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Effects of antioxidants used in the experimental premature ovarian insufficiency model on mitophagy proteins and them-associated MicroRNAs

Deneysel prematüre yumurtalık yetmezliği modelinde kullanılan antioksidanların mitofaji proteinleri ve bunlarla ilişkili MikroRNA'lar üzerindeki etkileri

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ABSTRACT

Aim: It was aimed to investigate the protective and therapeutic effects of quercetin and curcumin in POI by examining follicle count, immunohistochemical proteins, mRNAs and their related microRNAs.

Materials and Methods: In our study, an experimental premature ovarian insufficiency model was created on rats by means of cyclophosphamide. Then the tissues were examined by histopathological methods and histochemical staining. We studied the changes in parkin-dependent and independent mechanisms that occur with quercetin and curcumin. RNA isolation was performed on ovarian samples and cDNA synthesis, and qPCR processes were performed. The difference between the obtained findings and the groups was evaluated statistically.

Results: Cyclophosphamide depleted primordial follicles, which is the main pool for fertility, on ovarian tissue and induced atresia ($p<0.001$). Curcumin and quercetin acted on parkin-dependent pathways, resulting in significant improvement in the ovarian follicle pool ($p<0.001$).

Conclusion: The significant increase in the primordial follicle pool and the decrease in the rate of atretic follicles observed in the groups given curcumin and quercetin indicate that curcumin and quercetin have protective and therapeutic roles in the pathogenesis of POI by regulating mitophagy in the parkin-mediated pathway on the ovary.

Keywords: Cyclophosphamide, curcumin, quercetin, MiRNA, POI, parkin, FUNDC1.

ÖZ

Amaç: *Deneysel olarak Prematür Ovaryen Yetmezlik (POI) oluşturulmuş sıçanlar üzerinde; quercetin ve curcumin ile parkin bağımlı ve bağımsız mekanizmalarda meydana gelen değişiklikler inceledi. Folikül sayısı, immünohistokimyasal proteinler, mRNA'lar ve bunlarla ilişkili mikroRNA'ları inceleyerek POI'de quercetin ve curcuminin koruyucu ve tedavi edici etkilerini araştırmak amaçlandı.*

Gereç ve Yöntem: *Çalışmamızda sıçanlarda siklofosfamid aracılığıyla deneysel prematüre over yetmezliği modeli oluşturuldu. Daha sonra dokular histopatolojik yöntemler ve histokimyasal boyama ile incelendi. Over örneklerinde RNA izolasyonu yapıldı ve cDNA sentezi ve qPCR işlemleri gerçekleştirildi.*

Bulgular: *Elde edilen bulgular ile gruplar arasındaki fark istatistiksel olarak değerlendirildi.*

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***Sonuç**: Kurkumin ve kuersetin verilen gruplarda gözlenen primordial folikül havuzunda önemli artış ve atretik folikül oranında azalma, kurkumin ve kuersetin'in yumurtalıkta parkin aracılı yoldaki mitofajiyi düzenleyerek POI patogenezinde koruyucu ve terapötik rollere sahip olduğunu göstermektedir.*
***Anahtar Sözcükler:** Siklofosfamid, kurkumin, kuersetin, MiRNA, POI, Parkin, FUNDC1.*

INTRODUCTION

Premature ovarian insufficiency (POI) is a disease that progresses with amenorrhea in women and is followed by loss of function of the ovaries before the age of 40. It may present with hot flashes, loss of fertility and sleep disturbances in patients. It also carries a risk in terms of osteoporosis and cardiovascular diseases in the long term. The pathogenesis of this disorder, which is seen in approximately 1-2% of women, has not been fully elucidated. Studies have focused on this issue since single gene mutations, and some chromosomal abnormalities have been associated with POI. Mitophagy molecules, which play a role in the elimination of damaged mitochondria, are thought to be effective in the development of POI. Many of the mutations associated with POI function within the mitochondria (1,2).

Mitochondria have many important cellular functions such as lipid synthesis, nucleic acid and protein biogenesis, calcium metabolism and homeostasis, cell cycle regulation, reactive oxygen species (ROS) production, and antioxidant protection. Since it was understood that mitochondria, the main producers of intracellular ROS, have a role in POI, the focus has been on mitochondria-targeted antioxidants (3,4). It is thought that mutations in some identified genes may be associated with mitochondrial DNA (mtDNA), mitochondrial RNA (mtRNA) and mitochondrial protein synthesis and may cause ovarian failure through mitochondrial dysfunction. Among the exogenous antioxidants, curcumin and quercetin, which are flavonoids, can be used as mitochondria-targeted antioxidants (5–7).

In order to maintain mitochondrial quality and continue optimal energy production, it is necessary to eliminate dysfunctional mitochondria. Damaged mitochondria are removed by the ubiquitin-proteasome pathway, which removes mitochondrial outer membrane proteins, or by the autophagy-lysosome pathway, which leads to degradation in all organelles. In the autophagy-lysosome pathway, damaged mitochondria are selectively excluded through a specific pathway called mitophagy. Studies over the past few years have shown that microRNAs (miRNAs) regulate a wide variety of biological processes, including autophagy (8,9).

Many studies have focused on different miRNAs to elucidate this autophagic stage. Of these, miR-181a has been identified as a new mitophagy inhibitor (10). Another study reported that miR-137, a hypoxia-sensitive miRNA, inhibited hypoxia-induced mitophagy by targeting two mitophagy receptors, FUN14 domain-containing protein 1 (FUNDC1) and NIP3-like protein X (NIX). *PTEN-induced putative kinase 1* (PINK1) and Parkin also play crucial roles in mitochondrial quality control and mitophagy (11). In addition to the PINK1/Parkin pathway, mitochondrial proteins also play a role in triggering mitophagy by regulating mitochondrial clearance. *BCL2 Interacting Protein 3 Like* (BNIP3L)/NIX and FUNDC1 have been identified as mitophagy receptors in mammalian cells. Recent studies have suggested that FUNDC1, a mitochondrial outer membrane protein, activates autophagosomes and is a receptor for mitophagy in damaged mitochondria (12,13).

MiR-30e is a member of the miR-30 family. It is thought that this family, like miR-302/367, is involved in various biological processes such as self-renewal and reprogramming of stem cells and can regulate apoptosis (14).

We studied the changes in parkin-dependent and independent mechanisms that occur with quercetin and curcumin; It was aimed to investigate the protective and therapeutic effects of quercetin and curcumin in POI by examining follicle count, immunohistochemical proteins, mRNAs and their related microRNAs. Investigating the responses of follicle pools to curcumin and quercetin in the preventive and treatment groups using different methods and elucidating little-known mitophagy pathways are of great importance in the pathogenesis of POI.

MATERIALS AND METHODS

1. Animal protocols

For the study, 36 female, 8-week-old Wistar type adult rats between 180-260gr were used. Animals were kept in steel cages in pairs, in a light and dark

room (07:00-19:00 o'clock) between 12:12 hours at room temperature (22 ± 2 °C); access to water and food was provided as needed.

A total of 36 rats from 6 groups, 6 rats in each group, were used. The rats, which were divided into 6 groups to form POI, were induced by intraperitoneal injection of 70 mg/kg cyclophosphamide on day 1 followed by 30 mg/kg every three days for a total of 5 times. The control group received only propylene glycol, the substance to be used to dissolve flavonoids.

In the cyclophosphamide group, intraperitoneal injection of 70 mg/kg cyclophosphamide was performed on day 1, followed by cyclophosphamide induction with 30 mg/kg every three days, a total of five times.

In the curcumin I group, whose drying activity was investigated, 100 mg/kg/day curcumin solution dissolved in propylene glycol was administered via an orogastric cannula for 4 weeks from the first day of cyclophosphamide administration.

In the quercetin I group, whose drying activity was investigated, 30 mg/kg/day quercetin solution dissolved in propylene glycol was administered via an orogastric cannula for 4 weeks from the first day of cyclophosphamide administration.

The curcumin II group, in which the therapeutic efficacy was investigated, was rested for 3 weeks after the end of the cyclophosphamide administration to form the POI model. Then, 100 mg/kg/day curcumin solution dissolved in propylene glycol was administered via orogastric cannula for 4 weeks.

The quercetin II group, in which the therapeutic efficacy was investigated, was rested for 3 weeks after the end of the cyclophosphamide administration to form the POI model. Then, 30 mg/kg/day quercetin solution dissolved in propylene glycol was administered via orogastric cannula for 4 weeks.

At the end of the experiment, all subjects were sacrificed, and their right ovaries were placed in 10% formalin solution for histological examination, and their left ovaries were placed in liquid nitrogen for molecular analysis and stored at -80^{o}C.

2. Histochemical and Immunohistochemical Analysis

Ovarian tissues embedded in paraffin cassettes were taken on a slide with a thickness of 4 micrometers with a microtome and immunohistochemistry staining was performed with hematoxylin and eosin staining.

Paraffin-embedded ovarian tissues were serially sectioned with a microtome (Leica; 149MULTI0C1) at a thickness of 5 µm. One of every 10 sections was taken on the slide and the whole tissue was processed. Primordial follicle, primary follicle, atretic and normal secondary and tertiary follicles and corpus luteum were counted separately on tissues stained with hematoxylin and eosin.

3. Total RNA Synthesis

Hybrid R Total RNA isolation kit Hybrid-R™ (GeneAll 305-101) was used for RNA isolation. 1000 µl Trizol (RiboEx, 301-001) was placed in a microcentrifuge tube. Ovarian tissues were crushed in liquid nitrogen with mortar in a mortar and powdered, and the frozen tissue powder was transferred into trizol. After 5 minutes of incubation at room temperature, it was centrifuged at 12,000 G for 10 minutes at +4^{o}C and the supernatant was transferred to a clean tube. Chloroform at 1/5 of the liquid volume was added and shaken vigorously. It was then centrifuged at 12,000 G for 15 min at +4^{o}C. The liquid phase was transferred to the new tube. 1 volume of RBI buffer was added and shaken. 700µl of the mixture was added to Type-F minicolumn and centrifuged at 12.000G for 30 seconds. 500 µl of SWI buffer was placed in the minicolumn by pouring the remaining liquid. It was centrifuged at 12.000G for 30 seconds. This time, 500 µl of RNW buffer was placed in the minicolumn and centrifuged at 12.000G for 30 seconds. RNAse-free water was added to the middle of the column (50-100 µl) and incubated for 1 min and centrifuged at 12.000G for 1 min to elute the RNA. Samples were labeled and stored at -80^{o}.

4. cDNA synthesis

Roche Transcriptor first strand kit (Roche, 04897030001) was used for cDNA synthesis from RNA. 1500 ng of RNA was used for each sample. From 4.0 µl 5X reaction buffer, 0.5 µl RNAse inhibitor, 2.0 µl dNTP mix, 0.5 µl reverse transcriptase and 1500 ng total RNA were transferred to the PCR tube. 1.0 µl of RT primers were used for miRNA cDNA synthesis, while 1.0 µl of polyT primer supplied with the kit was used for mRNA cDNA synthesis. The mixture was completed with ddH2O to 20µl and incubated at 25^{o}C for 5 minutes, 42^{o}C for 60 minutes and 85^{o}C

for 5 minutes in a thermal cycler, and the obtained cDNAs were stored at -20°C.

5. qPCR analysis

During qPCR procedures, add 10 µl of WizPure qPCR Master (SYBR) (WizBio, W1711), 1.5 µl of 10 µM forward and reverse primer mix (500 nmol) and 2 µl of cDNA for each sample, making a total volume of 20 The remainder was made up with ddH2O in µl. All procedures were done on ice. Mixtures prepared for each gene were transferred to the wells of MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Cat. No: 4346906) in 3 replicates and covered with Optical Adhesive Film (Applied Biosystems, Cat. No: 4360954). The PCR cycle was programmed as 1x 95°C – 1 min, 45 x (95°C-10sec, 60°C-15sec, 72°C-15sec), 72°C-5min, 40°C-10sec and the reaction took place in the LC480 (Roche) device. Relative quantitation was calculated according to the 2-ΔΔCt method using GAPDH (mRNA) and u6 (miRNA) reference genes in order to quantify (in folds) the changes in gene expressions at the end of the qPCR process.

6. Follicle Counts

Follicle stages were defined as primordial follicle, primary follicle, secondary follicle and tertiary follicle according to Pedersen & Peters morphological criteria (15). In addition, these follicles were considered morphologically normal if no obvious signs of degeneration were noted, such as shrunken oocyte, irregularity of the granulosa cell layer, condensed nuclear chromatin, and/or cell swelling. The total number of follicles was recorded without counting the follicles in the successive section for the second time, and the percentage of each follicle type among the total follicles was calculated.

7. Statistical Evaluation

The difference between the groups was analyzed with Sidak's multiple comparison test, and the difference between the groups was analyzed with the Mann-Whitney U test. For statistical analysis, GraphPad Prism 9 program was used. Values $p<0.05$ were considered statistically significant.

8. Ethical Approval

All protocols in this study were carried out with the approval of Manisa Celal Bayar University Faculty of Medicine Animal Experiments Local Ethics Committee numbered 01.06.2021/192.

RESULT

1. Follicle Count Findings

According to the follicle count analysis, 64.62% of all follicles in the control group consisted of primordial follicles, while atretic secondary and tertiary rates were only 0.47%. In the cyclophosphamide group, while the primordial follicle rate decreased dramatically to 29.1%, on the contrary, the atretic secondary follicle rate increased significantly to 8.98% and the atretic tertiary rate was 5.26%.

Another remarkable change is in the corpus luteum ratios, corpus luteum ratio was 1.42% in the control group and 7.74% in the cyclophosphamide group.

In addition, primary and secondary follicle rates were higher in the Cyclophosphamide group compared to the control group. The percentage distribution of follicles and corpus luteum according to the groups is shown in Table-1.

2 Immunohistochemical Findings

2.1 Parkin Immunoreactivity

As a result of staining the tissue samples of the control group with parkin primary antibody, it was observed that parkin immunoreactivity was higher in atretic secondary (174.67 ± 7.55) and tertiary (185 ± 8.17) follicles than in primary, secondary and tertiary follicles (109.2 ± 4.71, 134.6 ± 10.6, 119 ± 8.1, ($p<0.001$), respectively). The difference between atretic follicles and other follicle groups was statistically significant.

In the cyclophosphamide-treated group, similar parkin immune reactivity was observed in primary, secondary and tertiary follicles (120.6 ± 6.75, 133.8 ± 3.71, 127.8 ± 10.65, respectively). On the other hand, parkin immunoreactivity in secondary and tertiary atretic follicles was also higher than the control group and this difference was statistically significant (284 ± 18.55, 305 ± 17.33, $p<0.001$, respectively).

It was found remarkable that parkin immunoreactivity in secondary (108.34 ± 2.36) and tertiary atretic follicles (120 ± 4.09) in the curcumin I group decreased to the levels of the control group ($p<0.001$). The decrease in immunoreactivity approaching the control group was also observed in the quercetin I, curcumin II and quercetin II groups and was statistically significant ($p<0.05$, $p<0.001$, $p<0.001$,

respectively). H-Score distributions of the groups stained with parkin antibody are shown in Table-2.

As a result of staining tissue samples from the control group with the FUNDC1 primary antibody, it was determined that immunoreactivity in primary, secondary and tertiary follicles (109.4 ± 3.14; 110.8 ± 4.12 108.6 ± 2.66, respectively) was quite low. FUNDC1 immunoreactivity in atretic secondary follicles (185 ± 8.17) and in atretic tertiary follicles (113.2 ± 10.84) was found to be present.

When the FUNDC1 immunoreactivity of the other groups was compared with the control group, it was determined that there was no statistically significant difference between the groups. ($p>0.05$ in all groups)

After the POI model was created by cyclophosphamide administration, BNIP3L immunoreactivity was found to be high in atretic secondary and tertiary follicles, whereas immunoreactivity in other groups (curcumin I, curcumin II, quercetin I and quercetin II) showed a decrease approaching the control level and this decrease was statistically significant (in all groups; $p<0.001$). H-Score distributions of the groups stained with BNIP3L antibody are shown in Table-3.

In immunohistochemical staining, it was determined that BNIP1 immunoreactivity increased in primary, secondary and tertiary follicles in curcumin and quercetin applied groups compared to the control group and Cyp group ($p<0.05$). No significant change was observed in atretic follicles compared to the Cyp group ($p=0.135$). H-Score distributions of the groups stained with BNIP1 antibody are shown in Table-4.

Immunoreactivity Findings are shown in Figure 1-Figure-5.

2.2 miRNA Findings

An average 2.5-fold increase in MiR181a expression was observed with cyclophosphamide application ($p<0.001$) and expression levels in other groups were found to be close to control. Expression of Mir-181a in experimental groups are shown in Figure 6.

An average 1.7-fold increase in MiR-137 expression was observed with cyclophosphamide application ($p<0.05$), and a significant decrease in its expression was observed in the curcumin-II group ($p<0.001$), but expression levels in the other groups were detected close to the control. Expression of MiR-137 in experimental groups are shown in Figure 6.

An average 2.5-fold increase in MiR30a expression was observed with cyclophosphamide application ($p<0.001$) and expression levels were detected close to control in the curcumin-I group, but a decrease in expression level was measured in other groups. Expression of MiR-30a in experimental groups is shown in Figure 6.

Table-1. Follicle Percentages in Experimental Groups

	Prim.Fol.	Primary Fol.	Sec. Fol	Ter. Fol.	CL	Atretic Sec. Fol.	Atretic Ter. Fol.
Control	64,62	18,08	9,91	5,03	1,42	0,47	0,47
Cyp	29,10	24,46	20,43	4,02	7,74	8,98	5,26
Curcumin-I	51,35	18,02	2,70	6,31	8,11	9,01	4,50
Kuercetin-I	48,62	19,27	6,42	5,50	8,26	8,26	3,67
Curcumin-II	43,90	15,24	10,37	6,10	13,41	7,32	3,66
Kuercetin-II	56,41	12,82	7,69	2,56	7,69	9,40	3,42

Prim: Primordial, Sec: Secondary, Ter: Tertiary CL: Corpus Luteum, Fol: follicle.

Table-2. H-Score Distributions of the Groups Stained with Parkin Primary Antibody

Parkin	Control	CYP	Cu-I	Que-I	Cu-II	Que-II
Primary	109.2 ± 4.71	120.6 ± 6.75	112 ± 2.2	205 ± 10.96	134 ± 10.68	204 ± 16.56
Secondary	134.6 ± 10.6	133.8 ± 3.71	139 ± 5.84	210 ± 6.33	178 ± 18.78	208 ± 10.3
Tertiary	119 ± 8.1	127.8 ± 10.65	153 ± 4	228 ± 9.28	157 ± 12.89	118 ± 96.42
Atretic Secondary	174.67 ± 7.55	284 ± 18.55	108.34 ± 2.36	192 ± 7.49	186 ± 8.61	234 ± 14.63
**Significance: Cu-I (p<0.001); Que-I (p<0.05); Cu-II (p<0.001); Que-II (p<0.001)*						
Atretic Tertiary	185 ± 8.17	305 ± 17.33	120 ± 4.09	225 ± 8.95	127 ± 104.2	101 ± 82.85
**Significance: Cu-I (p<0.001); Cu-II (p<0.001); Que-II (p<0.001)*						
Corpus Luteum	187.8 ± 14.27	157 ± 7.78	150 ± 3.17	216 ± 9.7	204 ± 8.61	194 ± 5.84

*Compared with the control group

Table-3. H-Score Distributions of Groups Stained with BNIP3L Primary Antibody

BNIP3L	Control	CYP	Cu-I	Que-I	Cu-II	Que-II
Primary	112 ± 5.1	210 ± 12.3	134 ± 7.2	205 ± 10.8	140 ± 9.5	198 ± 14.6
Secondary	130 ± 8.4	220 ± 15.6	142 ± 6.8	208 ± 12.1	150 ± 10.2	200 ± 11.9
Tertiary	118 ± 6.2	225 ± 11.7	139 ± 5.9	210 ± 9.8	148 ± 12.4	197 ± 13.3
Atretic Secondary	175 ± 7.8	295 ± 17.2	120 ± 4.1	115 ± 3.9	123 ± 4.7	118 ± 5.2
**Significance: Cu-I, Cu-II, Que-I, Que-II (all p<0.001)*						
Atretic Tertiary	185 ± 9.1	310 ± 18.4	122 ± 5.2	118 ± 4.6	125 ± 6.1	120 ± 5.8
**Significance: Cu-I, Cu-II, Que-I, Que-II (all p<0.001)*						
Corpus Luteum	188 ± 13.2	165 ± 8.4	152 ± 5.3	214 ± 11.2	205 ± 9.4	192 ± 8.6

*Compared with the control group

Table-4. H-Score Distributions of Groups Stained with BNIP1 Primary Antibody

BNIP1	Control	CYP	Cu-I	Que-I	Cu-II	Que-II
Primary*	107.2 ± 2.49	126.2 ± 7.09	143 ± 8.13	309 ± 16.56	123.6 ± 5.82	327 ± 16.62
Secondary*	114 ± 2.61	104.4 ± 3.93	167 ± 10.3	228 ± 13.64	251 ± 8.61	211 ± 8.61
Tertiary*	120.8 ± 6.05	142 ± 10.3	260 ± 14.5	271 ± 14.97	271 ± 15.94	197 ± 11.23
Atretic Secondary	200 ± 4.09	255 ± 15.17	287 ± 10.78	278 ± 16.92	289 ± 14.29	255 ± 13.79
Atretic Tertiary	270 ± 14.72	291 ± 9.7	316 ± 14.63	358 ± 5.1	230 ± 11.41	275 ± 19.5
Corpus Luteum	138.6 ± 6.92	237 ± 14.36	270 ± 10.49	258 ± 9.8	212 ± 12.89	224 ± 9.7

**Significance: Increase in primary, secondary, and tertiary follicles of Cu-I, Cu-II, Que-I, and Que-II groups compared to control and CYP groups (p<0.05).*

Figure-1. Parkin Immunoreactivity of Control (a, c, e) and Cyclophosphamide (b, d, f) Groups
While immunoreactivity is quite low in primary (P), secondary (S) and tertiary (T) follicles in the control group, it is observed that immunoreactivity is high in atretic follicles of the cyclophosphamide group. Ti. Theca interna, Cl: Corpus luteum.

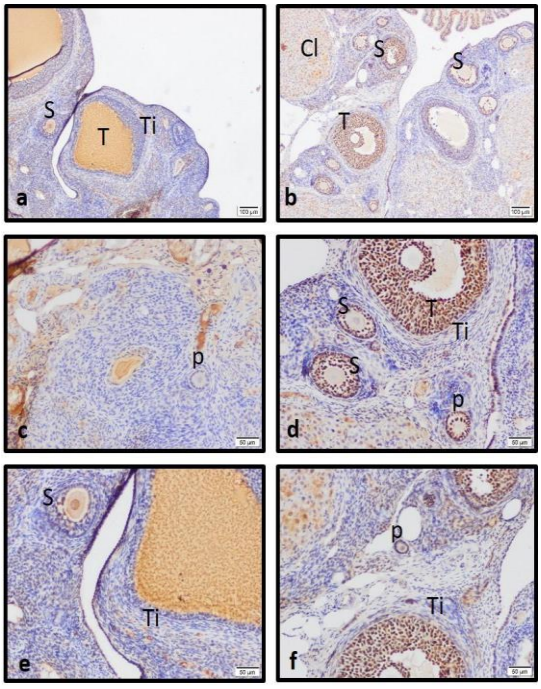


Figure-2. Parkin Immunoreactivity of Curcumin I (a, c, e) and Quercetin I (b, d, f) Groups
While immunoreactivity is observed to be much less in the primary (P), secondary (S) and tertiary (T) follicles in the curcumin I group, immunoreactivity is observed to be higher in the quercetin I group follicles. Ti. Theca interna.

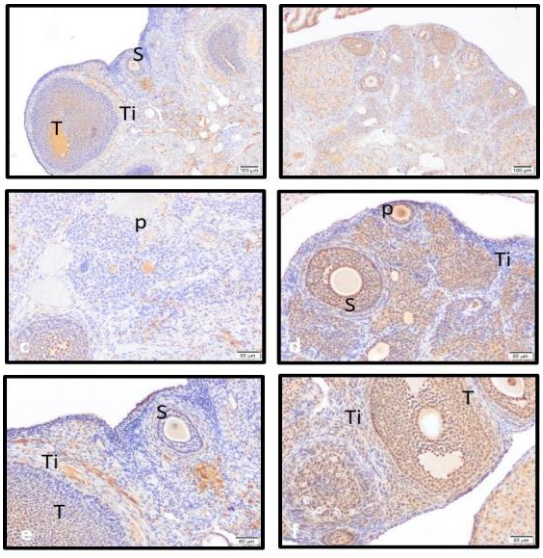


Figure-3. Parkin Immunoreactivity of Curcumin II (a, c, e) and Quercetin II (b, d, f) Groups
While immunoreactivity is observed to be much less in primary (P), secondary (S) and tertiary (T) follicles in the curcumin II group, immunoreactivity is observed to be higher in quercetin II group follicles. Ti. Theca interna, Cl: Corpus luteum.

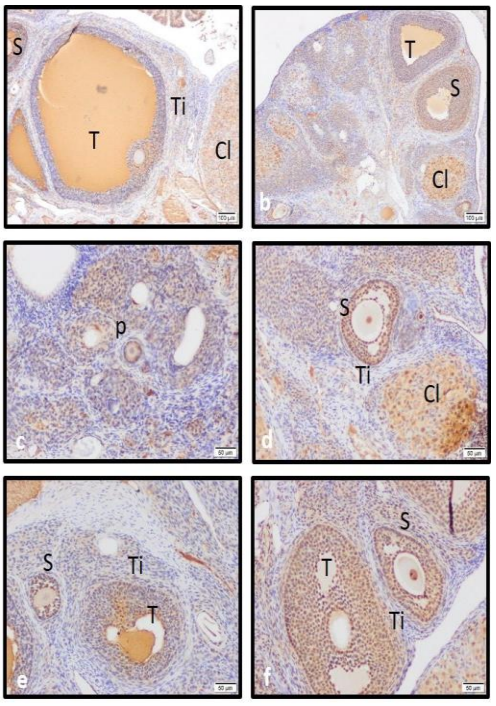


Figure-4. BNIP3L Immunoreactivity of Control (a, c, e) and Cyclophosphamide (b, d, f) groups
While immunoreactivity is quite low in primary (P), secondary (S) and tertiary (T) follicles in the control group, it is observed that immunoreactivity is high in atretic follicles of the cyclophosphamide group. Ti. Theca interna, Cl: Corpus luteum.

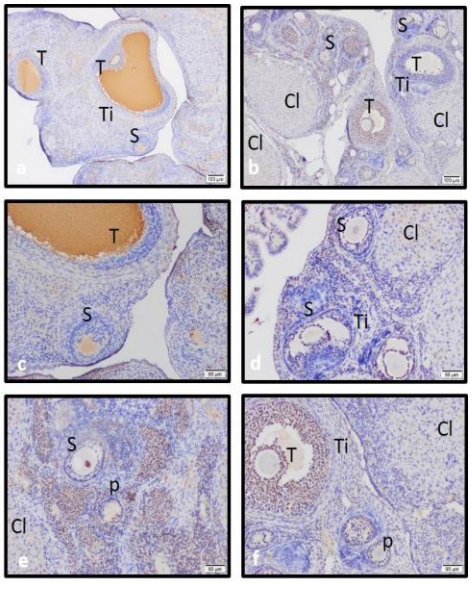


Figure-5. BNIP1 Immunoreactivity of Control (a, c, e) and Cyclophosphamide (b, d, f) Groups
While immunoreactivity was quite low in primary (P), secondary (S) and tertiary (T) follicles in each group, immunoreactivity was higher in atretic follicles of the cyclophosphamide group. Ti. Theca interna, Cl: Corpus luteum.

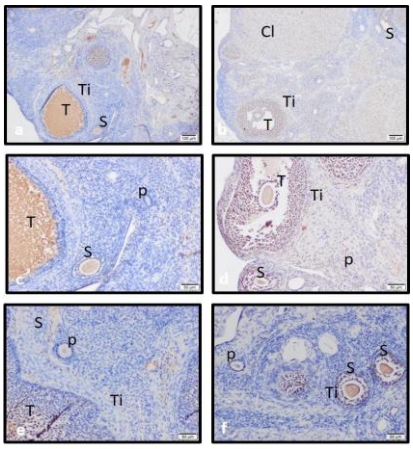
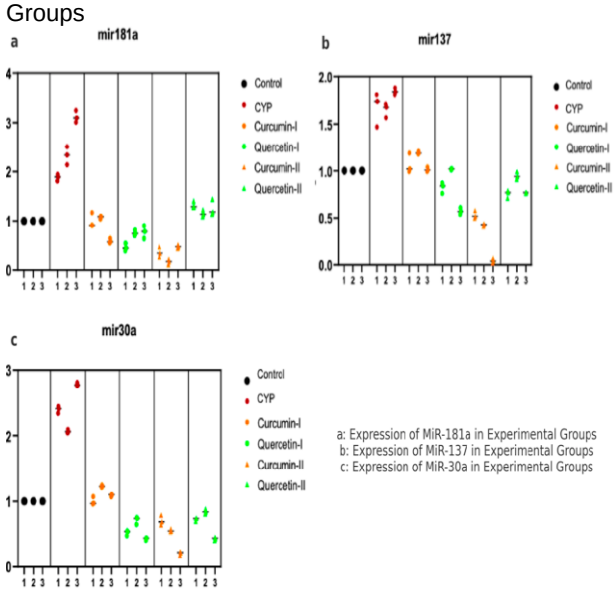


Figure-6. Expression of miRNAs in Experimental Groups



DISCUSSION

Premature ovarian failure is an important disorder that affects women of reproductive age and is accompanied by serious long-term complications. Mitophagy molecules, which play a role in the elimination of damaged mitochondria, are thought to be effective in the development of POI. In addition, mutations in many genes involved in mitochondrial function have also been identified as causes of POI. However, in mice, treatment with coenzyme Q10, a product of the Krebs cycle that plays an important role in mitochondrial biology, has been shown to prevent the onset of POI in both genetic and aging mouse models (16). Although there have been several studies examining the effects of mitochondrial dysfunction on ovarian functions and follicle development in recent years, the role of mitophagy in the POF model and the effects of quercetin and curcumin on mitophagy are not fully known (17,18). Although three mechanisms play a role in the realization of mitophagy (19), Parkin-mediated mitophagy is the most known pathway, and FUNDC1 and BNIP3L also play a role in the parkin-independent mitophagy mechanism.

In our study, a dramatic decrease in the number of primordial follicles in the Cyp group compared to the control group was observed, along with a significant increase in the number of atretic follicles. Increased follicular atresia is believed to be responsible for accelerating follicular depletion leading to ovarian dysfunction. A previous study has confirmed that granulosa cell apoptosis is a trigger for follicular atresia (20). Granulosa cell apoptosis is thought to be the initiator of follicular atresia. Apoptosis of granulosa cells occurs much earlier than the formation of follicular atresia, and the phenomenon of follicular atresia can only be observed when the apoptotic granulosa cells in the follicle reach a certain level (21), POI is characterized by accelerated atresia and continuous transformation of primordial follicles into large preovulatory follicles; For this reason, it is an organized and complex process that progresses with a decrease in fertility due to premature depletion of primordial follicles (22). In previous studies, pathological examination of the ovaries of women treated with cyclophosphamide revealed a decreased primordial follicle pool, damage to ovarian blood vessels, and ovarian atrophy. Although the mechanisms underlying these changes remain unclear, studies have shown that chemotherapy can cause a significant loss of both primordial follicles and growing follicles. Some researchers have also hypothesized that increased activation of follicles in the primordial pool leads to accelerated follicular atresia, resulting in premature depletion of the primordial follicle reserve. Kalich-Philosoph et al. (23) found that the mechanism in cyclophosphamide-induced loss of ovarian reserve is accelerated primordial follicle activation,

resulting in an exhaustion effect and follicle depletion. While cyclopofamide is toxic to dividing cells, it also activates dormant follicles, allowing them to grow and multiply (20,23). It has also been shown that cyclophosphamide can induce primordial follicles into a prematurely growing follicle pool and eventually cause the ovarian reserve to shrink (24). The increased proportions of primary, secondary and tertiary follicles in the total follicle pool in the Cyp group reveal that follicular development continues under cyclophosphamide application. In addition, the decreased number of primordial follicles and the increase in the number of atretic follicles seen in the Cyp group suggest that cyclophosphamide consumes the primordial follicles, which are the main pool for fertility, on the ovarian tissue and induces atresia.

Primordial follicle rates in the preventive and treatment groups in which curcumin and quercetin were applied were significantly higher than in the Cyp group. Additionally, it was determined that the rates of atretic tertiary follicles decreased compared to the Cyp group. It is known that the primordial follicle pool, which is the most important reserve for fertility, is constantly activated and transformed into growing follicles, while the rest is still dormant. Therefore, it is believed that the maintenance of dormant primordial follicles may determine the reproductive lifespan of females (25). Curcumin and quercetin are widely used phytochemicals. These molecules are known to have analgesic, anti-carcinogenic and anti-inflammatory effects. In the study conducted by Melekoğlu et al., where they examined the effects of curcumin and capsain on POI caused by the application of cyclophosphamide, they concluded that curcumin and capsain improved ovarian functions by reducing the level of inoxidative stress markers (26). The significant increase in the primordial follicle pool, which is the most important source of fertility, and the decrease in the rate of atretic tertiary follicles observed in the treatment and preservation groups reveal that curcumin and quercetin may have protective and therapeutic roles in the pathogenesis of POF.

In this study, in the preparations we examined immunohistochemically, it was observed that the expression in atretic follicles was increased in sections stained with parkin primer antibody. This increase was especially evident in the Cyp group. Considering that parkin is a molecule that controls the quality of mitochondria and ensures the elimination of damaged mitochondria through mitophagy, it can be suggested that mitophagy is effective through parkin in the elimination of atretic follicles. Parkin shows its effect through PINK1. PINK1 is produced and transferred to all mitochondria. In healthy mitochondria, PINK1 is rapidly degraded through proteolysis and remains at very low levels. However, in damaged mitochondria, this process is interrupted, causing the accumulation of PINK1 and the recruitment of parkin to mitochondria with PINK1 accumulation (27). It has been reported that PINK1 and Parkin proteins are absolutely necessary for a healthy mitochondrial cycle, and their depletion causes loss of mitochondrial function, degeneration in neurons and a decrease in ovarian cells. (28,29). Zhang et al. reached similar results; has demonstrated that hypothermia provides neuroprotection via increasing PINK1/Parkin-mediated mitophagy. (30). It has also been shown that Parkin can selectively translocate to damaged mitochondria in a cell and that its overexpression can eliminate damaged mitochondria through mitophagy (31). Considering the findings in our study, increased parkin immunoreactivity, especially in atretic follicles; This suggests that parkin-mediated mitophagy is active in follicles.

In the curcumin and quercetin applied groups, increased parkin immunoreactivity was detected in the Quercetin I, Quercetin II and Curcumin II groups, although it was more prominent in the Quercetin I and Quercetin II groups in the primary and secondary follicles compared to the Cyp group. In this study, parkin immunoreactivity observed in atretic follicles in the control and treatment groups showed a significant decrease compared to the Cyp group. In another previous study, it was shown that cisplatin, a chemotherapeutic agent, increased PINK1 and parkin levels and caused damage to epithelial cells from proximal convoluted tubules. It is known that mitophagy is a protective mechanism that is absolutely necessary for the cell by eliminating damaged mitochondria. However, when mitophagy is triggered above a certain level, cellular autophagy is induced. In the same study, the demonstration of numerous autophagosomes containing mitochondria in epithelial cells from proximal convoluted tubules was associated with cellular autophagy triggered by mitophagy. It has also been reported that the addition of curcumin prevented the cisplatin-induced increase in the levels of PINK1 and parkin, which are mitophagy-

related proteins (32). It is thought that the high parkin immunoreactivity observed in the atretic follicles in the cyp group in this study may cause cellular autophagy to be triggered by similarly high mitophagy. A decrease in parkin immunoreactivity in atretic follicles was observed with the application of curcumin and quercetin. When evaluated together with the increase in the primordial follicle pool shown in the treatment and protective groups, curcumin and quercetin balanced the high mitophagy caused by cyclophosphamide and reduced it to a protective level; It is thought that it protects the viability of the cell by ensuring mitochondrial clearance through increased mitophagy in primary and secondary follicles and has a positive effect on ovarian function.

In our study, miR-181a expression, which is associated with parkin, was found to be higher in the Cyp group than in the control group. A decrease in miR-181a expression was observed in the protective and treatment groups, approaching the control group. It has been reported that chemotherapeutic agents such as cyclophosphamide, which are regularly administered in the treatment of T-cell leukemia/lymphoma, upregulate miR181a expression together with AKT activation (33). It has been reported that increased expression of miR-181a suppresses proliferation and causes apoptosis in granulosa cell (34). The increase in miR-181a expression in gonads exposed to cyclophosphamide, when evaluated together with the decrease in the follicular pool observed in the Cyp group, suggests that it may cause apoptosis in granulosa cells. The decrease in miR-181a expression observed in the protective and treatment groups compared to the Cyp group may have contributed to the improvement in the follicular pool by removing its inhibitory effect on parkin.

The significant increase in miR-137 expression observed in the Cyp group suggests that cyclophosphamide may also have an effect on ovarian tissue through miR137. Additionally, an increase in BNIP3L immunoreactivity was observed in atretic follicles in the cyclophosphamide group. In the groups treated with curcumin and quercetin, BNIP3L immunoreactivity in atretic follicles decreased, approaching the control group. This suggests that BNIP3L may also affect ovarian mitophagy other than the parkin-dependent pathway. The FUNDC1 pathway was not found to be active.

While miR-30a expression increased in the cyclophosphamide-treated group compared to the control group, a miR-30a expression approaching the control group was detected in the protective and treatment groups. Recently, Chevalier and colleagues revealed that miR-30a-5p regulates BNIP3L-dependent mitophagy (35). The significant increase in miR30 observed in the Cyp group in our study may be associated with increased cell apoptosis.

In immunohistochemical staining, it was determined that BNIP1 immunoreactivity increased in primary, secondary and tertiary follicles in the curcumin and quercetin applied groups compared to the control group and Cyp group. No significant change was observed in atretic follicles compared to the Cyp group. BNIP1, a member of the BH3 protein family, is known to function as an apoptosis modulator in both the endoplasmic reticulum (ER) and mitochondria. Although it has been demonstrated that mitochondria are very important in the process of apoptotic cell death, it has been shown that anti-apoptotic and pro-apoptotic proteins such as BCL-2, BCL-X(L), BAX and BAK are also found in the ER (36–39). BNIP1 is also a functional partner of RNF186, which is involved in stress-mediated apoptosis, and a substrate in the ubiquitin process (40). The increase in BNIP1 immunoreactivity observed on growing follicles may be a result of increased mitophagy at the cellular level through mitochondria and ERs. The high level of BNIP1, a new molecule that plays a role in mitophagy and autophagy, in all groups in atresic follicles was considered as an indication that it functions in atresia. At the same time, parkin and BNIP1 expressions on primary, secondary and tertiary follicles are similar. Considering the autophagy effect of BNIP1, it can be considered as an indicator of the transition to autophagy after mitophagy together with parkin. Although its relationship with parkin has not been fully demonstrated before, the fact that it is parallel in our study suggests that they act together.

CONCLUSION

When the decrease in parkin immunoreactivity in atretic follicles with curcumin and quercetin application is evaluated together with the increase in the primordial follicle pool, curcumin and quercetin balance the high mitophagy caused by

cyclophosphamide and reduce it to a protective level; It is thought that it protects the viability of the cell and has a positive effect on ovarian function by ensuring mitochondrial clearance through increased mitophagy in primary, secondary and tertiary follicles.

Disclosure Statement

The authors declare that they have no conflict of interest. The authors declare that they have no direct or indirect financial relationships with any sponsor. The authors declare that the content has not been published or submitted for publication elsewhere other than as a brief abstract in the proceedings of a scientific meeting or symposium.

Conflict of Interest

The authors confirm no conflict of interest.

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