

Original Research The Effect of Quercetin on Ovary Functions in Rats with Cyclophosphamide Induced Ovary Damage

Ceren Yildiz Eren^{1,*}, Hulusi Goktug Gurer¹, Ozlem Ozgur Gursoy¹, Oktay Yilmaz², Ece Tunc², Serap Unubol Aypak³, Gamze Sevri Ekren Asici³

¹Department of Obstetrics and Gynecology, Clinic Eskisehir Acibadem Hospital, 33400 Eskisehir, Turkey

²Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03030 Afyon, Turkey

³Department of Veterinary Biochemistry, Faculty of Veterinary Medicinr, Aydin Adnan Menderes University, 09100 Aydın, Turkey

*Correspondence: cyeren@yaani.com (Ceren Yildiz Eren)

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Abstract

Background: Premature ovarian failure describes women under 40 who usually present with amenorrhea, hypergonadotropic hypogonadism, and infertility. Quercetin is an antioxidant flavonol. Quercetin's oxidative, kinase and cell cycle inhibitor activities are known. Our study aimed to examine the efficacy of Quercetin on premature ovarian failure. **Methods**: Forty-eight regular-cycled adult female Wistar rats weighing 200 ± 40 grams, 10-12 weeks old, were used in the study. They were randomly divided into four groups with 12 animals. Four groups are Control, Cyclophosphamide, Cyclophosphamide + Quercetin (100 mg/kg) and Quercetin (100 mg/kg) groups. At the end of the experiment, the ovarian tissues were quickly removed. Follicles were counted to determine the ovarian reserve. Serum was extracted, and an Anti-Müllerian hormone analysis was performed. RT-PCR (reverse transcriptase–polymerase chain reaction) from ovarian tissue performed mRNA expression analysis of the *Ddx4* gene. **Results**: As a result of Cyclophosphamide administration, it was determined that there was a decrease in both early-stage follicles and total follicles. This decrease was also statistically significant (p < 0.05). Anti-Müllerian hormone levels were significantly lower in the group given Cyclophosphamide group compared to the control group, and those of the Cyclophosphamide + Quercetin were very close to that of the control group. Anti-Müllerian hormone (AMH) levels were also significantly lower in the Cyclophosphamide group compared to the level of the control group by Quercetin treatment. **Conclusions**: Our study may prove that Quercetin can protect ovarian function against Cyclophosphamide-induced ovarian damage.

Keywords: quercetin; cyclophosphamide; premature ovarian failure; anti-mullerian hormone; RT-PCR

1. Introduction

Premature ovarian failure (POF) is used to describe women under 40 who usually present with amenorrhea, hypergonadotropic hypogonadism, and infertility. POF is a diagnosis that should be considered for women of reproductive age and means menopause before the age of 40, affecting approximately 1% of women. The main problems are anovulation and hypoestrogenism, characterized by elevated follicle stimulating hormone (FSH) and luteinizing hormone (LH). Although the etiology is unknown in most patients, it may be caused by autoimmunity, enzymatic disorder, infection, or iatrogenic [1]. Although high-dose and long-term expensive treatments are used in assisted reproduction treatment for these patients in infertility clinics, successful pregnancy is around 5% [2].

Quercetin is an antioxidant flavonol that belongs to the flavonoid group. It is frequently found as Quercetinglycoside [3,4]. Quercetin-3-O-glycoside mainly acts as a pigment in flowers, vegetables, and fruits. This flavonoid, abundant in plants, is a more potent antioxidant than vitamins C and E [5]. Its primary sources are grapes, strawberries, cherries, apples, citrus fruits, onions, and black tea [6,7]. It shows a relatively higher bioavailability than other phytochemicals [8]. However, the concentration of Quercetin can vary from one plant to another and even between different parts of the same plant [9]. Antiinflammatory and antioxidant effects; Quercetin's oxidative, kinase, and cell cycle inhibitor activities are known [10]. Its apoptosis-inducing effects are crucial to its anticancer potential. Quercetin is also effective on immunity and inflammation [11].

POF animal models have been used in many studies to determine the molecular mechanisms of POF and to develop therapeutic agents [12,13]. Currently, animal models of POF are made using various anticancer drugs. Cyclophosphamide is one of these drugs. Cyclophosphamide shows its effect through ovarian atrophy and depletion of primordial follicle stock [14].

The phosphatidylinositol 3-kinase signaling pathway regulates the survival of mammalian primordial follicles. The balance between positive and negative regulation of



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this pathway determines the condition of primordial follicles [15]. Agents such as Cyclophosphamide may disrupt this balance, leading to the activation of dormant primordial follicles [16]. Quercetin is thought to have protective effects against follicle loss caused by chemotherapy by regulating the phosphatidylinositol 3-kinase signaling pathway [17].

Our study aimed to examine the efficacy of Quercetin on ovarian function in POF-rat model.

2. Materials and Methods

2.1 Animals

Forty-eight regular-cycled adult female Wistar rats weighing 200 ± 40 grams, 10-12 weeks old, were used in the study. The subjects were kept in the light for half of the day (12 hours) and the dark for the other half (12 hours) and in a room with an average constant temperature of 22 °C. The subjects were fed with standard pellet feed and given city water. They were randomly divided into four groups with 12 animals. While forming the groups, the minimum numbers to obtain statistically significant results were taken into consideration. The relevant Ethics Committee approved the protocol regarding the procedures performed on animals. All work and procedures were carried out following the Guide for the Care and Use of Laboratory Animals.

2.2 Study Design and Collection of Samples

The animals used in the study were randomly divided into four groups, with 12 animals in each group prospectively.

Group 1 (Control Group): Rats were given intraperitoneal (IP) serum physiological 0.9%, 1 mL/kg every day from days 1 to 8 and then once on day 15.

Group 2 (Cyclophosphamide): Rats were given Cyclophosphamide 100 mg/kg/day IP on days 1 to 7 and serum physiological 0.9%, 1 mL/kg from days 8 to 15.

Group 3 (Cyclophosphamide + Quercetin): Rats were given Cyclophosphamide 100 mg/kg/per dose IP on days 1 to 7 and Quercetin 100 mg/kg IP serum dissolved physiologically on days 1, 8 and 15.

Group 4 (Quercetin): Rats have given Quercetin 100 mg/kg IP serum dissolved physiologically on days 1, 8, and 15.

At the end of the experiment, which lasted for 21 days, the rats in all groups were euthanized by taking intracardiac blood under intramuscularly administered ketamine (45 mg/kg) + xylazine (5 mg/kg) anesthesia, and the ovarian tissues were quickly removed. The excised tissues were fixed with appropriate fixatives, and then embedded in paraffin blocks. 5–6 mm thick sections taken from paraffin blocks were stained with hematoxylin-eosin and examined, and follicles were counted to determine the ovarian reserve for histopathological analysis. The histopathological changes were recorded according to the groups, and the follicles were separated and counted according to the groups. The blood taken for biochemical analysis was centrifuged at 5000 rpm for 15 minutes, their serum was extracted, and AMH (Anti-Müllerian hormone) analysis was performed. In addition, mRNA expression analysis of the Ddx4 gene was performed by RT-PCR (reverse transcriptase–polymerase chain reaction) from ovarian tissue.

2.3 Histopathological Analysis

Researchers carried out a histopathological examination. The researcher was unaware of the groups during the study. The removed ovaries were kept in 10% formalin for 72 hours, then cleaned with alcohol and xylene, increasing from 70% to 100%. The samples were coated with paraffin at 60 °C after cleaning. 5 μ m thick sections were taken from the paraffin blocks for analysis. For follicle count, sections were stained with hematoxylin-eosin and examined under a photomicroscope (Nikon Eclipse i5, Nikon, Tokyo, Japan).

2.4 Follicle Count

Classification of follicles was made according to previously established and accepted standards. Primary and secondary follicles were grouped as "early-stage follicles", which was used in statistical analysis. Two experienced pathologists did follicle count. Pathologists were blinded to study groups. Finally, the number of follicles at each stage was calculated by multiplying with a correction factor of five to represent the entire ovary. Results are presented as the number of follicles per developmental stage.

2.5 Serum AMH Levels Measurement

AMH was measured 24 hours after Cyclophosphamide administration as an essential indicator of follicle reserve. Approximately 800 μ L of blood samples were taken from the animals to measure AMH in serum. The samples were centrifuged at 1000 \times g for 15 minutes at 4 °C, and the serum was separated. The resulting serum was stored at -80 °C until analysis. The manufacturer's instructions made an AMH measurement kit with Shanghai SunRed Biological Technology brand Rat AMH ELISA measurement kit (Catalogue No: 201-1-1246, Shanghai, China). Absorbance reading was done on Chromate 4300 brand Elisa reader device (Awareness Technology, Inc. Martin Hwy. Palm, FL, USA), and results are given as ng/mL.

2.6 Real Time-PCR

Primarily, ovarian tissues were homogenized for analysis. For this purpose, a tissue homogenizer (Next Advance, New York, NY, USA) was used. According to the manufacturer's instructions, RNA isolation was performed using the PureLink RNA Mini Kit (Invitrogen, Waltham, MA, USA).

Accordingly:

The appropriate volume of lysis buffer containing 2mercaptoethanol was added to the sample. The lysate below was placed in the collection tube and centrifuged for two minutes at 12,000 g.

1.5 volumes of 100% ethanol and tissue lysate were added to an appropriately sized RNase-free tube and vortexed.

700 μ L of the sample was transferred to the cartridge (Spin Cartridge) and transferred to the collection tube, centrifuged at 12,000 g for 15 seconds at room temperature, and the liquid part was discarded. 350 μ L wash buffer one was added to the cartridge (Spin Cartridge) and centrifuged for 15 seconds at 12,000 g at room temperature.

 $80 \ \mu L$ of DNase mixture was added to the surface of the cartridge membrane and incubated for 15 minutes at room temperature.

Once more, wash buffer one was added and centrifuged. 500 μ L of washing buffer two was added to the cartridge with ethanol, centrifuged at room temperature, and discarded the liquid part.

The cartridge was placed in a recovery tube (Recovery Tube), added RNase-free water, and incubated for one minute at room temperature. Centrifuged for 2 minutes at $12,000 \times g$.

The quantity and quality of purified total RNA were determined with the Quant-iT[™] RiboGreen[™] RNA Test Kit (Life Technology, Hercules, CA, USA) using a fluorescent microplate reader (UV absorbance 260 nm).

The obtained RNA was stored at -80 °C.

cDNA synthesis was done in a palm cycler device. Briefly vortex and centrifuge the mixture to collect the sample at the bottom of the microfuge tube. The reaction was incubated at +25 °C for 10 minutes and then at +42 °C for 60 minutes. It was then incubated at +99 °C for 5 minutes and cooled to +4 °C for 5 minutes. During the first incubation, the primer is annealed to the RNA template. The RNA is then reverse transcribed, resulting in cDNA synthesis during the second incubation (Table 1).

ACTIN Forward	GGGCAACATAGCACAGCTTCT	21
β -ACTIN Reverse primer	GCTTCACCACCACAGCTGAGA	21
DDX4 Forward primer	TGGTCTTGCTTCCAACCCTG	20
DDX4 Reverse primer	TCAAGTGTTCACACACCGCT	20

While interpreting the results, the concentration value of the target genes was proportioned to the concentration value of the reference (housekeeping) gene, and the variation of the results obtained compared to the control group was examined. In our study, the beta-actin gene was used for this purpose.

2.7 $2^{-\Delta\Delta Ct}$ Calculation

Accuracy and reliability in PCR analyses are related to the efficiency of PCR. For this purpose, the mRNA expression level of the target gene (Ddx4) is encountered with the reference gene (Beta Actin), and the $2^{-\Delta\Delta Ct}$ method is widely used. In our study, the Beta Actin expression values of the samples were transformed by the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was made on the values obtained [18].

2.8 Statistical Analysis

Mean and standard deviation is given as descriptive statistics. The Kruskal Wallis test examined the significant difference between the groups. When a statistically significant difference was detected between the groups, the Mann-Whitney U test determined which groups caused the difference. Spearman Correlation analysis was performed to determine the correlation between the groups in the research.

3. Results

5–6 mm thick sections taken from paraffin blocks were stained with hematoxylin-eosin and examined, and follicles were counted. The number of follicles was determined in each of the groups created. As a result of Cyclophosphamide administration, it was determined that there was a decrease in both early-stage follicles and total follicles. This decrease in the number of follicles was also statistically significant (p < 0.05). In the follicle count of the group given Cyclophosphamide + Quercetin, results were close to the control group.

Similarly, both early-stage follicle count and total follicles count in the group given only Quercetin were close to the control group. The difference between the values obtained from Cyclophosphamide + Quercetin and Quercetin alone group and the other groups was not statistically significant. The results and images obtained from the histopathological examination are presented in Figs. 1,2.



Fig. 1. Early stage and total follicles count. Results are presented as the number of follicles per developmental stage. Group 1 (Control), Group 2 (Cyclophosphamide), Group 3 (Cyclophosphamide + Quercetin), Group 4 (Quercetin).



Fig. 2. Images obtained as a result of the histopathological examination. Follicles painted in different forms. Yellow arrows show the different follicles painted in different forms.

At the end of the measurements, it was observed that the AMH levels were significantly lower in the group given Cyclophosphamide (p < 0.01). Similarly, AMH levels were higher in the Cyclophosphamide + Quercetin group than in the Cyclophosphamide group. However, the difference is not statistically significant. There was no significant change in AMH levels in the Quercetin group, but the levels were lower than in the control group (Fig. 3).



Fig. 3. AMH levels. AMH levels measured in the cyclophosphamide group were significantly lower than in the control group. After quercetin treatment, AMH levels increased to the level of the control group. Group 1 (Control), Group 2 (Cyclophosphamide), Group 3 (Cyclophosphamide + Quercetin), Group 4 (Quercetin). AMH, Anti-Müllerian hormone.

In our study, mRNA expression analysis of Ddx4 proteins was performed by RT-PCR. Analysis was made on values transformed by the $2^{-\Delta\Delta Ct}$ method. According to the results, there is a significant difference between the groups in Ddx4. In the Ddx4 analysis, the lowest gene expression was in the POF group induced by Cyclophosphamide, and the highest expression was in the Quercetin group. In further analysis, it was determined that the said difference was due to the difference between the POF group formed with Cyclophosphamide and the Cyclophosphamide + Quercetin and Quercetin groups (Table 2) (Fig. 4).



Fig. 4. RT-PCR results. The lowest gene expression was in the Cyclophosphamide-induced POF group. Group 1 (Control), Group 2 (Cyclophosphamide), Group 3 (Cyclophosphamide + Quercetin), Group 4 (Quercetin). RT-PCR, reverse transcriptase–polymerase chain reaction.

4. Discussion

The incidence of POF, one of the diseases that cause female infertility, is increasing. Data shows that the prevalence has increased to 1–3% in recent years [19]. Improving ovarian function and restoring fertility in this disease is highly debated. Although experts have suggested various treatment strategies, there is a commonly agreed treatment. One of the most commonly known causes of POF is iatrogenic. It is caused by chemotherapy, one of the long-term effects of cancer treatment. The mechanism of the damage done by chemotherapy to the ovary remains unclear. Chemotherapeutic drugs damage the ovary at different levels. Follicles at different developmental stages target these drugs [20].

Cyclophosphamide is an alkylating agent and is toxic to the ovary [21]. This agent affects primordial follicles, causing a critical decrease in ovarian reserve. Cyclophosphamide's other effects on the ovaries impair angiogenesis by damaging the microvascular network. Cyclophosphamide causes excessive activation of inactive primordial follicles by activating the PI3K/PTEN/Akt (phosphatidylinositol 3-kinase/dual protein-lipid phosphatase/serine-threonine kinase) signaling pathway, which results in a decrease in the number of primordial follicles [22,23]. Another effect of Cyclophosphamide is the induction of apoptosis in primordial follicles by increasing oxidative stress [24]. In conclusion, authorities agree that new treatment strategies are needed to eliminate the damage to the ovarian tissue in POF cases developed due to chemotherapy.

It was determined that Cyclophosphamide induced an increase in lipid peroxidation resulting from decreased reduced glutathione and increased malondialdehyde content and induced oxidative stress in the ovary [25]. In addition, pycnotic granulosa cell nuclei are typically seen in cells exposed to Cyclophosphamide, and caspase expression, which indicates follicular destruction, is increased

Table 2. *Ddx4* analysis by groups*.

Groups		n	Mean Rank	SD	χ^{2*}	р	Significant Difference**	
$Ddx4 (2^{-\Delta\Delta Ct})$	1. Control	12	28.322	2.5	19.34	<i>p</i> < 0.05	2 < 3, 4	
	2. POF (Cyclophosphamide)	12	19.036					
	3. Cyclophosphamide + Quercetin	12	25.605					
	4. Quercetin	12	23.331					

*Kruskal Wallis Test, **Mann Whitney U Test. POF, premature ovarian failure; SD, standard deviation.

[26,27]. These are findings proving apoptosis. It has been shown that there is an increase in NF- κ B expression, indicating the role of inflammation in Cyclophosphamide toxicity in the ovary [27]. As a result of these mechanisms, ovarian reserve decreased. This decrease causes a decrease in the number of follicles and AMH levels. Quercetin reverses this process triggered by oxidative stress, apoptosis, and inflammation.

Female reproductive cells are stored as primordial follicles in human ovarian tissue. Primordial follicles are generally inactive and can be activated under physiological conditions. This activation may be essential in treating chemotherapy-induced POF [28,29]. Quercetin's ability to improve ovarian function and inhibit ovarian oxidative stress via the PI3K/Akt/FoxO3a signaling pathway was studied in a model of POF. A POF model was created in rats with Cyclophosphamide and treated with Quercetin. Serum levels of hormones such as AMH, estradiol, and FSH were determined by an analysis performed on ovaries stained with hematoxylin and eosin. The results showed that Quercetin increased the levels of some hormones and oxidation markers, especially AMH. It was found that Quercetin can restore ovarian function and prevent oxidative stress by regulating the PI3K/Akt/FoxO3a signaling pathway [30].

Quercetin is one of the inhibitors of the PI3K pathway. The effects of Quercetin were researched in a study conducted to determine its protective effect against follicle loss caused by chemotherapy in animal experiments. Quercetin was determined to alleviate the numerical reduction of inactive primordial follicles induced by Cyclophosphamide. The same study showed the downregulation of the PI3K/Akt/FoxO3 pathway and the decrease in phosphorylation of proteins that stimulate follicle activation caused by Cyclophosphamide due to the use of Quercetin. Other findings obtained from the study are obtained from the study: prevention of apoptosis in early growing and early antral follicles and preservation of AMH levels secreted from these follicles [31].

In this study, histopathological examinations, follicle count, and AMH measurements were performed on ovarian tissue. The results were obtained from previous support studies. The highest AMH levels were in the control group. It was observed that AMH levels were significantly lower in the group given Cyclophosphamide, and this decrease was statistically significant. AMH levels were also higher in the Cyclophosphamide + Quercetin group than in the Cyclophosphamide group. However, the difference was not statistically significant. There was no significant change in AMH levels in the group given Quercetin alone, but it was lower than in the control group.

Quercetin has anti-inflammatory, antioxidant, and anticancer effects [32]. Some articles emphasize that the dosage is essential for its expected biological activity [33]. It has been reported that Quercetin supports apoptosis at high doses and shows antioxidant effects at low doses [11]. Quercetin was administered intraperitoneally at a dose of 20 mg/kg, and its protective effects were demonstrated against ovarian toxicity, and Cyclophosphamide induced POF [34]. However, in a study in which both 20 mg/kg and a relatively high dose of 100 mg/kg of Quercetin were administered, it was observed that the 100 mg/kg dose was more effective against Cyclophosphamide-induced POF. It was found that the 100 mg/kg dose improved inflammation and apoptosis compared to the 20 mg/kg dose [31]. Therefore, in our study, Quercetin was used at a dose of 100 mg/kg to reveal all its effects.

Our study aimed to detect *Ddx4* positive germ cells to determine the status of ovarian functions in terms of the primordial follicle pool. For this purpose, mRNA expression of the Ddx4 gene was analyzed by RT-PCR. Our results show a significant difference between the Cyclophosphamide alone group and the Cyclophosphamide + Quercetin and Quercetin alone groups. In another study similar to ours, rats with POF created with Cyclophosphamide were treated with platelet rich plasma (PRP), and the expression of the Ddx4 gene was studied [35]. Contrary to our study, no significant difference was found between the groups in terms of expression of the Ddx4 gene. It was stated that the possible reasons for this may be the inability to adjust the dose or the short 24-hour period between Cyclophosphamide administrations. In the light of the data obtained from this study, the ineffectiveness of the therapeutic agent is not related to the time of administration. However, determining the appropriate dosage is essential for the expected effect.

5. Conclusions

In conclusion, our study may provide evidence that Quercetin can protect ovarian function against Cyclophosphamide-induced ovarian damage. It also appeared that the dosage is essential in Quercetin administration. The effects of Quercetin on POF were examined using different analysis methods in our study. Therefore, the obtained results are considered to be highly reliable. The most important limitation of the study is that the effects of Quercetin were not examined through oxidative stress markers.

Our study was designed and conducted by the authors. In this sense, it is completely original. There are studies that were designed and conducted similarly to our study and their results support each other [36,37]. These studies, which were carried out with methods such as animal experiments and cell culture, will be the source of further studies.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Substantial contributions to the conception—CYE, OOG, HGG, OY. Design of the work—CYE, OY, SUA, GSEA. Analysis—CYE, HGG, ET, OOG. Interpretation of data for the work: HGG, OOG, CYE. Drafting the work or revising it critically for important intellectual content—all authors. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The relevant Ethics Committee (Experimental Animals Application and Research Center, Afyon Kocatepe University) approved the protocol regarding the procedures performed on animals. All work and procedures were carried out following the Guide for the Care and Use of Laboratory Animals (Number: AKUHADYEK-98-21/29 September 2021).

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Conflict of Interest

The authors declare no conflict of interest.

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