The role of Coenzyme Q_{10} on the Total Antioxidant Capacity of Mouse Vitrified Pre-Antral Follicles

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ABSTRACT

Occurrence of oxidative stress (OS) following *in vitro* culture is inescapable. Therefore, the aim of the present study was to determine the efficacy of Coenzyme Q_{10} (CoQ_{10}) supplementation medium on developments and Total Antioxidant Capacity (TAC) of mouse vitrified preantral follicles. Fresh and vitrified-warmed isolated preantral follicles from ovaries of 14-16 day-old mice were cultured in supplemented α- MEM medium with or without CoQ_{10}. On the twelfth day of culture period, ovulation was induced by adding 1.5 IU/ml Human Chorionic Gonadotropin (hCG). Rates of survival, antrum formation and developmental stages of released oocytes (GV, MI, MII) were evaluated. Separately, Total Antioxidant Capacity (TAC) levels were measured at initial time, 24, 48, 72 and 96 hours of culture period by Ferric Reducing/Antioxidant Power (FRAP) assay. The results showed that the mean diameter and rates of survival, antrum formation, ovulation and MII oocytes of fresh and vitrified-warmed isolated preantral follicles with pretreatment of CoQ_{10} were significantly higher than those of respective groups without pretreatment of CoQ_{10}. TAC levels significantly increased up to 96 hours in fresh and vitrified-warmed preantral follicles with pretreatment of CoQ_{10} compared with those which were cultured without CoQ_{10}. Supplemented culture medium with CoQ_{10} promotes TAC levels and development of both fresh and vitrified-warmed isolated preantral follicles.

Key words: Vitrification; Coenzyme Q_{10}; Preantral Follicles; Total Antioxidant Capacity.

INTRODUCTION

Many efforts have been made to improve ovarian tissue cryopreservation [1]. Cryopreservation of isolated ovarian follicles, as an alternative option, showed more advantages than ovarian tissue cryopreservation [2]. For example, the high ratio of volume to surface area of ovarian tissue causes temperature transition to derive slowly from the central region of it which in turn leads to ice crystal formation. Also, permeation of cryoprotecant agents (CPA) occur more easily and faster in follicles compared to ovarian tissue which reduce exposure time to CPA and decrease toxicity possibility. In addition, basal lamina of isolated follicles acts as a barrier to prevent cancer cell infiltration, therefore, decreases the risk of cancer cells return in women who undergo chemotherapy or radiation for malignant diseases [2-5].

In both conditions, ovarian tissue and follicle cryopreservation, follicles should be matured either *in vivo* or *in vitro* to achieve fertilizable oocyte for restoring fertility. Thus, *in vitro* maturation (IVM) systems must be improved. In recent years, several culture systems have been made to enhance developmental competence of isolated follicles derived from vitrified-warmed and fresh ovaries [2, 6-12]. Among the various factors that affect the developmental competence of *in vitro* cultured preantral follicles, production of Reactive Oxygen Species (ROS) is of particular importance [13, 14]. Although ROS is produced during normal cellular metabolism and acts as a signaling molecule in some

physiological processes, the excessive production of it leads to Oxidative Stress (OS) which can eventually cause damage to cells [15]. Excessive generation of ROS in the *in vitro* culture condition is a predictable event, because there are many exogenous factors such as visible light, oxygen concentration, handling etc., which induce ROS production [16-18]. It has been also shown that cryopreservation increases ROS generation [5, 6, 12].

Under the optimal *in vivo* conditions, excessive generation of ROS is neutralized by antioxidant capacity, thus ROS and antioxidants are in balance condition [19]. Total Antioxidant Capacity (TAC) of follicular fluid has been considered as a predictive marker of successful *in vitro* fertilization (IVF) [20]. It has demonstrated that TAC concentrations of follicular fluids were associated with higher fertilization rate [21]. Therefore, evaluation of TAC during *in vitro* culture is important for the evaluation of quality of cultured follicles [2, 6, 11, 12]. Recently, world wide concerns have been raised about preventing any side effects of OS by supplementing media with various antioxidants to enhance developmental competence of follicle, oocyte and embryo in *in vitro* conditions [2, 8, 11, 12, 22, 23].

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble quinon, which is found in all living cells and is structurally similar to vitamin K. CoQ_{10} has a vital role in mitochondrial electron transport chain system to synthesis adenosine triphosphate (ATP). Moreover, CoQ_{10} acts as an antioxidant to prevent the lipid peroxidation to stabilize cell membranes and preserve cell functions [24-26].

Until now, there have been no reports indicating the effects of CoQ_{10} on the developmental competence and TAC levels of fresh and vitrified-warmed preantral follicles. Given this information, the objective of the present study was to investigate the possible effects of CoQ_{10} supplementation during *in vitro* culture of mouse fresh and vitrified-warmed preantral follicles on developmental competence and TAC levels.

MATERIALS AND METHODS
Reagents
Alpha Minimum Essential Medium (α-MEM), Dulbecco phosphate-buffered saline medium (DPBS) and fetal bovine serum (FBS) were purchased from Gibco, UK. Unless otherwise specifically noted, all other chemicals were purchased from Sigma-Aldrich. Also all media were made with Mili-Q water.

Animals
In this experimental study, female *Naval Medical Research Institute* (NMRI) mice were obtained from *Razi Vaccine and Serum Research Institute* and were maintained in controlled conditions at 12–12-h light–dark cycles and temperature of $24^{\circ C}$. All procedures for animal use and care were approved by the Institutional Animal Care and Use Committee (IACUC) at Damghan University, and every effort was made to minimize animal distress.

Isolation of follicles and experimental groups
Animals were sacrificed by cervical dislocation before dissection of ovaries. Ovaries were placed in α-MEM medium supplemented with 0.22 g/l Sodium bicarbonate, 1% (v/v) penicillin and streptomycin, 10% FBS and 25 mM HEPES and incubated at 37°C in 5% CO_2 air for 20 minutes to equilibration.

Preantral follicles were isolated mechanically by using two 29G needles connected to insulin syringe under a stereomicroscope (Nikon-Japan). Preantral follicles with a diameter of 145-160µm containing a healthy germinal vesicle oocyte with 2-3 layers of granulosa cells and a theca layer were selected and randomly divided into two main groups: (1) Fresh (control) and (2) vitrified groups. Preantral follicles of each group were cultured with or without pretreatment of CoQ_{10}. Some of the preantral follicles of each group were used for maturation evaluation, and the others were considered for TAC analysis separately.

Vitrification and warming
The vitrification and warming procedures were based on the method described previously [2]. Briefly, isolated preantral follicles were initially incubated for 5 minutes in Equilibration Solution (ES) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) in DPBS with 20% FBS, then incubated in vitrification solution (VS; 15% EG, 15% DMSO and 0.5M sucrose and 20% FBS in DPBS) for another 30 seconds. After dehydration, preantral follicles were immediately drawn into a Pasteur pipette with minimum volume of VS (< 0.1µl)

and were individually placed on top of a polypropylene strip of cryotop carrier (Kitazato, Japan) and immediately submerged into liquid nitrogen (LN2). The thin strip of cryotop was covered with a cap and stored in LN2 tank for at least one week. All equilibration and vitrification steps were carried out at room temperature. For warming, the cap of cryotop was removed while it was still submerged in LN2, then the strip directly immersed in DPBS medium containing 1M sucrose. The preantral follicles were left in the warming solution (1M sucrose in DPBS) for 30 seconds and transfered into droplets of DPBS medium containing 0.5 and 0.25M sucrose at an interval of 3 minutes at room temperature. After that, preantral follicles were incubated into fresh α-MEM medium supplemented with 10% FBS at 37°C and 5% CO2 for another 10 minutes before being placed in culture medium.

In vitro culture of preantral follicles and ovulation induction

The isolated preantral follicles were placed separately in 20μL of α-MEM drops supplemented with 2.2g/l sodium bicarbonate, 5% FBS, 0.1 IU/ml rhFSH (recombinant human follicle stimulating factor), 1% ITS (Insulin-transferin-selenium), 10 ng/ml EGF (Epidermal Growth Factor) and 1% (v/v) penicillin and streptomycin with or without 50 μM CoQ_{10} overlaid with mineral oil in 60-mm culture dishes and incubated at 37°C in 5% CO_2 air. On the following day half the medium (10 μl) was replaced with the fresh medium every other day till day 12.

During cultivation period, follicle diameter was measured every other day until 4 days as the average of two perpendicular cross-sectional diameters between the outer edges of the basement membrane. Survival of follicles were analyzed morphologically with an inverted microscope (Nikone, Japan). A follicle was considered to be undamaged if it possessed a symmetrical oocyte surrounded by several layers of granulosa cells and intact basement membrane. Any dark-looking follicles with partially or completely naked oocytes, large spaces within the granulosa-oocyte complex, were graded as damaged. Visible lucent area in the granulosa cell mass around the oocyte of intact follicles was considered as antral-like cavity formation.

On the twelfth day, preantral follicles were submitted to induce ovulation and oocyte maturation. The ovulation medium consisted of α-MEM supplemented with 1.5 IU/ml hCG (human Chorionic Gonadotropin), 2.2g/l sodium bicarbonate, 5% FBS, 100 mIU/mL FSH, 1% ITS, 10 ng/mL EGF and 1% (v/v) penicillin and streptomycin. After 16–18 hours, Oocytes were assessed under an inverted microscope and graded for nuclear maturity. Oocytes have a clear germinal vesicle (GV) graded as GV; disappearance of the clear GV assessed as GV breakdown or metaphase I (GVBD or MI) oocytes and metaphase II (MII) oocytes were regarded by the appearance of first polar body.

Total Antioxidant Capacity (TAC) assay

The total antioxidant capacity of preantral follicles extracts were done by Ferric Reducing/Antioxidant Power (FRAP) assay according to Benzie and Strain [27]. Briefly, 15 preantral follicles were collected from each experimental group at different times of culture period (0, 24, 48, 72 and 96 hours) and homogenized with sonication at 50W for 1 minute and centrifuged at 10000 *g* for 20 minute at 4°C to prepare cellular supernatant. 100μl of cellular supernatant was added to 1 ml of fresh Ferric Reducing Antioxidant Power reagent (FRAP; Tripiridyl triazine; Merck) and incubated in 37°C for 10 minutes at dark condition. Reading of the blue-colored reagent was then taken at 595 nm every 20 second for 10 minutes. The standard curve was prepared using Fe^{2+} in the range of 100–1000 mM. FRAP value was expressed as mmol /ml. All experiments were repeated at least three times and the mean values were calculated.

Statistical analysis

All results are presented as mean ± S.D. SPSS ver.22 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of results. Statistical analyses were performed by one-way analysis of variance (ANOVA) and Tukey's HSD was used as post hoc test. The values of $p < 0.05$ were considered significant.

RESULTS

Fresh and vitrified-warm pre-antral follicles were cultured for 12 days with or without pretreatment of CoQ_{10}. Pre-antral follicles at different stages of growth were illustrated in

Figure 1. After four days of culture, proliferation of granulosa cells led to scatter and irregular appearance of preantral follicle. Between 6-8 days of cultivation period, lucent spaces among the granulosa cells were considered as antral cavity (Fig.1). The mean diameter and rates of survival, antral cavity formation, ovulation and developmental stages of ovulated oocytes were summarized in Table 1 and 2. The changes of TAC during culture period was showed in Figure2.

The mean diameter of preantral follicles on the initial time of cultivation period showed no significant differences among the various groups (Table 1; P<0.05). Vitrified-warmed preantral follicles diameter on the 2nd and 4th days (176.6 µm and 204.9 µm respectively) of culture period were significantly lower than those of fresh preantral follicles (229.2µm and 290.6µm respectively; Table 1; P<0.05). On the second and fourth days, the mean diameter of the fresh (280.5 µm and 345.7 µm respectively) and vitrified-warmed preantral follicles (201.0 µm and 247.8 µm respectively) with pretreatment of CoQ_{10} increased significantly compared to those of respective groups without CoQ_{10} (Table 1; P<0.05). The rates of survival (75.78%), antrum formation (62%) and ovulation (40.89%) of fresh preantral follicles were significantly higher than those of vitrified-warmed preantral follicles without pretreatment of CoQ_{10} (55.56%, 55.23% and 22.95% respectively; Table2; P<0.05). Whereas, these rates of vitrified-warmed preantral follicles with CoQ_{10} (77.78%, 75.56% and 44.44% respectively) significantly increased in comparison with vitrified-warmed preantral follicles without CoQ_{10} (P<0.05). However, there were no significant differences between these rates of vitrified-warmed preantral follicles with CoQ_{10} and those of fresh preantral follicles (Control; P<0.05). Also the highest rates of survival (91.11%), antrum formation (86.67%) and ovulation (71.11%) were obtained from fresh preantral follicles with pretreatment of CoQ_{10} (P<0.05; Table 2). Percentage of MII oocytes (30.67%) from the fresh preantral follicles after stimulation with hCG on day 12 was significantly higher than those of vitrified-warmed preantral follicles (21.62%; P<0.05), whereas, both of them were statistically lower than that of fresh preantral follicles with pretreatment of CoQ_{10} (48.89%; Table2; P<0.05). There was no significant difference between rate of MII oocytes of vitrified-warmed preantral follicles with CoQ_{10} pretreatment (37.78%) and that of control groups (Table2; P<0.05).

TAC levels in vitrified-warmed preantral follicles after 0, 24, 48, 72 and 96 hours of culture period with or without pretreatment of CoQ_{10} were shown in Figure 2. TAC levels significantly decreased in the fresh preantral follicles (control) during the culture period up to 48 hours (P<0.05) and remained constant without significant difference up to 96 hours. Whereas TAC levels increased significantly after 24 hours up to 96 hours in fresh preantral follicles with pretreatment of CoQ_{10}. TAC levels in vitrified-warmed preantral follicles and preantral follicles significantly decreased from initial time up to 72 hours; after that, increased significantly up to 96 hours. While, in CoQ_{10} pretreatment vitrified-warmed preantral follicles, TAC levels increased significantly up to 72 hours and remained constant without significant changes up to 96 hours (P<0.05). At the beginning of culture, there were no significant difference among TAC levels of all groups. At 24 hours, TAC levels of fresh preantral follicles with CoQ_{10} were significantly higher than those of other groups; whereas, TAC levels were similar in fresh, vitrified-warmed preantral follicles and CoQ_{10} pretreatment vitrified-warmed preantral follicles at the same time (P< 0.05).

At 48 hours, the TAC levels of fresh and vitrified-warmed preantral follicles with pretreatment of CoQ_{10} were significantly higher than those of corresponding groups without CoQ_{10}. These conditions were also observed at 96 hours. There was no significant difference between TAC levels of fresh and vitrified-warmed preantral follicle at 72 hours. However, these levels were significantly lower than those of fresh and vitrified-warmed preantral follicle with pretreatment of CoQ_{10} (P<0.05).

Groups	NO. of Follicles	Follicle Diameter (um ± SD)		
		0th day	2th day	4th day
Fresh Preantral follicles	78	154.0±6.7	229.2±53.1[a]	290.6±93.7[a]
Fresh Preantral Follicles+COQ10	45	152.1±6.8	280.5±31.4[b]	345.7±56.0[b]
Vitrified Preantral Follicles	83	154.2±6.9	176.6±10.0[c]	204.9±12.1[c]
Vitrified Preantral Follicles+COQ10	45	152.8±4.8	201.0±16.8[d]	247.8±30.0[d]

Table 1. Diameter of cultured fresh and vitrified preantral follicles in the presence of COQ_{10}

In all cases at least 3 experimental replicates were performed.
Different superscripts in the same column reflect different levels of significant difference (P<0.05)
COQ_{10}: Coenzyme Q_{10}

Table 2. Maturation rates of cultured fresh and vitrified pre-antral follicles in the presence of COQ_{10}

Groups	NO. of Follicles	Survived (% ± SD)	Degenerated (% ± SD)	Antrum Formation (% ± SD)	Ovulated Follicles (% ± SD)	MI oocytes (% ± SD)	MII Oocytes (% ± SD)
Fresh Preantral follicles	78	(75.78±3.88)[a]	(24.22±3.88)[a]	(62.00±8.69)[a]	(40.89±5.12)[a]	(11.55±2.89)[a]	(30.67±3.65)[a]
Fresh Preantral Follicles+CoQ_{10}	45	(91.11±3.85)[b]	(8.89±3.85)[b]	(86.67±6.67)[b]	(71.11±7.70)[b]	(22.22±3.85)[b]	(48.89±3.85)[b]
Vitrified Preantral Follicles	83	(59.56±8.07)[c]	(40.44±8.07)[c]	(55.23±6.67)[c]	(22.95±2.98)[c]	(1.33±2.98)[c]	(21.62±2.31)[c]
Vitrified Preantral Follicles+ CoQ_{10}	45	(77.78±3.85)[a]	(22.22±3.85)[a]	(75.56±13.88)[a]	(44.44±7.70)[a]	(11.11±3.85)[a]	(37.78±3.85)[a]

In all cases at least 3 experimental replicates were performed.
Different superscripts in the same column reflect different levels of significant difference (P<0.05)
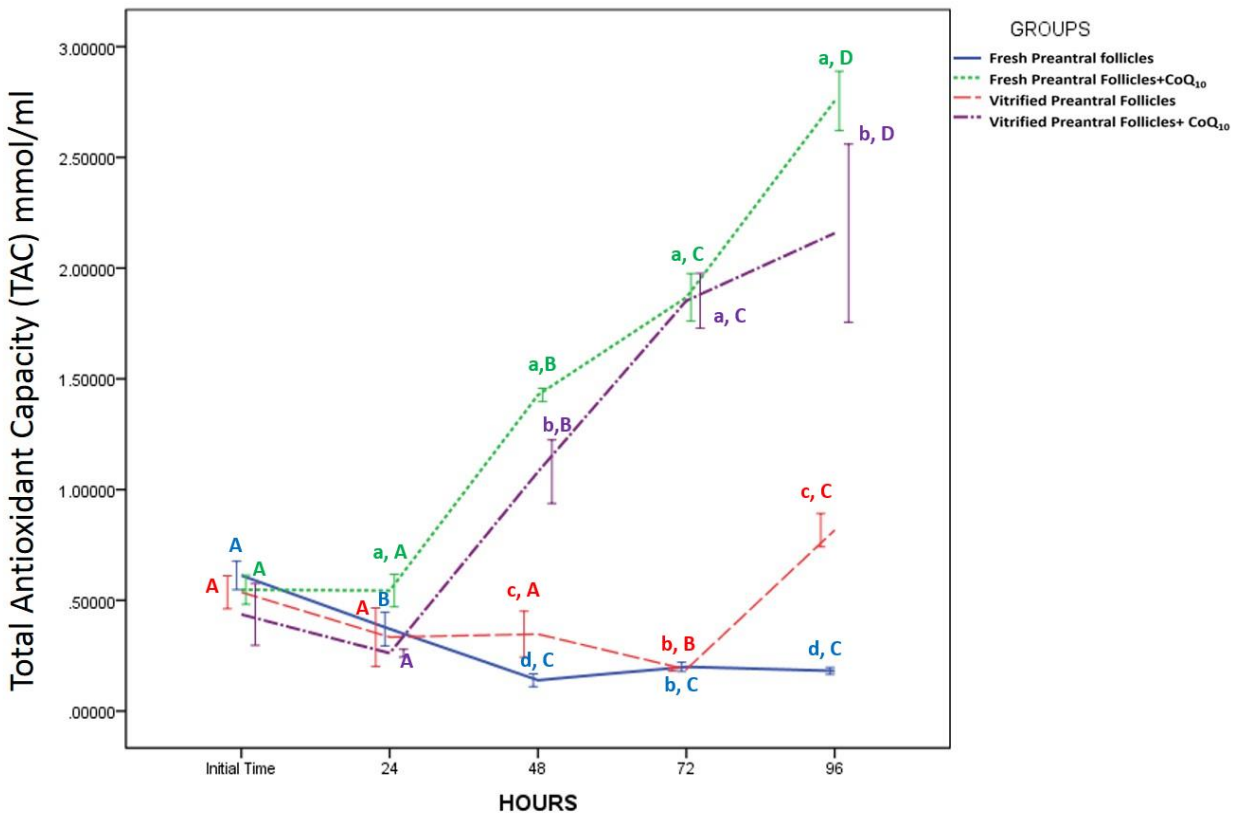COQ_{10}: Coenzyme Q_{10}; MI: metaphase I oocyte; MII: metaphase II oocyte.
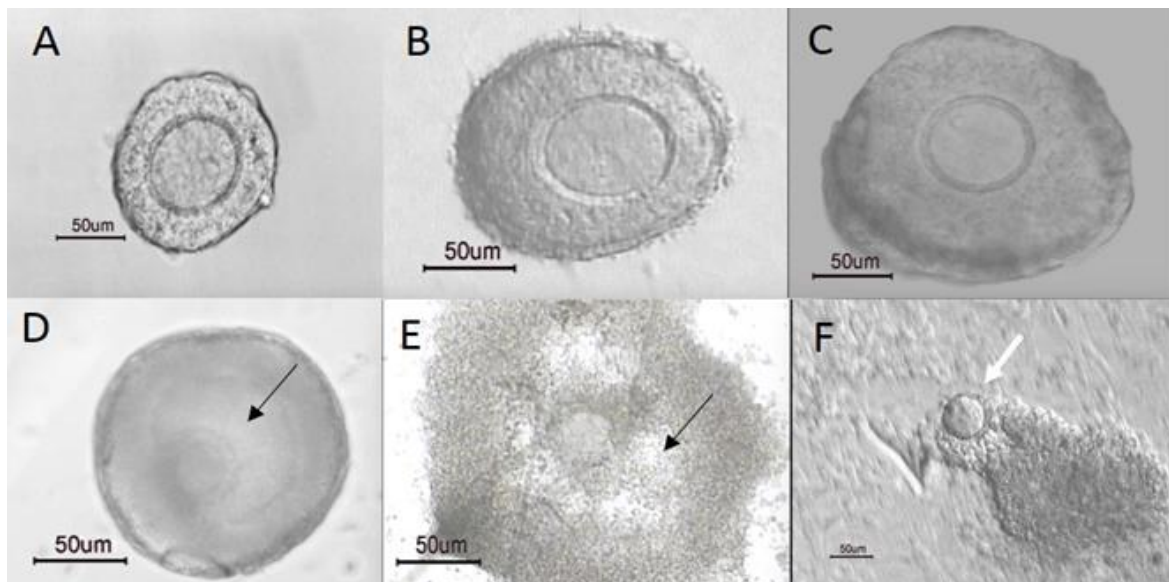
Figure 1:.Different stage of *in vitro* cultured mouse pre-antral follicles.
Cultured pre-antral follicle on initial day (A), day 2 (B), day 4 (C), day 8 (D), day 10 (E), Ovulated follicle (F)
Antral-like cavities are indicated by black arrow.

Ovulated MII oocyte indicated by white arrow

Figure 2. TAC levels of fresh and vitrified-warmed preantral follicles with or without pretreatment of CoQ_{10}.
In all cases 3 experimental replicates were performed.
Different super scripts (a, b, c and d) reflect different levels of significant differences at same times of culture among the different groups ($P<0.05$)
Different superscripts (A, B, C and D) reflect different levels of significant differences at different times of cultivation period within the same group ($P<0.05$).
TAC: Total Antioxidant capacity
COQ_{10}: Coenzyme Q_{10}

DISCUSSION

Vitrification is a well-organized method for cryopreservation of oocytes, embryos and ovarian tissue [2, 28-30]. In this study, we vitrified mouse isolated preantral follicles with the cryotop. Vitrification of preantral follicles compared with ovarian tissue has superior privilege such as high surface to low volume ratio leading to increased permeability of CPA which in turn reduced possibility of ice crystal formation and associated with successful vitrification. Furthermore, the possibility of potential return of cancer cells from auto-transplantation, in women with history of chemo and/or radiotherapy is also the main problem of vitrification of ovarian tissue [11, 28]. Hence, it seems that vitrification of isolated preantral follicles is more effective and safer than ovarian tissue for fertility preservation.

Cryopreservation makes several changes in cell physical and chemical characteristics; for instance, damage to cell membrane and cytoskeleton integrity, mitochondrial electron transport chain and mitochondrial depolarization which partly led to increase of production of ROS [29, 31-33]. ROS are produced in follicles physiologically and plays a critical role in ovulation process, but the overproduction of ROS caused damage to oocyte and granulosa cells through OS [34]. In this regards, Demant showed that after vitrification, mitochondrial activity of mouse oocytes changed [29]. Also, it has been demonstrated that the numbers and distribution of mitochondria

were affected by vitrification and warming which are critical factors for development of oocyte and embryo [33, 35]. In addition to cryopreservation, one of the major reasons of excessive ROS production in *in vitro* condition is relatively high oxygen concentration in the micro environment surrounding of the cells which may annoy the balance between the ROS production and antioxidant capacity leading to OS which in turn induced several different types of cell damages, such as membrane lipids peroxidation, oxidation of amino acids and nucleic acids, and apoptosis [14].

Supplemented culture medium with various antioxidants to minimize unsolicited effects of ROS have been widely used. It has been shown that several antioxidant including the mitochondrial cofactor and antioxidant increase endogenous antioxidants or mitochondrial bioenergetics [36]. In this context, Papis and his colleagues showed melatonin in an environment with high concentrations of oxygen and free radicals could improve developmental competence of bovine embryo [37]. Also, ALA supplementation of culture media has shown to improve survival rates, antrum formation and ovulation of fresh and cryopreserved preantral follicles in *in vitro* condition in partly via reduction of ROS and increase TAC [38]. Results of the present study showed CoQ_{10} supplementation medium improved the developmental competence of *in vitro* cultured fresh and vitrified-warmed preantral follicles after long-term *in vitro* culture and increased TAC levels of them up to 96 hours. CoQ_{10} is a vital factor in the electron transport chain system in the inner mitochondrial membrane. It is also known as a potent antioxidant which is involved in numerous aspects of cellular metabolism [39].

It seems that several features of CoQ_{10} activity may be involved in improving the development of vitrified-warmed and fresh preantral follicles. For instance, CoQ_{10} has a critical role in energy metabolism, as well as working as a liposoluble chain-breaking antioxidant for cell membranes in limiting membrane lipid peroxidation. So, in the present study, it seems, that CoQ_{10} improved development of *in vitro* cultured preantral follicles through supporting the growth and preserve the membrane stability. In this regard, recently it has been shown that adding CoQ_{10} to the culture medium of sperm cells of infertile men led to improvement in sperm functions [40]. Another part of the current study showed that TAC levels decreased in both vitrified-warmed and fresh preantral follicles; whereas, in the presence of CoQ_{10}, TAC levels of those increased significantly up to 96 hours later. In this regard, it has been demonstrated that CoQ_{10} can recycle and regenerate other antioxidants such as tocopherol and ascorbate [41]. Therefore, it seems that CoQ_{10} can increase TAC levels. In conclusion, fundamental functions of COQ_{10} in cell growth and metabolism, and its protective effects against OS make CoQ_{10} a good candidate for supporting development of fresh and vitrified-warmed isolated preantral follicles in *in vitro* condition.

ACKNOWLEDGEMENTS

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