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The ovoprotective effect of quercetin against methotrexate-induced injury by targeting Nrf2 signalling in female rats

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ABSTRACT

Although methotrexate (MTX) is a widely used anti-cancer drug in chemotherapy, its clinical use is limited due to its toxicity to many organs, including the ovaries. Quercetin (QUE), a natural flavonol, has known antioxidant and anti-inflammatory effects, but QUE's effects on endoplasmic reticulum stress (ERS) and nuclear factor erythroid 2-related factor 2 (Nrf2) pathways in MTX-induced ovotoxicity remain unclear. Therefore, this study was designed to investigate the therapeutic effect of QUE in MTX-exposed rats, including the ERS and Nrf2 signalling pathways. Five groups of six rats were formed as follows: control, MTX (20 mg/kg), MTX+low dose QUE (5 mg/kg), MTX+high dose QUE (10 mg/kg) and only high dose QUE (*per se*). Colourimetric methods were used to determine the levels of reproductive hormones in serum samples, and markers of oxidative stress (OS), inflammation, ERS, Nrf2 pathway and apoptosis in ovarian tissue. MTX administration resulted in dramatic histopathological findings in ovarian tissue and increased OS, inflammation, ERS and apoptosis associated with Nrf2 inhibition. Conversely, QUE treatment reversed the pathological biochemical and histological changes induced by MTX by modulating the Nrf2 pathway. Taken together, the results of this study provide the first evidences that QUE can ameliorate biochemical and histopathological findings in MTX-induced ovotoxicity. This needs to be supported by more comprehensive mechanistic studies before moving to clinical application.

Key words: ER stress; inflammation, methotrexate; Nrf2; ovotoxicity; oxidative stress; quercetin

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Introduction

Cancer is a global health concern that is defined by abnormal development and proliferation of degenerated cells (EL-BANA and KAMAL, 2020; ZHENG et al., 2022). Chemotherapy is the most commonly used approach to treating cancer (EL-BANA and KAMAL, 2020). Ideal chemotherapy aims to affect only cancer cells and not cause side effects on healthy cells in the organism. However, chemotherapeutic drugs have also deleterious effects, especially on rapidly proliferating normal tissues (ZHENG et al., 2022). Chemotherapy can cause significant gonadotoxic effects and have extremely devastating consequences, such as menstrual irregularities, metabolic problems and ovarian failure, which can lead to infertility (CHEN et al., 2022). Depending on the amount and kind of chemotherapeutic used during reproductive age, ovarian failure with loss of follicular reserve may occur (ALGANDABY, 2021). Methotrexate (MTX), a folic acid antagonist, that inhibits the dihydrofolate reductase enzyme involved in nucleic acid synthesis, is widely used in the treatment of leukaemia, osteosarcoma, lung, head and neck tumours (ERBOGA et al., 2015; ELSAWY et al., 2022). Like other chemotherapeutics, MTX has potent anticancer activity, but its side-effects on rapidly proliferating healthy tissues, including the gonads, are a concern for women (KARAPINAR et al., 2017; KILINC and UZ, 2021). Although the exact mechanism of MTX-induced tissue damage has not yet been established, current evidence points to the role of a complex mechanism involving oxidative stress (OS), inflammation and apoptosis (WANG et al., 2018; ELSAWY et al., 2022; MADKOUR et al., 2022). The lack of a drug in clinical use for MTX-induced tissue toxicity has accelerated research in recent years into the discovery of new molecules that can override MTX toxicity (ERBOGA et al., 2015; KARAPINAR et al., 2017; WANG et al., 2018; KILINC and UZ, 2021; ELSAWY et al., 2022; MADKOUR et al., 2022).

The nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway plays an important role in cellular adaptation to stress conditions, such as OS and inflammation (COMAKLI et al., 2023). Under physiological conditions, its cytoplasmic level is controlled by Kelch-like ECH-associated protein 1 (Keap1) (DARBAND et al., 2020). Elevated OS conditions abolish the Nrf2-Keap1 interaction by causing oxidation of Keap1 cysteine residues, which are critical for Keap1-Nrf2 interaction (KARUPPAGOUNDER et al., 2015), and Nrf2, transported into the nucleus, induces expression of antioxidant genes, notably heme oxygenase-1 (HO-1), NAD(P)H-quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (SHARMA et al., 2020; DEMIR et al., 2024). In recent years, it has been shown that the Nrf2 pathway, which is silenced by MTX, plays a critical role in the molecular mechanism of tissue damage (HASSANEIN et al., 2021; ATTIA et al., 2022; MATOUK et al., 2023; DEMIR et al., 2025), and that compounds with Nrf2 modulating properties may be of critical importance in the elimination of MTX-induced tissue damage (WANG et al., 2018; YOUNIS et al., 2021).

The potential of phytochemicals to eliminate pathophysiological conditions is increasingly being investigated due to their excellent therapeutic effects, minimal toxicity and ready availability (NAJAFI et al., 2022). Quercetin (QUE) is a natural flavonol that is found in a wide range of vegetables and fruits, ranging in colour from yellow to orange (YUKSEL et al., 2017; RASHIDI et al., 2021). QUE has antioxidant, anti-inflammatory, anticancer, immunomodulatory and cytoprotective effects, with the structural advantage of its five hydroxyl groups (RASHIDI et al., 2021; NAJAFI et al., 2022; ONUOHA et al., 2023). As a natural antioxidant, QUE has exhibited ovoprotective effect in experimental ovarian damage models caused by cytotoxic agents, through scavenging reactive oxygen species (ROS), improving the activities of antioxidant enzymes, and increasing the secretion of estradiol, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (RASHIDI et al., 2021). Interestingly, QUE has also been shown to be a natural modulator of Nrf2 (DARBAND et al., 2020; SHARMA et al., 2020; COMAKLI et al., 2023). Previous studies have shown the beneficial effects of QUE against MTX-induced renal, hepatic and pulmonary toxicity (ERBOGA et al., 2015; EL-BANA and

KAMAL, 2020; AK et al., 2023). Therefore, we hypothesized that QUE treatment might exhibit therapeutic effects against MTX-induced ovotoxicity. The aim of this study was to demonstrate the therapeutic effects of QUE, including the Nrf2/HO-1 pathway, on MTX-related ovotoxicity, by biochemical and histological parameters.

Materials and methods

Chemicals. QUE and MTX were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved with 10% dimethyl sulfoxide (DMSO) (TAN et al., 2020; ALGANDABY, 2021) and sterile saline (ERBOGA et al., 2015; YUKSEL et al., 2017), respectively.

Animals. Thirty Sprague-Dawley rats, 190±10 g, were obtained from the Surgical Practice and Research Centre of Karadeniz Technical University (Trabzon, Türkiye). The rats were housed in a standard environment with a room temperature of 23±1°C, 40-60% humidity, and 12 h light-dark cycle. After the seven-day adaptation period, the oestrus stages of the rats were evaluated by vaginal smear, and only rats in the oestrus period were included in the experiments (MENTESE et al., 2022). All experimental procedures were performed in strict accordance with the ARRIVE guidelines and the National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by Animal Research Ethics Committee of Karadeniz Technical University (Protocol Number: 2021/64).

Experimental procedure. Five groups of six rats were formed and all treatments given intraperitoneally, as follows: control, MTX (20 mg/kg), MTX+low dose QUE (5 mg/kg), MTX+high dose QUE (10 mg/kg) and only high dose QUE (*per se*). The control group had an injection of saline on day 1 and 10% DMSO for the next 3 days. The MTX group had an injection of MTX (20 mg/kg) on day 1 and 10% DMSO for the next 3 days. The MTX+QUE groups had an injection of MTX (20 mg/kg) on day 1 and QUE (5 and 10 mg/kg) for the next 3 days. The only high dose QUE group had an injection of saline on day 1 and QUE (10 mg/kg) for the next 3 days. The doses of MTX (ERBOGA et al., 2015; YUKSEL et al., 2017) and QUE (ERBOGA et al., 2015; SHARMA et al., 2020; ALGANDABY, 2021) were determined on the basis of previous studies. One day after the last treatment (AYAZOGLU DEMIR et al., 2022; MENTESE et al., 2022), the rats were sacrificed under deep anaesthesia. Ovarian tissues were quickly collected for histological and biochemical analyses. After blood sampling, serum samples were obtained by centrifugation at 1800xg.

Biochemical analysis. Ovarian tissues were homogenized in phosphate buffered saline and centrifuged at 1800xg for 10 min at 4°C. The bicinchoninic acid colourimetric method was used to determine the protein content of the supernatants (SMITH et al., 1985). The lipid peroxidation (LPO) levels of the supernatants were determined by the colourimetric method, on the basis of the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) (MIHARA and UCHIYAMA, 1978). Briefly, the supernatant was mixed with TBA, incubated in a boiling water bath for 1 h and then cooled. The mixture was centrifuged at 1800xg for 10 min and absorbance was measured at 532 nm with a microplate reader (Molecular Devices, Versamax, CA, USA) (YULUG et al., 2019). Serum levels of the anti-mullerian hormone (AMH), FSH and LH were estimated using ELISA kits (USCN, Wuhan, China) according to the manufacturer's protocols. Tissue levels of all the other biochemical parameters were estimated using ELISA kits (Finetest, Wuhan, China) according to the manufacturer's protocols (ABO EL-MAGD et al., 2021; NEGM et al., 2022).

Histological examination. Ovarian tissue sections were fixed in 10% buffered formalin, embedded in paraffin, cut into 5 µm slices and stained with haematoxylin and eosin (H&E). The stained slides were examined and photographed by a medical pathologist who was unaware of the experimental history of the groups. The pathological findings were calculated semiquantitatively, using the following scoring system: 0; no symptoms, 1: frequency of symptoms <33%, 2: frequency of symptoms 33-66%, and 3: frequency of symptoms >66% (MENTESE et al., 2022; AYAZOGLU DEMIR et al., 2023).

Statistical analysis. Statistical analyses were performed using SPSS 23.0, and the Kolmogorov-Smirnov test was used to test whether the data conformed to a Gaussian distribution. One-way ANOVA, followed by Tukey's post-hoc test were used to analyse Gaussian distributed biochemical results. The Kruskal-Wallis and Mann-Whitney U tests were used to analyse non-Gaussian distributed histological results. A P-value of <0.05 was considered to be significant.

Results

The effects of QUE treatment on OS biomarkers in the ovarian tissue of MTX-intoxicated rats. As shown in Table 1, MTX administration increased MDA levels in ovarian tissue by 512%, while dramatically reducing SOD levels by 40%, GPx levels by 33% and GSH levels by 65% compared to the control group. Among these parameters, only MDA (42%) was statistically significant, although treatment with low-dose QUE appeared to numerically improve these parameters. However, high doses of QUE resulted in significant normalization of MDA (82%), SOD (97%), GPx (67%) and GSH (210%) levels in comparison to the MTX group. Furthermore, the OS biomarkers did not differ statistically significantly between the high-dose QUE group and the control group.

The effects of QUE treatment on Nrf2 signalling in the ovarian tissue of MTX-intoxicated rats. The data in Fig. 1 showed that the administration of MTX suppressed the levels of Nrf2 (by 44%), HO-1 (by 39%) and NQO-1 (by 79%) in the ovarian tissue of rats when compared to control values. However, the effects of MTX on the levels of Nrf2

Table 1. The effects of MTX exposure and QUE supplementation on the OS parameters of the ovarian tissue of rats

	Control	MTX	MTX+QUE (5 mg/kg)	MTX+QUE (10 mg/kg)	QUE (10 mg/kg)
MDA (nmol/mg protein)	17.1±1.5	104.3±28.3$^{\#\#\#}$	60.3±14.4$^{\#\#\#,***}$	19.3±3.2***,¶¶¶	16.9±4.1
SOD (ng/mg protein)	130.6±19.7	78.0±28.1$^{\#}$	103.4±26.5	153.7±27.7**,¶	159.1±26.8
GPx (pg/mg protein)	7.2±1.2	4.8±0.8$^{\#}$	6.0±1.9	8.0±0.9*	8.9±2.9
GSH (µg/mg protein)	48.0±6.5	16.9±11.4$^{\#\#\#}$	25.6±11.8$^{\#\#}$	52.4±6.8***,¶¶¶	55.5±9.1

P-values according to the one-way ANOVA test, and the post-hoc Tukey test. Data are expressed as mean±SD
Compared with the control group: $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001
Compared with the MTX group: *P<0.05, **P<0.01, ***P<0.001
Compared with the MTX+QUE (5 mg/kg) group: ¶P<0.05, ¶¶P<0.001

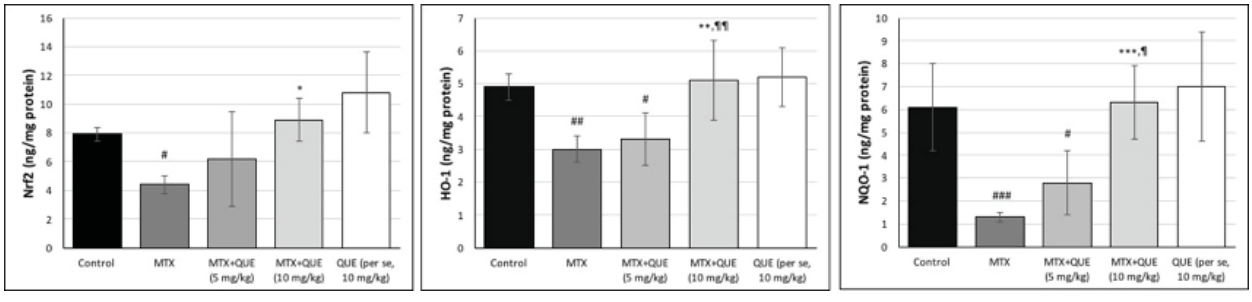

Fig 1. The effects of MTX exposure and QUE supplementation on the Nrf2/HO-1 signalling pathway of the ovarian tissue of rats

P-values according to the one-way ANOVA test, and the post-hoc Tukey test. Data are expressed as mean±SD
Compared with the control group: #P<0.05, ##P<0.01, ###P<0.001
Compared with the MTX group: *P<0.05, **P<0.01, ***P<0.001
Compared with the MTX+QUE (5 mg/kg) group: ¶P<0.05, ¶¶P<0.01

(102%), HO-1 (70%) and NQO-1 (385%) were reversed by the administration of high doses of QUE. Furthermore, the biomarkers of the Nrf2 pathway did not differ statistically significantly between the high-dose QUE group and control group.

The effects of QUE treatment on inflammation biomarkers in the ovarian tissue of MTX-intoxicated rats. As illustrated in Fig. 2, there were increases in the levels of high mobility group box 1 (HMGB1) (66%), nuclear factor kappa B (NF-κB) p65 (224%), tumour necrosis factor-alpha (TNF-α) (274%) and myeloperoxidase (MPO) (373%) in the MTX group in comparison to the control group. The administration of low-dose QUE resulted in a significant decrease in the levels of HMGB1 (21%), NF-κB p65 (51%), TNF-α (32%) and MPO (56%) in comparison to the MTX group. Nevertheless, treatment with high-dose QUE led to a statistically significant decrease in the levels of inflammatory biomarkers in comparison with the MTX group (42%, 71%, 70% and 75%, respectively). Furthermore, the inflammation biomarkers did not differ statistically significantly between the high-dose QUE (*per se*) group and the control group.

The effects of QUE treatment on ERS and apoptosis biomarkers in the ovarian tissue of MTX-intoxicated rats. As shown in Table 2, ERS, as indicated by high levels of glucose-regulated protein 78 (GRP78) (122%), activating transcription factor 6 (ATF6) (600%) and CCAAT-enhancer-binding protein homologous protein (CHOP) (620%), and apoptosis, as indicated by lower B-cell lymphoma 2 (Bcl-2) (57%) and higher Bcl-2-associated X protein (Bax) (100%) and caspase-3 (200%) lev-

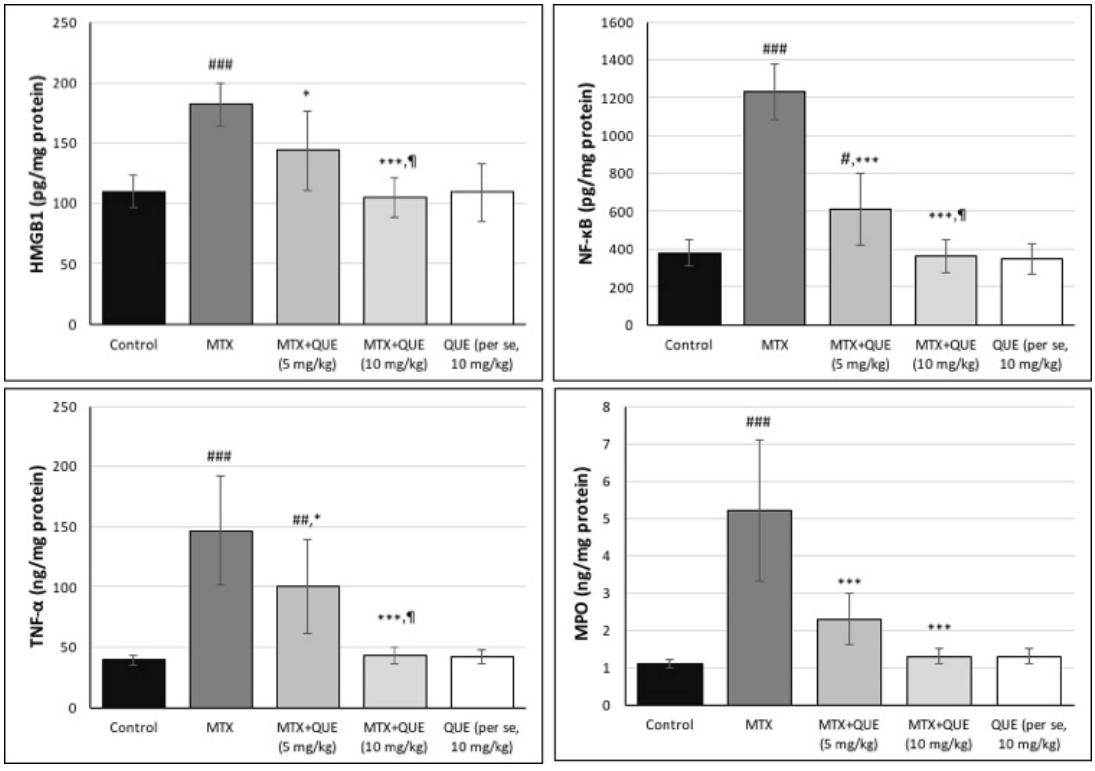

Fig 2. The effects of MTX exposure and QUE supplementation on inflammation parameters of ovarian tissue of rats

P-values according to the one-way ANOVA test, and the post-hoc Tukey test. Data are expressed as mean±SD
Compared with the control group: #P<0.05, ##P<0.01, ###P<0.001
Compared with the MTX group: *P<0.05, ***P<0.001
Compared with the MTX+QUE (5 mg/kg) group: ¶P<0.05

Table 2. The effects of MTX exposure and QUE supplementation on ERS and apoptosis parameters of the ovarian tissue of rats

	Control	MTX	MTX+QUE (5 mg/kg)	MTX+QUE (10 mg/kg)	QUE (10 mg/kg)
GRP78 (ng/mg protein)	2.3±0.7	5.1±2.2$^{\#\#}$	3.4±0.9	2.0±0.5**	2.0±0.3
ATF6 (ng/mg protein)	0.3±0.03	2.1±0.8$^{\#\#\#}$	1.0±0.3$^{\#,***}$	0.3±0.04***,¶	0.3±0.06
CHOP (ng/mg protein)	0.5±0.2	3.6±1.1$^{\#}$	2.7±2.2	0.5±0.1*	0.5±0.1
Bax (ng/mg protein)	0.7±0.2	1.4±0.6$^{\#}$	0.9±0.2	0.7±0.3*	0.7±0.2
Bcl-2 (pg/mg protein)	268±105	116±21$^{\#}$	144±43	267±106*	239±49
Caspase-3 (ng/mg protein)	0.6±0.06	1.8±0.8$^{\#\#\#}$	0.9±0.3**	0.6±0.08***	0.6±0.2

P-values according to the one-way ANOVA test, and the post-hoc Tukey test. Data are expressed as mean±SD
Compared with the control group: #P<0.05, ###P<0.001
Compared with the MTX group: *P<0.05, **P<0.01, ***P<0.001
Compared with the MTX+QUE (5 mg/kg) group: P<0.05

els, was induced in the ovarian tissue of MTX-only rats when compared with the control group. Treatment with low dose QUE only significantly decreased the levels of ATF6 (52%) and caspase-3 (50%) compared with the MTX group. However, treatment with high-dose QUE restored the levels of ERS and apoptosis biomarkers statistically significantly compared to the MTX group (61%, 86%, 86%, 50%, 131% and 67%, respectively). Furthermore, the ERS and apoptosis biomarkers did not differ statistically significantly between the high-dose QUE (*per se*) group and the control group.

The effects of QUE treatment on serum reproductive hormones levels in MTX-intoxicated rats. As demonstrated in Fig. 3, a significant decrease in the levels of AMH (61%), FSH (68%), and LH (72%) was observed in the serum of rats administered with MTX when compared with the control group. Treatment with low-dose QUE was observed to result in a significant restoration of FSH (103%) and LH (124%) levels in comparison with the MTX group. However, treatment with high-dose QUE resulted in statistically significant restoration of reproductive hormones levels in comparison with the MTX group (162%, 229% and 214%, respectively). Furthermore, the serum reproductive hormone biomarkers did not differ statistically significantly between the high-dose QUE (*per se*) group and the control group.

The effects of QUE treatment on histopathological alterations in the ovarian tissue of MTX-intoxicated rats. As demonstrated in Fig. 4, the

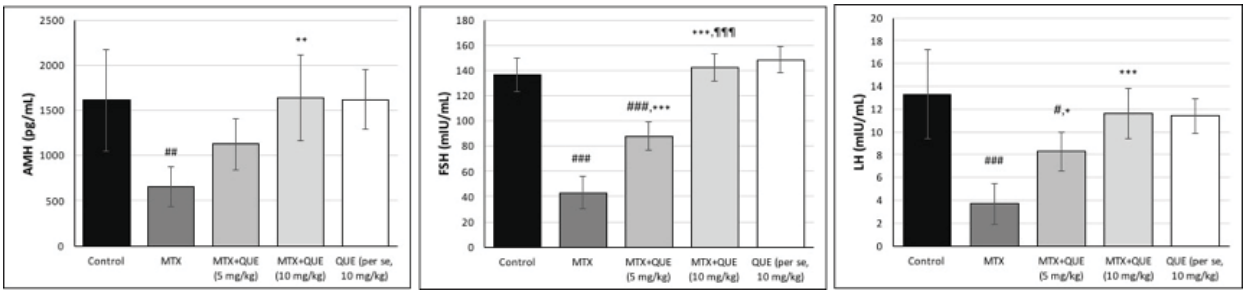

Fig. 3. The effects of MTX exposure and QUE supplementation on the serum hormone parameters of rats

P-values according to the one-way ANOVA test, and the post-hoc Tukey test. Data are expressed as mean±SD
Compared with the control group: #P<0.05, ##P<0.01, ###P<0.001
Compared with the MTX group: *P<0.05, **P<0.01, ***P<0.001
Compared with the MTX+QUE (5 mg/kg) group: P<0.001

histopathological features of the ovarian tissues obtained from all the study groups were examined and the results are quantified in Table 3. The administration of an MTX injection resulted in the observed damage to the ovaries, as indicated by an increase in vascular congestion, oedema, haemorrhage, follicular degeneration and leukocyte infiltration. Nevertheless, treatment with a high dose of QUE resulted in a substantial restoration of all the aforementioned pathological findings when compared with the MTX group. Furthermore, no substantial histopathological variations were evident in the ovarian tissues of rats between the control group and those treated with high dose QUE.

Table 3. The incidences of pathological findings in the ovaries of all groups

	Control	MTX	MTX+QUE (5 mg/kg)	MTX+QUE (10 mg/kg)	QUE (10 mg/kg)
Vascular congestion	0 (0-0.25)	2 (1-3)$^{\#\#}$	1 (0-2)	0 (0-0.25)**	0 (0-0.25)
Oedema	0 (0-0.25)	1.5 (1-2.25)$^{\#\#}$	1 (0-2)	0 (0-0.25)**	0 (0-0.25)
Haemorrhage	0 (0-0)	1 (0-1.25)$^{\#}$	0 (0-1)	0 (0-0)*	0 (0-0)
Follicular degeneration	0 (0-0)	1 (0-1.25)$^{\#}$	0.5 (0-1)	0 (0-0)*	0 (0-0)
Leukocyte infiltration	0 (0-0)	1 (0-2)$^{\#}$	0 (0-1)	0 (0-0)*	0 (0-0)

P-values according to Kruskal-Wallis variance analysis and the Mann-Whitney U test
Data are expressed as medians with a 25th and 75th percentile interquartile range (IQR)
Compared with the control group: #P<0.05, ##P<0.01
Compared with the MTX group: *P<0.05, **P<0.01

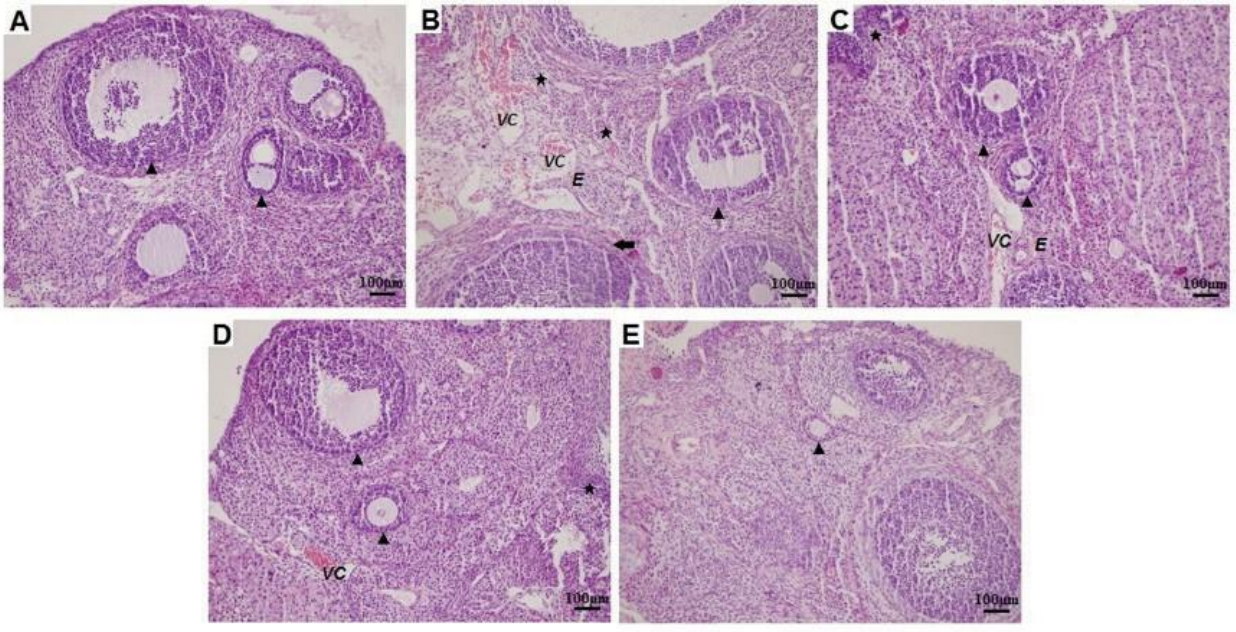

Fig. 4. Photomicrographs of histological sections of ovarian tissue in the (A) Control, (B) MTX, (C) MTX+QUE (5 mg/kg), (D) MTX+QUE (10 mg/kg) and (E) QUE (10 mg/kg, per se) groups (H&E, 200x) (filled triangle: germinal epithelium, black arrow: follicular degeneration, black star: inflammatory cell infiltration, VC: vascular congestion, E: oedema)

Discussion

Although chemotherapy improves patient survival, reproductive toxicity is a concern, especially for younger patients. Chemotherapy may cause ovotoxicity in the early period, and infertility due to ovarian failure in the chronic period in female patients (ZHENG et al., 2022). However, there is no approved drug in clinical use for chemotherapy-induced ovotoxicity (CHEN et al., 2022). These side effects do not allow the continuation of treatment in the chronic period (YUKSEL et al., 2017). For this reason, studies on the discovery of new molecules that will eliminate chemotherapy-induced ovotoxicity, ovarian failure and infertility have gained momentum in recent years (CHEN et al., 2022). This study was therefore designed to investigate the therapeutic effects of QUE against the MTX-induced toxic effects in ovarian tissue.

It was found that MTX treatment alone dramatically depleted the levels of blood reproductive hormones, causing serious pathological findings in the ovarian tissue. It is widely accepted that AMH, produced by granulosa cells, is a highly effective ovarian reserve marker (KARAPINAR et al., 2017). These findings were consistent with earlier reports that indicated the occurrence of ovotoxicity in response to the administration of MTX (HORTU et al., 2020; AKA et al., 2022; MADKOUR et al., 2022). However, QUE treatment, notably at the 10 mg/kg dosage, significantly reduced the adverse effects of MTX on serum reproductive hormones and histological architecture. Consistent with our results, it has been previously reviewed that QUE exerts an ovoprotective effect against ovarian injury induced by cytotoxic agents (RASHIDI et al., 2021).

The main proposed mechanism for MTX-induced tissue damage is OS (ERBOGA et al., 2015; KARAPINAR et al., 2017; ELSAWY et al., 2022). Inability to neutralize endogenous and/or exogenous ROS by the antioxidant system causes OS (DARBAND et al., 2020). As a result of OS, cell damage and ultimate cell death occur as a result of oxidation of biomolecules such as lipid, protein, carbohydrate and DNA (MENTESE et al., 2014). Membrane lipids undergo LPO as a result of ROS attack, resulting in unstable and reactive end products, such as MDA (COMAKLI et al., 2023). SOD, GPx and GSH have been identified as essential elements of the cell's antioxidant defence system. A reduction in antioxidant defence capacity has been demonstrated to result in a diminished ability to resist OS (YUKSEL et al., 2017; DEMIR et al., 2024). Consistent with the findings of analogous reports (KARAPINAR et al., 2017; HORTU et al., 2020; AKA et al., 2022; ELSAWY et al., 2022; COMAKLI et al., 2023), the present study demonstrated that the administration of MTX led to a depletion of the antioxidant system within ovarian tissue, resulting in a substantial increase in LPO levels. However, QUE treatment reduced the OS levels and restored the capacity of the antioxidant system. QUE has radical scavenging, chain breaking and metal chelator properties, resulting from its five hydroxyl groups (YUKSEL et al., 2017; RASHIDI et al., 2021). It was thought that the antioxidant potential of QUE, arising from its structural properties, plays a role in the elimination of MTX-related ovotoxicity. These results were found to be in accordance with reports demonstrating the antioxidant efficacy of QUE in experimental models (ERBOGA et al., 2015; YUKSEL et al., 2017; DARBAND et al., 2020; SHARMA et al., 2020; ONUOHA et al., 2023).

Recent years have seen mounting evidence demonstrating the role of inflammation in augmenting tissue damage caused by MTX (WANG et al., 2018; ELSAWY et al., 2022; MADKOUR et al., 2022). HMGB1 is a nuclear protein involved in nuclear processes, including replication and DNA repair (FANG et al., 2021). Besides its nuclear functions, it plays an important role in eliciting the tissue damage response by acting as an extracellular alarmin (KARUPPAGOUNDER et al., 2015). HMGB1 binds to toll-like receptor 4 and receptor for advanced glycation end products causing the activation of the NF-κB pathway, and ultimately, the release of pro-inflammatory cytokines, such as TNF-α and interleukin-6, is accelerated (KARUPPAGOUNDER et al., 2015; CHAI et al., 2021). In view of this, the inhibition of the HMGB1/NF-κB axis has been identified as a viable target mechanism for inflammatory pathologies (KARUPPAGOUNDER et al., 2015; FANG et al.,

2021). MPO, an indirect indicator of leukocyte infiltration, is a significant factor in the assessment of tissue injury (MENTESE et al., 2022). The present study corroborated the findings of earlier research in this field (KARAPINAR et al., 2017; WANG et al., 2018; HORTU et al., 2020; MADKOUR et al., 2022), demonstrating that systemic administration of MTX leads to a significant increase in the levels of inflammation in ovarian tissue. Nonetheless, QUE treatments were observed to be efficacious in reducing MTX-induced ovarian inflammation. These findings served to corroborate the anti-inflammatory properties of QUE, a conclusion that has previously been demonstrated in other studies, attributable to its capacity for HMGB1 inhibition (KARUPPAGOUNDER et al., 2015; LI et al., 2016; FANG et al., 2021).

The ER is an essential organelle in which processes, such as protein synthesis, folding and modification are carried out (EISVAND et al., 2022). However, impairment of ER function due to increased ROS and inflammation is referred to as ERS (endoplasmic reticulum stress) (ALSHAMMARI et al., 2021). Increased ERS in cells is recognised by sensor proteins, including ATF6, which consequently instigate the unfolded protein response (UPR) pathway. In the context of physiological conditions, this pathway is inactive due to interaction with ATF6-GRP78 (SUGANYA et al., 2014; EISVAND et al., 2022). An increase in ERS leads to the dissociation of ATF6 from GRP78, resulting in the activation of this UPR pathway (DEMIR et al., 2022). After the released ATF6 is activated by cleaved in the Golgi, it migrates to the nucleus where it regulates the expression of various proteins to normalize ER conditions (EISVAND et al., 2022). First of all, an attempt is made to eliminate the protein load in the lumen by increasing the synthesis of chaperone. If this load is not reduced, then the process of CHOP-induced apoptosis is activated (ALSHAMMARI et al., 2021). The elevated levels of CHOP result in the restriction of Bcl-2 and GSH levels, concomitant with an escalation of Bax and ROS levels. This progression ultimately leads to the activation of caspase-3, thus precipitating the process of apoptosis (SUGANYA et al., 2014). Consistent with the conclusions of previous reports (PAUL et al., 2015; WU et al., 2017; LV et al., 2020), the findings of this study demonstrated that the administration of MTX resulted in elevated levels of ERS and apoptosis markers within ovarian tissue. The elevated levels of the ERS markers are seen to be a consequence of the MTX-induced UPR. Nevertheless, ERS-induced apoptosis was observed, as evidenced by increased CHOP and apoptosis biomarker concentrations. This phenomenon can be attributed to the failure of UPR activation to restore ER homeostasis. The present study demonstrated that QUE treatments resulted in a reduction of ERS and apoptosis levels in ovarian tissue, which is consistent with the outcomes of other studies indicating that QUE exerts inhibitory effects on ERS and anti-apoptotic effects in various experimental models (LIU et al., 2013; SUGANYA et al., 2014; ALSHAMMARI et al., 2021; WANG et al., 2022).

Nrf2 is a critical regulator of over 200 cytoprotective genes that form a vital line of defence against OS and inflammation in cells (WANG et al., 2018; DARBAND et al., 2020). HO-1, which is responsible for degradation of heme, has also been known to induce the expression of other antioxidant enzymes, including SOD (LOBODA et al., 2016). NQO1 is another significant enzyme that functions as part of the antioxidant mechanism by converting NAD(P)H to $NAD(P)^+$ (OH et al., 2014). Consistent with the findings of recent studies (HASSANEIN et al., 2021; ATTIA et al., 2022; MATOUK et al., 2023; DEMIR et al., 2025), the results of this investigation demonstrated that the administration of MTX resulted in the inhibition of the Nrf2 pathway in ovarian tissue. Nonetheless, treatment with QUE, particularly at a dose of 10 mg/kg following MTX, resulted in substantial alleviation of pressure on the Nrf2 pathway in ovarian tissue. These findings were in alignment with previous reports suggesting the capacity of QUE to modulate the Nrf2 pathway (KARUPPAGOUNDER et al., 2015; DARBAND et al., 2020; SHARMA et al., 2020; ALSHAMMARI et al., 2021; AHMED et al., 2022; COMAKLI et al., 2023). It has been established that Nrf2 exerts a pivotal regulatory function in the inactivation of the HMGB1/NF-κB pathway (KARUPPAGOUNDER et al., 2015; WARDYN et

al., 2015). Consequently, it is hypothesised that the therapeutic efficacy of QUE is, at least in part, attributable to the suppression of OS and inflammation levels through the modulation of the Nrf2 pathway.

Conclusions

It was confirmed in this study that MTX causes ovotoxicity by increasing OS, inflammation, ERS and apoptosis in ovarian tissue, together with the inhibition of the Nrf2 pathway. QUE (especially at the dose of 10 mg/kg) treatment, on the other hand, modulated the Nrf2 pathway and reversed these pathological mechanisms. QUE can be considered as a therapeutic molecule for the elimination of MTX-related ovotoxicity. These results may be promising for the protection of reproductive health for many women undergoing cancer treatment today. This inference needs to be supported by more comprehensive mechanistic studies before clinical application.

Ethics approval

All experimental procedures were performed in strict accordance with the ARRIVE guidelines and the National Institutes of Health guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the Animal Research Ethics Committee of Karadeniz Technical University (Protocol Number: 2021/64).

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Declaration of competing interest

No potential conflicting interest was reported by the authors.

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SAŽETAK

Metotreksat (MTX) se široko upotrebljava kao antikancerogeni lijek u kemoterapijskim protokolima. Klinička uporaba MTX-a ograničena je prvenstveno njegovim toksičnim učinkom na mnoge organe, uključujući jajnik. Kvercetin (QUE), prirodni flavonoid, poznat je po svojemu antioksidacijskom i protuupalnom učinku, no nejasan je njegov učinak na stres endoplazmatskog retikula (ERS). Također, nejasan je i njegov učinak na toksičnost koju metotreksat izaziva na jajnicima putem nuklearnog faktora eritroid 2-povezanog faktora 2 (Nrf2). Cilj je istraživanja stoga bio procijeniti terapijski učinak kvercetina na štakore izložene metotreksatu, uključujući ERS i signalne puteve Nrf2. Istraživanje je obuhvatilo pet skupina u kojima je bilo po pet ženki štakora: kontrolna skupina, skupina koja je bila izložena MTX-u (20 mg/kg), skupina koja je dobivala MTX i malu dozu QUE-a (5 mg/kg), skupina koja je dobivala MTX i veliku dozu QUE-a (10 mg/kg) i skupina koja je dobivala samo veliku dozu QUE-a (*per se*). Kako bi se utvrdile razine reproduktivnih hormona u serumu i biljezi oksidacijskog stresa (OS), upale, ERS-a, signalnih puteva Nrf2 i apoptoze u tkivu jajnika, primijenjene su kolorimetrijske metode. Primjena MTX-a znatno je utjecala na histopatološke pokazatelje u tkivu jajnika s obzirom na porast oksidacijskog stresa, upale, ERS-a i apoptoze što je bilo uzrokovano inhibicijom faktora Nrf2. S druge strane, primjena QUE-a poništila je patološke biokemijske i histološke promjene uzrokovane MTX-om, i to modulacijom signalnog puta Nrf2. Ukupno gledajući, rezultati istraživanja nude prve dokaze o tome da QUE može ublažiti biokemijske i histopatološke promjene nastale zbog toksičnog djelovanja MTX-a na tkivo jajnika. Ove bi rezultate trebalo potkrijepiti sveobuhvatnijim istraživanjima patofizioloških mehanizama, prije njihove kliničke primjene.

Ključne riječi: stres endoplazmatskog retikula; upala; metotreksat; Nrf2; ovotoksičnost; oksidacijski stres; kvercetin