Original Article

Expression of Apoptosis-Related Genes in Cultured Ovarian Tissue of Mouse in Presence of Coenzyme Q_{10}

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Abstract

Objective: Oxidative stress affects the development of ovarian follicles during *in vitro* culture thus applying an antioxidant is necessary. The aim of this study was to investigate the effect of coenzyme Q_{10} (CoQ_{10}) on the expression of apoptosis-related genes of mouse ovaries during *in vitro* culture.

Materials and methods: The immature mouse ovaries were cultured in the presence of 50 µM CoQ_{10} for 7 days. Histological examinations were performed and the 17 beta-estradiol concentration was measured on the seventh day of culture. The relative expression of *Caspase* 3, *Bax, Bad,* and *Bcl* 2 genes were investigated by real-time RT-PCR.

Results: The rates of normal follicles in the presence of CoQ_{10} was significantly increased compared to the control group. Also, in CoQ_{10}-trated group a significant increase in the level of 17 beta-estradiol was seen compared to the control group. The mRNA expression of anti-apoptotic gene *Bcl*2 was significantly increased while the expression of pro-apoptotic genes (*Caspase*3, *Bax* and *Bad*) significantly declined in CoQ_{10} treated group compared to those of control group.

Conclusion: The supplementation of the ovarian culture media with CoQ_{10} improved the follicular development through alteration in expression of apoptosis-related genes and stimulated the production of estradiol.

Keywords: Apoptosis; Coenzyme Q_{10}; In Vitro Culture; Ovarian Tissue

Introduction

In vitro culture of ovarian tissue is an alternative strategy for follicular growth and development to improve fertility potential (1-4). During *in vitro* culture of follicles and ovarian tissue the high level of reactive oxygen species (ROS) are produced by oxidative stress that affects the survival of ovarian follicles and their development (5, 6). Therefore, many attempts have been focused on the application of an antioxidant to reduce the harmful effects of ROS (7).

There are two main categories of antioxidants: enzymatic such as superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic ones including vitamin E and C, selenium, and CoQ_{10} that are used to attenuate the effects of oxidative stress (8, 9).

CoQ_{10} as a non-enzymatic antioxidant is distributed throughout several tissues of the body, especially in the heart, kidney, liver, and muscle tissues (10). CoQ_{10} is a critical component of the electron transfer chain in mitochondria (11).

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According to previous studies, CoQ_{10} improves the survival and the developmental competence of follicles, oocytes, and embryos (12-14). Also, another series of studies showed that the supplementation of culture media with CoQ_{10} increases follicular total antioxidant capacity, superoxide dismutase, and glutathione peroxidase levels (13, 15). Heydarnejad et al. demonstrated that CoQ_{10} could alter the expression of apoptotic-related genes such as *Caspase-3* and *Bax* in sheep oocytes and cumulus cells during in vitro culture of cumulus oocytes complex (16). In addition, it was revealed that by several investigators, CoQ_{10} could increase the mitochondrial mass and improve the distribution and polarization of mitochondrial membrane in bovine and mouse oocytes, also it could reduce the mitochondrial malfunction in the blastocysts (17-20).

During in vitro culture of ovarian tissue, apoptosis as a programmed cell death may be initiated by extrinsic or intrinsic factors that triggered the related pathways (7, 21). Both of these pathways could affect the viability and development of oocytes and follicles (22). Several investigators showed that the usage of CoQ_{10} in vitro or in vivo could decrease the incidence of oocyte apoptosis by altering the expression of genes related to apoptosis (16). While based on our knowledge there was no report to evaluate the improvement of ovarian tissue culture by using CoQ_{10} and evaluating the changes in the follicular expression of apoptosis genes. Therefore, the aim of the present study was to investigate the effect of CoQ_{10} on the follicular development and expression of genes involved in apoptosis during mouse ovarian tissue culture.

Materials and methods

Animal: In this experimental study, two weeks old Naval Medical Research Institute (NMRI) mice (n=9) were kept under a controlled condition (20-24°C, 40-50% humidity and 12/12h light: dark cycles) in the animal house of Tarbiat Modares University. All stages of the research were carried out with the approval of the Ethics committee for Animal Research of Tarbiat Modares University (Ref NO: 1399.008). The mice were sacrificed by cervical dislocation, and their ovaries were removed and dissected using two insulin syringe needles under stereomicroscope (Olympus; Japan). The collected ovaries were washed in 30 µl droplets of alpha-minimal essential medium (α-MEM; Gibco, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, UK), 100 IU/mL-1 penicillin (Pan biote, Germany), 100 µg/mL-1 streptomycin (Pan biote, Germany), 0.23 mMol sodium pyruvate (Sigma-Aldrich, Germany) and 2.2 g/L sodium bicarbonate (Carl Roth, Germany).

Experimental design: The ovaries were randomly divided into control and experimental groups (n = 9 in each group). In the experimental group the ovaries were cultured in media supplemented with 50 µM CoQ_{10} based on the previous research (13). After one week of *in vitro* culture, the morphology of ovaries, the level of 17 beta-estradiol in collected cultured media, and the expression level of genes related to apoptosis were studied.

In vitro culture of ovaries: In both groups the ovaries were cultured on the insert (0.4-µm pore size; Greiner Bio-One, Germany) in the 24-well plates (SPL, Korea) containing 300 µl of α-MEM supplemented with 10% FBS, 1% insulin, transferrin and selenium (ITS; Gibco, USA), 100 mIU/mL recombinant FSH (rFSH; Homa pharmed; Iran) in the CO2 incubator (5% CO_2, 100% humidity, 37°C) for one weethe k. In experimental group 50 µM CoQ_{10} (Nono kimia; Kore) was added to the culture media (12, 14). Every other day, the development of ovarian follicles was evaluated under an inverted microscope (Labomed, USA) and, half of the culture media (150 µl) was renewed. The collected culture medium was stored at -20°C until measurement of the 17-β-estradiol level.

Histological study: To evaluate the morphology of cultured ovaries in both groups (n=5 in each group) of the study, they were subjected to tissue processing including a fixation with Bouin's solution, dehydration with increasing concentration of alcohol (70%, 80%, 90%, 100%), clearing with Xylene and impregnation and embedding with paraffin wax. After serial sectioning at 5 µm thickness, the sections with 5 intervals were collected, stained using hematoxylin and eosin (H&E), and studied under a light microscope. The normal follicles had an intact oocyte surrounded by regular layers of granulosa cells with no condensed nucleus and degenerated follicles showed shrinkage oocyte which was surrounded by disorganized and detached granulosa cells. To avoid duplicate counting of the follicles, only follicles with a visible nucleus in the oocyte were evaluated (7) and classification of follicles was performed based on the previous studies (23). The preantral follicles according to their sizes were divided into small (80-130 µm), medium

(131-210 μm) and large follicles with prominent antrum cavities (211-330 μm).

Hormonal assay: The level of 17-β-estradiol (E2) in the collected culture media (n = 3 in each group and in three repeats) at the end of the culture period was measured using the electrochemiluminescence immunoassay kit (ECLIA; Abbott architect i2000SR, UK).

Real time RT-PCR: At the end of the culture period, the RNA of cultured ovarian tissues in studied groups (3 ovaries in each group in 3 replications) were extracted by TRIzol ® Kit (Thermo Fisher Scientific, Bremen, Germany) following the manufacturer's protocol. RNA quantification was evaluated by spectrophotometric read at 260 nm. cDNA preparation was obtained using cDNA synthesis kit (Thermo Fisher Scientific, Bremen, Germany) according to the manufacturer's instructions. Relative mRNA expression levels were investigated by a StepOnePlus real-time PCR system (Applied BioSystems, USA) using QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Relative quantification analysis was performed using the $2^{-\Delta\Delta CT}$ method. The primers of the *Bcl-2*, *Bax*, *Bad* and *Caspase*-3 genes were designed by AlleleID software (Primier Biosoft, USA) and the elongation factor 1 (*Ef1*) gene was used as a housekeeping gene (24) (Table 1).

Statistical analysis: All data were analyzed by SPSS for the windows statistical package program (version 24, Chicago, IL, USA). The normal distribution of data and equality of variances were investigated with the Shapiro test and Levent test respectively. Then, an independent sample t-student test (parametric conditions) was used to compare the follicular count, hormone level, and gene expression level, between the two studied groups. A *P*-value less than 0.05 was considered statistically significant. The data are presented as mean ± SD. Each experiment was repeated at least three times.

Results

Morphology of ovaries: The morphological observations of cultured ovaries under an inverted microscope during cultivation time in both groups of study are shown in Figure 1. As these figures showed the growing follicles in the peripheral area of the ovarian tissue are prominent. The central region of the cultured ovarian tissue in both groups had a dark and dens appearance.

Histological examination of studied ovaries using hematoxylin and eosin staining is demonstrated in Figure 2. Normal follicles at different developmental stages were seen in both groups and the morphology of ovarian sections is almost similar in both groups. In the central regions of ovarian tissue, some degenerated follicles were seen especially in the control group and in the periphery of the ovaries in both groups. The large preantral follicles were observed in treated ovaries with CoQ_{10}.

The rates of survived and degenerated follicles at different developmental stages in both groups are summarized in Table 2. As results showed the ovaries that were cultured in the presence of CoQ_{10} had a significantly higher mean percentage of normal follicles (85.40 ± 4.22%) and lower degenerated follicles (14.60 ± 4.29%) in comparison with the control group ($P < 0.05$). These rates regarding to primordial, medium preantral, and large preantral follicles were not significantly different between these two groups ($P > 0.05$) while these rates had significantly increased in relation to primary (83.74 ± 2.99% vs 76.73 ± 1.98%) and small preantral follicles (67.52 ±.13% vs 42.32 ± 4.36%) in CoQ_{10} treated and control groups respectively ($P < 0.05$).

Table 1: The sequences of designed primers

Target gene	Accession number	Primer sequences	PCR product size (bp)
Bad	NM-0011285423.1	F: 5′-GGAGCAACATTCATCAGCAG-3′ R: 5′-TACGAACTGTGGCGACTC-3′	91
Bcl-2	NM-177410	F: 5′-TGCTGCTATCCTGCCAAG-3′ R: 5′-GTCTGTGTTCTTCATCGTTACTTC-3	108
Caspase-3	NM-00981003	F: 5′-GCTGACTTCCTGTATGCTTA-3′ R: 5′-GTTGCCACCTTCCTGTTA-3′	164
Bax	NM-0075227.3	F: 5′-GCGAATTGGAGATGAACT-3′ R: 5′-CAGTTGAAGTTGCCATCA-3′	129
Ef1	NM-010106.2	F: 5′-AGTCGCCTTGGACGTTCTT-3′ R: 5′-CCGATTACGACGATGTTGATGTG-3′	124

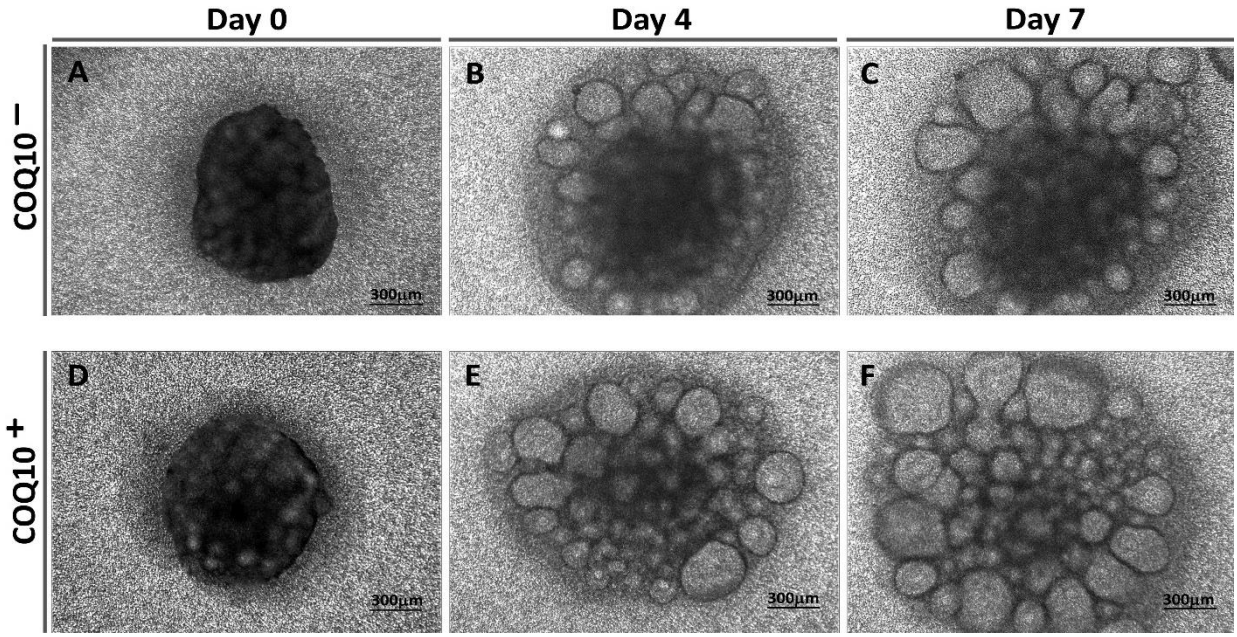

Figure 1: Photomicrographs of mouse ovaries under an inverted microscope on days 0, 5, and 7. A-C: Control, D-F: COQ10 treated group.

Hormonal assay: The average E2 levels in collected culture media after 7 days of in vitro culture of ovaries in both groups is shown and compared in Table 3. The level of E2 in the group treated with CoQ_{10} was significantly higher (8994.66 ± 1341.41 pg /ml**)** compared to that of the control group (5260.33 ± 1911.83 pg /ml; $P < 0.05$).

Real time RT-PCR result: The relative expression of target genes to the housekeeping gene (*Ef1*) in both studied groups is presented in Figure 3. As this figure shows the relative expression of *Bcl-2*, *Bax*, *Bad* and *Caspase-3* genes in control group were 0.97 ± 0.11, 1 ± 0.5, 0.99 ± 0.7 and 1.02 ± 0.99 and these ratios in CoQ_{10} treated group were 6.84 ± 1.03, 0.64 ± 0.01, 0.48 ± 0.07 and 0.46 ± 0.09 respectively ($P < 0.05$). In addition, the ratio of *Bcl-2*/*Bax* expression in the control and CoQ_{10} treated group were 0.98± 0.16 and 10.64 ± 1.6 respectively ($P < 0.05$).

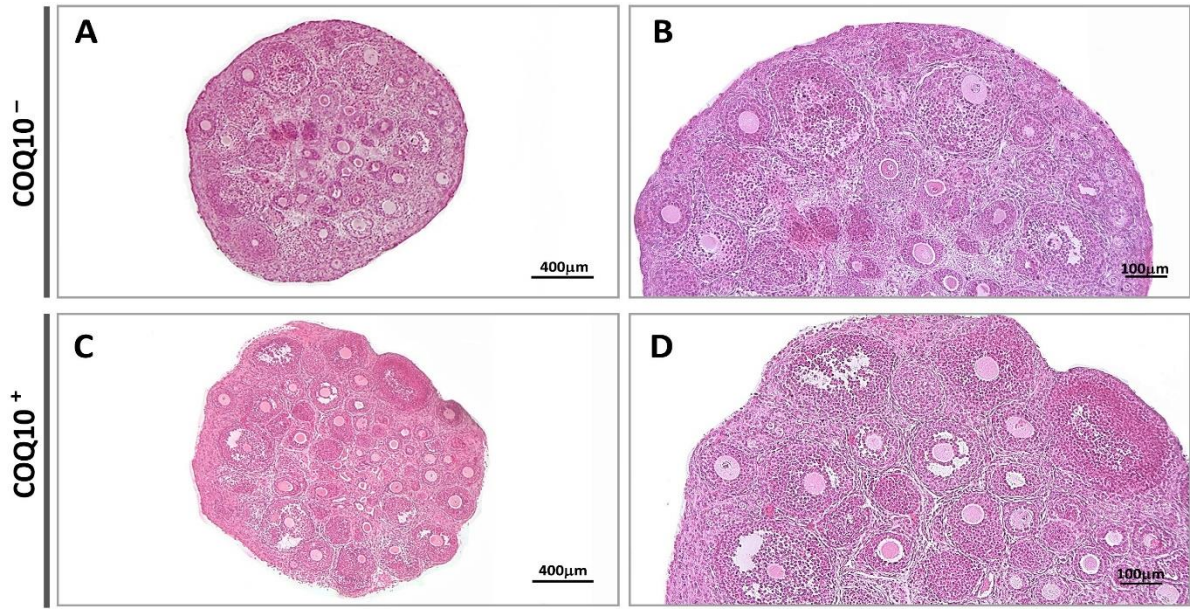

Figure 2: Photomicrographs of mouse ovarian tissue sections stained using hematoxylin and eosin after seven days of in vitro culture. A and B: Control; C-D: treated group with 50 µM COQ10.

Table 2: The mean percentages of normal follicles at different developmental stages in the studied groups

Groups		Total No. (Mean% ± SD)	No. Primordial (Mean% ± SD)	No. Primary (Mean% ± SD)	No. Small preantral (Mean% ± SD)	No. Medium preantral (Mean% ± SD)	No. Large preantral (Mean% ± SD)
Control	Normal	929	587	155	124	34	25
		(77.74 ± 5.76)	(95.44 ± 1.56)	(76.73±1.98)	(42.32 ± 4.36)	(73.37 ± 3.81)	(80.64 ± 4.62)
	Degenerated	266	28	47	169	16	6
		(22.26 ± 5.76)	(4.56 ± 1.56)	(23.27 ± 1.98)	(57.68 ± 4.36)	(29.63 ± 3.81)	(19.36 ± 4.62)
COQ10	Normal	1033	591	170	131	77	64
		(85.40 ± 4.22) *	(95.01 ± 1.00)	(83.74 ± 2.99) *	(67.52 ± 4.13) *	(66.37 ± 2.87)	(85.33 ± 2.18)
	Degenerated	177	31	33	63	39	11
		(14.60 ± 4.29) *	(4.99 ± 1.00)	(16.25 ± 2.99) *	(32.48 ± 4.130 *	(33.63 ± 2.87)	(14.67 ± 2.18)

COQ10: Coenzyme Q10;
*: Indicate significant differences compared with the control group in the same column (P<0.05).
Small preantral follicles (80-130 μm), medium preantral follicles (131-210 μm), and large preantral follicles (211-330 μm).

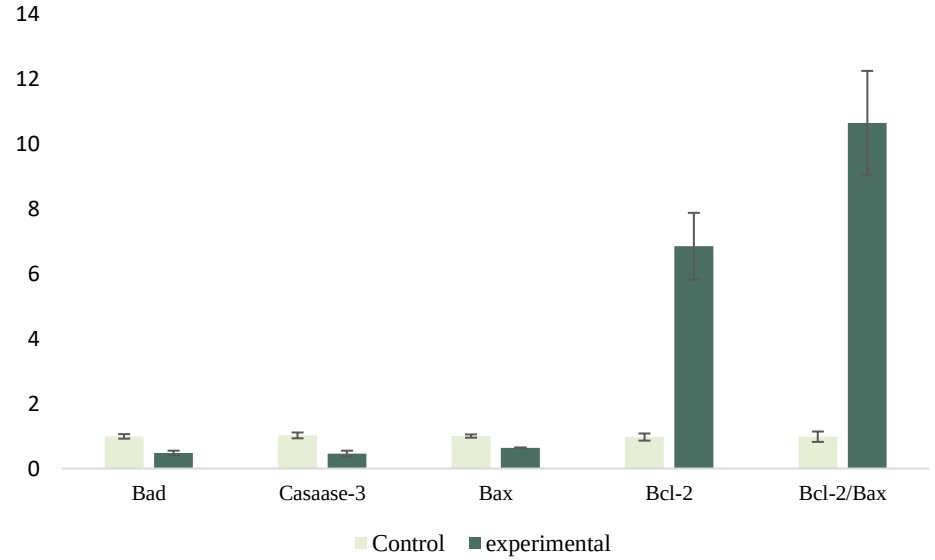

Figure 3: The comparison of relative expression of Bad, Caspase-3, Bax, and Bcl-2 genes to housekeeping genes in both studied groups. *: Indicate significant differences compared with the control group (P<0.05).

Table 3: The concentration of 17-β-estradiol (pg mL-1) on Day 7 of culture.

Groups	(Mean% ± SD)
Control	5260.33 ± 1911.83
COQ10	8994.66 ± 1341.41*

COQ10; Coenzyme Q10, *: Indicate significant differences compare with the control group (P<0.05)

The relative expression of pro-apoptotic genes *Caspase*-3, *Bax* and *Bad* was significantly decreased, while the expression ratio of *Bcl-2* and *Bcl-2*/*Bax* was significantly higher in experimental groups in comparison with the control group (P <0.05).

Discussion

The present study, for the first time, investigated the effect of CoQ_{10} on the expression of apoptosis-related genes in cultured ovarian tissue. Histological examination showed CoQ_{10} decreased degeneration and improved follicular survival rate. Also, CoQ_{10} improved the development of small preantral follicles and increased the number of medium and large preantral follicles by decreasing the rates of degeneration in the primary follicles and small preantral follicles. In addition, it seems that CoQ_{10} preserves the storage of primordial follicles by gradually activating these follicles and turning them into primary follicles.

One of the possible reasons for this effect of CoQ_{10} on the survival and development of ovarian follicles is its antioxidant properties and its ability to scavenge free radicals. Although in the present study we have not analyzed the ROS level in cultured ovaries, in agreement with this suggestion several previous studies have shown that the supplementation of culture media with 50 µM CoQ_{10} decreases ROS production and increases antioxidant capacity which in turn improved follicular survival and development (13, 15). Similarly, Ben Meir *et al*. showed that injection of 22 mg of CoQ_{10} for 12-13 weeks in older mice increased oocyte quality, ovulation rate, and ovarian reserves by increasing the number of primordial, preantral, and antral follicles (18).

CoQ_{10} also improves cytoplasmic maturation by affecting mitochondrial function by improving distribution patterns of mitochondrial, increasing the expression of mitochondrion-associated genes involved in the respiratory chain and metabolism-related genes (16, 18-20, 25). Also it improves nuclear maturation via reduction in DNA fragmentation, restoring spindle assembly, chromosome alignment, and actin organization (19, 25-27). In this regard, it has been investigated that the presence of 30 µM CoQ_{10} in the maturation media of sheep oocytes increased the gene expression of Growth Differentiation Factor-9 (GDF-9) as well as follicular development (16, 28).

The other part of this study demonstrated that CoQ_{10} stimulate granulosa cell to secret more 17-β-estradiol as follicular functional marker. Similarly, previous studies have shown that CoQ_{10} affects the proliferation of granulosa cells by increasing the expression of genes related to cell proliferation such as *Bone Morphogenetic Protein 15* (*BMP15*) and *Proliferating Cell Nuclear Antigen* (PCNA) (29, 30). On the other hand, Delkhosh *et al.* showed that administration of 22 mg CoQ_{10} in cyclophosphamide-treated mice increased Follicle Stimulating Hormone (FSH) receptor expression levels (29) and FSH stimulates the secretion of estradiol from granulosa cells (31).

Other part of the present study showed that CoQ_{10} decreased the expression of pro-apoptotic genes (*Bax*, *Bad*, *Caspase-3*) and increased the expression of the anti-apoptotic gene (*Bcl-2 and Bcl-2/Bax ratio*). Similarly, Heydarnejad *et al.* demonstrated the supplementation of sheep oocytes maturation media with CoQ_{10} decreased the expression of pro-apoptotic genes such as *caspase*-3 and *Bax* in oocytes and cumulus cells (16). Another in vivo study revealed that injection of CoQ_{10} into aged female mice and pigs reduced cell death and Caspase-3 level and oocytes apoptosis (18, 19, 26). The reduction of pro-apoptotic gene expression in the CoQ_{10}-treated group may be related to reducing to ROS production (13, 15), or inhibition of cytochrome C release and inhibition of Caspase activity (32).

Conclusion

The supplementation of the ovarian culture media with CoQ_{10} improved the follicular development through alteration in expression of the apoptosis-related genes and stimulate the production of estradiol in granulosa cells which in turn promoted follicular development.

Conflict of Interests

Authors declare no conflict of interests.

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