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ORIGINAL ARTICLE

Quercetin Versus Folic Acid: A Comparative Evaluation of Protective Effects on Methotrexate-Induced Ovarian Damage

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Abstract

Background: Methotrexate (MTX) is a chemotherapeutic and immunosuppressive agent that acts as a folic acid reductase inhibitor and is widely used in various clinical settings. Despite its therapeutic efficacy, MTX has been shown in some experimental models to induce oxidative stress–mediated damage in ovarian tissue. This study aimed to evaluate and compare the protective effects of quercetin and folic acid against MTX-induced ovarian injury.

Methods: Forty-two female Wistar albino rats were randomly divided into six groups (n=7): Control, QRC (quercetin), FA (folic acid), MTX (methotrexate), MTX+QRC (methotrexate+ folic acid), and MTX+FA (methotrexate+folic acid). Methotrexate was administered intraperitoneally at1mg/kg on days 1, 3, 5, and 7. quercetin(50mg/kg) and folic acid(20mg/kg) were administered orally on days 2, 4, 6, and 8. On day 9, ovarian tissues were collected for histopathological, immunohistochemical and biochemical analyses. Statistical analysis was conducted using one-way ANOVA and Kruskal-Wallis tests with Bonferroni and Dunn's post hoc corrections ($p<0.05$).

Results: Methotrexate significantly increased follicular degeneration, inflammation, vascular congestion, hemorrhage, edema, and fibrosis ($p<0.001$), as well as malondialdehyde (MDA) and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) ($p<0.001$). It also significantly reduced anti-Mullerian hormone (AMH) levels, superoxide dismutase (SOD) and catalase (CAT) activities, and all follicle counts including primordial follicles ($p<0.001$). Both quercetin and folic acid treatments ameliorated these effects. Quercetin showed greater efficacy in reducing TNF-α and IL-1β levels ($p<0.01$) and restoring antioxidant enzyme activities($p<0.05$), while folic acid was more effective in preserving AMH levels ($p<0.01$) and increasing primordial and Graafian follicle numbers ($p<0.05$).

Conclusion: Although methotrexate remains an effective therapeutic agent, its ovarian toxicity poses a potential risk to long-term fertility. The present findings indicate that quercetin and folic acid exert protective effects against MTX-induced ovarian damage through distinct mechanisms: quercetin primarily by attenuating oxidative stress and inflammation, and folic acid by supporting folliculogenesis and preserving ovarian reserve.

Keywords: Ovarian reserve, reproductive toxicology, methotrexate, quercetin, folic acid, inflammation.

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INTRODUCTION

Methotrexate (MTX) is a chemotherapeutic agent that functions as a folic acid reductase inhibitor, thereby suppressing DNA synthesis (1). However, MTX may cause a wide range of adverse effects, including gastrointestinal symptoms, hematologic toxicity, hepatotoxicity, and genotoxicity. Despite its high success rate in medical management, MTX is a cytotoxic drug, and one of its potential side effects is oxidative stress–induced ovarian damage. Such damage may reduce the ovarian follicle reserve and, in the long term, lead to infertility (2).

Ectopic pregnancy (EP) is a significant obstetric emergency that most commonly occurs in the fallopian tubes and accounts for approximately 2% of all pregnancies. Its incidence has been increasing due to the rising prevalence of pelvic inflammatory diseases and the widespread use of assisted reproductive technologies (3). EP can lead to life-threatening clinical conditions, particularly in cases of rupture, and remains one of the leading causes of maternal mortality during the first trimester. Therefore, rapid diagnosis and appropriate treatment are of critical importance (4). In hemodynamically stable patients, MTX is widely used as a medical treatment option, providing a fertility-preserving alternative to surgical intervention (3). In most patients with early-diagnosed, unruptured EP, MTX enables successful medical management (1). Nevertheless, the disadvantages associated with its toxicity, particularly oxidative stress mediated ovarian injury, have increased interest in recent years toward antioxidant strategies aimed at mitigating these effects (3,5,6).

Quercetin (QRC) is a naturally occurring flavonoid known for its potent antioxidant, anti-inflammatory, and cytoprotective properties (7). One of its most prominent features is its strong antioxidant capacity. QRC is considered one of the most effective scavengers of reactive oxygen species (ROS), including reactive nitrogen species such as nitric oxide (8). In various animal models, including those simulating ischemia-reperfusion injury, quercetin has demonstrated protective effects across multiple tissue types (9). By inhibiting lipid peroxidation and neutralizing oxygen-derived free radicals, QRC reduces oxidative damage and prevents cellular apoptosis (10). Furthermore, studies have shown that quercetin protects ovarian follicle reserves, inhibits apoptosis in granulosa cells, and supports overall ovarian function under oxidative stress conditions (11).

Folic acid (FA), a water-soluble vitamin, is a key cofactor in DNA and RNA synthesis, and its deficiency is associated with adverse reproductive outcomes. FA also possesses antioxidant properties, contributing to its ability to reduce oxidative stress–induced cellular damage (12). Because MTX inhibits folate metabolism, FA is often co-administered as a protective agent to minimize cytotoxic effects on healthy cells during MTX therapy (13). In particular, in multi-dose treatment regimens, supplementation with FA (or leucovorin) is recommended to antagonize the cytotoxicity of MTX on normal tissues and to mitigate treatment-related side effects (14).

MTX administration triggers inflammatory cascades through the increased expression of pro-inflammatory cytokines such as IL-6 and TNF-α, involving the activation of the NF-κB pathway, followed by stimulation of JAK/STAT3 signaling (5). Upon phosphorylation, the JAK/STAT complex translocates into the nucleus, binds to promoter regions of target genes, and initiates transcription of those involved in the inflammatory response (15). Inflammation is a physiological reaction of the body to internal or external stimuli (16). Activation of macrophages and lymphocytes, which leads to a coordinated cytokine response, is a hallmark of chronic inflammation (17). In response to antigens, inflammatory cells release molecules known as cytokines, which regulate immune and inflammatory processes. IL-1β and TNF-α are among the key pro-inflammatory cytokines, and MTX has been shown to increase their expression.

Antioxidants offer a promising strategy for minimizing chemotherapeutic drug-induced toxicity by mitigating oxidative stress. Particularly during or prior to MTX therapy, antioxidant supplementation has been shown to reduce the formation of free radicals and improve therapeutic outcomes. These molecules play a critical role in preventing oxidative damage at the cellular level. Folic acid (FA), the synthetic form of folate, is a water-soluble vitamin with known antioxidant properties and various physiological roles. Quercetin (QRC), on the other hand, is a naturally occurring flavonoid found abundantly in plants, known for its potent free radical–scavenging capacity.

Although MTX-induced ovarian toxicity is a clinically significant side effect with potential implications for fertility, there is currently no standardized preventive therapeutic approach. While several studies in the literature support the protective role of FA against MTX-induced ovarian damage, comparative studies evaluating QRC and FA in this context are lacking. Therefore, the present study aimed to evaluate and compare the protective and therapeutic potential of QRC and FA in an experimental model of MTX-induced ovarian toxicity.

MATERIALS AND METHODS

Animals and Experimental Groups

The study was designed and conducted in compliance with the ARRIVE guidelines for in vivo animal research. Animal Experiments Local Ethics Committee approved our experimental guidelines.

The experiment was initiated using male Wistar albino rats aged twelve weeks. All animals were housed under controlled conditions at 25°C, maintained on a 12-hour light/dark cycle. They had unrestricted access to standard laboratory chow and water. The animals were randomly assigned to six experimental groups, each consisting of seven rats.

Six distinct categories of rats were set up: Control (CONTROL), quercetin (QRC), folic acid (FA), methotrexate (MTX) methotrexate+quercetin (MTX+QRC) and methotrexate+ folic acid (MTX +FA). Rats in the control group were treated with saline for a period of 8 days. MTX (Kocak Pharma, 50 mg/mL. Istanbul, Turkey) was administered intraperitoneally (i.p.) at a dose of 1 mg/kg/day (18,19). to the respective animal groups on days 1, 3, 5, and 7 of the experimental protocol. FA (Sigma, Saint Louis, USA) and QRC (Cat no: Q4951 Sigma Aldrich, USA) were administered via oral gavage at doses of 20 mg/kg (20) and 50 mg/kg (21), respectively, on days 2, 4, 6, and 8 to the relevant groups. No animals were killed in our research application. On the ninth day of the protocol, following the collection of ovarian tissue and blood samples under general anesthesia induced by intraperitoneal administration of xylazine (10 mg/kg) and ketamine (60 mg/kg), the rats were humanely euthanized via cervical dislocation. (Fig.1).

Ovarian Morphology Evaluation

The left ovary were fixed in 10% neutral buffered formalin for 72 hours, followed by a graded ethanol dehydration process. Subsequently, the tissues were embedded in paraffin blocks to prepare them for histological analysis. Tissue slices with a thickness of 5 μm were cut and mounted on slides, then subjected to deparaffinization in xylene and rehydration through graded alcohols. Histological staining was carried out using Masson's trichrome and hematoxylin–eosin methods. Histological evaluations and image acquisition were carried out with an Olympus BX51 light microscope integrated with a DP71 digital imaging system (Olympus, Tokyo, Japan).

Histopathological evaluation was performed using a semi-quantitative scoring system previously described in the literature (22). Each slide was examined independently by two blinded histologists who were unaware of the experimental groups. The following parameters were evaluated and scored on a scale from 0 to 3: 1-Follicular degeneration, 2-Inflammation, 3-Hemorrhage, 4-Edema, 5-Vascular congestion. The scoring criteria were defined as follows: 0 = None, 1 = Mild, 2 = Moderate, 3 = Severe. For each animal, ten randomly selected fields were assessed, and the mean score was calculated to represent the group value.

Follicle Morphological Classification and Counting

Serial sections were obtained from paraffin-embedded ovarian tissue blocks starting from the point where follicular structures began to appear. In each experimental group, serial ovarian sections were analyzed for follicle count. Based on well-established morphological parameters, follicles were independently evaluated and categorized by two blinded histologists into five stages: primordial, primary, preantral, antral, and atretic.

The staging of follicles relied on granulosa layer thickness and specific cellular morphology. Criteria for identifying viable (healthy) follicles included the presence of an uninterrupted basal lamina, a well-defined oocyte containing a germinal vesicle, and a clearly discernible nucleolus. In contrast, follicles exhibiting degenerative features such as dead granulosa cells and pyknotic nuclei were considered damaged or atretic.

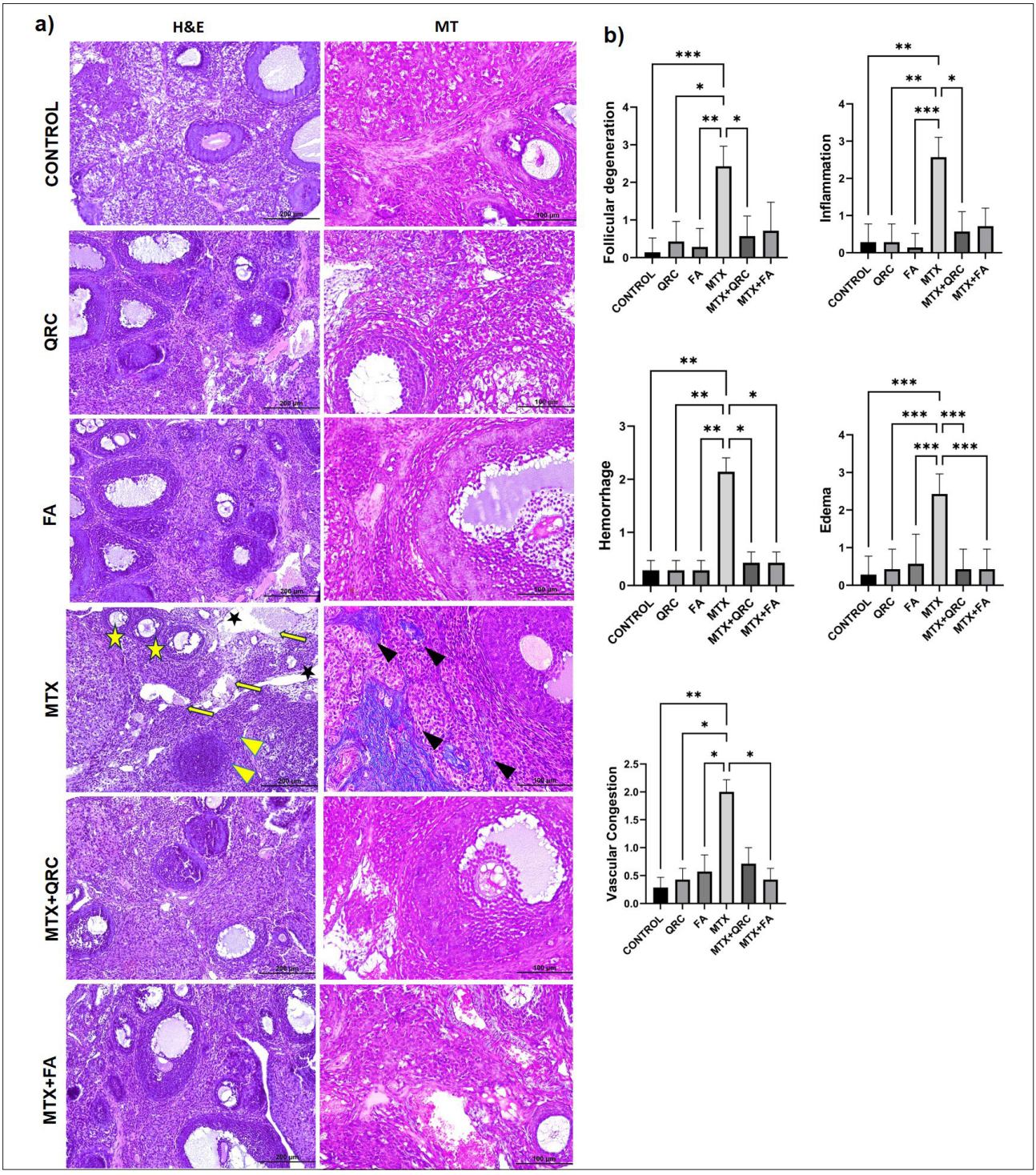

Figure 1a: Histology of ovary sections (Olympus BX51, Tokyo, Japan. H&E; X200: Scale bar; 100 μm, MT; X400: Scale bar; 200 μm). In the CONTROL group, normal ovarian morphology is observed with well-preserved primary, secondary, and antral follicles. No signs of degeneration, inflammation, or fibrosis are present.In the QRC and FA-treated groups, follicular structures are preserved, and stromal areas appear normal, without evidence of degeneration or inflammation.In the MTX group, prominent histopathological alterations are evident, including follicular atresia, cytoplasmic vacuolization, nuclear pyknosis, and degenerative changes in granulosa cells (yellow star). Vascular congestion (black star), dense inflammatory cell infiltration (yellow arrowhead), and extensive fibrosis (black arrowhead) are seen in the stroma (yellow arrow). In the MTX+QRC and MTX+FA groups, mild follicular degeneration and stromal congestion are observed. Partial preservation of follicular structures is noted. b) Comparison of ovarian histoscoring results. Statistically significant differences between groups are indicated by asterisks in each graph: *p<0.05, **p<0.01, *p<0.001.

Immunohistochemistry

The immunohistochemical staining kit (Thermo Ultravision Detection System, Fremont, USA, TP-125-HL) was used in conjunction with the streptavidin-biotin-peroxidase method. This method was used to identify the expressions of TNF-α, IL1β, IL6 and AMH in ovary tissue. Ovarian tissue blocks were sectioned into 5 μm-thick slices and subsequently processed through deparaffinization and rehydration steps. Sections were washed in phosphate-buffered saline (PBS) at room temperature, followed by a 5-minute treatment with 3% hydrogen peroxide to quench endogenous peroxidase activity. After a second PBS wash, antigen retrieval was carried out by incubating the sections in 10% sodium citrate buffer (pH 6.0) at 95°C for 5 minutes using a microwave oven. The slides were then left to cool at room temperature for 20 minutes prior to immunostaining. Primary antibodies used in this study included: TNF-α (Tumor Necrosis Factor Alpha Antibody, Abcam, Massachusetts, USA; catalog no: bs-6434R; dilution 1:150), IL-1β (Interleukin-1 Beta Antibody, Bioss, Massachusetts, USA; catalog no: bs-1685532; dilution 1:200), and IL-6 (Interleukin-6 Antibody, Bioss, Massachusetts, USA; catalog no: bs-0782R; dilution 1:200) and AMH (Antimullerian Hormone, Santa Cruz Bıotechnology, Oregon, ABD -sc 1667529; 1:150) were treated with the sections for an entire night at 4°C. Following incubation with biotinylated secondary antibodies, sections were treated with a streptavidin–peroxidase complex. The chromogenic reaction was then developed using the DAB (3,3'-Diaminobenzidine) substrate system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Mayer's hematoxylin was used for counterstaining. Images were captured from ten distinct regions of each ovarian section using a DP71 digital camera mounted on an Olympus BX51 light microscope (Tokyo, Japan). The immunoreactivity intensities of TNF-α, IL-1β, IL-6, and AMH were quantified using the ImageJ software program (NIH, Washington, USA).

Enzyme-Linked Immunosorbent Assay

The right ovary was immediately sectioned on a chilled mold to maintain tissue integrity. The ovarian specimens were placed into Eppendorf tubes and stored at −80°C until analysis. For biochemical evaluations, the frozen tissues were homogenized in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) to prepare tissue homogenates. The homogenized samples were then centrifuged at 4°C for 10 minutes at 1500 × g. The resulting supernatants were aliquoted and used for enzyme-linked immunosorbent assay (ELISA) analyses.

To assess the antioxidant and AMH capacity of the ovarian tissue, the levels of Glutathione Peroxidase (GSH-Px, Sunred Bio, Cat. No. 201-11-5104; Shanghai, China), Superoxide Dismutase (SOD), and Catalase (CAT) (Sunred Bio, Cat. No. 201-11-5106; Shanghai, China) were measured using commercial ELISA kits. Additionally, lipid peroxidation levels were evaluated by quantifying malondialdehyde concentrations (MDA, Sunred Bio, Cat. No. 201-11-0157; Shanghai, China).

Statistical Analyses

The results obtained from analyses were evaluated using the GraphPad Prism 9.0 statistical program. The Shapiro-Wilk test was performed to assess the normality of the data distribution. For comparisons involving multiple groups, one-way analysis of variance (ANOVA) and the Kruskal-Wallis test were utilized. Post hoc analyses were conducted using the Bonferroni test for ANOVA and the Dunn test for the Kruskal-Wallis test, both of which identified significant differences among the variables. A p-value below 0.05 was considered statistically significant for all analyses.

RESULTS

Histopathological Findings

In this study, H&E staining was used to evaluate the ovarian tissue histoarchitecture and follicle counts across all experimental groups, while MT staining was employed to assess the general appearance of the connective tissue.

In the Control group, primary and secondary follicles were clearly observed. The granulosa cell layers appeared regular with intact nuclear and membrane structures. The oocyte morphology was healthy, with no signs of degeneration, vacuolization, or necrosis in the granulosa cells. Cellular morphology was homogeneous with well-defined cell borders. Stromal architecture appeared normal with appropriate cell density. No pathological

findings such as inflammatory cell infiltration, edema, or hemorrhage were observed. The vascular structures were intact, showing no signs of congestion or dilation. A similar preservation of ovarian histoarchitecture was observed in the groups treated with QRC and FA.

In contrast, in the MTX group, most ovarian sections exhibited disrupted structural integrity, with follicular degeneration, atretic changes, and signs of inflammation. The granulosa cells showed compaction, cellular degeneration, pyknotic nuclei, and vacuolization in some areas. In the follicles containing oocytes, nuclear structures were either deformed or could not be clearly distinguished. The stroma demonstrated dense inflammatory cell infiltration, and the vasculature exhibited marked congestion and focal areas of edema. In the MTX+QRC group, compared to the MTX group, degenerative changes in the granulosa cells were reduced, and the number of atretic follicles was lower. Stromal areas also showed reduced signs of congestion, edema, and perivascular inflammatory infiltration. In the MTX+FA group, although there was no significant follicle loss compared to the MTX group, deterioration in follicle quality (including degeneration and irregularity) was noted. The stromal edema, vascular congestion, and cell infiltration in this group were similar to the findings in the MTX+QRC group. Assessment of collagen fiber density in MT-stained sections for stromal fibrosis revealed an evident increase in collagen accumulation in the MTX group, whereas all other groups showed similar staining patterns, with no apparent differences (Fig. 1a).

Histopathological Scoring

Histopathological evaluation revealed that MTX administration caused significant tissue damage in the ovarian tissue. In the MTX group, the scores for follicular degeneration ($p<0.001$), inflammation ($p<0.01$), hemorrhage ($p<0.01$), edema ($p<0.001$), and vascular congestion ($p<0.01$) were significantly higher compared to the Control group.

In groups treated with quercetin (MTX+QRC) and folic acid (MTX+FA), significant improvements were observed in these parameters. In the MTX+QRC group, scores for follicular degeneration, inflammation, and hemorrhage ($p<0.05$) as well as edema ($p<0.001$) were significantly reduced compared to the MTX group. Similarly, in the MTX+FA group, scores for hemorrhage and vascular congestion ($p<0.05$) and edema ($p<0.001$) were also significantly decreased compared to the MTX group. These findings suggest that both QRC and FA exert protective effects against MTX-induced ovarian damage. However, QRC demonstrated a more pronounced improvement in follicular degeneration and hemorrhage scores than FA (Fig. 1b).

Follicle Count Analysis

All follicle types, including primordial, primary, preantral, antral, Graafian, and atretic follicles, were counted in each group. MTX administration significantly reduced all follicle scores compared to the control group ($p<0.05$ for all; a). Co-administration of QRC (MTX+QRC) and FA (MTX+FA) improved follicle scores significantly compared to MTX ($p<0.05$; b, c). Among treatment groups, MTX+FA provided slightly better preservation in primordial, antral, preantral, and Graffian follicles, while MTX+QRC showed a more pronounced reduction in atretic follicle scores. A statistically significant difference was observed between MTX+QRC and MTX+FA in certain follicle types (d). (Table 1).

Immunohistochemical Findings

Immunohistochemical evaluation revealed a marked increase in the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in the MTX group, whereas AMH expression, an indicator of ovarian reserve, was significantly reduced. Strong positive immunoreactivity for TNF-α, IL-1β, and IL-6 was observed in granulosa cells and stromal regions of ovarian tissue in the MTX group, while minimal staining was noted in the Control, QRC, and FA groups. Co-administration of quercetin or folic acid with MTX (MTX+QRC and MTX+FA groups) led to a notable reduction in the expression of these inflammatory markers compared to MTX alone, with TNF-α and IL-1β suppression being more pronounced in the MTX+QRC group. In contrast, the reduced AMH expression in the MTX group was partially restored in both treatment groups. Notably, AMH immunoreactivity was more intense in the MTX+FA group, suggesting that folic acid may be more effective than quercetin in preserving ovarian reserve (Fig. 2).

Table 1. Number of follicles in different stages of ovarian follicular development

Follicle Score	CONTROL	QRC	FA	MTX	MTX+QRC	MTX+FA	*p*
Primordial follicle	80.33 ±4.844[a]	79.50 ±3.017	82.13 ±5.193	29.33 ±5.574[abc]	71.33 ±11.38[bd]	76.67 ±12.04[cd]	0.001
Primer follicle	46.01 ±7.043[a]	43.17 ±3.710	47.83 ±5.419	27.67 ±7.230[abc]	40.67 ±8.262[b]	41.00 ±7.589[c]	0.017
Preantral follicle	33.17 ±5.879[a]	35.67 ±4.590	47.83 ±5.913	18.33 ±3.011[abc]	29.67 ±7.005[b]	31.50 ±3.564[c]	0.001
Antral follicle	31.67 ±5.164[a]	32.00 ±5.550	43.00 ±8.672	19.67 ±4.926[abc]	28.83 ±8.589[bd]	35.50 ±3.391[cd]	0.001
Graffian follicle	9.67 ±1.211[a]	10.83 ±3.061	13.83 ±2.714	4.33 ±1.366[abc]	8.00 ±2.449[bd]	9.67 ±2.503[cd]	0.001
Atretic follicle	7.00 ±1.414[a]	7.17 ±1.602	6.33 ±1.211	8.83 ±1.941[abc]	6.00 ±1.414[b]	7.33 ±0.816[c]	0.034

The data is presented as mean ± median (min–max). Superscript letters indicate significant differences between groups ($p<0.05$); a: CONTROL vs MTX, b: MTX vs MTX+QRC, c: MTX vs MTX+FA d: MTX+QRC vs MTX+FA

According to the quantitative analysis of immunoreactivity levels, the MTX group exhibited a significant increase in TNF-α, IL-1β, and IL-6 levels compared to the control group ($p<0.001$). This increase supports the notion that MTX induces a strong inflammatory response in ovarian tissue. In both the MTX+QRC and MTX+FA groups, the levels of these pro-inflammatory cytokines were significantly reduced compared to the MTX group ($p<0.01$ or $p<0.001$). Notably, quercetin treatment resulted in a more pronounced reduction in TNF-α levels compared to folic acid ($p<0.05$). While IL-1β and IL-6 levels were significantly decreased in both treatment groups, quercetin appeared to exert a stronger suppressive effect on IL-1β. These findings indicate that both QRC and FA are effective in attenuating MTX-induced inflammation, although quercetin may exhibit a more dominant protective effect for certain inflammatory markers (Fig.3).

Lipid Peroxidation Level, Antioxidant and AMH Findings

Biochemical analysis of oxidative stress markers in ovarian tissue revealed a significant increase in MDA levels and a significant decrease in SOD and CAT enzyme activities as well as AMH levels in the MTX group ($p<0.01$, $p<0.001$), indicating pronounced oxidative damage and reduced ovarian reserve following methotrexate administration.

In contrast, MTX+QRC and MTX+FA groups exhibited a significant reduction in MDA levels, accompanied by increased SOD and CAT enzyme activities and AMH levels compared to the MTX group ($p<0.01$, $p<0.001$). These findings suggest that both quercetin and folic acid exert protective effects by reducing oxidative stress and preserving ovarian function. Notably, quercetin was more effective in restoring SOD and CAT activity toward control levels, whereas folic acid showed a greater effect in improving AMH levels, reflecting better preservation of ovarian reserve (Fig.4).

DISCUSSION

Numerous studies have reported that MTX exerts toxic effects on female reproductive organs through mechanisms involving oxidative stress (1,4,5,23). It has also been shown that MTX, when administered at various doses, significantly reduces antioxidant levels in the ovary and induces severe structural and functional damage by increasing oxidative stress (4,6,24).

Histological analyses performed using H&E and Masson's trichrome staining revealed that MTX adminis-

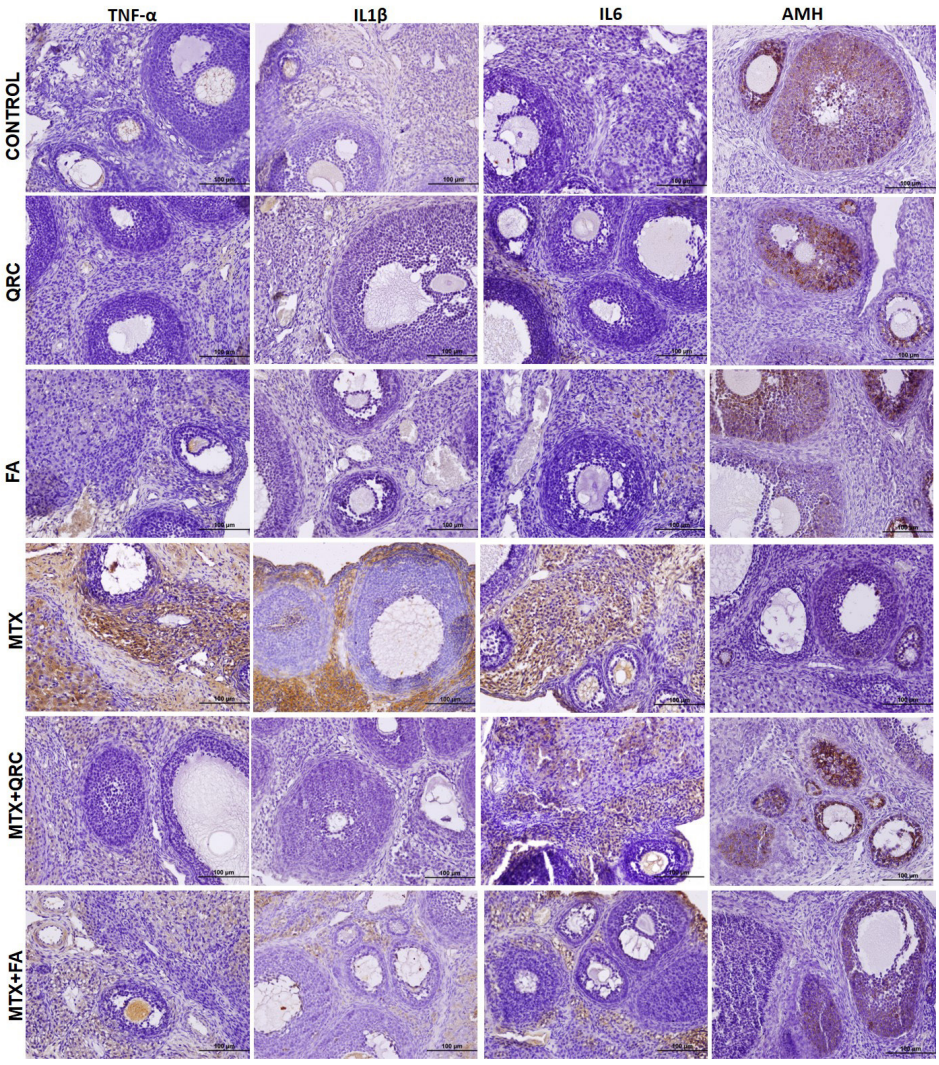

Figure 2: Immunohistochemical staining of pro-inflammatory markers (TNF-α, IL-1β, and IL-6) in ovarian tissue sections. TNF-α expression was markedly increased in the MTX group compared to the control group, while a clear reduction was observed in the MTX+QRC and MTX+FA groups. IL-1β levels were highest in the MTX group among all groups, whereas both MTX+QRC and MTX+FA treatments significantly decreased IL-1β expression. Similarly, IL-6 immunoreactivity was elevated in the MTX group and attenuated in the MTX+QRC and MTX+FA groups. Overall, MTX administration led to a significant upregulation of all inflammatory markers, whereas co-administration of QRC or FA reduced these effects to varying degrees, indicating their protective anti-inflammatory potential.

tration caused pronounced structural disruption in the ovarian histoarchitecture. These alterations included granulosa cell compaction, degeneration, pyknotic nuclei, vacuolization, prominent stromal inflammatory cell infiltration, vascular congestion, and edema. Similar findings have also been reported in previous studies (5,25,27). In contrast, co-administration of QRC and FA significantly alleviated this structural damage. Notably, quercetin appeared more effective in preserving follicular integrity and limiting degenerative changes. This protective effect of quercetin may be attributed to its potent antioxidant capacity and its ability to prevent granulosa cell dysfunction by reducing mitochondrial oxidative stress (8,10,11,28). On the other hand, folic acid has also been shown to mitigate ovarian damage and has demonstrated the potential to restore ovarian function, particularly in cases of radiation- or toxin-induced dysfunction (14,29). These findings indicate that both agents confer structural protection against MTX-induced ovarian injury and may play a supportive role in preserving ovarian function.

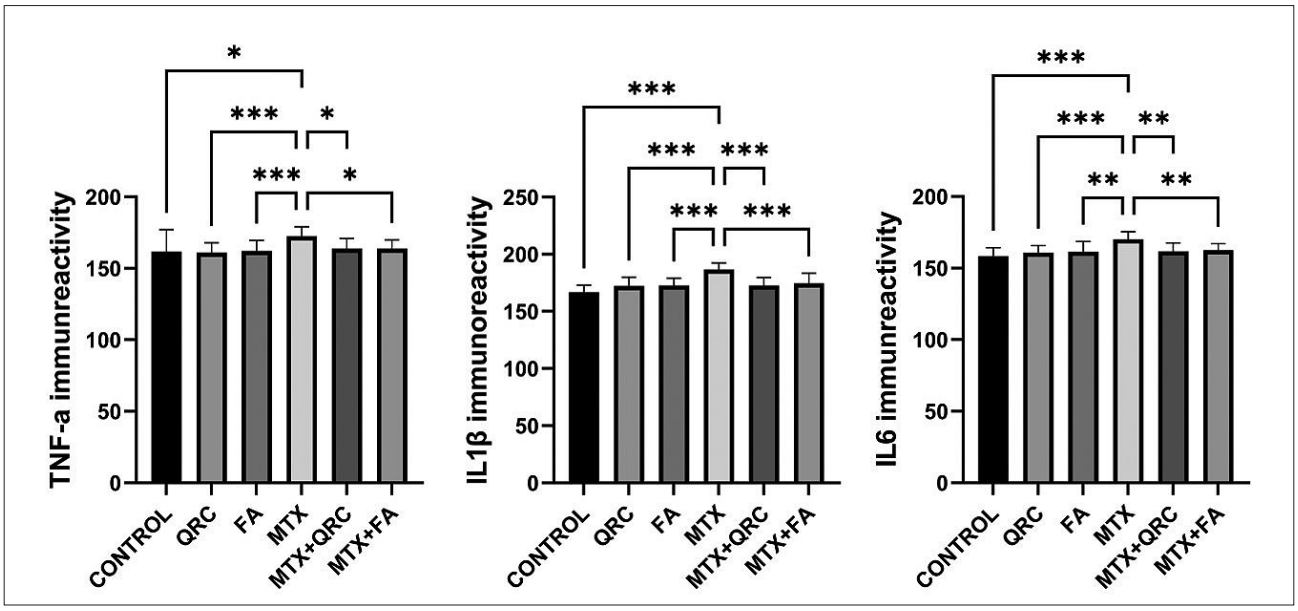

Figure 3: Quantitative analysis of TNF-α, IL-1β, and IL-6 immunoreactivity levels in ovarian tissues across all groups. The MTX group showed a significant increase in all markers compared to the control (p<0.001). Co-administration of quercetin (MTX+QRC) or folic acid (MTX+FA) significantly reduced these elevated levels. Data are presented as mean ± SD. *p<0.05, **p<0.01, **p<0.001.

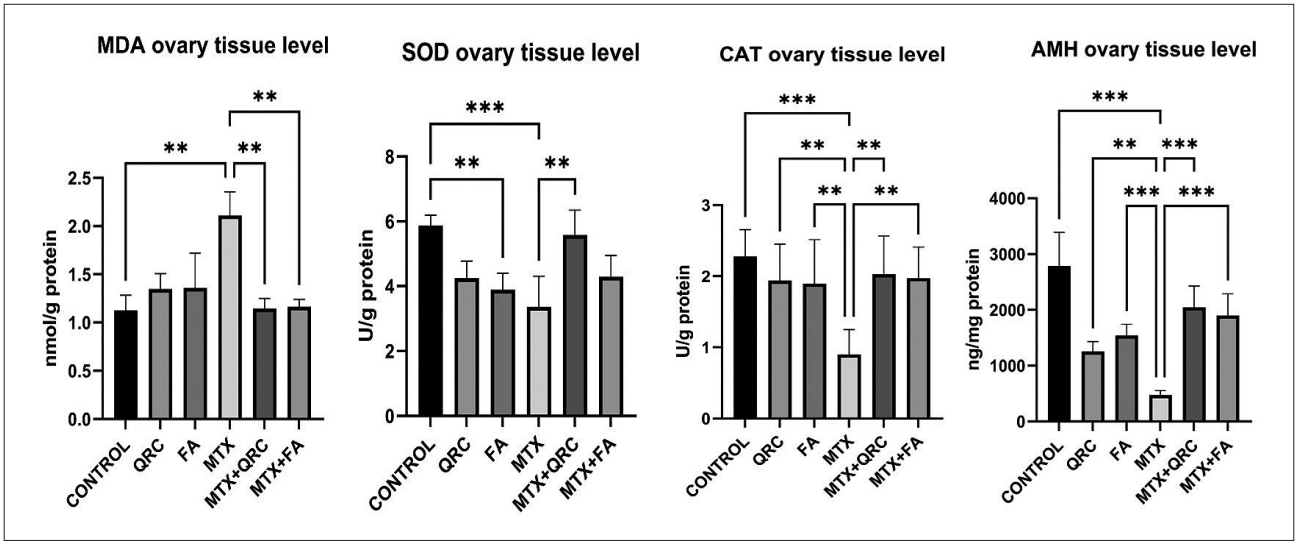

Figure 4: Tissue levels of MDA, SOD, CAT, and AMH in ovarian tissue samples across all groups. MTX administration significantly increased MDA levels while reducing SOD, CAT, and AMH levels, indicating oxidative stress and impaired ovarian reserve. Co-treatment with quercetin or folic acid ameliorated these effects. Data are expressed as mean ± SD. *p <0.05, **p<0.01, ***p<0.001.

MTX administration resulted in a significant reduction in all follicle types within the ovarian tissue, indicating widespread follicular damage. This finding suggests that MTX affects not only growing follicles but also primordial follicles, the key component of the ovarian reserve, highlighting the extensive and profound nature of the ovarian injury.Treatment with QRC and FA significantly reduced follicular loss and contributed to the preservation of ovarian structure. In previous studies, QRC was shown to restore ovarian reserve function by attenuating tissue damage in a premature ovarian failure (POF) model (11,28). In the present study, comparison of the treatment groups revealed that the MTX+FA group exhibited higher scores, particularly in primordial, antral, and Graafian follicles, suggesting that FA may exert a more pronounced protective effect on folliculogenesis. Conversely, the lower atretic follicle scores in the MTX+QRC group indicate that QRC may be more effective in preventing follicular atresia.These findings imply that the protective mechanisms of QRC and FA may vary across different stages of follicular development, and that both agents may possess complementary therapeutic potential in the management of MTX-induced ovarian toxicity.

Exposure to MTX was associated with upregulated expression of inflammatory mediators such as TNF-α, IL-1β, and IL-6, while simultaneously reducing AMH levels, thereby contributing to both inflammation and impaired ovarian reserve. These adverse effects were significantly attenuated in the groups treated with QRC or FA. Notably, QRC demonstrated a more pronounced suppressive effect on TNF-α and IL-1β expression, consistent with previous studies highlighting its strong anti-inflammatory properties (6,10). On the other hand, FA treatment was found to be more effective than QRC in preserving ovarian reserve, as indicated by the greater improvement in AMH levels. Indeed, the anti-inflammatory potential of FA has also been documented in prior randomized controlled trials (13,30). The present findings support the protective role of FA in maintaining ovarian reserve, while also demonstrating that QRC possesses not only anti-inflammatory effects but also a partial capacity to restore ovarian function.

Furthermore, biochemical analyses showed that elevated MDA levels and reduced SOD, CAT enzyme activities, as well as lower AMH levels in the MTX group, served as clear biochemical indicators of oxidative stress and ovarian functional impairment. The accumulation of high levels of reactive oxygen species (ROS) is known to contribute significantly to oxidative stress by inducing mitochondrial dysfunction, a mechanism well established in the literatüre (6,31). Both treatment agents improved these parameters; however, QRC was more effective in enhancing antioxidant enzyme activities, whereas FA demonstrated greater efficacy in restoring AMH levels. In another POF model induced by cyclophosphamide, QRC was shown to inhibit oxidative stress and reduce the number of atretic follicles (32). In a recent systematic review, the beneficial effects of FA supplementation on oxidative stress markers were comprehensively reported (30). Together, these findings suggest that both QRC and FA offer protective effects against MTX-induced ovarian damage, as evidenced by histological and molecular assessments. However, these protective effects may involve distinct molecular targets and mechanisms, highlighting their complementary roles in ovarian protection.

In conclusion, although methotrexate (MTX) is an effective therapeutic agent, the present study demonstrates that it can induce significant structural, biochemical, and molecular damage to ovarian tissue, including follicular depletion, oxidative stress, and inflammation. The administration of quercetin (QRC) and folic acid (FA) significantly attenuated these adverse effects through distinct but complementary mechanisms. QRC showed greater efficacy in reducing oxidative and inflammatory responses, whereas FA was more effective in preserving ovarian reserve, as reflected by higher AMH levels and improved primordial follicle counts. These findings suggest that both agents may provide protective benefits against MTX-induced ovarian toxicity, supporting their potential as adjuvant strategies to safeguard reproductive function. However, the absence of a combined MTX+FA+QRC treatment group represents a limitation of the present study, and future investigations should address this gap to explore potential synergistic effects of these antioxidants. Further studies, particularly in clinical settings, are warranted to validate these protective effects and assess their translational relevance.

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Abbreviations List

AMH: Antimullerian Hormone
CAT: Catalase
DAB: 3,3'-Diaminobenzidine
EP: Ectopic pregnancy
FA: Folic acid
GSH-Px: Glutathione Peroxidase
H&E: hematoxylin–eosin
IL-1β: Interleukin-1 Beta
IL-6: Interleukin-6 Antibody
MDA: Malondialdehyde
MT: Masson's trichrome
MTX: Methotrexate
PBS; phosphate-buffered saline
QRC: Quercetin
ROS: reactive oxygen species
SOD: Superoxide Dismutase
TNF-α: Tumor Necrosis Factor Alpha

Ethics Approval and Consent to Participate

The rats were obtained and accommodated at the Experimental and Clinical Research Center of Erciyes University. The study was designed and conducted in compliance with the ARRIVE guidelines for in vivo animal research. Erciyes University Animal Experiments Local Ethics Committee approved our experimental guidelines (decision no: 22/174 date: 08.08.2022).

Consent for Publication

Not applicable. This study did not include any identifiable individual data. All analyses were performed on anonymized samples obtained from routine laboratory investigations, and no personal or identifiable patient information was used.

Availability of Data and Materials

The data supporting the findings of this study are available from the corresponding author upon request.

Competing Interests

The authors declare that they have no conflict of interest.

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Author Contributions

Idea/Concept: HTY, AY. Desıgn: HTY, KTK. Control/Supervısıon AY, ÖG, Data Collectıon And/Or Processıng: ÖG, ÖCM, HTY. Analysıs And/Or Interpretatıon: ÖG, ÖCM,. Lıterature Revıew: HTY, KTK. Wrıtıng The Artıcle: HTY. Crıtıcal Revıew: AY, HTY. References And Fundıngs anh Materıals. HTY, AY.

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