on the embryo development potential of both fresh and vitrified oocytes but could decrease spindle damage; R addition–modulated apoptosis and positively affect the gene expression of vitrified/warmed oocytes[6] The potential effect of LC supplementation on in vitro maturation, especially in oocyte in many studies has been surveyed, for instance, Somfai T et al. in 2011 assessed the effects of L-carnitine as lipid metabolism and mitochondrial activity on nuclear maturation and in vitro fertilization of porcine follicular oocytes during. They found that L-carnitine could significantly increase oocyte cleavage and mitochondrial activity and significantly decrease lipid droplets' density, but further development to the blastocyst stage was not improved [14]. The oocyte quality will affect further successful embryo development, and there is a link between oocyte metabolism and successful ATP production that is highly dependent on mitochondrial activity. So, Dunning KR et al. reviewed studies investigating the beneficial effect of LC on oocyte and embryo development and the importance of β-oxidation through metabolic inhibitors. Overall, they found that the date of using dietary LC for female infertility treatment and oocyte quality improvement is limited. However, the ultimate result of this review on the effect of LC on oocyte maturation and embryo growth was satisfactory[15]. In contrast, Phongnimitr et al. reported that LC supplement has no beneficial effect on blastocyst development. Still, they found that LC could increase ATP content compared to the control group, which depended on mitochondrial health and could positively impact lipid droplets by dislocating them from the peripheral area to the inner cytoplasm. Finally, they concluded that LC supplementation during IVM redistributed lipid droplets in oocytes; if they survived vitrification, their developmental competence was similar to that of nonvitrified oocytes [16]. Also, it has been seen that LC could successfully decrease the number of apoptotic oocytes in pigs, intracellular ROS, and increase GSH levels in pig and sheep oocytes[17, 18]. GSH indicates a mature oocyte cytoplasm with well-established beneficial effects on blastocyst formation and quality[19]. In the present study, we found that the average difference of intracytoplasmic GSH levels in the study group was significantly higher than the control group, and L –carnitine could successfully increase oocyte intracytoplasmic GSH levels and therefore, high intracellular GSH levels after adding L –carnitine to vitrification media was observed.

Conclusion

To sum up, the addition of LC to the cryopreservation media could successfully increase mitochondrial activity and GSH levels. Cryopreservation of oocytes is a crucial step for conserving the female portion; however, despite decades of research, it remains a challenge in virtually all species due to the complex structure of the oocyte. Conventional slow freezing commonly leads to intracellular ice crystallization and cell damage. Although vitrification of human oocytes is gaining popularity, it is still challenging due to oocytes' complex structure and sensitivity to chilling. Vitrification is a relatively inexpensive and straightforward method of cryopreserving oocytes; however, despite the tireless efforts over the past 20 years, vitrification has yet to achieve convincing results capable of widespread application. Researchers must achieve more consistent results and establish a "universal" protocol that can be applied for the cryopreservation of oocytes at different developmental stages. So, maybe with refer to results obtained from the effect of LC supplements could do more research on human oocytes and standardized human oocyte protocol by adding some antioxidant agent like LCs.

Acknowledgment

None.

Conflict of interest

The authors further declare that they have no conflict of interest.

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