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Biological effects of L-carnitine on ovine oocyte maturation and embryo development

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Abstract

This study was conducted to determine the effects of L-carnitine on in vitro ovine maturation and early embryo development. In the first experiment, oocytes were matured in TCM-199 medium with different concentrations of L-carnitine (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM) and after fertilization, presumptive zygotes were cultured for 9 days on mCR2aa medium. In the second experiment, oocytes were matured in a maturation medium with various concentrations of L-carnitine (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM). After fertilization, presumptive zygotes were cultured in a culture medium containing various L-carnitine concentrations (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM). In vitro maturation (IVM) was carried out in a humid atmosphere of 5% CO2, 5% O2, and 90% N2 at 38.5 °C, and for in vitro culture (IVC), the concentration of O2 decreased to 5%. Morula and blastocyst development was evaluated on days 5 and 9, respectively. The results of the first experiment showed that the concentrations of 0.125, and 0.25 mM L-carnitine numerically led to an increase in the percentage of morula, blastocyst, and hatched blastocyst compared with control. The percentage of blastocyst formation increased at concentrations of 0.125 mM and 0.25 mM (31.97 ± 0.74 and 31.60 ± 1.39, respectively) compared with the control treatment (29.44 ± 2.42) ($p>0.05$). The results of the second experiment showed that the different concentrations of L-carnitine, simultaneously in the maturation and culture media of ovine embryos, similar results were observed when it was used only in the maturation medium, and the percentage of blastocyst formation increased at concentrations of 0.125 mM and 0.25 mM (35.62 ± 0.45 and 35.04 ± 1.70, respectively) compared to the control treatment (31.56 ± 3.39) ($p>0.05$). In conclusion, the use of L-carnitine in the media for oocyte maturation and embryo culture is recommended.

Keywords: Culture, Embryo, L-Carnitine, Maturation, Oocyte.

1. Introduction

The ovine embryo is recognized as a valuable source of information for studying mammalian embryogenesis mechanisms [1]. Despite advances in *in-vitro* embryo production technology (IVEP), the quantity and quality of *in vitro* embryos are lower than those of ***in vivo*** embryos [2-3]. IVEP in ovine is very low compared to other livestock species, such as cattle or pigs, and the low rate of IVEP is due to the low rate of nuclear and cytoplasmic maturation of the oocyte [4]. Therefore, oocyte competence is the ability of oocytes to reach nuclear and cytoplasmic maturity, achieve successful monospermic fertilization, and support the early development of the embryo [5].

The growth of *in vitro* fertilized embryos depends on the quality of the maturation and culture media [6]. The media in which the early stages of embryonic development take place has significant short-term and long-term effects on the subsequent development of the embryo [2]. Adding antioxidant supplements to the culture medium by increasing mitochondrial function and regulating ROS levels has positive effects on oocyte maturation and embryo growth [7]. One potential antioxidant, which plays an important role as a scavenger of ROS or as an agent to support nuclear maturation and fertilization, is L-carnitine [4].

L-carnitine is a water-soluble quaternary ammonium compound and a natural vitamin-like substance. This substance is synthesized from the amino acids lysine and methionine in the liver and is mainly present in the muscles and liver of all animals [8]. L-carnitine is known as an antioxidant with the capacity to neutralize free radicals derived from ROS, especially superoxide anion [3-9], and can protect cellular organelles such as mitochondria from the harmful effects of oxidative stress [3]. It is also responsible for the β-oxidation and transport of fatty acids and plays an important role in lipid metabolism [10]. Maturation, fertilization, and subsequent embryo development before implantation require the production of sufficient amounts of ATP [11]. In this regard, the role of carnitines as mitochondrial modulators has increased in both oocytes and embryos. The main physiological characteristics of carnitines are regulating the transfer of acyl-CoA to the mitochondrial matrix, limiting the rate of fatty acid

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oxidation, storing energy in the form of acetyl-carnitine, modulating the toxic effects of poorly metabolized acyl groups, and antioxidant activity [12].

However, information on the effects of L-carnitine during oocyte maturation and early embryo development in ovine is still limited [4]. Therefore, this study aimed to evaluate different concentrations of L-carnitine in IVM and IVC media until reaching the blastocyst stage.

2. Materials and methods

2.1. Location, chemicals, and media

All experiments were conducted at the Embryo Biotechnology Laboratory of the Iranian Research Organization for Science and Technology (IROST) at a constant temperature of 27-31 °C. All chemicals and media were provided by Sigma-Aldrich (USA) and Gibco (USA), and plasticware was purchased from Falcon (USA), unless stated otherwise. All stock solutions and media were prepared in sterile triple-distilled Milli-Q water and was filtered through 0.22 µm membrane filters.

2.2. Oocyte collection and *In Vitro* maturation

Ovaries were collected from a local slaughterhouse and transported to the laboratory within 3 h in a phosphate-buffered saline solution supplemented with gentamycin (50 µg/ml), at 25-30°C. The collected ovaries were cut to remove the surrounding adipose tissue. The ovaries were washed five times with physiological saline. Ovarian slicing using a surgical blade was used to collect the oocytes. Follicular fluid and COCs were placed in a small petri dish containing aspiration medium supplemented with TCM-199, L-glutamine (2 mM), Bovine Serum Albumin (BSA) (0.3%), and gentamicin sulfate (50 µg/ml). Then, under a stereomicroscope, COCs were collected and washed three times by gentle pipetting in the washing medium. The washing medium was composed of TCM-199, L-Glutamine (2 mM), Sodium Pyruvate (0.81 mM), Fetal Bovine Serum (FBS) (10%), and gentamicin sulfate (50 µg/ml). Then, COCs presenting three or more layers of cells and homogeneous cytoplasm were selected, and matured in groups of up to 15 COCs in 100 µl droplets of maturation medium, covered with mineral oil, and cultured for 24 h at 38.5°C, 20% O2, and 5% CO2. IVM medium, which included TCM-199 with 10% FBS, 10% ovine follicular fluid, 5 mg/mL follicle-stimulating hormone (FSH), 1 mg/mL estradiol- 17β, 0.81mM sodium pyruvate and 50 mg/mL gentamicin sulfate.

2.3. Sperm processing and *in vitro* fertilization

Spermatozoa were provided for fertilization as previously explained [13], and the cryopreserved semen of a ram was rapidly thawed at 37 ºC and washed twice using 10 ml IVF medium (i.e., Brackettand-Oliphant medium containing 10µg/mL heparin, 137.0µg/mL sodium pyruvate, and 1.942 mg/mL caffeine sodium benzoate). The samples were washed twice by centrifugation at 1000 rpm for 5 and 7 min at room temperature. A hemocytometer was used to calculate the concentration of spermatozoa and subsequently adjusted to 1.0 × 107/ml by dilution. Fifty µL of the sperm suspension was incorporated with a 50 µl droplet of the IVF medium. The 15 matured COCs were transferred to 100 µL of IVF medium droplets and incubated for 18 h at 38.5 ºC in a humidified atmosphere of 20% O2 and 5% CO2 in air.

2.4. *In vitro* culture

After fertilization, the presumed zygotes were carefully pipetted to remove the remaining cumulus cells and to adhere to the spermatozoa. Afterward, washing the oocytes was performed several times using the IVC medium that comprised of mCR2aa, the composition of which was 24.9 mM NaHCO3, 2.9 mM KCl, 108.3 mM NaCl, 2.5 mM hemi-calcium lactate, 1 mM glutamine, 0.5 mM sodium pyruvate, 0.5 mM glycine, 0.5 mM alanine, 1 mM glucose, 5 µgmL−1 phenol red, 50 µgmL−1 gentamycin, 2% basal medium eagle amino acids, and 1% MEM non-essential amino acids, containing 0.6% BSA and 10% FBS [14]. Presumptive zygotes were randomly distributed among the experimental groups. Embryonic development occurred in a humidified atmosphere of 5% CO2, 5% O2, and 90% N2 at 38.5 ºC. Morula and blastocyst development were evaluated on days 5 and 9 respectively.

2.5. Experimental design

2.5.1. Experiment 1; effect of L-Carnitine supplementation during IVM medium culture

COCs were matured *in vitro* at different concentrations of L-carnitine (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM) in TCM-199 medium. After 24 h of IVM, COCs were subjected to IVF and presumptive zygotes were cultured in mCR2aa medium. The experiment was replicated three times.

2.5.2. Experiment 2; effect of L-carnitine supplementation during IVM and IVC media culture

In the second experiment, COCs were matured in a maturation medium containing different concentrations of L-carnitine (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM). After fertilization between the mature oocytes and spermatozoa, the culture of presumed zygotes was carried out in a mCR2aa medium containing different concentrations of L-carnitine (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM). The percentages of cleavage, morula, blastocysts, and hatched blastocysts were evaluated on day 9 of culture. The experiment was replicated 3 times.

2.6. Statistical analysis

Three replicates were applied to each experimental group. SPSS statistical software (version 16) was utilized for quantitative data analysis. Mean ± standard error of the mean was used to express the data. Statistical analysis was carried out by the analysis of variance method followed by the ANOVA test. The results were considered statistically significant at $p \leq 0.05$.

3. Results

In the first experiment, L-carnitine supplementation at concentrations of 0, 0.125, 0.25, 0.5, 1, 2, and 4 mM was investigated in the maturation medium of ovine oocytes. The concentrations of 0.125, and 0.25 mM L-carnitine numerically led to an increase in the percentage of morula, blastocyst, and hatched blastocyst compared with control (*p>0.05*). The percentage of blastocyst formation increased at concentrations of 0.125 mM and 0.25 mM (31.97 ± 0.74 and 31.60 ± 1.39, respectively) compared with the control treatment (29.44 ± 2.42). However, concentrations higher than 0.5 mM L-carnitine did not improve early embryo development and caused a significant decrease in morula and blastocyst formation compared with 0.25 mM L-carnitine ($p<0.05$). Therefore, the concentrations of 0.25 mM L-car-

nitine in the oocyte maturation medium led to an increase in the percentage of blastocyst formation compared with the other groups (Fig. 1).

In the second experiment, when different concentrations of L-carnitine were used simultaneously in the maturation and culture media of ovine embryos, similar results were observed when it was used only in the maturation medium. The percentage of blastocyst formation increased at concentrations of 0.125 mM and 0.25 mM (35.62 ± 0.45 and 35.04 ± 1.70, respectively) compared to the control treatment (31.56 ± 3.39). Based on these results, concentrations of 0.125, and 0.25 mM L-carnitine are suggested in comparison with higher concentrations than L-carnitine (0.5 mM) in the maturation and culture media of ovine embryos (Fig. 2).

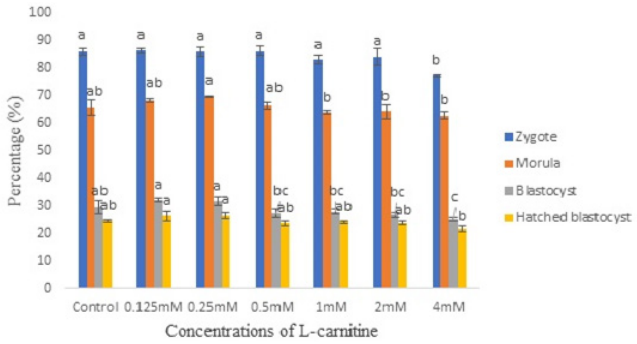

Fig. 1. Effects effect of L-carnitine on in vitro ovine maturation on preimplantation early embryo development.

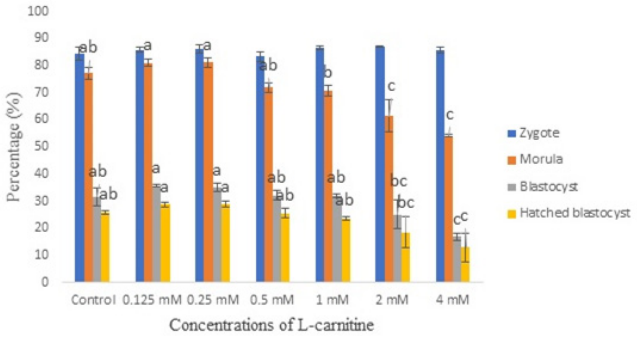

Fig. 2. Effect of L-carnitine on in vitro ovine maturation and early embryo development.

4. Discussion

Oocyte metabolism affects the competence of oocyte development, and the factors influencing oocyte metabolism are the culture media compounds in which the oocyte grows [15]. Contradictory results of using L-carnitine in the *in vitro* culture of embryos from different species have been reported. Some researchers noted that oocyte and embryo culture in the presence of L-carnitine did not affect oocyte maturation and the expanded blastocyst rate [16-17]. However, the role of L-carnitine in the IVM and IVC of many species, including mice [18], pigs [19], canian [11], camel [20]and bovine [16-21] has been reported.

Similar to the results of our study, the use of high concentrations of L-carnitine in the maturation medium of bovine [22] and pig [23] resulted in a decrease in the percentage of blastocyst formation. These reports indicate detrimental effects on oocyte viability. L-carnitine shows a high affinity for binding to Ca2+ molecules. Therefore, high L-carnitine levels can inhibit metabolic processes and enzyme activities that utilize Ca2+ as a second messenger, leading to changes in cellular homeostasis [24].

However, using an appropriate concentration of L-carnitine in the oocyte maturation medium improves the rate of blastocyst development. L-carnitine reduces oocyte cytoskeleton damage and promotes mouse embryo development *in vitro* [25]. These cases are mostly due to the antioxidant properties of L-carnitine, and definitely, oxidative stress affects the ability of oocyte growth. The increase in growth potential caused by the addition of L-carnitine to the culture medium is due to the increase in ATP production, which is closely related to oocyte quality [26]. Some researchers have indicated that L-carnitine supplementation during the IVM of ovine oocytes mitigated oxidative stress-induced embryotoxicity by reducing intracellular ROS levels and enhancing intracellular GSH, thereby improving the growth potential of oocytes and embryos and modifying the transcript levels of antioxidant enzymes [27]. In bovine oocyte maturation, the application of 0.6 mg/ml L-carnitine in the IVM medium did not significantly affect maturation and embryonic development rates in non-vitrified bovine oocytes but preserved the developmental competence of IVM oocytes post-vitrification at levels comparable to non-vitrified oocytes [16]. A concentration of 2.5 mM L-carnitine during bovine oocyte maturation revealed that supplementation improved the development of embryos from less meiotically competent oocytes and accelerated blastocyst formation from more competent ones. Overall, the impact of L-carnitine is specific and contingent on the meiotic and developmental competence of the oocytes [28].

You et al. [19], studied the effect of 10 mM (1.98 mg/mL) L-carnitine during the IVM of immature pig oocytes, finding that L-carnitine enhanced the developmental competence of SCNT embryos. This effect likely arises from increased intracellular GSH synthesis in the recipient ooplasm, leading to reduced ROS levels and stimulating nuclear reprogramming via elevated expression of POU5F1 and other transcription factors. The use of 10 mM L-carnitine in a pig oocyte maturation medium was shown to decrease intracellular lipid droplets and ROS levels, while also enhancing SOD1 gene expression [29].

Fathi and El-Shahat [20], reported that the inclusion of L-carnitine during the IVM of camel oocytes increased cleavage rates and embryo development (morula and blastocyst stages), and that L-carnitine during IVC enhanced developmental rates at the morula and blastocyst stages. Using 0.6 mg/mL L-carnitine during IVM led to improved maturation, fertilization, and development of canine embryos as noted by Moawad et al. [11].

Dunning et al. showed that L-carnitine can increase lipid metabolism and improve embryo development during oocyte maturation [26]. L-carnitine not only alters the energy metabolism of lipids but also reduces the number of lipid droplets [30]. It also regulates ATP production from lipids [11]. The improvement in embryo development and blastocyst rate may be due to the reduction of cytoplasmic lipid droplets, which reduces lipid peroxidation and ROS production in the embryo [10]. By reducing ROS, L-carnitine prevents apoptosis of granulosa cells, thus preventing oocyte degeneration [9].

Mitochondrial distribution in culture medium containing L-carnitine is associated with improved oocyte nuclear maturation and increased ATP production during

the MI-MII transition [31]. Since L-carnitine increases β-oxidation and β-oxidation guarantees the nuclear and cytoplasmic maturation of oocytes, therefore L-carnitine supports the nuclear and cytoplasmic maturation of oocytes *in vitro* [9].

L-carnitine has beneficial effects not only in maturation but also after fertilization [11]. Adding L-carnitine during embryo culture had no harmful effects on the rate of final blastocyst formation. These observations also show that compensatory pathways during embryo development (i.e., reduction of oxidative metabolism), along with the antioxidant properties of L-carnitine, can improve cellular oxidative stress in the embryo and even overcome oxidative damage that occurs during oocyte maturation [5]. Supplementation with 10 mM L-carnitine during *in vitro* embryo production enhances the developmental potential of ovine embryos and acts as an anti-apoptotic and proliferative agent for embryo development [8]. Jiang et al [21], utilized 2.5 mM L-carnitine to culture embryos derived from aged bovine oocytes, demonstrating that L-carnitine enhanced the development of zygotes to the blastocyst stage and improved the quality of the blastocysts. The resulting embryos exhibited fewer apoptotic cells in the blastocyst, decreased ROS levels, and increased GSH levels, along with upregulation of mRNA levels of SOD1 and GPX4 in the embryos. Carrillo-González and Maldonado-Estrada [32] examined the impact of 3.8 mM L-carnitine in the IVM medium and 1.5 mM in the IVC medium on the implantation rate of *in vitro* bovine embryos and reported significantly higher pregnancy rates at Days 45 and 72 in blastocysts following embryo transfer.

The beneficial effects of L-carnitine are attributed to its antioxidant capacity and ability to increase ATP production from intracellular lipid stores [11]. In a study by Takahashi et al., they observed that the level of embryo apoptosis was reduced after treatment with L-carnitine. Therefore, the improvement in blastocyst production quality in the presence of L-carnitine may be due to the anti-apoptotic activity of L-carnitine [33].

Increased ROS production may lead to cell damage, such as DNA fragmentation and apoptosis, and block embryonic development [18]. ROS may be produced as a byproduct of abnormally high metabolic activity [34]. ROS can cause oxidative damage to DNA, proteins, and membrane unsaturated fatty acids [35], and affect RNA transcription [9].

Using the appropriate concentration of L-carnitine during the IVM and IVC of ovine oocytes led to an increase in blastocyst formation. Therefore, it is recommended to use concentrations of 0.125 and 0.25 mM L-carnitine in the media of oocyte maturation and embryo culture.

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Conflict of interests
The authors declare no conflicts of interest.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human subjects were included in the present study.

Informed consent
The authors declare that no patients were included in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Abbase Darzi nia: Conceptualization, Writing – original draft; Mohammad Zandi: Supervision, Conceptualization, Formal Analysis, Methodology, Validation– review & editing; Annahita Ghaedrahmati: Validation, Writing – review & editing.

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