ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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RESVERATROL ALLEVIATES METHOTREXATE-INDUCED OVARIAN INJURY VIA SUPPRESSING OXIDATIVE STRESS AND APOPTOSIS IN RATS

RESVERATROL SIÇANLARDA OKSİDATİF STRESİ VE APOPTOZU BASKILAYARAK METOTREKSAT KAYNAKLI YUMURTALIK HASARINI HAFİFLETİR

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Öz

Amaç
Dişi sıçanlarda Metotreksat (MTX) kaynaklı yumurtalık hasarına karşı Resveratrolün (RES) antioksidan ve antiapoptotik etkilerinin değerlendirilmesi amaçlanmaktadır.

Gereç ve Yöntem
Çalışmada ratlar 7 gruba ayrıldı: Kontrol; MTX (15mg/kg-1. gün); MTX (1. ve 3. gün); MTX (1., 3. ve 5. gün); MTX (1. gün) + RES (20mg/kg); MTX (1. ve 3. gün) + RES; MTX (1., 3. ve 5. gün) + RES. Grup 4 ve 6'da sadece bir hayvan kaldığı ve grup 7'de hiç hayvan olmadığı için bu gruplar değerlendirme dışı bırakıldı. Yumurtalık hasarı, sıçanların yumurtalık dokularında hematoksilen-eozin (H-E) ve TUNEL boyaması yapılarak değerlendirildi. Yumurtalık dokularındaki toplam oksidan/antioksidan (TOS/TAS) durumu spektrofotometrik olarak değerlendirildi. Ayrıca kandaki DNA hasarı comet testi ile değerlendirildi.

Bulgular
Biyokimyasal analizler sonucunda MTX gruplarında, grup 2 ve 3'te kontrol grubuna göre TOS düzeyleri artarken, grup 5'te anlamlı olarak azaldı. Öte yandan, grup 5'te grup 2 ve 3'e göre TAS düzeyi anlamlı olarak arttı. H-E boyama sonuçları, MTX kaynaklı hasarın RES (grup 5) tedavisi ile düzeldiğini gösterirken; maksimum TUNEL pozitif boyama görüntüsü; 2. ve 3. gruplarda eşit miktarlarda, ardından 5. grupta gözlemlenmiştir. Ayrıca DNA hasarını gösteren comet skoru 2. ve 3. gruplarda anlamlı olarak artarken, 5. grupta önemli ölçüde azalmıştır.

Sonuç
Bu çalışmanın sonuçları doğrultusunda, Resveratrol'ün antioksidan özelliğinden dolayı Metotreksat'a bağlı yumurtalık hasarına karşı koruyucu etki sağlayabileceği görülmüştür.

Anahtar Kelimeler: Antioksidan, Metotreksat, Rat, Resveratrol, Yumurtalık hasarı

Abstract

Objective
The study aimed to evaluate the antioxidant and antiapoptotic effects of Resveratrol (RES) against Methotrexate (MTX)-induced ovarian damage in

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female rats and were divided into 7 groups:

Material and Method
Control; MTX (15mg/kg-1st day); MTX (1st and 3rd day); MTX (1st, 3rd and 5th day); MTX (1st day) + RES (20mg/kg); MTX (1st and 3rd day) + RES; MTX (1st, 3rd and 5th day) + RES. Since there was only one animal left in groups 4 and 6 and no animals in group 7, these groups were excluded from the evaluation. Ovarian damage was evaluated by performing hematoxylin-eosin (H-E) and TUNEL staining on ovarian tissues of rats. Total oxidant/antioxidant status in ovarian tissues was evaluated spectrophotometrically. In addition, DNA damage in blood was assessed by comet assay.

Results
As a result of biochemical analyzes, TOS levels increased in MTX groups, group 2 and 3, compared to the control group, while TOS level decreased significantly in group 5. On the other hand, TAS level increased significantly in group 5 compared to groups 2 and 3. H-E staining results showed that MTX-induced damage improved with RES (group 5) treatment. Maximum TUNEL positive staining image; it was observed in equal amounts in groups 2 and 3, followed by group 5. In addition, the comet score, which indicates DNA damage, increased significantly in groups 2 and 3, while it decreased significantly in group 5.

Conclusion
In line with the results of this study, it was observed that Resveratrol may provide protective effects against Methotrexate-induced ovarian damage due to its antioxidant properties.

Keywords: Antioxidant, Methotrexate, Ovarian damage, Rat, Resveratrol

Introduction

Methotrexate (MTX), which is used in the treatment of a wide range of diseases ranging from neoplasia to psoriasis, is a folic acid antagonist and an antiproliferative agent that inhibits DNA and RNA synthesis by binding the dihydrofolate reductase enzyme (1,2). MTX, while inhibiting the synthesis of nucleic acids, thymidylates and proteins, it suppresses growth and proliferation in malignant and some non-cancerous cells, causing toxic effects especially on rapidly dividing cells (3).

The toxic effects of MTX may vary from patient to patient. Therefore, MTX is used in a wide dose range depending on the indication it is used in (4). Some of the most well-known side effects of MTX are listed as haematological, hepatic and pulmonary toxicity (5). It is thought that oxidative stress resulting from the effect of MTX may be responsible for most of the side effects (6-8). Karri and Vanithakumari (3) examined the histology of the ovaries, uterus, cervix, and vagina to reveal the mechanism of action of MTX, and the study results showed that dose-dependently, MTX limits preantral and antral follicular growth in the ovary. Studies have shown that the use of MTX can cause toxic effects on tissues and should be used together with antioxidant substances (8,9).

Resveratrol (RES), a polyphenol compound, is a powerful antioxidant with anti-aging properties that attracts attention in the health sector (10). The synthesis of RES, which is a natural stilbene, is made by the stilbene synthase enzyme (11). RES is found mainly in grape skin, peanut, raspberry, mulberry, plum and some plants. RES is known to be a phytoallexin produced by plants, especially as a result of pathogens attacking plants, injury or exposure to ultraviolet (UV) light. Studies have shown that RES inhibits capillary occlusion by showing antioxidant activity and inhibits platelet aggregation in capillaries by modulation of apoliprotein and lipid synthesis. The majority of research on RES has focused on cancer, and it has been determined that this compound has stopping and preventing properties in many stages of cancer (12, 13). However, RES has shown that it prevents the formation of helicobacter pylori, which causes gastritis, ulcers and cancer in the stomach, and the development of malignant cells in mammals (14). Ortega and Duleba (15) examined the effects of resveratrol on the ovaries in their study and reported that resveratrol increases follicular reserve and prolongs ovarian life. Furthermore, it has been shown in different studies on human cancers (such as breast, cervix, uterus, ovary) that RES exhibits anticancer properties as well as antioxidant properties (16).

In our study, we aimed to evaluate the protective effect of RES, which is a powerful antioxidant, on rat ovary against oxidative organ damage, biochemically, histochemically and immunohistochemically, after repeated and single dose administration of MTX to female rats.

Material and Method

Experimental Protocol

A total of 42 female Wistar Albino rats, weighing between 240-360 g, obtained from Mehmet Akif Ersoy University Experimental Animal Production and Experimental Research Center, were used in the study. Rats were housed in individual cages under standard light (12/12 h light/dark cycle) at 25°C in a well-ventilated area. Rats were fed with tap water and standard rodent chow ad libitum. Experiments were performed in accordance with the animal research guidelines of the National Institutes of Health and were approved by the Mehmet Akif Ersoy Animal Experiments Local Ethics Committee (Ethical number: 18.09.2019-545).

In our study, 7 different experimental groups, each consisting of 6 female rats, were formed. Group 1 served as the control group and treated with a single intraperitoneal injection (IP) of 0.9% saline (1 mL/kg) on the day 1st. Groups 2, 3, 4 and 5 were given a single dose of 15 mg/kg intraperitoneal MTX on the first day; Groups 5, 6 and 7 were given RES as an antioxidant at a dose of 20 mg/kg 1 hour before MTX administration by oral gavage. Groups 3, 4, 6 and 7 received repeated doses of MTX on the 3rd day, and group 4 and 7 on the 5th day. Groups 5, 6 and 7 were administered RES at the same time for 7 days (Table 1). 24 hours after the last administration, that is, on the 8th day, the animals were sacrificed by surgical anemia under anesthesia of 10% ketamine HCl (Ketalar; Alfamin) and 2% xylazine (Alfazin) administered intramuscularly in groups.

Hematoxylin-Eosin and TUNEL (terminal deoxynucleotidyl transferase (tdt) mediated nick-end labeling) Staining

Ovarian tissue samples taken from rats were fixed in 10% neutral formalin solution for at least 24 hours. Tissues were washed for 48 hours, dried and cleared in xylene and embedded in paraffin and cut into 5 µm sections. Replicate sections were either stained with hematoxylin (Merck, Cat No.109249) and eosin (Tekkim, Cat No.TK400109) or stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Abcam, Cat No. Ab206386). The ovarian sections analyzed (17) under the light microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

The staining score for H&E and IHC was evaluated as;

(-),0 none staining
(+), 1 mild staining
(++), 2 moderate staining
(+++), 3 intense staining.

Preparation of Ovarian Tissue Samples

Ovarian tissues of each group stored at -20 ºC were weighed separately after being brought to room temperature and diluted 10 times with 50 mM phosphate buffer (pH 7.4). Homogenization

Table 1 Animals and experimental design

Experiment Group	Number of Animals	Dose	Experiment Time
Group 1 (Control)	6	0.9% saline 1 mL/kg (1st day) i.p	7 days
Group 2 (MTX1)	6	15 mg/kg/day i.p. MTX (1st day)	7 days
Group 3 (MTX2)	6	15 mg/kg/day i.p. MTX (1st+3rd day)	7 days
Group 4 (MTX3)	6	15 mg/kg/day i.p. MTX (1st+3rd+5th day)	7 days
Group 5 (MTX1+RES)	6	15 mg/kg/day i.p. MTX (1st day)+ 20 mg/kg/day RES (during 7 days)	7 days
Group 6 (MTX2+RES)	6	15 mg/kg/day i.p. MTX (1st+3rd day) + 20 mg/kg/day RES (during 7 days)	7 days
Group 7 (MTX3+RES)	6	15 mg/kg/day i.p. MTX (1st+3rd+5th day) + 20 mg/kg/day RES (during 7 days)	7 days

was completed by treatment with Janke & Kuntel Ultraturrax T-25 (Germany) brand tissue shredder and then UW-2070 Bandeun Electronic (Germany) brand sonicator. Tissue samples were cooled by centrifugation at 10.000 rpm, 10 min. Supernatant was taken and transferred to eppendorf tubes.

Total Oxidants and Antioxidants

TAS (Total Antioxidant Status) and TOS (Total Oxidant Status) parameters were studied by spectrophotometric method using Rel Assay Diagnostic Assay kits and MultiskanGO (ThermoFisher Sci., Waltham, Massachusetts, USA) microplate reader in the supernatants obtained. TOS results were expressed in µmoL H_2O_2 equivalent/L (µmol H_2O_2 eq/L). TAS value of the samples tested was expressed as mmol Trolox equivalent/L (mmol Trolox eq/L). Determination of OSI, which is an indicative parameter of oxidative stress level and the ratio of TOS to TAS was calculated using the following formula:

$$\text{OSI (arbitrary unit)} = [(\text{TOS, µmol/L}) / (\text{TAS, µmol Trolox equivalent/L}) \text{ X } 100]$$

Comet Assay

Lymphocyte DNA damage was analyzed using the comet assay method. Briefly, 20 µL of whole blood sample was mixed with 150 µL of low melting point agarose (LMA) at 37 °C. 140 µL of the prepared mixture was placed on a slide pre-coated with normal melting agarose (NMA). The slides were incubated for 5 minutes at 4°C. The slides were then placed in the lysis solution for 1 hour. After lysis, it was placed in a tank filled with cold alkaline electrophoresis buffer (1 mmol/L EDTA and 300 mM NaOH, pH>13) for 30 min. Then, electrophoresis was performed at 25V, 300 mA for 25 minutes. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.4) for 5 minutes. 100 cells were randomly analyzed under a fluorescence microscope (Olympus BX-50, Japanese) by adding ethidium bromide on slides. According to the degree of damage, cells were classified into 5 categories, 0, 1, 2, 3, 4, from undamaged (no DNA migration) to severely damaged (DNA migrated) (18-20).

Statistical Analysis

Statistical analyzes were performed using the IBM SPSS 20.0 program. Results are given as mean ± standard error. Kruskal-Wallis test was used for semi-qualitative evaluation in histological analysis. Non-parametric Mann-Whitney U test was used for pairwise comparisons. One-way ANOVA (post hoc Tukey test) was used for comparison between groups in comet assay, TAS and TOS analyses. P values below 0.05 were considered significant.

Results

Histopathological Studies in Ovarian Tissue

The histology of the H-E stained ovarian tissues of the MTX and RES treated rats are presented in Figure 1. While normal histological structures were observed in the ovarian tissue sections of the control group, hemorrhagic areas were observed in some sections, albeit partially. When the ovarian tissues of the experimental group (Groups 2-3) given MTX were

Table 2 Grading of histological findings between groups

Groups	Group 1 Control (n=6)	Group 2 MTX1 (n=6)	Group 3 MTX2 (n=3)	Group 5 MTX1+RES (n=4)
Parameters/ Scores	- + ++ +++	- + ++ +++	- + ++ +++	- + ++ +++
Oocyte degenerations	-	+++	++	+
Zona pellucida degenerations in its structure	-	++	++	+
Mononuclear cell infiltrations	-	++	++	+
Decreases in the numberof follicles	-	+++	++	+
Vascular congestions	-	++	+++	++
Seperations between the granulosa cells of the follicles	-	++	+++	+
Hemorrhagic areas	-	+++	+++	+++
Follicular degenerations	+	++	+++	+

examined, compared to the control group; oocyte degenerations, separations between the granulosa cells of the follicles, degenerations in the zona pellucida structure, mononuclear cell infiltrations, hemorrhagic areas especially in the corpus luteum, vascular congestions, decreases in the number of follicles and follicular degenerations were observed (Table 2). Since the number of animals in group 4 decreased to one, the findings in this group could not be evaluated statistically, while the findings in group 2 and group 3 were evaluated. In the group given MTX and RES together (Group 5-6-7), compared to the damaged MTX group, many findings were improved except for the formation of hemorrhagic areas. Since the number of animals in group 6 decreased to one and there were no animals in group 7, the findings in these groups could not be evaluated, while the findings in group 5 were evaluated.

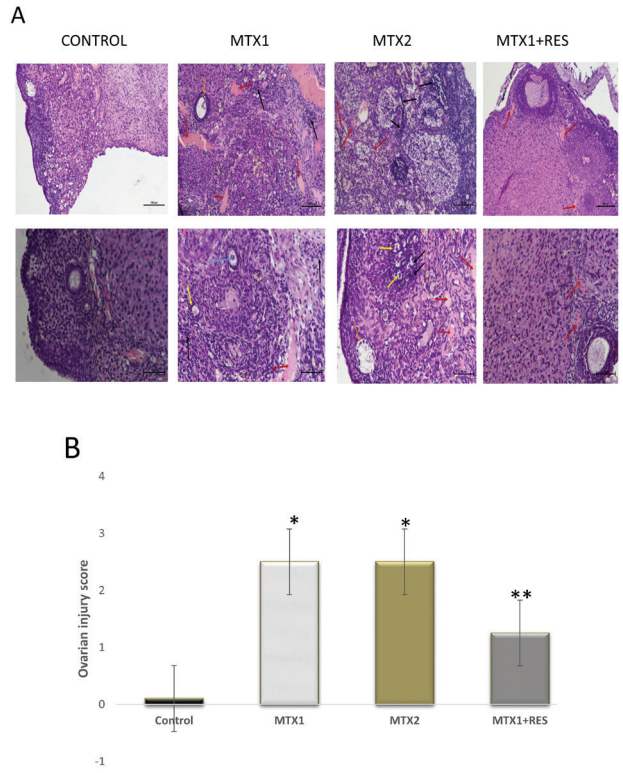

Figure 1:
(A) Histopathology (H&E staining) of ovarian tissues. In the control group, hemorrhagic findings were observed, albeit partially, apart from the normal histological structures. Black arrows in other groups; mononuclear cell infiltrates, yellow arrow; follicle degenerations, blue arrows; oocyte degeneration, orange arrow; Separation of granulosa cells, red arrows; shows hemorrhagic areas. (B) Quantification of ovarian injury based on a semi-quantitative morphological scoring system from 0 to 3. *p < 0.05 vs. control, **p < 0.05 vs. MTX1

TUNEL Staining of Ovarian Tissue Section

No positive staining was observed in the control group in the TUNEL staining of ovarian tissue sections. More positive staining was observed in the sections in group 2 and group 3 compared to group 1 (Figure 2). In the group given MTX and RES together (Groups 5-6-7); less positive staining was observed compared to the damaged MTX group. However, since the number of animals in group 6 decreased to one and there were no animals in group 7, the stainings in these groups could not be evaluated, whereas positive stainings were observed in group 5 and were evaluated. The highest positive staining in terms of staining degrees; observed in equal amounts in groups 2 and 3, then in group 5. Tunnel findings were consistent with histopathological findings.

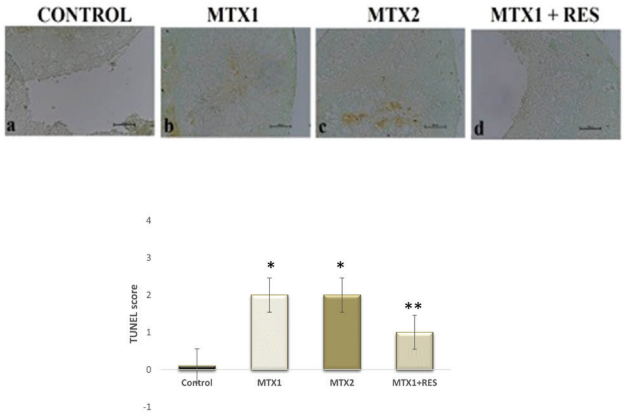

Figure 2:
TUNEL staining of ovarian tissue section. *p < 0.05 vs. control, **p < 0.05 vs. MTX1

TAS and TOS Levels in Ovarian Tissue

Ovarian tissue TAS, TOS and OSI analysis results of the experimental groups are given in Table 3. Compared to the control group, TOS level increased significantly in group 2 and group 3, where the increased dose of methotrexate was applied (*p=0.000, *p=0.001, respectively). A significant decrease was observed in TOS levels in the group given RES by applying a single dose of MTX (Group 5) compared to both the control and Group 2. (*p=0.001, **p=0.000, respectively). TAS levels were significantly decreased in all groups compared to control (*p=0.041, *p=0.001, *p=0.002, respectively). However, there was no significant change in TAS levels in group 5 that received a single dose of MTX and concomitant RES, compared to group 2 that received only MTX (p<0.05). OSI values, which are indicative of oxidative stress, increased significantly in group 2 and group 3 in which increasing dose of

Table 3 Analysis of TAS, TOS and OSI parameters of the experimental groups.

	TOS (μmol H_2O_2 eq/L)		TAS (mmol Trolox eq/L)		OSI (AU)	
Groups	**Mean ±SD**	**P**	**Mean ±SD**	**P**	**Mean ±SD**	**P**
Group 1	25.13±2.28	**p=0.000	1.12±0.08	**p=0.041	2.24±0.32	**p=0.000
Group 2	38.62±2.12	*p=0.000	0.99±0.05	*p=0.041	3.87±0.30	*p=0.000
Group 3	34.53±2.15	*p=0.001	0.85±0.08	*p=0.001	4.06±0.69	*p=0.000
Group 5	16.06±4.45	*p=0.001 **p=0.000	0.90±0.08	*p=0.002	1.80±0.62	**p=0.000

Data were presented as mean and standard deviation.
One way ANOVA (post hoc Tukey test) was used for comparison between groups. *p: Comparison with control group, **p: Comparison with Methotrexate1 group. Group 1: control, Group 2: MTX1, Group 3: MTX2, Group 5: MTX1+RES (Other groups were not included in the statistical analysis due to insufficient number of animals due to animal deaths).

Table 4 Comet score analysis of groups.

Groups	Comet score (Arbitrary Unit)	
	Mean ±SD	**P**
Group 1	121.50 ± 41.62	
Group 2	223.00 ± 92.61	*p=0.043
Group 3	342.00 ± 20.88	*p=0.000
Group 5	79.40 ± 23.96	**p=0.005

Data were presented as mean and standard deviation. One way ANOVA (post hoc Tukey test) was used for comparison between groups. *p: Comparison with control group. **p: Comparison with MTX1 group. Group 1: control, Group 2: MTX1, Group 3: MTX2, Group 5: MTX1+RES (Other groups were not included in the statistical analysis due to insufficient number of animals due to animal deaths).

methotrexate was applied compared to the control (*p=0.000, *p=0.000, respectively). In the group given RES by administering MTX1 dose (Group 5); A significant decrease was observed in OSI levels compared to MTX1 (Group 2) (**p=0.000).

Effects of Resveratrol on Methotrexate-Induced DNA Damage

Lymphocytes DNA damage in the groups was performed by the comet assay. The comet scoring of the groups is shown in Table 4. It was determined that the animals in the MTX administered groups (Group 2 and Group 3) had more DNA damage than the control group (p=0.043, p=0.000, respectively). In the group treated with RES (Group 5), DNA damage was found to be significantly reduced compared to the groups treated with MTX alone (p=0.005). DNA damage images of all groups are shown in Figure 3.

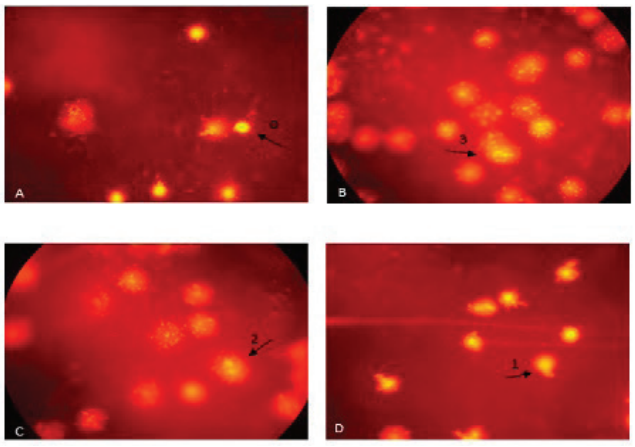

Figure 3: Evaluation of DNA damage by Comet Assay (A) control, (B) MTX1, (C) MTX2, (D)MTX1+RES

Discussion

Cancer is a leading cause of death in the worlwide. Drugs used in cancer treatment can cause infertility as a side effect (21). Many antioxidants have been tried in previous studies to prevent or reduce the side effects of these drugs. The main objective of the current study is to elucidate the mechanisms underlying MTX-related ovarian toxicity and to explore the possible ameliorative and protective effects of RES.

Methotrexate is used in the treatment of many diseases, especially cancer. However, it causes damage not only to pathological cells, but also to healthy cells. MTX use may cause oxidative stress-induced damage to tissues and organs (22, 23). Oxidative stress is a condition caused by excessive accumulation of ROS in tissues as a result of disruption of the oxidant/antioxidant balance. Negative effects of MTX are also observed in the testes and ovaries, as well as the kidney and liver, which are vital organs (24-27).

In our study, we preferred to use RES as one of the strongest antioxidants and confirmed its protective effect against MTX damage in ovarian tissue. Resveratrol is a natural polyphenol synthesized by plants as a phytoalexin that is activated under stress conditions such as ultraviolet radiation and fungal infection (28). This naturally occurring polyphenol found in grapes, strawberries and herbs has been suggested to exhibit antioxidant and anti-inflammatory activities and is a longevity agent. Many studies have examined the use of RES as a therapeutic agent to treat various pathological and metabolic disorders (15). In previous studies, the protective effects of resveratrol on MTX-induced liver damage (29), intestinal damage (30, 31) and ileum damage (32) has been reported.

In our study, a large number of animal deaths were observed in the groups that received two and three doses of MTX. For this reason, group 4, group 6 and group 7 were excluded from the evaluation. The cause of animal deaths encountered in the use of repeated doses of MTX has been investigated in the literature. Senol et al. (33) reported that the most common side effect of the use of chemotherapeutic agents is bone marrow suppression and infection due to neutropenia, which is the most common cause of death. Gonderen and Kapucu (34) also reported in their study that neutropenia is the most important side effect of chemotherapy and increases the death rate. We think that our causes of death may also be in this direction.

First, the damaging effect of MTX on the ovaries was evaluated histopathologically in this study. In the H&E staining of rat ovaries in the MTX groups, oocyte degenerations, separations between the granulosa cells of the follicles, degenerations in the structure of the zona pellucida, mononuclear cell infiltrations, hemorrhagic areas especially in the corpus luteum, vascular occlusions and reductions in the number of follicles were observed. Evaluating the protective effects of coenzyme Q on MTX-induced ovarian and uterine damage, Kiremitli et al. (35) also reported similar histopathological findings in the ovary after MTX administration. In our study, histopathological evaluation revealed that ovarian damage in the MTX + RES group was significantly milder than in the MTX group In addition, the decrease in the number of follicles seen in MTX groups increased in MTX+RES groups. Similar to our study in the literature, Ata et al. (36) reported the effects of RES on MTX-induced ovarian damage. In this study, RES was shown to protect the ovary from damage caused by MTX. In addition, it has been shown that resveratrol provides this effect with decreased MDA and increased SOD and tGSH levels. However, apoptosis and DNA damage were not evaluated in this study. In previous studies, protective effects of alpha lipoic acid and lycopene on MTX-induced ovarian damage have also been demonstrated (37, 38). In another study, Chen et al. (39) studied the ovaries of aging rats and suggested that RES increases the follicle reserve in this process and prevents atresia. Ozdemir et al. (40), on the other hand, used RES as an antioxidant against cyclophosphamide-induced ovarian damage as a chemotherapeutic agent in rats and evaluated its effects at a microscopic level. In the cyclophosphamide group, increased TUNEL labeling in theca cells of stratified primary, secondary and graaf follicles decreased as a result of RES application, and it was reported that this decrease was more pronounced in stratified primary follicles. In conclusion, it has been suggested that RES may be an option to preserve fertility in women using chemotherapeutic agents.

Additionally, we found that in rats treated with MTX, the apoptotic index, which is an indicator of ovarian damage, increased approximately twenty times with the TUNEL method compared to the control group, and this increase was significantly reduced by RES application. This result demonstrated histopathologically the protective effect of RES on MTX-induced ovarian damage.

Also, in this study, a statistically significant increase in TOS and OSI levels, which are oxidative stress markers, and a decrease in TAS levels were observed

in ovarian tissue in groups 2 and 3 compared to the control group. Compared to groups 2 and 3, OSI levels were significantly reduced with RES administration in group 5 and no significant increase in TAS levels was noted. Gunyeli et al. (41), in their study with Astaxanthin (AXA) against MTX induced ovarian damege, analyzed TOS, OSI, and TAS levels. TOS and OSI levels increased significantly with MTX application in ovarian tissue, and these values were shown to decrease with AXA application. At the same time, Gunyeli et al. (41) reported that TAS levels decreased with MTX and increased with AXA administration. Our findings supported previous reports that MTX administration induces oxidative stress in various tissues (42-44).

In our study, the comet method was used to reveal DNA damage. Compared to the control group, the comet score increased significantly in the MTX administered groups, while the comet score decreased significantly in the MTX+RES administered group. Alturfan et al. (45) examined the effect of resveratrol on different tissues against acrylamide-induced oxidative stress on rats and reported that while the level of 8-OHdG, which expresses DNA damage, was statistically increased in the group given acrylamide, this level decreased significantly in the group given RES. Our findings indicate that the DNA damage-reducing effect of RES is consistent with the literature.

Conclusion

As a result, we found that RES significantly protected the ovaries against damage due to a single dose of MTX. However, we experienced severe animal losses in the groups where we planned to see the effect of RES on the damage caused by repeated doses of MTX.

In conclusion, we think that the oral use of RES together with an important chemotherapeutic agent such as MTX will be successful due to its effects on reducing ovarian damage and preserving follicle reserve. These results may be promising for the protection of reproductive health for many women undergoing cancer treatment today. For this purpose, more comprehensive clinical studies are needed in the future.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
Experiments were performed in accordance with the animal research guidelines of the National Institutes of Health and were approved by the Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Date: 18.09.2019; No: 545).

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Availability of Data and Materials
Data available on request from the authors.

Authors Contributions
KKA and SY conducted the animal studies, wrote the article and contributed equally to this work. SY performed TAS - TOS analyzes, interpreted the data and participated in the statistical analysis. In addition to assisting with animal studies, CAA participated in TAS and TOS analysis, interpretation of results and article writing. MO undertook histological examinations. RA performed the comet assay. All authors have read and approved the article.

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