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# The effect of Coenzyme Q10 on mitochondrial biogenesis in mouse ovarian follicles during *in vitro* culture

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**Research Article**

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## Summary

The aim of this research was to investigate the effect of Coenzyme Q10 (CoQ10) on the expression of the Transcription Factor A Mitochondrial (*Tfam*) gene and mtDNA copy number in preantral follicles (PFs) of mice during *in vitro* culture. To conduct this experimental study, PFs were isolated from 14-day-old National Medical Research Institute mice and cultured in the presence of 50 µm CoQ10 for 12 days. On the 12th day, human chorionic gonadotropin was added to stimulate ovulation. The fundamental parameters, including preantral follicle developmental rate and oocyte maturation, were evaluated. Additionally, the *Tfam* gene expression and mtDNA copy number of granulosa cells and oocytes were assessed using the real-time polymerase chain reaction. The results revealed that CoQ10 significantly increased the diameter of PFs, survival rate, antrum formation, and metaphase II (MII) oocytes ($P < 0.05$). Moreover, in the CoQ10-treated groups, the *Tfam* gene expression in granulosa cells and oocytes increased considerably compared with the control group. The mtDNA copy number of granulosa cells and oocytes cultured in the presence of CoQ10 was substantially higher compared with the control groups ($P < 0.05$). The addition of CoQ10 to the culture medium enhances the developmental competence of PFs during *in vitro* culture by upregulating *Tfam* gene expression and increasing mtDNA copy number in oocyte and granulosa cells.

## Introduction

Female infertility is a significant concern today, with several factors contributing to it. As a result, an increasing number of people are using assisted reproductive technologies (ARTs), such as *in vitro* culture (IVC) of ovarian tissue, follicles, oocytes, and embryos. While ART has benefits for treating infertility, it can also lead to an increase in reactive oxygen species (ROS) during *in vitro* cultivation, resulting in oxidative stress (OS) due to the absence of an antioxidant defence system (Filatov *et al.*, 2020).

One potential antioxidant that can help neutralize free radicals, recycle other antioxidants, suppress OS, and protect mitochondria from oxidative damage is Coenzyme Q10 (CoQ10). This fat-soluble compound is synthesized naturally in the human body and is one of the most consumed nutritional supplements (Arenas-Jal *et al.*, 2020). Studies have confirmed that CoQ10 is beneficial for both *in vivo* and *in vitro* usage. For example, oral consumption of CoQ10 protects the ovarian follicular reserve, increases oocyte quality, and increases the number of cumulus cells (Rodríguez-Varela and Labarta, 2021). Furthermore, CoQ10 has been shown to initiate and improve follicular development by upregulating BMP-15, GDF-9, and FSHR, while reducing the amount of ROS by activating other antioxidants (Lee *et al.*, 2022).

Excessive ROS can cause severe mutations in mitochondrial DNA (mtDNA), leading to mitochondrial dysfunction (Kung *et al.*, 2021). A sufficient mtDNA copy number is necessary for successful follicle development, and insufficient mtDNA copy numbers can result in oocytes that cannot fertilize or are aged or degenerated (Busnelli *et al.*, 2021). It has been demonstrated that increasing mtDNA copy number in *in vitro* conditions can improve oocyte developmental competency (Mao *et al.*, 2012). Additionally, there is a relationship between mtDNA copy number in granulosa cells (GCs) and oocytes, and follicle growth and maturation (Lan *et al.*, 2020). Mitochondrial transcription factor A (TFAM) is the most prominent factor in regulating mtDNA transcription and protecting against OS (Mao *et al.*, 2012). Tfam plays an essential role in regulating mtDNA copy number in ovarian follicles, which is crucial for follicular survival. A balance between mtDNA and Tfam ratio is necessary, and Tfam, with factors such as NF-E2–related factor 2 (Nrf2), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and Sirtuin 1 (*SIRT1*) genes, upregulates mitochondrial biogenesis (Popov, 2020).

A *Tfam* knockdown experiment by Otten *et al.* showed that zebrafish embryos with knocked-down *Tfam* had low ATP and mtDNA content, leading to mitochondrial dysfunction and embryo abnormalities (Otten *et al.*, 2020). In addition, studies have shown that upregulation of

*Tfam* expression and subsequent elevation of mtDNA copy number can improve vitrified–thawed oocyte competency and quality (Amoushahi *et al.*, 2017, 2018; Ito *et al.*, 2020; Moshaashaee *et al.*, 2021).

Although some studies have shown promising results regarding the effects of COQ10 on folliculogenesis during *in vitro* culture (Hosseinzadeh *et al.*, 2015, 2017; Kashka *et al.*, 2015, 2016; Heydarnejad *et al.*, 2019), there is limited knowledge about its effect on mtDNA copy number and *Tfam* gene expression in ovarian follicles. Therefore, the aim of this study was to investigate the effect of CoQ10 on mtDNA copy number and *Tfam* gene expression in mouse oocytes and GCs of preantral follicles (PFs) during *in vitro* culture.

## Materials and methods

### Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Germany), unless otherwise specified.

### Animals

Female and male National Medical Research Institute (NMRI) mice were obtained from the Pasteur Institute of Iran. They were housed and cared for in accordance with the guidelines of our university's animal ethics committee. The animals were kept under controlled conditions (12-hour light/12-hour dark cycle, 20–25°C room temperature, and 40–50% humidity) and provided with standard water and laboratory chow. The animal research ethics committee of Damghan University approved this study (Ref. No. 34/2018).

### Preantral follicle isolation

Newborn female mice (14 days old, $n = 20$) were sacrificed, and their ovaries were removed and placed in droplets of α-minimum essential medium (α-MEM; Gibco, UK) supplemented with 0.22g/l sodium bicarbonate, 0.0036g/l sodium pyruvate, 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Preantral follicles were mechanically isolated using an insulin syringe's 29-gauge needle under a stereomicroscope at ×10 magnification (Talebi *et al.*, 2012). Eligible PFs were selected based on the following criteria: round-shaped follicles with diameters of 140–160 µm and containing intact oocytes with several layers of GCs. During the process, the medium was kept at 37°C.

### Experimental design

The collected PFs were assigned randomly to control and CoQ10-treated groups. PFs from both groups were cultured *in vitro* for 12 days. The study was conducted in two parts. First, follicular development was assessed, including follicular survival, growth, antrum formation, and ovulation. Second, mtDNA copy number and *Tfam* gene expression in GCs and oocytes were evaluated.

### In vitro culture of PFs

Isolated PFs were individually transferred using glass Pasteur pipettes into 25-µl drops of α-MEM supplemented with 1% insulin transferring selenium (ITS; Gibco, UK), 20 ng epidermal growth factor (EGF), 100 mIU recombinant human follicle-stimulating hormone (rhFSH; Cinnal-f, Iran), 5% v/v FBS, and 50 µM CoQ10 under sterile mineral oil, and then incubated under 5% $CO_2$, 37°C, and 95% humidity conditions for 12 days. Next, 10 µl of culture medium from each drop was replaced with fresh medium every other day. Follicle diameter was measured using an inverted microscope at ×10 magnification on the initial day, second, and fourth day of culturing, as described previously (Talebi *et al.*, 2012). In brief, the diameter of the follicles was measured by calculating the average of two perpendicular diameters using an inverted microscope equipped with a pre-calibrated optical micrometer. The evaluation of GC proliferation, growth, and antrum cavity formation was observed under a stereomicroscope over the 12-day culture period. The presence of any lucent space between GCs during *in vitro* culture was defined as an antrum cavity. Moreover, the survival rate was determined by observing morphological changes during the culture period. On the 12th day of the culture period, ovulation was induced by adding 1.5 IU/ml of human chorionic gonadotropin (hCG; IBSA, Switzerland) to the culture medium. After 18 h, the oocytes were classified into two different types: the first type was germinal vesicle breakdown (GVBD), where the germinal vesicle was absent, and the second type was metaphase II oocytes (MII), identified by the time the first polar body was ejected.

**Table 1.** Designed primer sequences used for real-time polymerase chain reaction

| Gene | Primer pair sequence (5′→3′) | Accession number | PCR product size (bp) |
|---|---|---|---|
| *Ef1* | Forward: AGTCGCCTTGGACGTTCTT<br>Reverse: CCGATTACGACGATGTTG ATGTG | NM_010106 | 124 |
| *Tfam* | Forward: AAGGGAATGGGAAAGGTAGA<br>Reverse: AACAGGACATGGAAAGCAGAT | NM_010106 | 76 |

**Table 2.** Mouse mitochondrial specific primer sequences

| PCR product size (bp) | Accession numbers | Primer pair sequence (5′→3′) | Gene |
|---|---|---|---|
| 79 | NM-013523.3 | GCTAGTGTGAGTGATAGGGTAG | *MTR* |
| | | CCAATACGCCCTGTAACAAC | *MTR* |

### Granulosa cell separation

For granulosa cell separation, the culture medium was supplemented with 0.02 g collagenase for 1 h. Oocytes were picked up using a mouth pipette and then transferred individually to 1.5-ml microtubes. Granulosa cells were separated from the bottom of the culture dish by adding 1 ml trypsin and centrifuging at 3000 rpm for 3 min.

### Real-time polymerase chain reaction (PCR)

Real-time PCR was used to determine *Tfam* relative mRNA expression in both oocytes and GCs. RNA was extracted separately from oocytes and GCs using the RiboEx® protocol (PCRlab, Germany). The samples were then quantified and qualified by spectrophotometer and electrophoresis gel, respectively. cDNA was synthesized using the TaKaRa cDNA synthesis kit (TaKaRa Bio, Japan) based on the manufacturing protocol. *Tfam* mRNA expression was assessed using the Rotor-gene 6000 (Qiagen) and QuantiTect SYBR Green RT-PCR kit (Qiagen,

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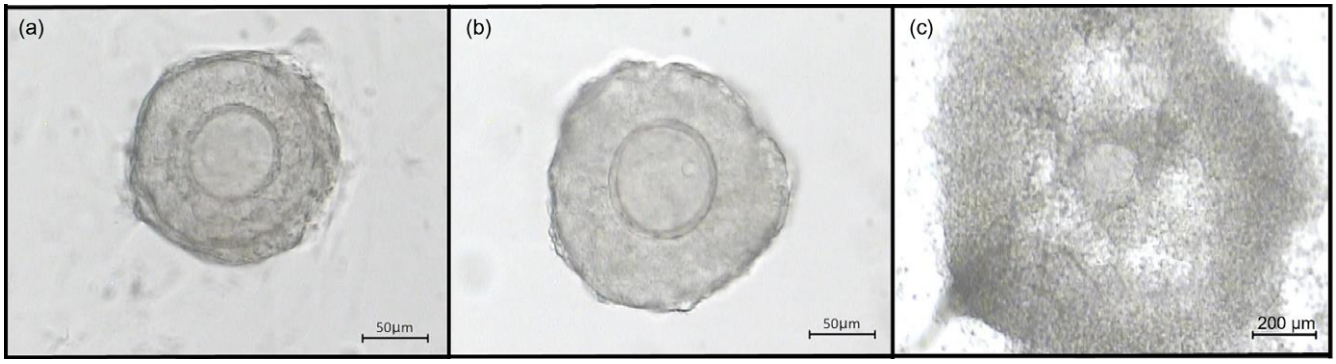


**Figure 1.** Images of the *in vitro* cultured mouse PFs during *in vitro* culture; PFs on the initial day (a), fourth day (b), 12th day (c).

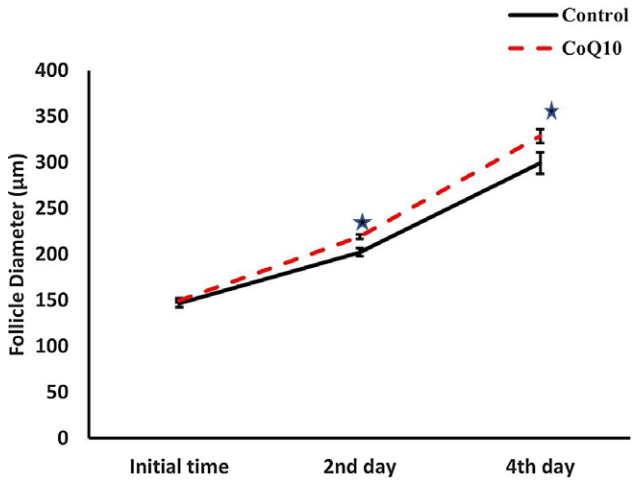


**Figure 2.** Diameter of PFs during the culture period. The stars indicate a significant difference compared with the control group ($P < 0.05$).

Hilden, Germany). The Livak method and 2-ΔΔCT were used to analyze *Tfam* expression. *Tfam* primers were designed using AlleleID software (Premier Biosoft, USA), and elongation factor 1 (*Ef1*) was used as a housekeeping gene (Table 1).

### *Quantification of mtDNA copy number*

DNA samples were extracted from oocytes and GCs following the procedure described by Ghorbanmehr *et al.* (2018). Briefly, 10 µl of lysis solution containing 50 mM Tris–HCl (pH 8.5), 0.1 mM EDTA, 0.5% Tween-20, and 200 µg/ml proteinase K (Roche, Germany) were mixed with the samples and incubated at 55°C. After proteinase K deactivation at 95°C for 10 min, the mouse mitochondrial DNA sequence was obtained from NCBI (NC_005089.1) and divided into 200-bp fragments with 50-bp overlaps. Primers were designed using Primer3 plus software to avoid duplication in the nuclear genome (Table 2) and synthesized by MWG DNA sequencing service in Germany (Ebersberg, Germany). The PCR product was extracted from agarose gel and cloned into the pTZ57R/T vector (Thermo Scientific Bio). Five serial dilutions of the plasmid were prepared, and real-time PCR was performed to quantify the mtDNA copy number in oocytes and GCs. Each extracted mtDNA sample was tested in triplicate using five points on the serial standard curve without template control. The cycling programme included initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

### *Statistical analysis*

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, IBM SPSS statistic 16, USA). After checking the normal distribution of data using the Shapiro test, the Mann–Whitney *U*-test was applied. Statistical significance was set at $P < 0.05$.

## Results

### *PFs development*

The growth of PFs was examined morphologically every other day during the *in vitro* culture period by an inverted microscope (Figure 1). The diameter of isolated PFs is summarized in Figure 2. The diameter at the initial time did not differ in both groups ($P > 0.05$). However, on the second and fourth day of cultivation, the follicle's diameter showed a significant increase in the presence of CoQ10 compared with the control group ($P < 0.05$; Figure 2). On the second day of culture, the isolated PFs attached to the bottom of the culture dish through granulosa cell proliferation and developed a round and adhesive shape. On the fourth day, the number of GCs increased and surrounded the oocyte and formed a redundant form. From the sixth day onwards, the antrum cavity formed, and PFs with round and intact oocytes, zona pellucida, and regular perivitelline space were considered as surviving follicles. Degenerated follicles were the ones that released their oocytes before adding hCG or had a delay in granulosa cell proliferation. The survival rate of PFs in the presence of CoQ10 and the control group was 87% and 79%, respectively, which was significantly higher in CoQ10 compared with the control group ($P < 0.05$; see Table 3). The rate of antrum formation in the CoQ10-treated group and the control group was 71% and 58%, respectively, which was significantly higher in the treated group ($P < 0.05$; Table 3). Furthermore, the rate of ovulated follicles in the CoQ10-treated group (51%) was significantly higher than in the control group (40%; $P < 0.05$; Table 3). The maturation rate of oocytes derived from PFs is shown in Table 3. The percentage of released metaphase I (MI) and MII oocytes from PFs in the CoQ10-treated group was significantly increased compared with those in the control group ($P < 0.05$; Table 3).

### *Tfam gene expression*

The relative mRNA expression of *Tfam* was evaluated in oocytes and GCs. The relative expression of the *Tfam* gene in oocytes was considerably higher in the CoQ10-treated group compared with the control group ($P < 0.05$; Figure 3). Also, the relative mRNA

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**Table 3.** Maturation rates of cultured preantral follicles

| Groups | No. of follicles | Survived *n* (% ± SD) | Antrum formation *n* (% ± SD) | Ovulated follicles *n* (% ± SD) | Stages of oocyte development | |
|---|---|---|---|---|---|---|
| | | | | | MI oocytes *n* (% ± SD) | MII oocytes *n* (% ± SD) |
| Control | 180 | 143 (79.44 ± 4.19) | 105 (58.33 ± 3.34) | 73 (40.56 ± 4.19) | 17 (23.44 ± 3.39) | 56 (76.56 ± 3.39) |
| CoQ10 | 180 | 158* (87.78 ± 2.55) | 128* (71.11 ± 4.82) | 93* (51.67 ± 1.67) | 11* (11.80 ± 4.78) | 82* (88.20 ± 4.77) |

In all cases at least three experimental replicates were performed.
*Indicates different levels of significant difference with control groups ($P < 0.05$).
MI: metaphase I oocyte; MII: metaphase II oocyte.

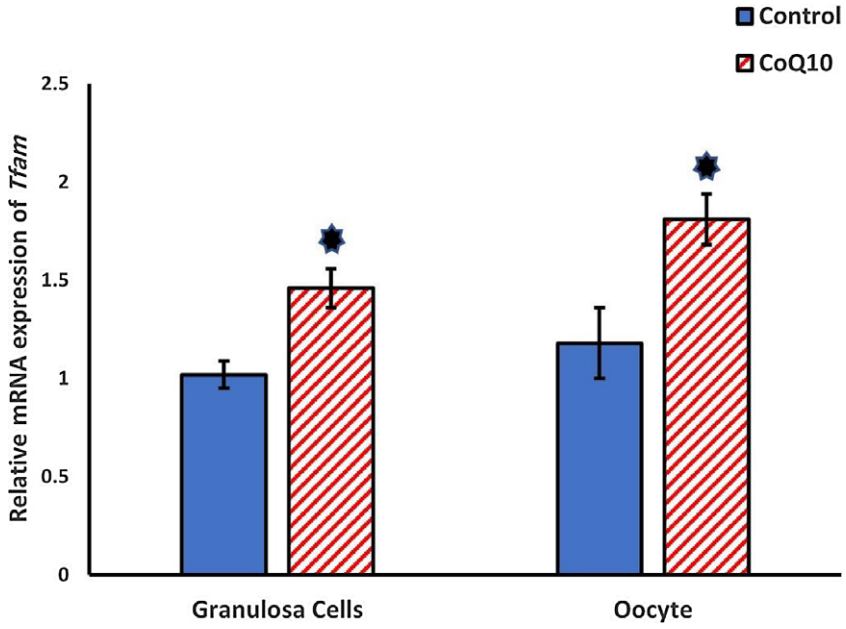


**Figure 3.** The relative mRNA expression of the *Tfam* gene. The stars indicate a significant difference compared with the control group ($P < 0.05$).

expression of the *Tfam* in GCs of the CoQ10-treated group was considerably higher compared with the control group ($P < 0.05$; Figure 3).

### *mtDNA copy number*

The mtDNA copy numbers in the oocytes and GCs of both the treated and control groups are presented in Figure 4. The mtDNA copy numbers in oocytes derived from the CoQ10-treated and control groups were 506,512 and 171,887, respectively. This value was significantly higher in the CoQ10-treated group ($P < 0.05$; Figure 4). Additionally, the mtDNA copy number of GCs derived from the CoQ10-treated and control groups were 484,589 and 314,786, respectively. The results indicated that the mtDNA copy number of GCs was significantly higher in the CoQ10-treated group compared with the control group ($P < 0.05$; Figure 4).

## Discussion

*In vitro* maturation is a commonly used assisted reproductive technology that faces several challenges, such as an increase in OS and ROS production during the culture period (Talebi *et al.*, 2012; Kashka *et al.*, 2016). While ROS plays a crucial role in follicle development and fertilization, an excessive amount can negatively affect different signalling pathways involved in oocyte and embryo development (Lu *et al.*, 2018; Misrani *et al.*, 2021). As a result, the addition of antioxidants to the culture medium is recommended. However, it is still unclear how antioxidants affect ovarian follicle formation and growth during *in vitro* culture. Antioxidants have been shown to be crucial for oocyte maturation and ovulation (von Mengden *et al.*, 2020). Therefore, this study aimed to examine the effect of CoQ10, a potent antioxidant, on *Tfam* gene expression and mtDNA copy number in ovarian follicles.

The study found that CoQ10 increases *Tfam* gene expression and mtDNA copy number in preantral follicle oocytes and GCs, thereby improving folliculogenesis during *in vitro* culture. Previous studies have shown that high ROS production during *in vitro* maturation interrupts follicle development and reduces its competency (Soto-Heras and Paramio, 2020). Conversely, CoQ10 can elevate follicle growth and development (Xu *et al.*, 2018). Lee *et al.* showed that CoQ10 promotes ROS reduction and oocyte quality improvement by inciting ovarian follicle stem cells (Lee *et al.*, 2021, 2022). The same group demonstrated that CoQ10 improves mouse embryo development *in vitro* by elevating bcl2 and sirt1 expression in cumulus cells, which reduces ROS (Lee *et al.*, 2022). Additionally, CoQ10 helps infertile individuals undergoing *in vitro* fertilization (IVF) by improving mitochondrial function and ovarian follicle maintenance (Özcan *et al.*, 2016). A study by Ma *et al.* (2020) found that CoQ10 reduced post-meiotic aneuploidy and increased oocyte maturation rates in aged women during *in vitro* maturation. Yang *et al.* (2021a, 2021b) observed that 50 μm CoQ10 suppressed OS, improved mitochondrial

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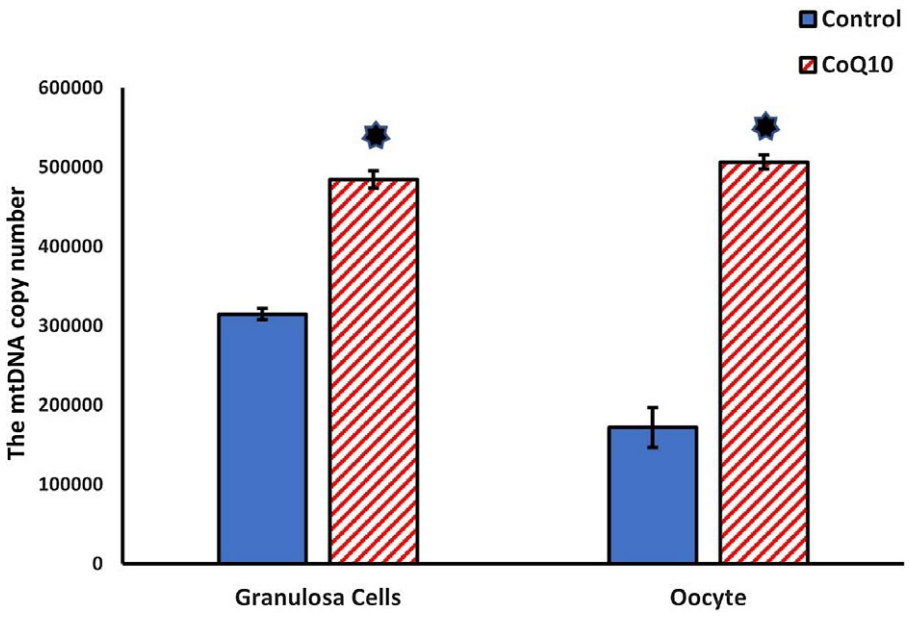


**Figure 4.** The mtDNA copy number of granulosa cells and oocyte. The stars indicate a significant difference compared with the control group ($P < 0.05$).

function, and upgraded meiotic maturation during *in vitro* cultivation, therefore increasing oocyte quality. These results are in line with our findings regarding the application of 50 µm CoQ10 to an *in vitro* culture medium.

However, Streacker and Whitaker (2019) confirmed that 100 µm CoQ10 was deleterious during *in vitro* maturation, while Maside *et al.* (2019) found that applying 100 µm CoQ10 negatively affected ART outcomes. In contrast, our results were compatible with studies that used 50 µm CoQ10 during *in vitro* culture medium. Furthermore, CoQ10 acts as an anti-apoptotic antioxidant, preventing cell death by reducing OS and balancing the amount of anti-apoptotic Bcl-2 and pro-apoptotic BAX factors (Delkhosh *et al.*, 2021).

It has been investigated that CoQ10 in oocytes and cumulus cells decreases the expression of apoptotic genes such as caspase-3 and Bax, while considerably increasing GDF9 expression (Heydarnejad *et al.*, 2019). Li *et al.* (2019) also observed that CoQ10 activated enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione (GSH), downregulated Bax and caspase-3, and increased *Bcl-2* gene expression. Among the anti-apoptotic factors, nuclear respiratory factor 2 (Nrf2) is vital in maintaining redox balance and regulating antioxidants and follicle maintenance (Smolková *et al.*, 2020).

As a result of increased OS, Nrf2, by binding to the antioxidant response element (ARE), activates protective factors such as SOD and suppresses OS. Conversely, activated NF-κB, as a major transcription factor, controls inflammatory and apoptotic responses regulated by Nrf2 during OS situations (Khodakarami *et al.*, 2022). Nrf2, via activating PGC-1α, regulates *Tfam* gene expression, which is essential for mtDNA transcription (Shimizu *et al.*, 2022). Similarly, CoQ10 can suppress NF-κB and increase *Nrf2* gene expression, leading to *Tfam* expression regulation (Li *et al.*, 2019). It is therefore reasonable to consider that CoQ10 has a beneficial effect on *Tfam* expression.

Based on previous studies, the Tfam level is regulated by PGC-1α and is dependent on NRF1 and NRF2, which can be stimulated by PGC-1α. It is worth mentioning that NRF2 expands the antioxidant defence system, and CoQ10, by upregulating NRF2, not only improves the antioxidant defence system but also increases *Tfam* expression by activating the *PGC-1α* gene (Li *et al.*, 2019; Deng *et al.*, 2020). Furthermore, there is a relationship between *Tfam* expression and mtDNA copy numbers, whereby an increase in *Tfam* increases the mtDNA copy number (Lan *et al.*, 2020), which agrees with our results. It has been shown that good quality embryos have higher mtDNA copy numbers compared with bad quality ones (Cecchino and Garcia-Velasco, 2019). Matured oocytes contain 150,000 copies of mtDNA, while infertile ones have fewer mtDNA copy numbers, indicating that growing follicles are in great need of mtDNA (Rahimi Darehbagh *et al.*, 2022). A lack of efficient mtDNA copy numbers may cause unpredictable ART outcomes. In this case, Yang *et al.* (2021a, 2021b) found that the mtDNA and gDNA ratio was noticeable for IVF outcomes. They observed that good quality embryos had a higher rate of mtDNA/gDNA in their cumulus cells compared with those with a low mtDNA/gDNA ratio. These findings are compatible with our observation regarding mtDNA copy number and follicle development. In conclusion, supplementing CoQ10 to the PFs culture medium can improve follicle development by upregulating *Tfam* gene expression and increasing mtDNA copy number.

**Author contributions.** R.H.: Conducted experiments and collected data. S.Z.: Supervision, conceptualization, methodology, software, data curation, writing - original draft preparation. M.N.: Supervision, visualization, investigation. S.S.: Investigation and writing draft. All authors contributed to the finalization of the manuscript and approved the final draft.

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