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Research article

Effect of L.Carnitine supplementation to *in-vitro* culture media on maturation of bovine oocytes and embryos development

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Abstract:
This study was conducted to investigate the role of L.Carnitine in enhancing the developmental progress of in-vitro produced bovine embryos. A total of 794 cumulus-oocyte complexes (COCs) were collected by the slicing method from the genitalia of unknown age and breed slaughtered cows. The COCs were divided into four groups and matured in TCM-199 supplemented with four concentrations of L.Carnitine (0.0mg/ml, 0.3mg/ml, 0.6mg/ml and 1.2mg/ml), a concentration for each group. The results showed that the groups treated with 0.3 and 0.6 mg/ml recorded higher percentages of oocytes maturation (48.98% and 49.20%) respectively with significant (P<0.01) differences compared with control and 1.2mg/ml. Matured oocytes were fertilized by straws of frozen-thawed semen, and the resulted embryos were divided into four groups and cultured in media supplemented with the four concentrations of L.Carnitine (0.0mg/ml, 0.3mg/ml, 0.6mg/ml, and 1.2mg/ml) in order to investigate its effect on embryos developmental progress. Results showed that the concentrations (0.3 and 0.6 mg/ml) gave better percentages of embryo development (18.55% and 17.20%) respectively with significant (P<0.05) differences when compared with control and 1.2mg/ml. In conclusion, supplementation of in-vitro bovine oocytes maturation media and in-vitro embryo development media with 0.3 and 0.6 mg/ml of L.Carnitine improved significantly oocyte maturation and embryo developmental progress.
Keywords: L.Carnitine, bovine, oocytes, embryos

Introduction:

In-vitro production of embryos is one of the assisted reproductive technologies that helps in reducing infertility and supporting the studies about the reproductive phenomena *in-vivo* and *in-vitro*, because of the great need for the basic researches to understand the complexity of the physiological mechanisms of male and female reproductive cycles, to get better and successful results. For *In-vitro* production of bovine embryos, the oocytes can be obtained by two sources, from the ovaries of the slaughtered cows, and from the live donor cows via trans-vaginal follicular aspiration system that guided by ultrasound, and in the both sources, the oocytes need to be maintained *in-vitro* for about 24 hours in specific media in order to complete their maturation, which is crucial for fertilization and embryo developmental progress. Thus, many researches were established to enhance the culture media that improves embryo cleavage and development. Bovine oocytes and embryos contain large amounts of lipids in their cytoplasm (1). Those lipids are important source for energy. The metabolism of the fatty acids occur in the mitochondria by beta oxidation which is essential process that oxidize the fatty acids to produce ATP (2). L.Carnitine is a quaternary ammonium

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compound known to have dual important physiological actions, firstly it catalyzes the transport of the fatty acids from the cytosol to the mitochondria which accelerates fatty acids beta oxidation (3), and secondly it has the properties of powerful antioxidant (4) which reduces the formation of reactive oxygen species (ROS) and decreases their harmful effects (5). Thus, this research was designed to investigate the effect of supplementing the culture media of bovine oocytes and embryos with L.Carnitine on increasing oocytes maturation and enhancing the developmental progress of embryos.

Materials and methods:

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study.

All the chemicals that used for *in-vitro* production of embryos and the bovine serum albumin (BSA) are from the products of SIGMA-ALDRICH CHEMIE GmbH Steinheim-Germany. The TCM-199, the Dulbecco's Phosphate Buffer Saline (DPBS) and the antibacterial solutions are from the products of GIBCO. Heat inactivated fetal bovine serum (FBS) is from the products of biowest, South American origin. L.Carnitine is from the products of Chemicalpoint-Germany. Hormones FSH and LH (Pluset) is from the products of Calier-Spain. The chemicals that used in the research are NaHCO3, L.Glutamine, Kafein, Hparin and BO solution which needs the following ingredients to be prepared: NaCl, KCl, CaCl2 2H2O, NaH2PO4 2H2O, MgCl2 6H2O, Na Pyruvate and 0.5% phenol red.Ovaries from slaughterhouse were collected in thermos contain normal saline supplemented with 50 IU/ml penicillin + 50 µg/ml streptomycin in a temperature around 30-35ºC within thirty minutes after slaughtering and transferred during 2-3 hours to the lab. The ovaries were cleaned from the sticking blood by washing several times with fresh warmed normal saline.In order to recover the COCs, the ovaries were sliced in petri dish contain solution composed of 97ml of DPBS + 3 ml fetal calf serum (FCS) + penicillin streptomycin. COCs were graded under stereo microscope and all the COCs that surrounded by one layer or more of compact cumulus cells with homogenous cytoplasm were used in the study. For *in-vitro* Maturation, the media TCM-199 (1X) from GIBCO contain Earle's salts, 25mM Hepes and L.Amino acids, were supplemented with 10% FBS, 0.11gm NaHCO3, 0.015 mg L.Glutamin, 0.5 IU/ml Pluset and 50 IU/ml penicillin + 50 µg/ml streptomycin (the percentages of the additions is for 45 ml of maturation media). Four droplets 50 µl each of maturation media covered by mineral oil (density 0.84 g/ml SIGMA-Germany) were pre-prepared in 35mm petri dish and placed -before at least two hours of COCs grading- in a 5% CO2 incubator at 38.5ºC and humidified atmosphere. Each one of these 50 µl droplets contain specific concentration of L.Carnitine (0.0mg/ml, 0.3mg/ml, 0.6mg/ml and 1.2mg/ml) according to [6]. The COCs were washed two times in maturation media and distributed randomly on these 50 µl droplets of maturation media -5 COCs for each drop- for 22-24 hours incubation.For *in-vitro* fertilization, matured COCs were fertilized by freezed-thawed straws, using BO solution that prepared in the lab by these chemical ingredients. Firstly two stocks, A and B should be prepared. The composition of 250 ml of stock A is 2.1546 gm of NaCL, 0.0987 gm of KCL, 0.10855 gm of CaCL2 2H2O, 0.0840 gm of NaH2PO4 2H2O, 0.0697 gm of MgCL2 6H2O and 0.5% phenol red. The composition of 200 ml of stock B is 2.5873 gm of NaHCO3 and phenol red 0.5%. In order to prepare 100 ml of BO solution, we mixed 76ml of stock A + 24ml of stock B + 0.01375 gm of Na Pyruvate + 100 IU/ml penicillin + 100 µg/ml streptomycin. BO used to wash matured

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oocytes from the remnant of TCM-199 and to wash the thawed sperms from the remnant of freezing materials and also used in sperms capacitation media and as a fertilization media. To prepare oocyte washing media, 0.1 gm of BSA dissolved in 10 ml of BO solution. To prepare sperm washing solution (SWS) that contain ingredients induce sperm capacitation, we dissolved 0.01942 gm of Caffeine in 10 ml of BO solution + 0.2 gm of BSA + 5IU/ml of Heparin (stock 5000 IU/ml). Matured COCs washed twice by oocyte washing media, and at the same time denuded partially from the cumulus cells by gentile pipetting. Four straws from known fertile bulls were thawed in 37ºC water bath for 20 seconds and deposited in conical tube contain four ml SWS previously putted in water bath at 37ºC. The semen is centrifuged 1800 g for 5 minutes at room temperature. After centrifugation, the supernatant discarded and ½ ml of SWS were added on the sperms pellet, then the conical tube deposited in the incubator at 45º angle to allow the sperms to swim up. After one hour incubation, the motility of the sperms from the upper region of the SWS media were checked and the sperms were adjusted to calculate 1-3 million sperm/ml.Drops of SWS covered by mineral oil contains quite suitable number of sperms that can fertilize oocytes (25000-30000 sperm/oocyte) were established in 35mm petri dish and deposited in CO2 incubator at 38.5ºC in humidified atmosphere. For *in-vitro* culturing, the presumptive zygotes after 18-20 hours of fertilization were washed twice by TCM-199 and at the same time fully denuded from the cumulus cells and transferred to pre-prepared four 50 µl droplets of TCM-199 covered by mineral oil in 35mm petri dish. Each droplet supplemented with specific concentration of L.Carnitine (0.0mg/ml, 0.3mg/ml, 0.6mg/ml and 1.2mg/ml). The presumptive early embryos were cultured till 96 hours after fertilization in CO2 incubator at 38.5ºC in humidified temperature.

Results:

Table (1): effect of TCM-199 supplemented with different concentrations of L.Carnitine on bovine oocytes maturation.

Conc. of L.Carnitine mg/ml	No. of cultured oocytes	No. of matured oocytes	Percentage of maturation
Control 0.0	213	79	37.08%
0.3	198	97	48.98%
0.6	189	93	49.20%
1.2	194	48	24.74%
Total	794	317	39.92%
Chi-Square (χ^2)	---	---	8.955 **
**** (P<0.01).**			

Table 1 show the effect of adding three concentrations of L.Carnitine (0.3mg/ml, 0.6mg/ml, 1.2mg/ml) and the control on the maturation of oocytes that matured in TCM-199. The total number of oocytes that cultured in TCM-199 is calculate 794 divided into four groups, 213 oocytes as control group not treated with L.Carnitine, 198 oocytes treated with 0.3mg/ml of L.Carnitine, 189 oocytes treated with 0.6mg/ml and 194 oocytes treated with 1.2mg/ml. After 24 hours, the number of matured oocytes was 79 in control group, 97 in 0.3mg/ml group, 93 in 0.6mg/ml group and 48 in 1.2mg/ml group, and the percentages of maturation was 37.08%, 48.98%, 49.20% and 24.74% respectively with highly significant (P<0.01) differences between the percentages of the groups.The concentrations of L.Carnitine 0.3mg/ml and 0.6mg/ml groups recorded higher percentages of maturation with highly

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significant (P<0.01) differences over the control and 1.2mg/ml group of L.Carnitine, and the latest group recorded lesser percentage of maturation, with significant differences compared with control group. There is no significant differences in maturation between the percentages of 0.3 and 0.6mg/ml groups.

Table 2: effect of TCM-199 supplemented with different concentrations of L.Carnitine on bovine embryos development

Conc. of L.Carnitine mg/ml	No. of matured oocytes	No of live embryos after 72 hrs	No. of live embryos after 96 hrs	Chi-Square (χ^2)
Control 0.0	79	13 (16.45%)	7 (8.86%)	4.937 *
0.3	97	26 (26.80%)	18 (18.55%)	5.460 *
0.6	93	23 (24.73%)	16 (17.20%)	5.411 *
1.2	48	4 (8.33%)	0 (0.0%)	4.293 *
Chi-Square (χ^2)	---	6.157 **	4.382 *	---
*** (P<0.05), ** (P<0.01).**				

Table(2) showed the effect of L.Carnitine in enhancing the developmental potential of bovine embryos to reach the day four of *in-vitro* culturing. The table showed that there was 79 matured oocytes in the control group, and after 96 hours of fertilization we gain 7 live embryos which represents 8.86% from the total number of the matured oocytes in the control group.In concentration 0.3mg/ml group of L.Carnitine, the number of matured oocytes was 97, and we gained 18 live embryos after 96 hours of fertilization that represents 18.55% from the matured oocytes in this group. Also, at the 0.6mg/ml group there was 93 matured oocytes, and we gained 16 live embryos which represent 17.20% from the whole number of matured oocytes in that group. In 1.2mg/ml group of L.Carnitine concentration, there was 48 matured oocytes, and we did not gain any live embryos after 96 hours of fertilization. The statistical analysis of this table showed that there was significant (P<0.05) difference in the percentages of embryos development between the groups. L.Carnitine in concentrations 0.3 and 0.6mg/ml gave higher percentage of embryos development after 96 hours of fertilization with significant (P<0.05) difference compared with control group and 1.2mg/ml group, and the latter group does not gain any live embryos after 96 hours of fertilization with significant (P<0.05) difference compared with control group. There is no significant differences in embryos development between the groups that supplemented with 0.3 and 0.6mg/ml of L.Carnitine.

Discussion:

Tables 1 and 2 of the study investigated the effect of adding L.Carnitine to the *in-vitro* culture media on improving the maturation of bovine oocytes, and the development of early embryos that produced *in-vitro* by comparing the developmental progress of embryos in the culture media that supplemented and not supplemented with L.Carnitine. The most important step in embryos *in-vitro* production system, is *in-vitro* maturation of the oocytes, because it is crucial step for the success of fertilization, cleavage and development of the embryos (7,8). *In-vitro* developmental progress of embryos obtained from oocytes matured *in-vitro* is lower significantly compared with the developmental progress of the embryos that obtained from oocytes matured *in-vivo* (9).

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Lot of factors *in-vitro* such as, -gaseous concentrations in the atmosphere of the incubator, temperature and light variations, protein supplementation and growth factors in the maturation media, presence of large amounts of spermatozoa during fertilization, and the stress due to the manipulation of the oocytes- can influence on the maturational process of the oocytes which leads to retardation in embryos developmental progress (7,10). When the mammalian oocytes incubated for *in-vitro* maturation, they exposed to an atmosphere contain 5% CO2 and approximately 20% O2, which is consider as much as more than the double concentration of O2 that may they exposed to when they mature naturally in the oviduct *in-vivo* (11). Cells that expose to high levels of O2 produce considerable amounts of ROS, which known to have sever harmful effect on cells developmental progress and they may cause cell injuries (12). ROS formed naturally during the aerobic metabolism of the cells, and the cells neutralize the increased production of ROS when they are proceed in normal healthy physiological status (13). When the amounts of the ROS balanced in the cells, they play important role in signaling and regulating many physiological processes, such as, tissue regenerating, steroidogenesis, hormone signaling, intracellular redox regulation and embryogenesis (14). Contrary, the increased amounts of ROS due to the disturbances of the physiological process may cause severe damage to the cells which leads to cell changes in structure and functions, or may lead to cell death (15). During the oocyte maturation and early embryo cleavage *in-vivo*, the production of ROS is balanced by wide range of antioxidants naturally found in the female reproductive tract (16), but the oocytes and embryos that matured and cultured *in-vitro* usually exposed to high concentrations of ROS due to the existence of many different environmental factors than those in *in-vivo* maturation environment (13), and the synthetic media of *in-vitro* production of embryos often contains small amounts of antioxidants which encourage the increased production of ROS quantities due to the unbalance between the amounts of the antioxidants in the media and the increased production of ROS that formed through the metabolic activities of the cultured oocytes and embryos, furthermore, the formation of high concentrations of ROS in the media causes further depletion in the concentration of the antioxidants (17) which leads to increase the oxidative stress on the cells in the media, that consider the major cause of decreasing the oocytes maturation efficiency and further embryo development in many species (13,18), therefore, the useful of adding antioxidants to *in-vitro* embryo production media is to reduce the oxidative stress on oocytes and embryos by balancing the ROS formation in the media which reduce the losses in *in-vitro* produced embryos that may occur due to the oxidative stress (12,19). L.Carnitine has powerful antioxidant properties (4). L.Carnitine also has the capacity to facilitate lipid metabolism, which is consider as an endogenous energy source crucial for supporting oocytes maturation and developmental progress of embryos. Oocytes and pre-implantation embryos of ruminants are rich of lipids in their cytoplasm, which is degraded in endoplasmic reticulum and mitochondria into some extent during the maturation of COCs (20), and by the beta oxidation process, the lipids and the fatty acids are metabolized within the mitochondria to generate ATP which is essential energy supplement for the oocyte to undergo fertilization, cleavage and early embryo development, thus at this point, the amount of the cellular ATP that generated by beta oxidation pathway can determine the oocyte quality and ability to continue its cleavage and progress in further embryo development (21).For enhancing beta oxidation step, the fatty acids should be facilitated to enter actively into the mitochondrion. This entry is catalyzed by the enzyme carnitine palmitoyl transferase I (CPT1) which promotes the attachment of

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carnitine with the activated fatty acids to facilitate the transportation of the fatty acids from the cytosol into the mitochondria where the carnitine is separated by the enzyme carnitine palmitoyl transferase II (CPT2) and the fatty acids enters the beta oxidation cycle which produces Acetyl-Coenzyme A molecules that generates ATP by the Krebs cycle. In our study, we use L.Carnitine in three concentrations (0.3, 0.6 and 1.2) mg/ml in the maturation media of the bovine COCs and in the culture media of the early stage embryos, based on the concentrations that previously mentioned by (6) in mouse oocytes and (22) in bovine oocytes and embryos, In order to determine the effective dose that enhances the maturation of the COCs and the development of the bovine embryos by comparing the developmental progress of the embryos. The results showed that L.Carnitine on the concentrations 0.3 and 0.6 mg/ml improved significantly compared with the control, the maturation competence of the bovine oocytes and the developmental progress of the embryos till 96 hours of culturing. (23) indicated that the addition of L.Carnitine to the maturation media of mouse oocytes and culture media of embryos significantly increased the beta oxidation which improves oocytes maturational competence and developmental potential of embryos, because beta oxidation process has essential role in the nuclear and cytoplasmic maturation of oocytes. Enhancing the beta oxidation of lipids in oocytes and embryos increases ATP production, which is consider one of the major factors that contribute in promoting the maturational competence of oocytes and developmental potential of embryos cultured *in-vitro* (1,24). In mammalian oocytes and embryos, the lipids represents potential energy source for increasing the production of ATP, but the lipids needs transporter such as CPT1, which accelerates the transportation of fatty acids from the cytosol toward the site of beta oxidation in the cell (the mitochondria), thus the addition of L.Carnitine -which is cofactor of CPT1- to the *in-vitro* culture media seems effective way to increase the production of ATP in oocytes and embryos cultured *in-vitro* (25). As the beta oxidation of lipids accelerated by the addition of L.Carnitine to the *in-vitro* culture media, it causes increased production of ROS to reach high levels in the media due to the handling and culturing of the oocytes and embryos, which causes cells lipid peroxidation, DNA fragmentation and disturbances in RNA transcription with negative influences on protein synthesis (26,27), that leads to blockage in *in-vitro* embryos development or early embryonic death (26,28). L.Carnitine has antioxidant capacities by increasing the intracellular glutathione synthesis which have the ability to reduce ROS levels by the scavenging capability (29,30). The antioxidant activity of L.Carnitine suggested due to its effect in scavenging many deleterious ions of free radicals, such as 1,1diphenyl-2-picryl-hydrazyl free radical (DPPH), superoxide anion radical and hydrogen peroxide (4). Increased the glutathione levels in metaphase II oocytes enhances the maturational competence of mammalian oocytes and the developmental progress of embryos due to the protection against deleterious effect of free radicals (18). The study of (31) indicated that the addition of L.Carnitine at the concentrations 0.3 and 0.6 mg/ml to the maturation media of mouse oocytes increased significantly the intracellular glutathione levels in metaphase II oocytes in both concentrations groups. The maturation process of the oocyte occur in three stages, which are nuclear, cytoplasmic and molecular, and those are play crucial role in determining the quality of matured oocyte and its developmental competence, and the quality of the resultant embryo (32). It has been indicated that the addition of L.Carnitine to the *in-vitro* maturation media of the mammalian COCs, improved the glutathione levels during the period of maturation, which suggests that the effect of L.Carnitine is more prominent on the cytoplasm maturation of the cells rather than

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the nuclear maturation in porcine oocytes (29) and mouse oocytes (31). L.Carnitine improved the maturation competence of mammalian oocytes by improving the meiotic competence of the oocytes that resulted from enhancing the mitochondrial activity of the granulosa cells and stopping their apoptosis (33). Furthermore, addition of L.Carnitine to the *in-vitro* maturation media of oocytes increased the expression of mitogen-activated protein kinase (MAPK1) gene which increased the MAPK activity that in turn increased the MAPK phosphorylation levels in the oocyte (31). (34) mentioned that the addition of 0.1 - 0.5 mg/ml concentration of L.Carnitine to the *in-vitro* culture media, improved the maturation of bovine oocytes and the developmental potential of pre-implantation embryos. (31) mentioned that the addition of 0.6 mg/ml of L.Carnitine to the maturation media of immature mouse oocytes decreased the ROS levels in the maturation media. (35) mentioned that supplementation of oocytes maturation media and 8-cells embryos culture media with 0.6 mg/ml concentration of L.Carnitine, improved significantly the integrity of microtubules and chromosomes structures and decreased the levels of cells apoptosis. (6) mentioned that supplementation of the *in-vitro* culture media of mice oocytes and embryos with 0.3 mg/ml of L.Carnitine, blocked the hydrogen peroxide deleterious effects and decreased the levels of DNA damage. It seems that the effective antioxidant action of L.Carnitine works by both ways, direct action on scavenging the deleterious ROS and indirect action by increasing the levels of glutathione in the cells (30). Our results showed that the control group -that is not supplemented with L.Carnitine- have lower percentage of oocytes maturation and embryos development than the groups that supplemented with 0.3 and 0.6 mg/ml of L.Carnitine, which suggests that there is equilibration between the L.Carnitine antioxidant action and the ROS production, that leads to scavenging of ROS which decreases their effect at these concentrations of L.Carnitine. The results showed that the supplementation of L.Carnitine in higher concentration, 1.2 mg/ml to the *in-vitro* culture media resulted in lowest percentage of oocytes maturation and embryos developmental progress compared with the control group and the other groups that supplemented with 0.3 and 0.6 mg/ml. (25) mentioned that the addition of excessive L.Carnitine concentration to the *in-vitro* culture media of bovine oocytes and embryos may causes increased depletion of lipids density in the cells, which could be detrimental to the oocyte maturation and the developmental progress of embryos. (36) mentioned that the addition of high concentration of L.Carnitine, 2 mg/ml to the *in-vitro* maturation media of porcine oocytes causes inhibition to the meiotic progression which leads to decrease the percentage of matured oocytes.

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