Coenzyme Q\textsubscript{10} Improves Developmental Competence of Mice Pre-antral Follicle Derived From Vitrified Ovary

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ABSTRACT

The aim of this study was to investigate the \textit{in vitro} development of isolated pre-antral follicles derived from vitrified ovaries in the presence of coenzyme Q\textsubscript{10} (CoQ\textsubscript{10}). Mice pre-antral follicles derived from fresh and vitrified-warmed ovarian tissues were cultured individually in α-MEM medium supplemented with or without CoQ\textsubscript{10}, followed by adding human Chorionic Gonadotropin (hCG) to induce ovulation. The follicle development parameters and ovulated oocyte maturation were assessed. The developmental parameters of pre-antral follicles and oocyte maturation rates were significantly higher in CoQ\textsubscript{10} pretreatment groups of both vitrified and fresh samples compared to the respective CoQ\textsubscript{10} free groups. CoQ\textsubscript{10} improves the \textit{in vitro} development of pre-antral follicles derived from fresh and vitrified–warmed ovaries.

Keywords: Vitrification, Ovary, Pre-antral follicles, Coenzyme Q\textsubscript{10}.

INTRODUCTION

The Ovarian tissue cryopreservation is a good strategy to preserve fertility of women who are at risk of premature ovarian failure or need chemo and radiation therapy. Ovarian tissue cryopreservation is wrapped methods because it can be done in several protocols with many variable [1-3]. However, vitrification as simple, quick and economical method is more efficient than other approaches [4].

Vitrification as a part of assisted reproduction technique (ART) induces several destructive phenomena which among them, oxidative Stress (OS) is particularly important [5, 6]. OS occur as a result of an imbalance between the systemic production of reactive oxygen species (ROS) and its removal [7] which leading to cell toxicity, DNA damage and apoptosis [8]. The development of OS is one of the main reasons for the poor development of gametes and embryos in ART [5].

In \textit{in vivo} condition, ROS constantly be neutralized by enzymatic and non-enzymatic antioxidant agents. However, a small amount of ROS is required for setting various vital functions of gametes development, as a result, the activity of ROS must be kept at a normal balance [5, 7-9]. ROS generation following \textit{in vitro} condition is unavoidable [5, 6, 9]. Therefore, supplemented culture media with antioxidants were usually used. However the use and potential benefits of antioxidants during \textit{in vitro} culture is still debate [9, 10].

Coenzyme Q\textsubscript{10} (CoQ\textsubscript{10}) are well known for antioxidant properties in both oxidized and reduced form to eliminate free-radicals [11, 12]. CoQ\textsubscript{10} is a fat-soluble vitamin like agent involved in mitochondrial respiratory chain [9, 13]. It has been shown that, there is a direct correlation between seminal plasma CoQ\textsubscript{10} concentration and semen parameters [14]. Also, the previous results showed that administration of CoQ\textsubscript{10} in the infertile women had beneficial effects [12, 13, 15]. Presence of CoQ\textsubscript{10} in the human follicular fluid has been demonstrated [13]. Although the actions of CoQ\textsubscript{10} in the follicular fluid is unclear, total CoQ\textsubscript{10} levels were higher in follicular fluids correlated with mature oocyte and high quality embryos, suggesting a possible role in mechanisms of control and growth of follicles and protective role against OS [13].
Although, several antioxidants have been investigated on in vitro development of pre-antral follicles, it has not yet been determined whether adding the CoQ$_{10}$ in culture medium affect the developmental competence of pre-antral follicles derived from fresh and vitrified warmed ovaries. Therefore, the aim of this study was to investigate the rates of developmental competence of isolated mice pre-antral follicles during in vitro culture in the presence of CoQ$_{10}$.

**MATERIALS AND METHODS**

**Reagents**

All reagents were purchased from Sigma-Aldrich UK, unless otherwise stated and all media were made with Mili-Q water.

**Animals and experimental Groups**

In this experimental study, Female Naval Medical Research Institute (NMRI) mice were purchased from Razi Vaccine and Serum Research Institute and were cared for and used according to the Animal Ethics Committee. The mice were housed and bred in the Animal House under light and temperature-controlled conditions (12 h of light and 12 h of darkness; and 24°C) and they provided with food and water ad libitum. For all experiments, mice aged 14-16 days were sacrificed by cervical dislocation and their ovaries were removed and placed in α-minimal essential medium (α-MEM; Gibco, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, UK), 2.2 g/l sodium bicarbonate, 25 mM HEPES, 100 IU/ml penicillin, and 75 µg/ml streptomycin. The ovaries were randomly divided into two main groups: (1) Fresh (control) and (2) vitrified groups. Isolated pre-antral follicles of each groups were cultured in the presence or absence of CoQ$_{10}$ for evaluation of development parameters.

**Ovarian tissue cryopreservation and thawing**

The vitrification procedure was based on the method described previously [16] with some modifications. The ovary was placed in an equilibrium solution containing 7.5% v/v ethylene glycol (EG), 7.5% v/v dimethyl sulfoxide (DMSO) and 20% FBS in Dulbecco’s phosphate-buffered saline (DPBS) medium for 10 min and then they were placed for 2 min in the vitrification solution containing 15% v/v EG, 15% v/v DMSO, 20% FBS and 0.5M sucrose in DPBS medium. Then ovaries were placed in a Cryovial carrier and transferred to liquid nitrogen (LN2) immediately. All equilibration and vitrification steps were carried out at room temperature. For warming, the ovaries were left in the warming solution (1M sucrose in DPBS) for 5 min and transferred into droplets of DPBS medium containing 0.5 and 0.25M sucrose at an interval of 5 min at room temperature. Finally, ovaries were placed into fresh α-MEM medium supplemented with 10% FBS at 37°C in CO$_2$ incubator for another 20 min before submitted to isolation of their pre-antral follicle.

**Pre-antral follicle isolation**

Pre-antral follicles derived from fresh and vitrified-warmed ovaries were isolated mechanically as described previously [17] by using a 29 gauge needle under a stereomicroscope (Nikon, Japan) at 10× magnification. Isolated follicles were selected according to the following criteria: rounded structure with 140-160 µm diameters, containing visible centrally located oocyte surrounded with intact several layers of granulosa cells, intact basement membrane, at least one layer of theca cells and without antral cavity. During the operating procedures the culture medium was always kept at 37°C.

**In vitro cultured and assessment of developmental parameters:**

Isolated pre-antral follicles from fresh and vitrified ovaries were cultured in 25 µl drops of α-MEM medium supplemented with 5% FBS, 100mIU/ml recombinant human follicle stimulating hormone (rhFSH), 1% Insulin-transferin-selenium (ITS), 10ng/ml Epidermal growth factor (EGF), 100 IU/ml penicillin, 75 µg/ml streptomycin, 2.2g/l sodium bicarbonate and 50µM CoQ$_{10}$ under mineral oil in a humidified atmosphere of 5% CO$_2$ in air at 37°C in Falcon Petri dishes (60 x 15 mm) for up to 12 days. At 48h intervals, 15µl of culture medium from each drop was replaced by fresh medium.

**Assessment of developmental and morphological parameters**

The survival rate of cultured pre-antral follicles was assessed microscopically based on the morphology of the pre-antral follicle every
other day as described previously [18]. Briefly, follicles were considered as normal which had a central oocyte and several layers of granulose and theca cells and intact basement membrane without any signs of degeneration, such as dark oocyte or cumulus cells. Visible lustrous area in the granulosa cell mass around the oocyte considered as antral-like cavity formation. Follicles diameter was measured only in healthy follicles on day 2 and 4 of culturing period by measuring and averaging two perpendicular cross sectional diameters of each follicle at 100x magnification with pre-calibrated ocular micrometer under inverted microscope (Nikon, Tokyo, Japan).

**Induction of ovulation**

At twelfth day of cultivation period, ovulation was induced by changing culture medium containing 1.5 IU/ml human chorionic gonadotropin (hCG). 24-36h after that, ovulation was assessed and released oocyte was classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD) when the GV was absent, and metaphase II oocytes (MII) when the first polar body was extruded.

**Statistical analysis**

Statistical analysis was performed by using SPSS-ver.16 software package (SPSS Inc., Chicago, IL, USA). Data were statically analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD was used as post hoc test. Significant differences were considered at level of P < 0.05. Each experiment was carried out at least three replicates.

**RESULTS**

The mean diameter and rates of developmental parameters of isolated pre-antral follicles derived from vitrified and non-vitrified ovaries with or without pretreatment of CoQ10 are summarized in Tables I and II. Pre-antral follicles at different stage of development can be seen in Fig1. Every other day, the cultured follicles were morphologically evaluated. During culture period, pre-antral follicles initially became attached to the culture dish through proliferation of their granulosa and theca cells. After the fourth day, the follicles were out from the symmetric mode and form an irregular and diffuse appearance so that it was impossible to measure its diameter. Phenomenon analogous to ovulation occurred at 16-24 hours after hCG administration (Figure 1).

There were no significant difference among the mean diameter of pre-antral follicles of all groups (p<0.05) at the initial time of culturing. While, the diameter of pre-antral follicle is increased progressively during the culture period. As shown in Table I, the diameter of isolated pre-antral follicle derived from fresh ovaries (Control) on 2th and 4th day of culture period (229 and 290 µm respectively) was significantly higher than those of isolated pre-antral follicles derived from vitrified-warmed ovaries (161 and 178 µm respectively; P<0.05). The mean diameter of the fresh pre-antral follicles in the presence of CoQ10 on day 2 and 4 (280 and 345µm respectively) was significantly higher in comparison with other groups (P<0.05). The mean diameter of isolated pre-antral follicles derived from vitrified-warmed ovaries with pre-treatment of CoQ10 at 2th and 4th day (187 and 223 µm respectively) were significantly greater than those of isolated pre-antral follicles derived from vitrified ovaries in the absence of CoQ10 (178 and 161 µm respectively) while, these were significantly lower than those of pre-antral follicle derived from fresh ovaries (P<0.05).

The rates of survival (75%), antrum formation (62%) and ovulation (40%) of the fresh pre-antral follicles were significantly higher than those of pre-antral follicles derived from vitrified-warmed ovaries (48%, 58% and 31% respectively; P<0.05). Whereas, these rates of pre-antral follicles derived from vitrified-warmed ovaries which were cultured in the presence of CoQ10 (Survival rate; 65%, antrum formation; 58% and ovulation 31%) were similar to those of fresh pre-antral follicles (P<0.05). Also the highest rates of survival (91%), antrum formation (86%) and ovulation (71%) were obtained from fresh pre-antral follicles with pre-treatment of CoQ10 (Table 2). Percentage of MII oocytes resulting from fresh pre-antral follicles (30%) was significantly higher than those of pre-antral follicles derived from vitrified-warmed ovaries (14.0%; P<0.05), although both were statistically lower than that of fresh pre-antral follicles which were cultured in the presence of CoQ10 (48%; P<0.05). The percentages of MII oocytes were
similar between pre-antral follicles derived from vitrified-warmed ovaries with CoQ\textsubscript{10} pretreatment (26%) and that of control groups (30%; P<0.05).

Table 1. Diameter of cultured pre-antral follicles derived from fresh and vitrified ovaries in the presence of CoQ\textsubscript{10}

<table>
<thead>
<tr>
<th>Groups</th>
<th>NO. of Follicles</th>
<th>0\textsuperscript{th} day</th>
<th>2\textsuperscript{nd} day</th>
<th>4\textsuperscript{th} day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh (Control)</td>
<td>78</td>
<td>154.0±6.7</td>
<td>229.2±53.1\textsuperscript{a}</td>
<td>290.6±93.7\textsuperscript{a}</td>
</tr>
<tr>
<td>Fresh +CoQ\textsubscript{10}</td>
<td>45</td>
<td>152.1±6.8</td>
<td>280.5±31.4\textsuperscript{b}</td>
<td>345.7±56.0\textsuperscript{b}</td>
</tr>
<tr>
<td>Vitrified</td>
<td>87</td>
<td>153.4±5.3</td>
<td>161.1±14.2\textsuperscript{a}</td>
<td>178.7±17.7\textsuperscript{a}</td>
</tr>
<tr>
<td>Vitrified +CoQ\textsubscript{10}</td>
<td>60</td>
<td>152.9±5.4</td>
<td>187.9±21.6\textsuperscript{a}</td>
<td>223.7±27.1\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Different superscripts in the same column reflect different levels of significant difference (P<0.05)

CoQ\textsubscript{10}: Coenzyme Q\textsubscript{10}

Figure 1. Graphic representation of the in vitro cultured mouse pre-antral follicles
Pre-antral follicle on initial day (A), day 8 (B), day 12 (C), The MII oocyte ovulated after hCG stimulus on day 12 (D)
Antral-like cavities are indicated by black arrow.
Ovulated MII oocyte indicated by white arrow.

Table 2. Maturation rates of cultured pre-antral follicles derived from fresh and vitrified ovaries in the presence of CoQ\textsubscript{10}

<table>
<thead>
<tr>
<th>Groups</th>
<th>NO. of Follicles</th>
<th>Survived (% ± SD)</th>
<th>Degenerated (% ± SD)</th>
<th>Antrum Formation (% ± SD)</th>
<th>Ovulated Follicles (% ± SD)</th>
<th>MI oocytes (% ± SD)</th>
<th>MII Oocytes (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh (Control)</td>
<td>78</td>
<td>(75.78±3.88)\textsuperscript{a}</td>
<td>(24.22±3.88)\textsuperscript{a}</td>
<td>(62.00±8.69)\textsuperscript{a}</td>
<td>(40.89±1.12)\textsuperscript{a}</td>
<td>(11.55±2.89)\textsuperscript{a}</td>
<td>(30.67±3.65)\textsuperscript{a}</td>
</tr>
<tr>
<td>Fresh +CoQ\textsubscript{10}</td>
<td>45</td>
<td>(91.11±3.85)\textsuperscript{a}</td>
<td>(8.89±3.85)\textsuperscript{a}</td>
<td>(86.67±6.67)\textsuperscript{a}</td>
<td>(71.11±7.70)\textsuperscript{a}</td>
<td>(22.22±3.85)\textsuperscript{a}</td>
<td>(48.89±3.85)\textsuperscript{a}</td>
</tr>
<tr>
<td>Vitrified</td>
<td>87</td>
<td>(48.80±7.45)\textsuperscript{a}</td>
<td>(51.20±7.45)\textsuperscript{a}</td>
<td>(44.64±3.90)\textsuperscript{a}</td>
<td>(20.77±4.57)\textsuperscript{a}</td>
<td>(5.94±3.99)\textsuperscript{a}</td>
<td>(14.82±1.49)\textsuperscript{a}</td>
</tr>
<tr>
<td>Vitrified +CoQ\textsubscript{10}</td>
<td>60</td>
<td>(65.00±6.38)\textsuperscript{a}</td>
<td>(35.00±6.38)\textsuperscript{a}</td>
<td>(58.33±6.39)\textsuperscript{a}</td>
<td>(31.67±11.39)\textsuperscript{a}</td>
<td>(5.00±6.38)\textsuperscript{a}</td>
<td>(26.67±4.44)\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Different superscripts in the same column reflect different levels of significant difference (P<0.05)
CoQ\textsubscript{10}: Coenzyme Q\textsubscript{10}; MI: metaphase I oocyte; MII: metaphase II oocyte.
DISCUSSION

Cryopreservation of ovarian tissue is a promising technology for fertility preservation. But there are many difficulties ahead in this approach. Cryopreservation change the cell membrane, cytoskeletal structure and mitochondrial distribution and depolarization which in turn lead to increasing the production of ROS and inducing OS [19, 20]. It has been shown that mitochondrial defects are accompanying with failures in development of oocyte and embryos [21, 22]. Also, it has been shown that the ROS production of pre-antral follicles derived from vitrified and non-vitrified ovaries during in vitro culture periods had a tendency to be increased, whereas, ROS concentration of fresh pre-antral follicles were significantly lower than those of vitrified samples [18, 23]. It was suggested that mitochondrial electron-transport chain is a major source of ROS production [24]. Production of ROS in the in vitro culture is an unavoidable event, because exogenous factors such as visible light, oxygen concentration, handling and etc. as well as endogenous sources of ROS originating from cellular metabolism induce production ROS [9, 25]. Both enzymatic and non-enzymatic antioxidants form a front line of defense against cell damage caused by ROS [26]. It has been shown that the addition of various natural or synthetic antioxidants prevent or attenuate the OS in in vitro culture condition [10, 17, 18, 23, 27, 28]. In this regard, it has been demonstrated that supplemented medium with sodium selenite improved the rates of survival and developmental competence the pre-antral follicles via reduction of ROS [10]. Also, results from another studies indicated that the presence of alpha lipoic acid (ALA) in pre antral culture medium could enhance the rates of developmental parameters [17, 18]. These observations are in agreement with our results that showed CoQ_{10} as a potent antioxidant could improve the rates of survival, antrum formation and MII oocytes of pre-antral follicles derived from vitrified and fresh ovaries after long-term in vitro culture.

CoQ_{10} as a major cellular antioxidant is located in the mitochondrial respiratory chain [29] which transports electrons from complexes I and II to complex III which in turn lead to the stability of complex III and subsequent production of energy [22]. Whereas, not all aspects of CoQ_{10}’s mechanisms are completely understood. It has been shown that CoQ_{10} can act as a superior antioxidant against OS damages [30]. CoQ_{10} plays an important role in prevention of lipid peroxidation. Also CoQ_{10} reacts with superoxide and reduce to ubisemiquinone. Other ROS also react with CoQ_{10}, while the biological importance of these reactions is unclear [30]. The previous results showed that CoQ_{10} caused progress of oocyte maturation through changing in pattern of mitochondrial distribution and polarization, the polarity of the membrane and expression of genes that involved in the electron transport chain [15]. It has been demonstrated that administration of CoQ_{10} for aged mice leads to increasing the number of ovulated oocytes via improvement of oocyte mitochondrial function [31]. In another study showed that CoQ_{10} supplementation in infertile men, increased fertilization rates in the subsequent in vitro fertilization cycle [14]. It has been also found that supplemented media with CoQ_{10} increased spermatozoa motility [14]. CoQ_{10} intake in men who have idiopathic infertility improved acrosome reaction [32] and fertility rates [33]. CoQ_{10} may acts as a potent gene regulator and exert its effects through the induction of gene transcription involved in cell signaling and metabolism [34]. Also, it has been shown that CoQ_{10} is directly involved in apoptotic pathway and attributed it to its antioxidant properties [35].

In conclusion, present study demonstrate that supplementation of culture medium with CoQ_{10} has positive effects on development of mice pre-antral follicle. These effects may be due to at least in part to antioxidant properties of CoQ_{10}. Although more studies are needed to be continue.

ACKNOWLEDGEMENTS

This study was supported in part by grants from the Damghan University and institute of biological sciences of Damghan University. The authors are grateful for Mr. Abutaleb Koosha for their technical assistance.

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