Received: 13 April 2019 | Revised: 30 July 2019 | Accepted: 12 September 2019

DOI: 10.1002/jbt.22398

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Upregulation of FSHR and PCNA by administration of coenzyme Q10 on cyclophosphamide-induced premature ovarian failure in a mouse model

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Abstract

Cyclophosphamide (CTX) has been broadly used in the clinic for the treatment of autoimmune disorders and ovarian cancer. The process of chemotherapy has significant toxicity in the reproductive system as it has detrimental effects on folliculogenesis, which leads to an irreversible premature ovarian failure (POF). Coenzyme Q10 (CoQ10) has positive impacts on the reproductive system due to its antioxidant properties, protecting the cells from free-radical oxidative damage and apoptosis. However, little is known about the possible synergistic effect of CTX and CoQ10 on the expression of genes involved in folliculogenesis, such as proliferation cell nuclear antigen (PCNA) and follicle-stimulating hormone receptor (FSHR). A total of 32 NMRI mice were applied and divided into four groups, including healthy control, CTX, CTX + CoQ10, and CoQ10 groups. The effects of CoQ10 on CTX-induced ovarian injury and folliculogenesis were examined by histopathological and real-time quantitative reverse transcription-polymerase chain reaction analyses. The rates of fertilization (in vitro fertilization), embryo development, as well as the level of reactive oxygen species (ROS) in metaphase II (MII) mouse oocytes after PMSG/HCC treatment were also assessed. Results showed that the treatment with CTX decreased the mRNA expression of PCNA and FSHR, IVF rate, and embryo development whereas the application of CoQ10 successfully reversed those factors. CoQ10 administration significantly enhanced histological morphology and decreased

ROS levels and the number of atretic follicles in the ovary of CTX-treated mice. In conclusion, it seems that the protective effect of CoQ10 is exerted via the antioxidant and proliferative properties of this substance on CTX-induced ovarian damage.

KEYWORDS
cyclophosphamide, coenzyme Q10, ovary, gene expression, in vitro fertilization

1 | INTRODUCTION

Much progress has been made in the field of cancer therapy while the advent of chemotherapeutic agents has increased the life expectancy of patients dealing with cancer.[1] On the other hand, chemotherapy-induced damage to the ovary has raised many concerns as the side effects of radiation or chemotherapy on the ovary cells are generally irreversible. It has been reported that the administration of anticancer drugs such as cyclophosphamide (CTX) can lead to premature ovarian failure (POF) in women.[2] One of the complications of POF is the overproduction of reactive oxygen species (ROS), which could cause damage to ovarian tissue and decrease the ovarian reserve (ie, follicle depletion).[2]

CTX (Figure 1A), *N*,*N*-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-amine 2-oxide), as an alkylating anticancer drug, is frequently used not only for the treatment of breast or ovarian cancer but also for many autoimmune disorders, including rheumatoid arthritis, systemic lupus erythematosus, etc.[1] It has been implicated that CTX, in the presence of cytochrome P450, is metabolized into two unstable and transient intermediates namely,

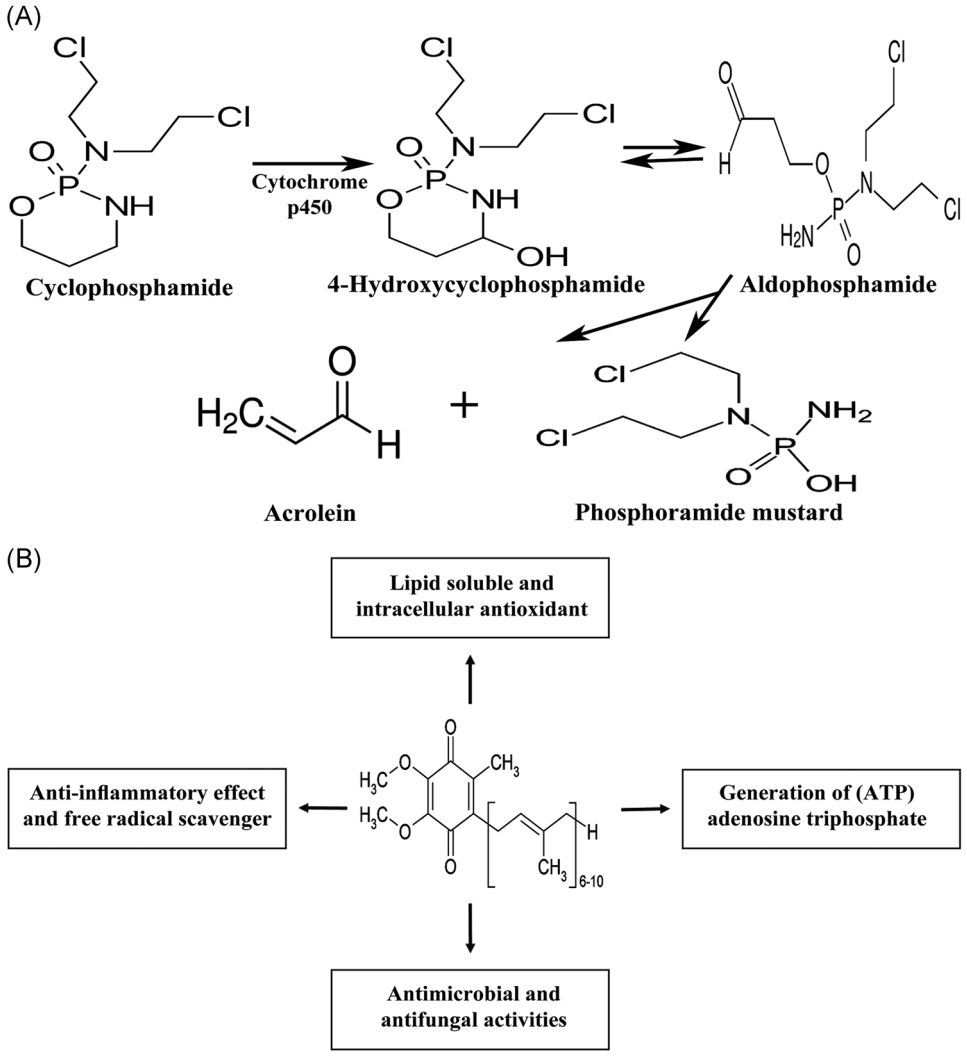

FIGURE 1 The chemical structures of cyclophosphamide, 4-hydroxycyclophosphamide, aldophosphamide, acrolein, phosphoramide mustard (A) and the chemical structure and biological activities of coenzyme Q10 (B)

4-hydroxycyclophosphamide and aldophosphamide which are both, in turn, converted into two stable toxic compounds, namely acrolein (propenal, C_3H_4O) and phosphoramide mustard (H_6N_3OP) (Figure 1A).[1-3] Between these two chemical agents, acrolein, as a highly electrophilic and α, β-unsaturated aldehyde, has greater toxicity as it generates higher levels of ROS.[4]

The overproduction of ROS is a hallmark of oxidative stress, which occurs when the balance between pro-oxidant and antioxidant contents of the cells is impaired. ROS encompass a group of diverse chemical agents such as alkoxyl (RO•) and hydroxyl radicals (•OH), as well as superoxide anion ($^{\bullet}O_2^-$) and non–free-radical species such as hypochlorous acid (HOCl), hypobromous acid (HOBr), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). These molecules are capable of causing lipid peroxidation and DNA damage, which could result in cell injury, cellular dysfunction, and apoptosis.[5-9]

CTX can elevate the production of ROS through some signaling pathways such as PI3K/Akt/mTOR pathway, and some molecular complexes including the NADPH complex ($NADPH/NADP^+$) and mitochondrial electron respiratory chain.[10-12] Furthermore, several studies have elucidated that the active metabolites generated during the metabolism of CTX can lead to chromosomal aberrations, sister chromatid exchange, DNA adducts, single-strand breaks, and the formation of DNA-DNA crosslinks.[13,14] Animal studies have also shown that CTX could disrupt the ovarian follicle reserve.[2,15] In mice, primary follicles, antral follicles, as well as granulosa cells are the most sensitive cells to CTX-induced ovarian damage, while antral follicles and granulosa cells of more mature follicles are targeted by CTX in rats.[16] On the other hand, an increase in the levels of ROS can cause some alterations in the spindle morphology and microtubule arrangement of oocytes (MT), leading to a decrease in the quality of oocytes.[2] Considering the deleterious effects of CTX on the process of folliculogenesis, there is a need for the determination of genes involved in this phenomenon.

Myriad studies have indicated that natural antioxidants can prevent oxidative DNA damage and lipid peroxidation. Therefore, they bind to free radicals and subsequently, neutralize them to lower their destructive impacts on the cellular functions.[7,8,17] Since antioxidants play an essential role in human health, they could be employed for the mitigation of adverse effects of anticancer drugs on the reproductive system.

Coenzyme Q10 (CoQ10) (Figure 1B), known as ubiquinone, is a lipid-soluble electron transporter, which possesses ten isoprenyl units in its side chain.[18,19] CoQ10 transports electrons from complexes I and II to complex III in the mitochondrial respiratory chain. Thus, CoQ10 is a key component involved in the synthesis of adenosine triphosphate (ATP), and it is essential for the stability and action of complex III.[20,21] CoQ10 also exists in other biological membranes, and as a major intracellular antioxidant, it plays a crucial role in the protection of membrane phospholipids and proteins present in the mitochondrial membrane from ROS-induced oxidative damage.[22] It has been reported that CoQ10 has therapeutic effects on liver tissues of rats with hepatocellular carcinoma thereby the modulation of the expression of nuclear factor-κB (NF-κB).[23] It has been implicated that CoQ10 is capable of protecting pancreatic β-cell line MIN6 via the suppression of the activation of caspase-3, DNA fragmentation, and the release of cytochrome c from mitochondria, resulting in the protection of β cells through against the apoptotic properties of staurosporine.[24] Numerous investigations reported that CoQ10 supplementation facilitates the process of DNA repair and decreases oxidative DNA damage.[25] In line with these investigations, it has been reported that the administration of CoQ10 could not only improve mitochondrial energetics of oocytes but also prevent follicle loss in aged animal models.[21] It has been revealed that human follicular fluid contains CoQ10 as it can enhance the oocyte maturation and embryo grade during in vitro fertilization (IVF).[26]

To the best of our knowledge, no study has been conducted so far to elucidate the impact of CoQ10 on the alteration of genes that contribute to the process of folliculogenesis, including proliferating cell nuclear antigen (PCNA) and follicle-stimulating hormone receptor (FSHR) in CTX-induced ovarian failure. Therefore, the aims of this study were as follows: (1) investigating the effect of CoQ10 on histomorphometry and the mRNA expression of PCNA and FSHR genes in the ovarian tissue of mice treated with CTX and CoQ10, and (2) assessment of ROS levels in MII oocytes as well as evaluation of the IVF rate and embryo development after the induction of ovulation.

2 | MATERIALS AND METHODS

2.1 | Animal procedures

In this experimental study, 32 female and 10 male NMRI mice (8-10-week old) with a weight of 20-25 g were used. The animals were housed in an animal laboratory with controlled temperature (25°C ± 2°C), humidity (30%-60%), and photoperiod (12 hours light: 12 hours dark) and they had free access to chow and tap water.

Male rats were used for IVF process. Female mice were randomly divided into four groups ($n = 8$ mice per group) as follows:

(G1): healthy control group (G1) in which mice only received sesame oil.

(G2 or CTX group): cyclophosphamide (Sigma-Aldrich, Germany) group in which mice daily received an intraperitoneal injection of CTX at a dose of 20 mg/kg[27] for 21 days.

(G3 or CTX + CoQ10 group): the induction of POF in this group was similar to G2, while mice were also treated with IP injection of CoQ10 (Sigma-Aldrich) at the dose of 22 mg/kg every other day for 21 days.[21,28]

(G4 or CoQ10 group): healthy control group treated with CoQ10 at a dose of 22 mg/kg every other day for 21 days.

CoQ10 was dissolved in sesame oil[21] and administered every other day because the half-life of CoQ10 is about 33 hours in the blood.

2.2 | Histopathological evaluation

On the last day of the experiment (day 21), some ovarian samples were fixed in 10% formaldehyde at 4°C overnight. The samples were then paraffin-embedded, serially sectioned at 5-μm thickness,

deparaffinized, and finally stained with hematoxylin and eosin (H&E). The numbers of primordial, pre-antral, antral, and cystic follicles were counted according to our previous study.[29] Briefly, in each group, 20 sections of every 10th section were randomly examined.[30] To avoid double-counting of the same follicles, one of them was counted where the dark staining nucleolus was seen inside its nucleus.[30] All sections were observed under an optical microscope (Nikon, Japan) at ×100 magnification.

2.3 | RNA extraction, cDNA synthesis, and qRT-PCR

In each group, total RNA was extracted from some ovarian samples using TRIzol reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Then, based on our previous study, total RNA extraction was then treated with DNase I (Invitrogen) to eliminate traces of genomic DNA contamination.[7] The details were the same as described previously.[7,8,31] The complementary DNA (cDNA) was synthesized from the extracted mRNA templates using a cDNA synthesis kit (Thermo Scientific, EU) according to the manufacturer's instructions. Table 1 shows the sequence of specific primers for PCNA, FSHR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an endogenous control.[8,32] The real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed, based on our previous studies, in 20 mL reaction volume containing 10 mL SYBR Green Master Mix (Sigma-Aldrich), 2 mL forward and reverse primers, 1 mL cDNA, and 7 mL RNase/DNase free water.[8] The relative expression ratio of each target gene was determined by the Pfaffl method, and the C_T values were normalized against the expression rate of GAPDH as an internal control gene. All reactions were carried out in triplicate.

2.4 | Ovarian stimulation

At the end of the treatment period (day 21), mature NMRI female mice were superovulated by intraperitoneal injection of 7.5 IU pregnant mare serum gonadotropin (PMSG) (Sigma-Aldrich) followed by the administration of 7.5 IU human chorionic gonadotropin (HCG) (Sigma-Aldrich) 48 hours later.[33] Ovulation occurred within 14 hours of post-HCG injection. During the ovulation period, the treatment procedures with CTX and CoQ10 were carried out.

TABLE 1 Primer sequences used in real-time reverse transcription polymerase chain reaction

Genes	Primer sequences (5′-3′)	Product size, bp
GAPDH	F: GGAAAAGAGCCTAGGGCAT R: CTGCCTGACGGCCAGG	64
PCNA	F: GGAAGCTTAGAGTAGCTCTCATC R: GGGAATTCGTGACAGAAAAGACCTC	174
FSHR	F: AGGTACAGCTCTGCCATGCT R: GTACGAGGAGGGCCATAACA	171

Abbreviations: F, forward; FSHR, follicle-stimulating hormone receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferation cell nuclear antigen; R, reverse.

2.5 | ROS assay in MII oocytes

After the ovulation period, the animals were killed by cervical dislocation. Then, the uterine tubes and ovaries were separated and placed in α-minimum essential medium (α-MEM; WelGENE, Korea) supplemented with antibiotics and 5% fetal bovine serum (Gibco, UK). Then, oocytes were removed by autopsy, and after washing, MII oocytes were isolated and used for the ROS assay and IVF. However, the levels of ROS in freshly isolated MII oocytes ($n = 10$ for each group, with three replications) were measured based on the method reported in our previous studies.[8,32] Briefly, in the first stage performed in a dark place, 10 pooled MII oocytes were incubated in Assay Buffer (40 mmol/L Tris-HCl, pH 7 at 37°C) containing 5 mmol/L 2′,7′-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) for 25 minutes. In the second stage, incubated oocytes were washed with PBS and sonicated at 50 W for 3 minutes, then immediately centrifuged at 3000 rpm at 4°C for 12 minutes. Finally, the supernatants were collected and monitored by a spectrofluorometric method at excitation and emission wavelengths of 480 and 520 nm, respectively.[8,32] Data related to the ROS levels were expressed as mM H_2O_2.

2.6 | In vitro fertilization

This step was performed based on our previous study.[34] First of all, the cauda epididymis of male NMRI mice (8-10-week old, $n = 10$) was dissected and immediately placed into human tubal fluid (HTF) medium (Sigma Aldrich), containing 5 mg/mL of bovine serum albumin (BSA) (Gibco), at 34°C for 90 minutes until sperm capacitation. Then, isolated oocytes were transferred into the HTF medium (supplemented with 15 mg/mL BSA) containing capacitated spermatozoa and incubated for a period of 6 hours. In the last step, after the insemination process, the oocytes were transferred into the global medium supplemented with 5 mg/mL BSA and cultured for 5 days (120 hours). On day 5, the rate of IVF, as well as the numbers of two-cell embryos (cleavaged) and hatched blastocysts were evaluated. During the IVF period, the culture media was not supplemented with either CoQ10 or CTX.

2.7 | Statistical analysis

Data were analyzed by the SPSS software version 20 (SPSS Inc). The values of different groups were compared using one-way analysis of variance followed by Tukey's post hoc test. The results were expressed as the mean and standard deviation (mean ± SD). Differences between groups were considered statistically significant when the P value was less than .05.

3 | RESULTS

3.1 | Histopathological evaluations

In the healthy control and CoQ10-treated groups, the structure and morphology of ovarian tissues were histologically typical and normal (Figure 2A and 2D). Mice treated with CTX showed an abnormal morphological structure such as atrophy and space in ovarian stroma. However, after the administration of CoQ10 (G3), the rate of abnormal morphological structures was markedly decreased when compared with the CTX-treated group. On the other hand, the histological analysis performed by H&E staining showed that in ovarian tissue of CTX-treated mice, the numbers of primordial, primary, pre-antral, and antral follicles were significantly decreased compared with the healthy control group, while the number of atretic follicles was increased considerably ($P < .05$) (Table 2). However, the treatment with CoQ10 prevented follicle loss and also decreased the number of atretic follicles in comparison with that of the CTX group ($P < .05$).

3.2 | Relative mRNA Expression of the PCNA and FSHR Genes

To investigate the effects of CTX and CoQ10 on the expression of PCNA and FSHR, as two important genes involved in the process of folliculogenesis, the qRT-PCR analysis was performed to measure the level of gene expression. According to Figure 3, CTX led to a statistically significant decrease in mRNA expression of the PCNA gene compared with the healthy control group ($P < .05$). But, in the CTX group treated with CoQ10, the expression of PCNA was significantly elevated as compared with the CTX-treated group ($P < .05$). On the other hand, the level of the gene expression of FSHR was significantly reduced in the CTX-treated group compared with the control group, while the mRNA level of the FSHR gene was significantly higher in the CoQ10-treated group than that of the CTX control group ($P < .05$). There was no significant difference in the expression of the PCNA and FSHR genes between both healthy control groups (ie, G1 and G4) ($P > .05$).

3.3 | Effect of CTX and CoQ10 on Intracellular ROS Level in isolated MII Oocytes

The deleterious effects of CTX and the impact of the antioxidant properties of CoQ10 on MII oocytes, isolated from super-ovulated mice, were evaluated by the measurement of intracellular ROS levels in oocytes using the spectrofluorimetric method (at excitation and emission wavelengths of 480 nm and 520 nm, respectively). According to Table 3, the intracellular ROS level in MII oocytes, isolated from the CTX-treated group was significantly higher than the healthy control group ($P < .05$). On the other hand, the level of ROS in MII oocytes, obtained from the CoQ10-treated group was significantly diminished as compared with the CTX-treated group ($P < .05$). Furthermore, there was a significant difference between healthy control groups (ie, G1 and G4) in this regard ($P < .05$).

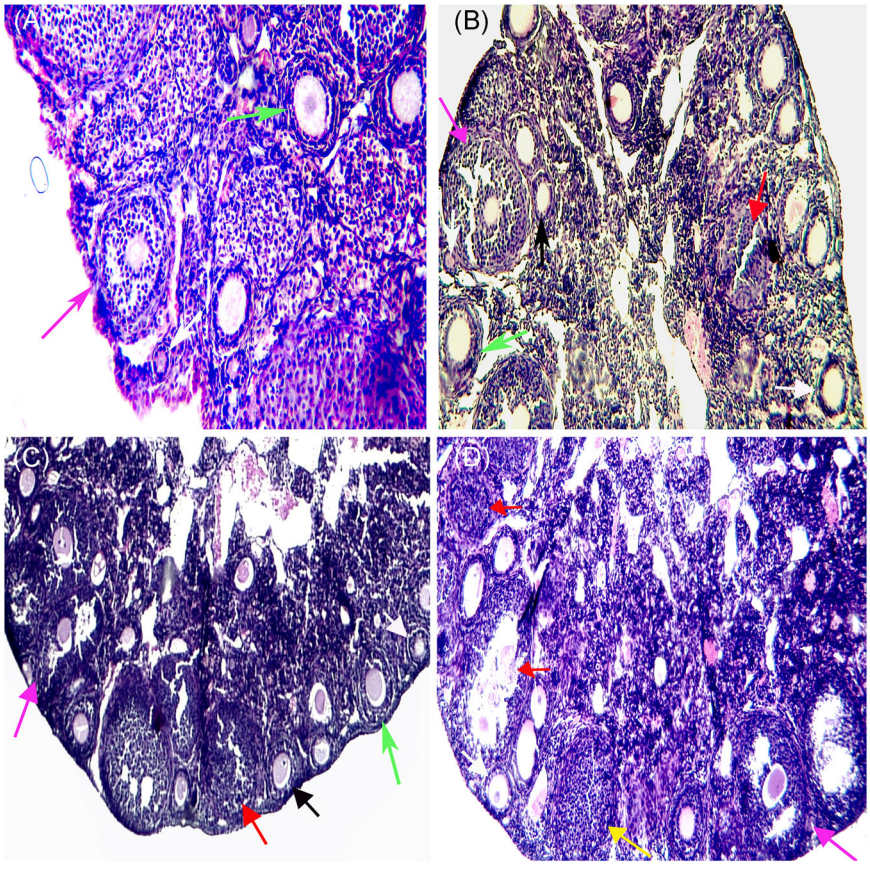

FIGURE 2 Representative picture of ovarian histology stained with hematoxylin and eosin (H&E) in different groups of the study. A, Control group in which standard ovarian architecture is evident. B, Cyclophosphamide control group in which severe ovarian damage is apparent. C, The cyclophosphamide group treated with coenzyme Q10 in which there was an improvement in the ovarian structure. D, Treated control group in which typical ovarian architecture is seen. Black, red, white, and pink arrows show primary, atretic, primordial, and antral follicles, respectively. The yellow arrow shows corpus lutetium and green arrows show preantral follicle. Scale bar = 100 μm

TABLE 2 The numbers of primordial, primary, preantral, antral, and atretic follicles, in all groups of the study

Groups	Primordial follicles (*n* ± SD)	Primary follicles (*n* ± SD)	Preantral follicles (*n* ± SD)	Antral follicles (*n* ± SD)	Atretic follicles (*n* ± SD)
Control	321.32 ± 9.71	185.11 ± 6.59	83.51 ± 3.25	41.02 ± 2.12	22.01 ± 1.01
CTX	215.28 ± 7.32*	101.13 ± 6.42*	39.32 ± 2.24*	21.07 ± 2.32*	75.32 ± 4.41*
CTX + CoQ10	271.52 ± 7.51*,**	152.64 ± 5.97*,**	57.02 ± 2.93*,**	32.22 ± 3.02*,**	54.11 ± 3.93*,**
CoQ10	332.14 ± 10.09	188.47 ± 6.17	83.66 ± 3.41	42.81 ± 1.98	19.25 ± 1.81

Note: Values are given as mean ± SD.
Abbreviations: CoQ10, coenzyme Q10; CTX, cyclophosphamide; SD, standard deviation.
*$P < .05$ shows significant differences in the CTX and CTX + CoQ10 groups compared with the control group.
**$P < .05$ show significant differences in the CTX + CoQ10 group compared with the CTX group.

3.4 | Effect of CTX and CoQ10 of fertilization rate and embryo development

Table 4 shows the fertilization rate and embryo development. Moreover, Figure 4 depicts the development of embryos.

According to Table 4, the fertilization rate of MII oocytes, isolated from mice treated with CTX was significantly lower than that of the healthy control group ($P < .05$), while the fertilization rate of MII oocytes obtained from female mice treated with CoQ10 (CoQ10) was significantly increased compared with the CTX-treated group (CTX) ($P < .05$). Additionally, there was a significant difference between healthy control groups ($P < .05$). On the other hand, the embryo development, including the rate of two-cell (cleavaged) and hatched blastocysts, was considerably reduced in the CTX-treated group compared with the healthy control group ($P < .05$). However, the embryo development at all stages mentioned earlier was markedly increased in the CoQ10-treated group (G3) when compared with the control group ($P < .05$). Interestingly, there was no significant difference between healthy control groups (ie, G1 and G4) in this regard ($P > .05$)

4 | DISCUSSION

In the present study, the impact of CoQ10, as an antioxidant supplement, on cyclophosphamide-induced ovarian failure and the process of folliculogenesis was investigated by the measurement of critical genes (eg, PCNA and FSHR) involved in this phenomenon, as well as the evaluation of the rate of IVF and ROS levels in female mice.

The histomorphometric results obtained from our study showed that the number of follicles at all stages (ie, primordial, primary, preantral, and antral) was significantly reduced in the cyclophosphamide-treated group compared with the healthy control group, while the number of atretic follicles was markedly elevated. On the other hand, the treatment with CoQ10 led not only to an increase in the number of ovarian follicles at all stages but also to a decrease in the number of atretic follicles.

It has been reported that some of the available treatments applied to patients with cancer including chemotherapy could destroy the ovarian reserve; thus, they result in ovarian failure and subsequently infertility.[2] Apoptosis has been considered one of the mechanisms involved in follicle loss when ovarian cells are exposed to cyclophosphamide.[15] Studies suggested that cyclophosphamide is capable of inducing apoptosis through the overproduction of ROS and causing oxidative stress in ovarian cells, inducing them to undergo programmed cell death.[2]

Furthermore, active metabolites of cyclophosphamide, namely phosphoramide mustard and acrolein, could cause DNA adducts, single-strand breaks, DNA crosslink formation, sister chromatid exchange, chromosomal aberrations, and finally apoptosis.[2,14] One study reported that in cyclophosphamide-induced POF, the expression of anti-apoptotic proteins such as Bcl-2 is considerably

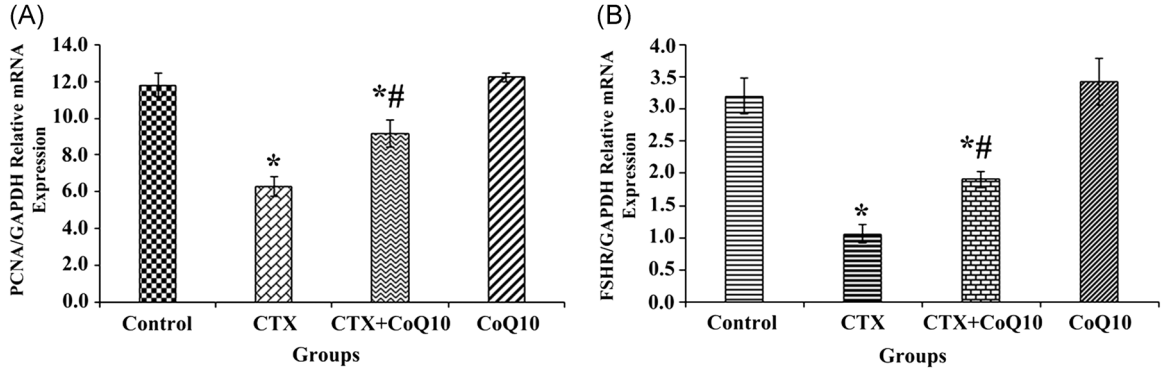

FIGURE 3 The mRNA expression levels of PCNA and FSHR in the ovarian tissue evaluated by qRT-PCR, on day 21 of the experiment. A, The mRNA expression of PCNA. *Significant differences compared with the control group ($P < .05$). #Significant differences compared with the cyclophosphamide control group ($P < .05$). Values are given as mean ± SD. B, The mRNA expression of FSHR. *Significant differences compared with the control group ($P < .05$). #Significant differences compared with the CTX control group ($P < .05$). Values are given as mean ± SD. CoQ10, coenzyme Q10; CTX, cyclophosphamide; FSHR, follicle-stimulating hormone receptor; mRNA, messenger RNA; PCNA, proliferation cell nuclear antigen; qRT-PCR, real-time quantitative reverse transcription-polymerase chain reaction; SD, standard deviation

decreased, while the expression of pro-apoptotic proteins including Bax is markedly increased. Hence, it would be conceivable that CTX could increase follicular atresia by the induction of apoptosis in follicular granulocyte cells[35] (Figure 5).

Also, in animal models of cancer treated with CTX, an excessive generation of ROS leads to the upregulation of caspase-3 in ovarian follicles in a dose-dependent manner, subsequently resulting in follicle depletion.[2] In the study conducted by Meng et al,[15] they showed a substantial follicle loss (primordial/primary), abnormal ultrastructure, and DNA fragmentation in xenografted human ovarian tissue after the intraperitoneal administration of cyclophosphamide (0.2 mg/g, 1 hour after xenotransplantation) in female immunodeficient BALB/c nude mice within 2 weeks post-transplantation. They also reported that chemotherapy could harm primordial/primary follicles by either induction of apoptosis in the granulosa cells and oocytes or indirectly damaging ovarian stromal cells characterized by vascular damage and cortical fibrosis.[15]

TABLE 3 Intracellular ROS levels (mM H_2O_2) in retrieved MII oocytes after PMSG/HCG treatment in all groups of the study

Groups	ROS (mean ± SD)
Control	47.14 ± 2.62
CTX	71.53 ± 1.99*
CTX + CoQ10	60.30 ± 1.46*,**
CoQ10	41.01 ± 2.16***

Note: Values are given as mean ± SD.
Abbreviations: CoQ10, coenzyme Q10; CTX, cyclophosphamide; HCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; ROS, reactive oxygen species; SD, standard deviation.
*$P < .05$ show significant differences in the cyclophosphamide and cyclophosphamide + CoQ10 groups compared with the control group.
**$P < .05$ show significant differences in the CTX + CoQ10 group compared with the CTX group.
***$P < .05$ show significant differences in the healthy treated group (CoQ10) compared with the control group.

Several studies showed that in primordial and small primary follicles, cyclophosphamide seemingly targets oocytes to induce apoptotic in those cells, whereas in larger follicles, cyclophosphamide stimulates apoptosis in granulosa cell, leading to the death of oocyte.[16,35]

Tsai-Turton and his colleagues reported that the pre-activated form of cyclophosphamide, 4HC, is capable of inducing apoptosis in COV434 human granulosa cells in a dose-dependent manner through an increase in ROS levels, a decrease in the concentration of intracellular glutathione (GSH), and the activation of caspases-3 and -9.[16] On the other hand, one study reported that the numbers of primordial, pre-antral, and antral follicles were increased in 9-month-old mice, which were subcutaneously administered CoQ10 (three times per week) for 12 to 13 weeks. Such an increase in the number of various types of follicles mentioned earlier may be due to the decreased rate of atresia or lack of tendency toward regeneration in aging conditions.[21]

It seems that the treatment with CoQ10 could be useful in the reduction of the adverse effects of cyclophosphamide, most likely through its antioxidant and anti-apoptotic activities.

Our results also indicated that cyclophosphamide significantly decreased the mRNA expression of PCNA in ovarian tissue compared with the healthy control group, while its expression increased in the CTX + CoQ10 group when compared with the CTX-treated group (Figure 3A).

PCNA, a non-histone acidic nuclear protein with a molecular weight of 36 kDa, differentially expressed in fetal and adult ovaries in several mammals or arthropods[8,32] (Figure 5).

Also, PCNA has been considered a proliferation marker in the granulosa cells that can act as a primary regulator during the development of ovarian follicles.[36,37] It has been implicated that the expression of PCNA is increased during the growth and maturation of follicles and oocytes.[38] Interestingly, PCNA has an essential role during biological processes such as DNA replication, DNA repair, sister chromatid cohesion, DNA damage

TABLE 4 Fertilization rate and embryo development in all of the study's groups

Groups	Number of MII oocytes	Number of fertilized (% ± SD)	Number of cleavaged (% ± SD)	Number of hatched blastocysts (% ± SD)
Control	108	74 (68.51 ± 0.4)	46 (62.16 ± 1.1)	16 (34.78 ± 0.6)
CTX	79*	41 (51.89 ± 0.2)*	19 (46.34 ± 0.9)*	4 (21.05 ± 0.4)*
CTX + CoQ10	93*,**	55 (59.13 ± 0.7)*,**	29 (52.72 ± 1.6)*,**	8 (27.58 ± 0.3)*,**
CoQ10	116	88 (75.86 ± 0.5)***	57 (64.77 ± 1.3)	21 (36.84 ± 0.8)

Note: Values are given as mean ± SD.
Abbreviations: CoQ10, coenzyme Q10; CTX, cyclophosphamide; SD, standard deviation.
*$P < .05$ show significant differences in the cyclophosphamide and cyclophosphamide + CoQ10 groups compared with the control group.
**$P < .05$ show significant differences in the CTX + CoQ10 group compared with the CTX group.
***$P < .05$ show significant differences in the healthy treated group (CoQ10) compared with the control group.

prevention, cell cycle control, and cell survival.[39] It has been shown that PCNA can be detected at all stages of the process of folliculogenesis, and could be considered a marker of the number of ovarian follicles.[37]

One study showed that the mating of healthy female rats with cyclophosphamide-treated male rats results in the alterations of the profile expression of DNA repair genes during pre-implantation development.[14] The results obtained from the comet assay highlighted that the rate of transcription for some selected members of the nucleotide excision repair family such as PCNA and xeroderma pigmentosum complementation (XPE) is dramatically increased in one-cell stage and 8-cell embryos sired by cyclophosphamide-treated male rats as compared with the control rats[14]; however, the increased expression of PCNA in one-cell embryos sired by cyclophosphamide-treated males may be due to the alteration in control of the cell cycle or the enhanced transcription-coupled repair.[14]

Although the accurate mechanism of CoQ10, which leads to the enactment of PCNA expression, is still unknown, we suggest that CoQ10 may have proliferative effects. However, this issue should be studied in the future.

In the present experiment, the expression level of FSHR in the CTX-treated group was markedly lower than that of the healthy control group (Figure 3B). However, the expression of FSHR was

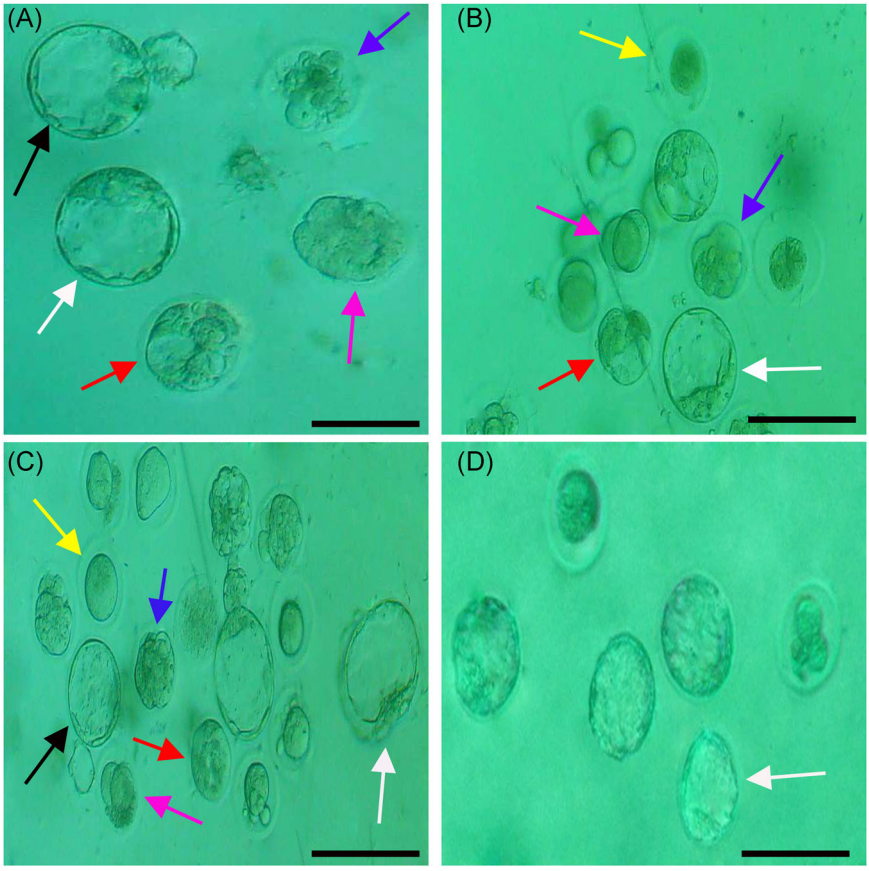

FIGURE 4 Effects of cyclophosphamide and coenzyme Q10 (CoQ10) on in vitro fertilization rate and embryo development. A, Healthy control group. B, Cyclophosphamide control group. C, Cyclophosphamide group treated with CoQ10. D, Healthy control group treated with CoQ10. Black, white, red, yellow, blue, and pink arrows show hatched blastocysts, blastocysts, early blastocysts, oocytes (metaphase I), morula, and arrested embryo, respectively

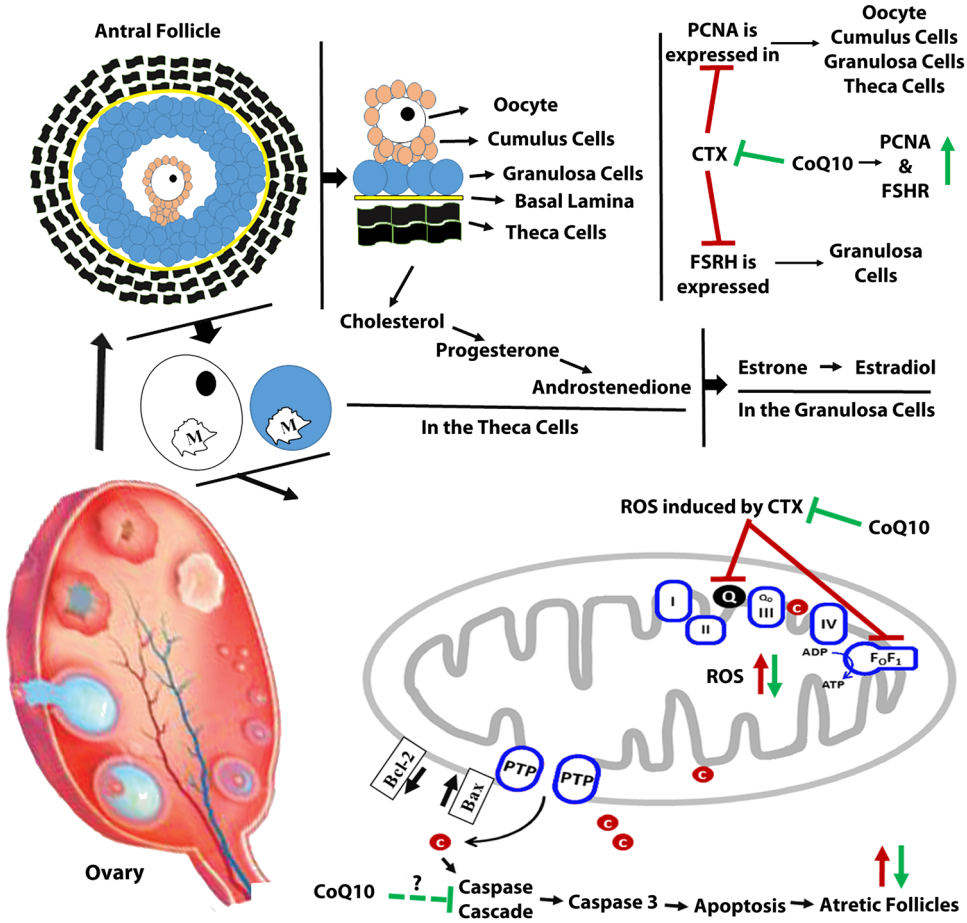

FIGURE 5 Effect of CTX and CoQ10 on the mitochondrial function and the expression of PCNA and FSHR genes. The exogenous generation of ROS in response to CTX adversely affects the functionality of mitochondria by the prevention of the synthesis of CoQ10, which is considered an essential molecule in the respiratory chain, and the activity of respiratory chain complexes and ATP synthase (F0F1). It is now known that the overproduction of endogenous ROS (excessive production of mitochondrial ROS) induces apoptosis via the mitochondrial pathway (intrinsic apoptosis pathway), accompanied by the activation of pro-apoptotic proteins such as Bax and the suppression of anti-apoptotic proteins such as Bcl-2. These phenomena can stimulate MOMP that results in the release of cytochrome *c* (C) from the intermembrane space into the cytosol. When cytochrome centers the cytoplasm, it binds to Apaf-1 to form a big protein complex called apoptosome. The apoptosome initiates the caspase cascade leading to caspase-3 activation and eventually cell death. Based on the above statements, it would be plausible that the number of atretic follicles would be increased. PCNA is differentially expressed in oocytes, granulosa cells, cumulus cells, and theca cells and it plays a significant role in DNA repair. It seems CTX could decrease the mRNA expression of PCNA in ovarian tissue thereby an increase in the level of ROS. The administration of CTX decreased the expression of FSHR, which is exclusively expressed on the granulosa cells. The mRNA expression of follicle-stimulating hormone receptor (FSHR) decreased in the ovarian tissue after being affected by CTX. Hence, the decrease of 17β-estradiol (E2) concentration in serum of cyclophosphamide-treated mice, which has been reported in other reports, may be due to this fact. Regarding the above statements, it appears that further investigations are needed to shed light on the understanding of the mechanism underlying the upregulation of crucial genes in response to CoQ10. Apaf-1, apoptotic protease-activating factor 1; Bcl-2, B-cell lymphoma 2; CoQ10, coenzyme Q10; CTX, cyclophosphamide; FSHR, follicle-stimulating hormone receptor; MOMP, mitochondrial outer membrane permeabilization; mRNA, messenger RNA; PCNA, proliferation cell nuclear antigen; ROS, reactive oxygen species

significantly increased in the CTX + CoQ10 group when compared with the CTX-treated group.

FSH, as a key hormone in mammalian reproduction, is necessary for gonadal development, maturation at puberty, and gamete production during the fertile phase of life.[40] In female, binding of FSH to its cognate receptors (ie FSHR), which are exclusively expressed on the granulosa cells from as early as the primary/pre-antral stage of folliculogenesis (Figure 5), leads to the development and proliferation of the granulosa cells and pre-antral follicles, stimulates the estradiol production in GCs, and regulates the expression of FSHR on the granulosa cells.[41] FSHR, with a molecular weight of 80 kDa, belongs to the family of the G protein-coupled receptors characterized by seven membrane-spanning α helices. The binding of ligands to the extracellular domain of the G protein-coupled receptors, induces a conformational change which allows the cytosolic domain of the receptors to bind to a G protein-linked with the inner face of the plasma membrane.[8,32,40]

On the other hand, upon binding of FSH to its cognate receptors, the follicular transition (from pre-antral to antral) occurs, and cytochrome P450 enzymes are activated. The binding of FSH to

FSHR also increases the transcription of CYP19A1 in the granulosa cells, which are involved in converting theca cell-derived androgens such as androstenedione and testosterone into estrogen and estradiol, respectively.[8,32,40]

The stimulation of follicle growth through the PMSG injection could cause a remarkable increase in the expression of FSHR, whereas the administration of HCG, followed by the injection of PMSG, to induce super-ovulation could significantly decrease the mRNA expression of the FSHR gene.[42,43] Therefore, in our study, the effect of CoQ10 on the expression of FSHR and PCNA was performed before the injection of PMSG/HCG in cyclophosphamide-induced ovarian toxicity.

Liu et al established a murine model of POF in 8-week-old female C57BL/6 mice while they were treated with the intraperitoneal injection of CTX (70 mg/kg) in the first day of the experiment. The injection of CTX continued at a dose of 30 mg/kg every 3 days for 3 weeks to induce cyclophosphamide-induced ovarian damage. The immunohistochemical analysis of their study revealed that there was no significant difference in the expression of FSHR between the CTX-treated and control groups.[44] In our study, mice received cyclophosphamide daily since it has been reported that the half-life of CTX is about 3 to 12 hours in the blood circulation[45]; however, mice in the study of Liu et al received cyclophosphamide every three days, suggesting that discrepancies in result of the expression of FSHR could be due to this phenomenon. In our study, therefore, it is suggested that CoQ10 by upregulating the expression level of FSHR mRNA, could prevent the follicular regression. However, the accurate mechanism is unclear.

Therefore, further studies on the other genes involving folliculogenesis will be needed to understand the mechanism better.

On the other hand, several studies have reported that the serum concentrations of 17-β-estradiol and progesterone are lower in mice treated with CTX, while the levels of LH and FSH are significantly higher in these mice when compared with healthy mice[35,44] (Figure 5).

Some studies demonstrated that the process of steroidogenesis is enhanced when FSH binds to its cognate receptor (ie, FSHR), leading to the elevation of 17-β-estradiol.[8,32] It seems that the decreased concentration of the 17-β-estradiol hormone in CTX-induced ovarian damage may stem from the downregulation of the FSHR gene expression (Figure 5). Nevertheless, the precise mechanism underlying the reduction of 17-β-estradiol in CTX-induced ovarian damage is not fully understood.

One study reported that the administration of CoQ10 at a dose of 150 mg/kg (daily, for 4 months) to infertile women, who suffered from amenorrhea, reduced oxidative stress in the neuroendocrine system, and it was able to improve the function of diminished hypothalamic-pituitary-ovarian axis (HPO).[46] On the other hand, Gui et al indicated that the expression of the sex hormone receptors, including the estrogen receptor α (ERα), estrogen receptor β (ERβ), progesterone receptor (PR), as well as androgen receptor (AR) does not show any significant difference among the experimental groups.[47] However, investigating the effects of CTX on the other genes involved in steroidogenesis will be performed to access the accurate mechanism.

Another part of our results showed the level of ROS in oocytes, isolated from super-ovulated mice treated with cyclophosphamide was significantly increased compared with the control group. However, the treatment with CoQ10 inhibited the overproduction of ROS, which was induced by the exposure of mice to cyclophosphamide.

It has been reported that cyclophosphamide can enhance the expression of oxidative stress markers such as ROS, reactive nitrogen species (RNS), and 3-nitrotyrosine (3-NT).[1] Hence, cyclophosphamide is capable of tilting the redox system in favor of the production of pro-oxidant agents. It is widely accepted that antioxidant therapy is a promising strategy for the decrease of oxidative stress and it plays a vital role in the improvement of the ovarian function and oocyte quality.[19]

Some experimental evidence demonstrates that the overproduction of ROS could harm oocyte maturity, fertilization, embryo quality, and pregnancy rate, while an optimum level of ROS is necessary to support adequate oocyte development.[48] ROS has direct and indirect effects on the biological processes occurred in oocytes including maturation and fertilization potential. Such effects might be exerted spontaneously or through assisted reproduction technology (ART).[48] During recent years, the relationship between ROS levels and the IVF outcome has been well established.[48] In the study carried out by Liang et al,[19] they showed that the addition of CoQ10 (100 μM) to the culture media could greatly improve the quality of H_2O_2-treated embryo as this effect is confirmed by the reduced rate of ROS, DNA damage, and apoptosis in the embryo at the blastocyst stage.

Our findings also showed that in the CTX-treated group, the numbers of MII oocytes, 2-cell embryos (cleavaged), hatching blastocyst embryos, as well as the rate of fertilization, were significantly lower than the healthy control group. In line with this, the treatment of CTX-treated mice with CoQ10 increased the number of earlier-mentioned parameters.

Although chemotherapy is usually accompanied by a reduction in fertility, the preservation of fertility ability among young adults suffering from cancer is an urgent need for these patients.[15] The use of antioxidant agents as a complementary therapy for the mitigation/prevention of the adverse effect of anticancer drugs has been investigated remarkably.

However, there are limited reports on oocytes or embryo development during or after chemotherapy. The administration of cyclophosphamide at doses of 4, 20, and 40 mg/kg to pregnant CBA/Ca-mice revealed that the number of blastocyst cells was significantly lower than the control group when it was administered at all doses. Moreover, the results of the in vitro differentiation of 84-hour blastocysts that were subsequently cultured for a further 120 hours showed that cyclophosphamide at doses of 20 and 40 mg/kg inhibited blastocyst hatching, decreased the attachment rate of the embryos to glass coverslips, and reduced the rate of inner cell mass, as well as the number of embryos with expanded trophoblast.[13]

Accordingly, cyclophosphamide, at all doses, increased the number of aberrant cells, chromosomal aberrations, chromosome breaks, and chromosomal rearrangements. It can also inhibit the synthesis of histone proteins and DNA replication in 84-hour blastocysts.[13]

The study conducted by Koike et al[49] reported that ovarian stimulation, immediately, after the injection of cyclophosphamide (400 mg/kg) dramatically decreased the average number of retrieved oocytes, the average number of oocytes which are developed to MII oocytes, and the fertilization rate as compared with the control group, while the rate of morula and blastocyst formations remained the same as the control group. Notably, two weeks and one month after the administration of cyclophosphamide into mice, the number of retrievable oocytes was decreased by 50% and increased by 90%, respectively, while the number of MII oocytes did not significantly change when compared with the control group. Of note, Koike and his colleagues did not perform histological examinations to observe the influence of cyclophosphamide on ovarian tissue.[49]

In the early year of 1971, Fritz and Hess[50] reported that the administration of cyclophosphamide (30 mg/kg, intravenously) before the implantation of blastocysts (ie before day 6 or 7) in rabbit, did not cause any change in the total number of implants compared with the control group, while the administration of cyclophosphamide on the 6th, 7th, or 8th days of pregnancy increased the number of early embryonic deaths (embryonic resorption). Correspondingly, the injection of cyclophosphamide on the 9th, 10th, and 11th days of gestation (after the period of implantation) elevated the number of fetal death.[50] It has been revealed in a clinical trial study that the pre-treatment with CoQ10 (200 mg, orally, three times a day) for 2 months before IVF-ICSI (intracytoplasmic sperm injection) cycle increased the rate of fertilization, as well as the numbers of retrieved oocytes and high-quality embryos in young women suffering from poor ovarian response or low ovarian reserve. It also improved the clinical pregnancy and the chance of live birth.[18] Liang et al[19] showed that the quality of blastocyst, along with the cleavage and blastocyst formation rates are significantly improved when the embryo is cultured in a medium containing 100 μM CoQ10. In the research project conducted by Gardner et al,[51] they exhibited a direct correlation between the blastocyst quality and blastocyst hatching. Besides, their results also demonstrated that the treatment with CoQ10, at a concentration of 100 μM, can enhance the mRNA expression of some genes, such as fibronectin 1, integrin α5, and cyclooxygenase 2, which are involved in the early blastocyst formation and blastocyst hatching.[19]

5 | CONCLUSION

The present study indicates that the administration of CoQ10 attenuates CTX-induced ovarian dysfunction, which is considered the leading cause of infertility in women. To the best of our knowledge, this is the first report showing that CoQ10, as an antioxidant compound, is capable of downregulating the increased amount of ROS in MII oocytes, increasing the mRNA expression of PCNA and FSHR, as well as enhancing the rate of IVF and the embryo development.

6 | SUGGESTIONS

According to the results, it is suggested that other genes involved in the process of folliculogenesis should be investigated to show which genes contribute to maturation and the development of the embryo in response to CTX or CoQ10 therapy. It is also warranted that further studies must be carried out to elucidate and interpret the relationship between FSHR expression and a decreased level of 17-β-estradiol.

ACKNOWLEDGMENTS

The authors would like to thank all staff of the Al-Zahra Hospital for their assistance in providing the lab equipment.

ETHICS STATEMENT

The ethical code of this study is IR.TBZMED.REC.1396.555.

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How to cite this article: Delkhosh A, Delashoub M, Tehrani AA, et al. Upregulation of FSHR and PCNA by administration of coenzyme Q10 on cyclophosphamide-induced premature ovarian failure in a mouse model. *J Biochem Mol Toxicol*. 2019;33:e22398. https://doi.org/10.1002/jbt.22398