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EFFECT OF CoQ10 ADDITION TO MATURATION MEDIUM AFTER VITRIFICATION OF BOVINE OOCYTES

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ABSTRACT
This study was conducted to investigate the effect of CoQ10 on embryo production *in vitro* after vitrification / thawing of bovine oocytes. The treatment was divided into five groups (control, 0, 20, 40 and 80 µM). The percentages of oocytes viability after vitrification / thawing were 89. 62, 90.78, 90.38 and 89.72 % for 0, 20, 40 and 80 µM respectively. Results revealed that the differences were significant ($P < 0.05$) among the groups in maturation, fertilization and early embryonic development in *vitro*. The early embryonic development (2 to 4cell) was 36, 33.33 and 25 % for control, 40 µM and 0 µM respectively. On the other hand no embryonic development was detected in groups 20 and 80 µM.

KEYWORD: CoQ10, bovine oocytes, in vitro, embryo production.

INTRODUCTION

Mammalian ovaries contain a large number of oocytes enclosed in primordial follicles. Ovarian cyclic activity induces some of these follicles to initiate growth towards ovulation. However, In growing follicles, only a subset of oocytes are capable to support maturation , fertilization and early embryo development , as shown through embryo *in vitro* production (Mermillod *et al.*, 2008) Cryopreservation of oocyte and sperm is an essential tool for biotechnology, improvement of genetic breeding, and assisted reproduction technology (Leibo, 1990). Cryopreservation of embryos can be used to establish genome resource banks for non-domestic ungulates (Dixon *et al*., 1991). During cryopreservation, gametes are exposed to three types of stresses mechanical, thermal and chemical, which can result in compromised cell function and death (Smith *et al*., 2011). Vitrification is a popular method for cryopreservation of bovine embryos due to its simplicity and success rate; however, vitrification of bovine oocytes is still challenging due to their complex structure and sensitivity to chilling (Massip , 2003 ; Chang *et al*., 2009). Germinal vesicle (GV) stage bovine oocytes have homogenous lipid droplets that show little change following cooling; however their large size and low surface: volume ratio makes it difficult for water and CPAs to move across the plasma membrane (Seidel, 2006), Oocytes at the metaphase-II (MII) stage have undergone both cytoplasmic and nuclear maturation, including extrusion of first polar body and alignment of chromosomes on the meiotic spindle. Like MII oocytes, MI-stage oocytes have a metaphase-I plate and a functional spindle Parks and Ruffing (1992). Coenzyme Q10(CoQ10), also known as ubiquinone or ubidecarenone, is a lipophilic molecule classified as a fat soluble quinine (Crane, 2001). It plays an important role in cellular metabolism. It is the only lipid soluble antioxidant synthesized endogenously and is present in all cellular membranes and in blood. It also serves as an important lipid-soluble antioxidant, scavenges free radicals and inhibits oxidation of lipid as well as a membrane stabilizer. (Kwong *et al*., 2002). In addition to playing a key role in the mitochondrial electron transport chain, it is a critical Coenzyme in the synthesis of adenosine triphosphate (ATP). Therefore, those organs with the highest energy requirements such as the heart and the liver have the highest CoQ10 concentrations (Okamoto *et al.* 1989; Aberg *et al*., 1992). The aim of this study is to examine the addition of CoQ10 to maturation medium to improve the bovine in vitro embryo production before vitrification the oocyte.

MATERIALS & METHODS

The chemical materials are from Siqma Company only CoQ10 and hayluronadase from siqma company USA. The ovaries were collected from AL- Shualla local abattoir in Baghdad from cows and heifers. Ovaries were collected and placed into glass tubes contained normal saline solution (0.9% NaCl) supplemented with (100IU/mL penicillin and 100 µg/mL streptomycin), and placed at 30 - 35°C. Ovaries were transported to the laboratory of Higher Institute of Infertility Diagnosis and Assisted Reproductive Technologies at AL-Nahrain University in Baghdad within less than 2 h. (Rezk, 2009). Oocytes were collected by 18-gauge hypodermic needle attached with a sterile disposable 5-10 mL syringe contain 0.5 mL of culture medium supplemented with 20 IU/mL heparin to prevent clotting in follicular fluid. After oocyte retrieval, content of each syringe was poured into a Petri dish. Then this content examined under dissecting microscope for oocytes collection using modified pasture pipette and washed for three times with SMART medium (De Smedt *et al.*, 1992).

Preparation of CoQ10

A powder of Coenzyme Q10 (Coenzyme Q10, M.W. 863.358, Sigma, USA) was used for preparation of CoQ10. Stock solution was prepared by dissolving 0.0862 g in 1 mL of DMF (*N*,*N*-Dimethylformamide, M W 73.09, USA, Sigma).

For preparation of low concentration treated group (G2; 20µM), 2 µL of stock solution was diluted with 0.998 mL of SMART medium. However, addition 4 and 8 µL of stock solution to 0.996 and 0.992 mL of SMART medium to prepare high concentration group (G3; 40, G4; 80µM respectively).

Preparation of vitrification and thawing solutions

The equilibration solutions consisted of 7.5% (v/v) dimethyl sulphoxide (DMSO) with 7.5% (v/v) propanediol (PrOH) were prepared by adding the corresponding volume of CPA to culture medium containing 10% Bovine Serum Albumin (BSA). Vitrification solutions consisting of 15% (v/v) DMSO with 15% (v/v) PrOH which were added to culture medium supplemented with 10% BSA. Thawing solutions contain sucrose (0.5M) which was added to culture medium containing10% BSA (Kuwayama *et al.*, 2005 and Kuwayama, 2007).

Vitrification and Thawing

The vitrification and warming procedures were performed according to Kuwayama *et al.* (2005) and Kuwayama, (2007) with some modifications. Normal and viable immature oocytes or embryos (three oocytes or embryos) were equilibrated in 0.5 mL of ES at room temperature for 15 minutes. After that, they were placed into 0.5 mL of VS. Then oocytes were placed on the Cryotop strip in a small drop of VS and the Cryotop immersed into LN_2 directly. For thawing, the protective cover was removed from the Cryotop while it is still submerged in LN_2. Stepwise removal of the cryoprotectant was done by transferring the oocytes or embryos through a descending concentration of thawing solution at room temperature. The strip was immersed directly into the thawing solution of 0.5M (sucrose) for 3 minute. The thawed oocytes were washed twice with culture medium.

***In vitro* Maturation**

Oocytes were washed three times in culture medium containing 5% BSA to remove substances in follicular fluid, than about 5-10 oocytes per droplet (1mL) from culture medium allocated to this group (SMART)with different concentrations of CoQ10, supplied with 10 IU/mL hCG, 5 IU/mL PMSG and 1µg/mL Estradiol and cultured in four well Petri dish and covered their by paraffin oil was incubated for about 24 h in CO_2 incubator ($5\%CO_2$) at 38.5°C with high humidity (95%).

Preparation of sperm

For *in vitro* fertilization, frozen straws semen was taken from Artificial Insemination Department, Ministry of Agriculture. Two bulls with a proven history of successful IVF were used throughout the study. Sperm was prepared as described by Parrish *et al.* (1988). Two straws were thawed in a water bath at 38°C for 1 min. For swim-up separation of the motile fraction, the thawed semen (0.2 mL) was layered under 1 mL of Sperm-TALP that was freshly supplemented with 0.06% BSA, 0.036% heparin and 2.5 mM caffeine in eppendorf tube. The motility of the sperm after thawing was determined under a light microscope (400×). After 1 hr at 39°C under 5% CO_2 in air, 750 µL from the top of the medium of each vial was collected and pooled in a sterile centrifuge tube. The swim-up separated sperm were centrifuged at 500 RPM (36°C) for 6 min. The sperm pellet was resuspended in fresh 5 mL of Sperm-TALP medium and centrifuged again. The final sperm pellet was suspended to ~200 µL with IVF medium and incubated for 15 min at 39°C under 5% CO_2 in air. During this time sperm concentration was estimated according to Smith and Mayer, (1955) using Neubaure hemocytometer chamber. The concentration was adjusted to 50 million sperm/mL using the fertilization medium for dilution. A ~2 µL aliquot of this sperm suspension was introduced to each 100 µL fertilization droplet containing ~20 oocytes to give a final the motile spermatozoa. After activation they were added to the oocytes at the concentration of approximately 1 x 10^6 motile sperm/oocyte, then covered by liquid paraffin and incubated at 38.5°C in a moist atmosphere 5% CO_2 with high humidity for 24hr.

***In vitro* fertilization (IVF)**

The IVF medium was prepared by adding heparin and to the Fert-TALP medium. Four 100 µL droplets of this medium were placed in a 35 mm 4-well culture plates dish and overlaid with Paraffin oil. Prior to insemination, the IVM oocytes were washed three times in washing medium and transferred in groups of 20-25 to the fertilization droplets.

***In vitro* embryonic culture (IVC)**

Embryos were washed for three times by using culture medium allocated to this group (SMART) and were cultured in 1mL of the medium (SMART) in 4-well culture plates dish each 5 zygotes/well culture plates and was covered by liquid paraffin 38°C in a moist atmosphere of 5% CO_2 and 95% humidity in CO_2 incubator for 24 h.

Statistical analysis

The Statistical Analysis System- SAS (2012) was used to analyze the data. Chi-square test was used to detect the significant differences among percentages. $P \leq 0.05$ was considered significant.

RESULTS

Seven hundred and nineteen bovine oocytes were used in this experiment, in the control group the percentage of maturation, fertilization, cleavage and early embryonic development is 71.12, 55.44, 44.64 and 36 % respectively. The oocyte viability percentage after the vitrification / thawing oocytes were not differed among the treatments. In the group (20 µM), the higher percentage was found (90.78%) and the lower percentage (89.62 %) was found in group (0 µM) (Figure 1). Significant differences ($P<0.01$) in the percentages of IVM of bovine oocytes were found among the groups. The highest percentage of IVM (Figure 2), IVF (Figure 3), cleavage (Figure 4) and early embryonic development (2 and 4 cell stage) (Figure 5) was observed in group CoQ10 (40 µM) 51.77, 35.61, 42.30 and 33.33 % respectively, and the lowest percentage was (41.22, 24.07, 23.07 and 0 %) in group CoQ10 (80 µM) for IVM, IVF, cleavage and early embryonic development (2 and 4 cell stage) respectively.

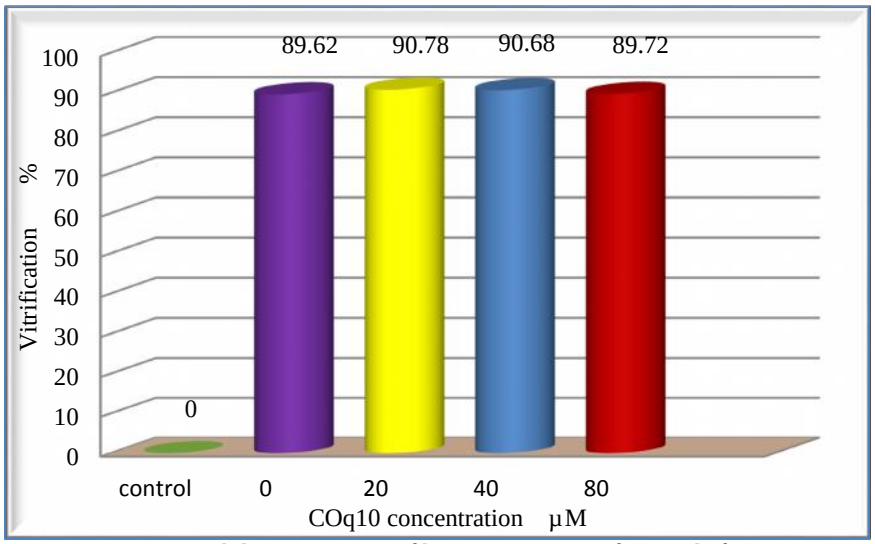

FIGURE 9: Viability percentage of bovine oocyte vitrification before IVM.
Chi-square value-- χ^2 15.093 ** Significant differences (P<0.01) ** among groups.

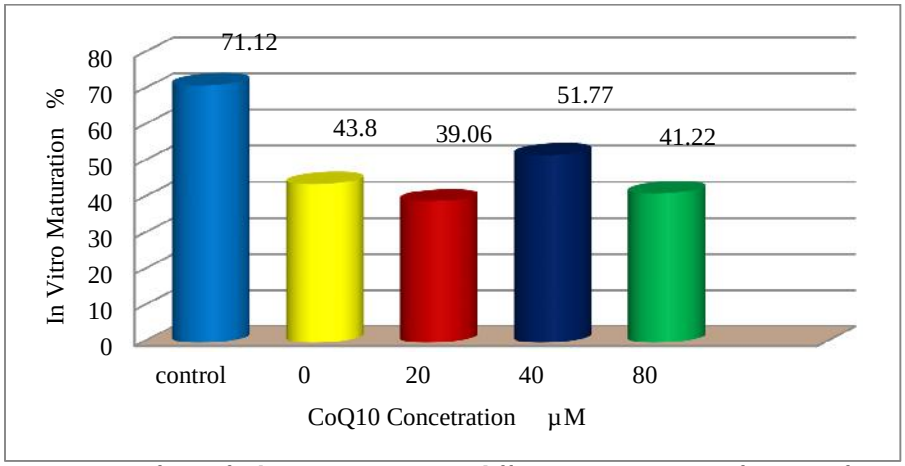

FIGURE 2: percentage of IVM for bovine oocytes using different concentration of CoQ10 after vitrification.
Chi-square value-- χ^2 8.327 ** Significant differences (P<0.01) ** among groups.

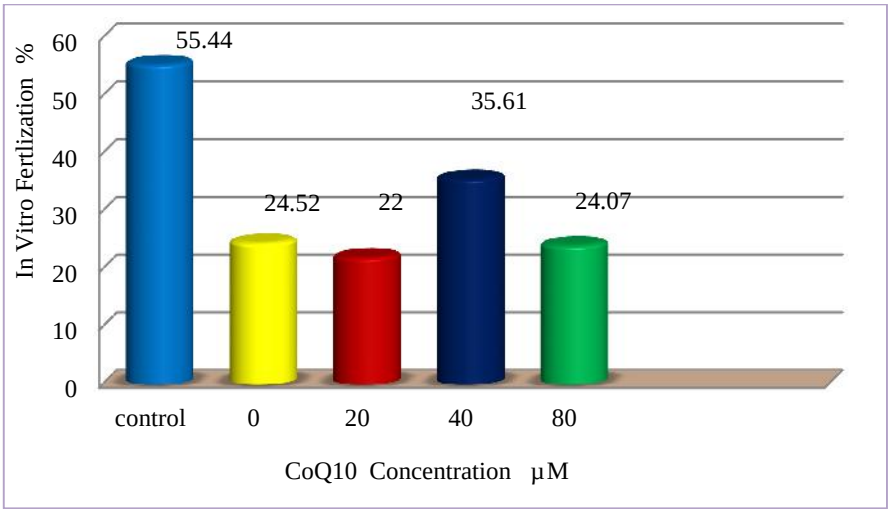

FIGURE 3**:** percentage of IVF for bovine oocytes using different concentration of after vitrification and IVM *in vitro*.
Chi-square value-- χ^2 10.806 ** Significant differences (P<0.01) ** among groups.

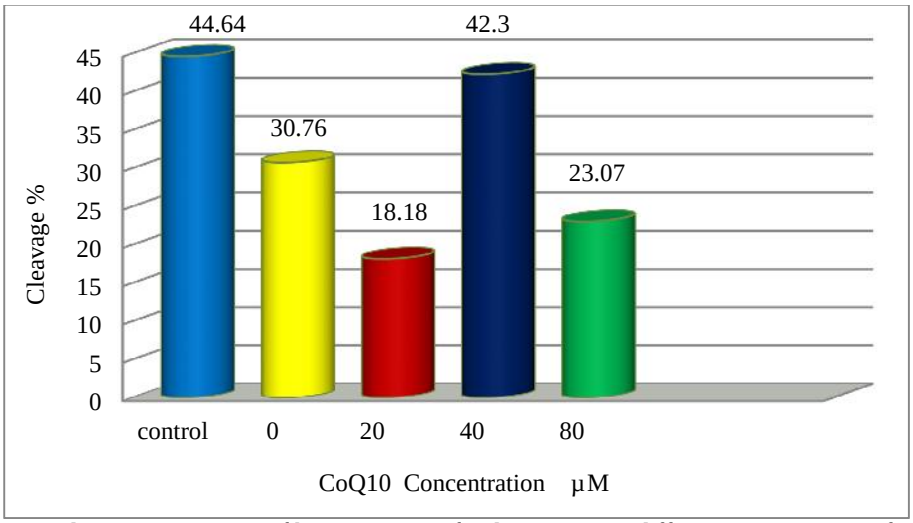

FIGURE 4: Cleavage percentage of bovine oocytes fertilization using different concentration of CoQ10. Chi-square value-- χ^2 8.559 ** Significant differences (P<0.01) ** among groups.

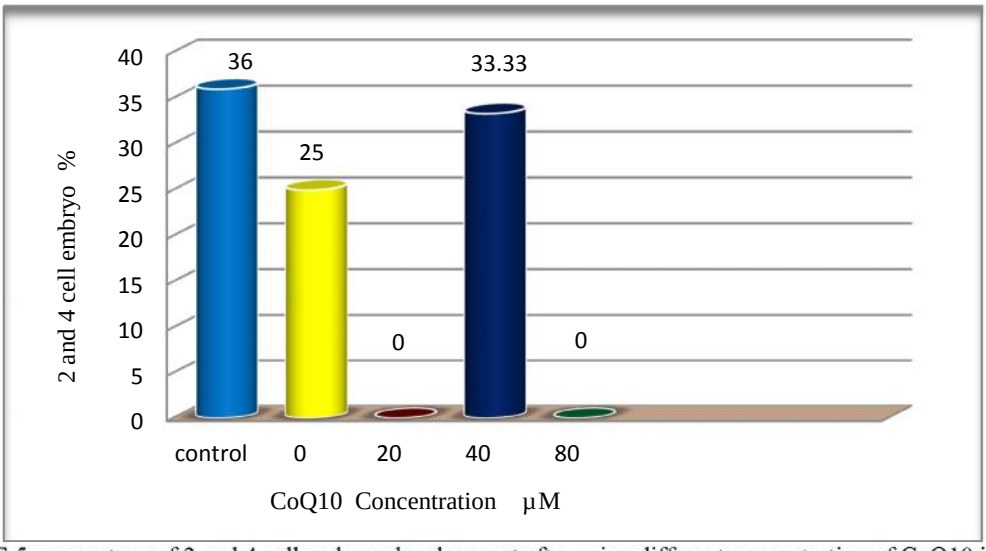

FIGURE 5: percentage of 2 and 4 cell embryo development after using different concentration of CoQ10 in culture *medium*

Chi-square value-- χ^2 9.742 ** Significant differences (P<0.01) ** among groups.

DISCUSSION

The oocytes were divided into five groups according to CoQ10 addition (control, 0, 20, 40 and 80µM) to maturation medium. Addition of CoQ10 to culture medium after vitrified/ thawing oocytes was performed to identify the ability of CoQ10 at different concentrations to reduce the stress of thawing. Non- significant differences were found among treatments (89.62, 90.78, 90.38 and 89.72 %) for 0, 20, 40 and 80 µM groups. The IVM in the control group is 71.12 %, which differed from the other groups of the treatments. The percentage of IVM is 51.77 % in the 40 µM group and the lowest percentage was found in the 20 µM treatment 39.06 %. Subsequently both of groups (20 and 80µM) had failed to reach the fertilization. MI-stage oocytes have a metaphase-I plate and a functional spindle. In contrast, chromatin of germinal vesicle (GV) oocytes is still in the diplotene phase of prophase-I without a meiotic spindle. Oocytes at the metaphase-II (MII) stage have undergone both cytoplasmic and nuclear maturation, including extrusion of first polar body and alignment of chromosomes on the meiotic spindle. The DNA of the oocyte is condensed into chromosomes that are aligned along the equatorial region of the meiotic spindle and are susceptible to disruption (Parks and Ruffing, 1992). The functional integrity of both the sperm and the oocytes are required for successful fertilization and embryo development. In most species IVF has become a valuable tool for assessing sperm functionality and for studying the success or failure of the gamete interaction. However *In vitro* fertilization was used to evaluate bull fertility (Gordon and Liu, 1990). A relationship between *in vivo* bull fertility and *in vitro* sire effects on embryonic development was suggested because of the bulls of high *in vivo* fertility, embryos that cleaved to the 2-cell stage earlier and had a higher chance of developing to the morula / blastocyst stage in culture than those sired by lower fertility bulls (Eid *et al.*, 1994). The meiotic spindle apparatus damages in the oocyte of several

species (Rojas *et al.*, 2004; Succu *et al.*, 2008). It is known that the several ultra structural of the cell affected by vitrification and structural change (Hyttel *et al.*, 2000).The temporary loss or clumping of microtubules might have been induced by vitrification on MI oocytes, because of Microtubules were well organized near the spindles in the MI and MII stages (Hochi *et al.*, 1997).

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