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ORIGINAL ARTICLE

The impact of Nrf2/HO-1, caspase-3/Bax/Bcl2 and ATF6/IRE1/PERK/GRP78 signaling pathways in the ameliorative effects of morin against methotrexate-induced testicular toxicity in rats

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Abstract

Background Methotrexate (MT) is a broadly used chemotherapeutic drug however its clinical use is confronted with several forms of toxicities containing testicular damage. The current study assessed the ameliorative effects of morin on MT-induced testicular damage with the investigation of its mechanism and the potential involvement of oxidative stress, inflammation, apoptosis and endoplasmic reticulum stress in such protection.

Methods The animals were divided into 5 distinct groups (7 rats in each group). Group 1 was control group, group 2 received MT-only (20 mg/kg bw), group 3 received orally morin-only (100 mg/kg bw), group 4 received MT (20 mg/kg bw) + morin (50 mg/kg bw) and group 5 received MT (20 mg/kg bw) + morin (100 mg/kg). In this study, morin was administered orally for 10 days, while MT was administered intraperitoneally on the 5th day.

Results MT intoxication was linked with augmented MDA while decreased GSH levels, the enzyme activities of glutathione peroxidase, superoxide dismutase, and catalase and mRNA levels of *HO-1* and *Nrf2* in the testis tissues. MT injection caused inflammation in the testicular tissue via up-regulation of *MAPK14, NFκB*, *TNF-α* and *IL-1β*. MT application also caused apoptosis and endoplasmic reticulum stress in the testis tissue via increasing mRNA transcript levels of *Bax*, *caspase-3*, *PERK*, *IRE1*, *ATF-6*, *GRP78* and down-regulation of *Bcl-2*.

Conclusion Treatment with morin at a dose of 50 and 100 mg/kg considerably mitigated oxidative stress, inflammation, apoptosis and endoplasmic reticulum stress in the testicular tissue indicating that testicular damage related to MT toxicity could be modulated by morin administration.

Keywords Apoptosis · Endoplasmic reticulum stress · Methotrexate · Morin · Testicular injury

Introduction

Methotrexate (MT) is a chemotherapeutic that is used to treat autoimmune and inflammatory diseases [1]. It is a folic acid antagonist and generally used for the treatment of osteosarcoma, psoriasis, leukemia and breast cancer [2]. MT inhibits the activity of dihydrofolate reductase, an enzyme that catalyzes the conversion of dihydrofolate to the tetrahydrofolate which is required for the synthesis of the nucleotides. MT was also shown to inhibit *de novo* purine biosynthesis. MT, for that reason, inhibits the synthesis of DNA, RNA and protein and eventually causes to apoptosis in the cells or tissues [3, 4]. However, MT possess toxicities not only towards cancerous cells but also healthy organs and tissues such as liver, kidney [5], brain [6] and testis [7]. One approach to weaken the adverse effects of MT in medicinal

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application could be to protect the organs from unwanted side effects. For that reason, the protective effects of natural biomolecules with antioxidant activities that could ameliorate severity of MT-induced toxicity may possibly be beneficial for therapeutic applications.

Antioxidants are broadly used as nutrients and have been studied for their efficacy in mitigating toxicities towards several types of drugs [8, 9]. Among antioxidants, flavonoids are natural biologically active compounds identified in plants and bee products that possess high antioxidant capacity [9]. Morin is a natural polyphenolic antioxidant found in most fruits, vegetables, dietary herbs, nuts, and seeds [10, 11]. A substantial amount of study reported that morin is a biologically active compound that exhibits a comprehensive spectrum of pharmacological activities with low cytotoxicity [12]. The pharmacological activities of morin include free radical scavenging in a broad range of cancer types, anti-inflammatory, anti-oxidant, anti-autophagic and anti-apoptosis [13–16]. In addition, it has been reported that morin-5'-sulfonic acid sodium salt, a derivative of morin, reduces cyclophosphamide-induced genitourinary system toxicity in rats [17].

The current study was designed to examine the efficacy of morin on mitigating oxidative stress, inflammation responses, apoptosis, autophagy and endoplasmic reticulum stress against MT-induced testicular damage in rats.

Material and method

Chemicals

Methotrexate (50 mg/5 mL injectable solution) was obtained Koçak Farma (İstanbul, Turkey). Morin hydrate (CAS no: 654055-01-3) and all reagents used throughout the study were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

Animals

Adult male Wistar albino rats (280–300 g) were purchased from Experimental Research Center, Bingol University (Bingol, Turkey). The animals were kept under standard laboratory conditions (45 ± 5% humidity, 24 ± 1 °C, a 12 h light: 12 h dark cycle), in the above mentioned centre. Food and drinking water were provided *ad libitum* to the animals. All experimental protocols were done in accordance with guidelines for animal care and it has been approved by the Animal Experimentation Ethics Committee of the Bingol University (Protocol No: 2022-E.53,634).

Experimental groups

Animals were divided in to 5 groups, each group consist of 7 rats.

Group I (Control): Rats received 0.9% saline via oral gavage for 10 days and a single intraperitoneal (i.p.) injection of saline on day 5 only.

Group II (Morin): Rats received 100 mg/kg morin via oral gavage for 10 days and a single intraperitoneal (i.p.) injection of saline on day 5 only [11].

Group III (MT): Rats received saline via oral gavage for 10 days and a single i.p. dose of 20 mg/kg MT dissolved in saline on day 5 only [18].

Group IV (MT + Morin-50): Rats received 50 mg/kg morin via oral gavage for 10 days and a single i.p. dose of 20 mg/kg MT on day 5 only.

Group V (MT + Morin-100): Rats received 100 mg/kg morin via oral gavage for 10 days and a single i.p. dose of 20 mg/kg MT on day 5 only.

All rats were killed under the sevoflurane (Sevorane 100% liquid, Abbott Laboratories, İstanbul, Turkey) anesthesia 24 h after the last treatment. The testis tissues were quickly removed, rinsed with ice-cold saline, washed, and kept frozen in liquid nitrogen. These tissues were then stored at -20 °C for use in biochemical and molecular analysis.

Examination of lipid peroxidation levels in testis tissue

The levels of lipid peroxidation (LPO) in the testicular tissue were determined through measurement of the absorbance of the colour formed by the reaction of malondialdehyde (MDA) with thiobarbituric acid at 532 nm. In order to measure it, the testis tissue was homogenised in 1.15% potassium chloride (KCl). The obtained homogenates were subsequently centrifuged at 1000 rpm at 4 °C for 15 min. MDA levels were measured following the procedure developed by Placer, Cushman [19]. MDA levels were expressed as nmol/g tissue.

Analysis of antioxidant markers in testis tissue

To investigate the antioxidant status in testis tissue, the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) as well as the levels of glutathione (GSH) were analysed. SOD activity was measured in line with the method developed by Sun, Oberley [20] and results were expressed U/g protein. The activity of CAT was determined via the method of Aebi [21] and the results were presented as katal/g protein. GPx activity was assayed by the method developed by Lawrence and Burk [22] and the activity was expressed as U/g protein.

Table 1 Primer sequences

Gene	Sequences (5'-3')	Length (bp)	Accession no
NF-κB	F: AGTCCCGCCCCTTC-TAAAAC R: CAATGGCCTCTGTG-TAGCCC	106	NM_001276711.1
IL-1β	F: ATGGCAACT-GTCCCTGAACT R: AGTGACACT-GCCTTCCTGAA	197	NM_031512.2
TNF-α	F: CTCGAGT-GACAAGCCCGTAG R: ATCTGCTGGTAC-CACCAGTT	139	NM_012675.3
Nrf2	F: TTTGTAGATGAC-CATGAGTCGC R: TCCTGCCAAACTT-GCTCCAT	161	NM_031789.2
HO-1	F: ATGTCCCAGGATTT-GTCCGA R: ATGGTACAAGGAG-GCCATCA	144	NM_012580.2
Bax	F: TTTCATCCAGGATC-GAGCAG R: AATCATCCTCTG-CAGCTCCA	154	NM_017059.2
Bcl-2	F: GACTTTGCAGAGAT-GTCCAG R: TCAGGTACTCAGT-CATCCAC	214	NM_016993.2
Caspase-3	F: ACTGGAATGT-CAGCTCGCAA R: GCAGTAGTC-GCCTCTGAAGA	270	NM_012922.2
MAPK14	F: GTGGCAGT-GAAGAAGCTGTC R: GTCACCAGGTACA-CATCGTT	170	NM_031020.2
ATF-6	F: TCAACTCAGCAC-GTTCCTGA R: GACCAGTGACAG-GCTTCTCT	130	NM_001107196.1
PERK	F: GATGCCGAGAAT-CATGGGAA R: AGATTCGAGA-AGGGACTCCA	198	NM_031599.2
IRE1	F: GCAGTTCCAGTA-CATTGCCATTG R: CAGGTCTCTGTGAA-CAATGTTGA	163	NM_001191926.1
GRP78	F: CATGCAGTTGT-GACTGTACCAG R: CTCTTATCCAGGC-CATATGCAA	143	NM_013083.2
β-Actin	F: CAGCCTTCCTTCTTGGG-TATG R: AGCTCAGTAACAGTC-CGCCT	360	NM_031144.3

The levels of GSH were measured by the method developed by Sedlak and Lindsay [23]. GSH levels were expressed as nmol/g tissue. Total protein amount of testis tissue was determined by the method of Lowry, Rosebrough [24].

Quantitative real-time PCR analysis in testis tissue

Total RNA isolation from testis tissue was carried out by use of QIAzol Lysis Reagent (Qiagen, Cat: 79,306, Germany). cDNA was synthesised from total RNAs with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ Cat: 4,368,814, USA). The synthesised cDNAs were utilised for the determination of the mRNA transcript levels of the tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), nuclear factor kappa B (NF-κB), mitogen-activated protein kinase 14 (MAPK14), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), Bcl-2-associated x protein (Bax), B-cell lymphoma-2 (Bcl-2), cysteine aspartate specific protease-3 (caspase-3), glucose-regulated protein 78 (GRP78), activating transcription factor-6 (ATF-6), inositol requiring enzyme-1 (IRE1) and double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) genes. RT-PCR primers were designed using the Oligo 6.0 primer design program according to the Rattus norvegicus sequence. The designed primers were synthesized by Sentebiolab (Turkey). In the qRT-PCR stage, a mixture was prepared using primers of RNase free water and cDNAs, SYBR Green PCR Master Mix. Following that, the analyses were performed with ROTOR-GENE Q (Qiagen, Germany) in triplicate. *β-actin* was used for normalization purposes as a housekeeping gene. Primer sequences are provided in Table 1.

Statistical analysis

The results were given as mean ± standard deviation. GraphPad Prism 5.0 software was used for the data analysis. Data were analysed using one-way ANOVA with Tukey's post hoc tests for multiple comparisons. $P < 0.05$ was considered as significantly different.

Results

Effects of morin on MT-induced oxidative stress and lipid peroxidation

The impacts of MT and morin treatments on oxidative injury and antioxidant markers in testis tissue were given in Fig. 1. The levels of MDA substantially increased in the MT-supplemented group in comparison to the normal control group. Conversely, antioxidant parameters of testis tissue (GSH, SOD, CAT and GPx) were remarkably reduced

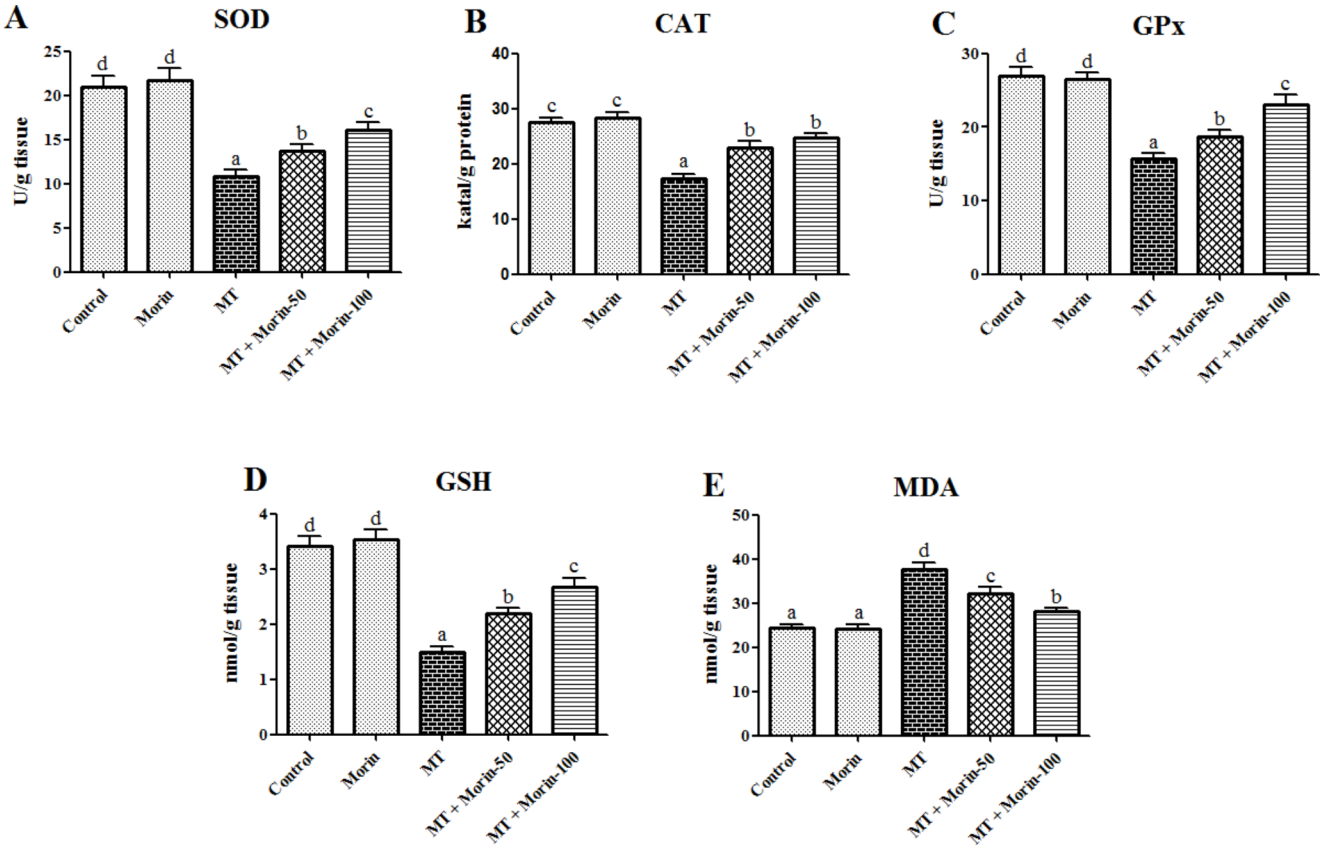

Fig. 1 Morin decreases MT-induced oxidative stress parameters in testis tissue. Morin increased (A) SOD, (B) CAT, (C) GPx and (D) GSH, and decreased (E) MDA level in MT-induced rats. Values are expressed as mean ± SD. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$)

($p < 0.05$). In MT + Morin-50 and MT + Morin-100 groups testis MDA levels were noticeably reduced in comparison to the MT-intoxicated rats ($p < 0.05$). On the contrary, the levels of GSH, GPx, SOD, and CAT were significantly augmented as compared to the MT-treated group. The rats supplemented with morin only did not elucidate significant changes in the testis oxidative damage and antioxidant biomarkers as compared to the control rats.

Effects of morin on MT-induced NFκB, TNF-α, IL1β, *MAPK14*, Nrf2 and *HO-1* gene expressions

The expression levels of *NFκB*, *TNF-α*, *IL1β*, *MAPK14*, *Nrf2* and *HO-1* in the testis of MT-treated rats were determined with qRT-PCR. The expression levels of *NFκB*, *TNF-α, IL1β* and *MAPK14* were up-regulated ($p < 0.05$) in the MT treated group compared to the control. The administration of 50 and 100 mg/kg dose of morin remarkably down-regulated the levels of these markers as compared to the MT only treated group. The expression levels of the *HO-1* and *Nrf2* were greater ($p < 0.05$) in the MT + morin-50 and MT + morin-100 groups as compared to the MT group. Conversely, their mRNA transcript levels were considerably lower as compared to the MT group. The rats fed with morin only did not reveal significant changes in the mRNA transcript level of *NFκB, TNF-α*, *IL1β*, *Nrf2* and *HO-1* in the testis of MT-treated rats as compared to the control rats (Fig. 2).

Effects of morin on MT-induced apoptotic gene expressions

The impacts of the MT treatments and morin supplementation on the mRNA levels of *Bax*, *Bcl-2* and *Caspase-3* were studied for testicular tissue. The mRNA levels of *Bax* and *Caspase-3* were significantly up-regulated by MT treatment compared to untreated group while their expressions were reduced in the MT + morin-50 and MT + morin-100 groups compared to the MT group ($p < 0.05$). Moreover, the expression of *Bcl-2* were down-regulated by MT treatment whereas supplementation with different dose of morin up-regulated its expression suggesting anti-apoptotic role of morin in MT-induced testis toxicity (Fig. 3).

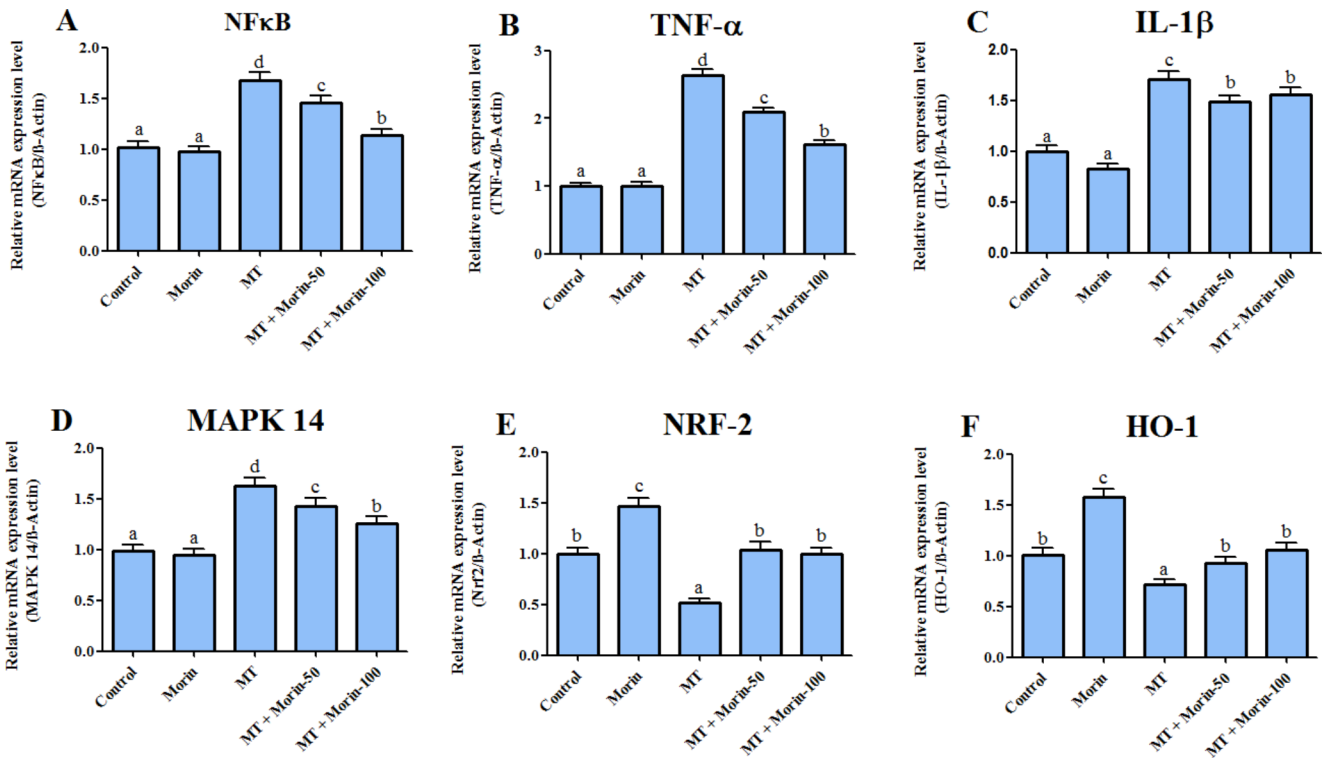

Fig. 2 Ameliorative effects of morin and MT treatments on inflammation parameters and Nrf-2/HO-1 mRNA expression levels in testis tissue. (A) NF-κB mRNA transcript levels, (B) TNF-α mRNA transcript levels, (C) IL-1β mRNA transcript levels, (D) MAPK 14 mRNA transcript levels, (E) Nrf-2 mRNA transcript levels, (F) HO-1 mRNA transcript levels. Values are expressed as mean ± SD. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$)

Effects of morin on MT-induced endoplasmic reticulum stress

The mRNA transcript levels of *PERK*, *IRE1*, *ATF-6* and *GRP78* genes, the indicators of ER stress in testicular tissues of rats, were also studied. It was shown that MT treatment caused ER stress via up-regulating levels of *ATF-6*, *GRP78*, *IRE1* and *PERK* in testes tissue while treatment of morin at 50 mg/kg and 100 mg/kg doses down-regulated levels of these genes. The rats fed with morin only did not reveal significant changes in the mRNA transcript level of these genes (Fig. 4).

Discussion

Methotrexate is an antagonist and analogue of folate and is frequently used for the treatment of a wide array of malignant and non-malignant diseases [25]. Despite its widespread use, MT causes severe toxicities towards organs and tissues such as liver, kidney, brain and testis even at low

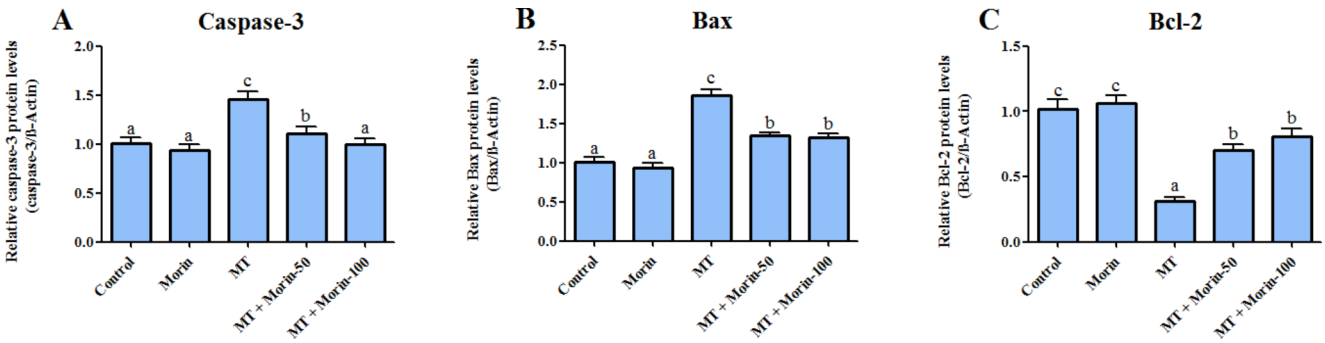

Fig. 3 Ameliorative effects of morin and MT treatments on Caspase-3, Bax and Bcl-2 mRNA transcript levels in testis tissue. (A) Caspase-3 mRNA transcript levels, (B) Bax mRNA transcript levels, (C) Bcl-2 mRNA transcript levels. Values are expressed as mean ± SD. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$)

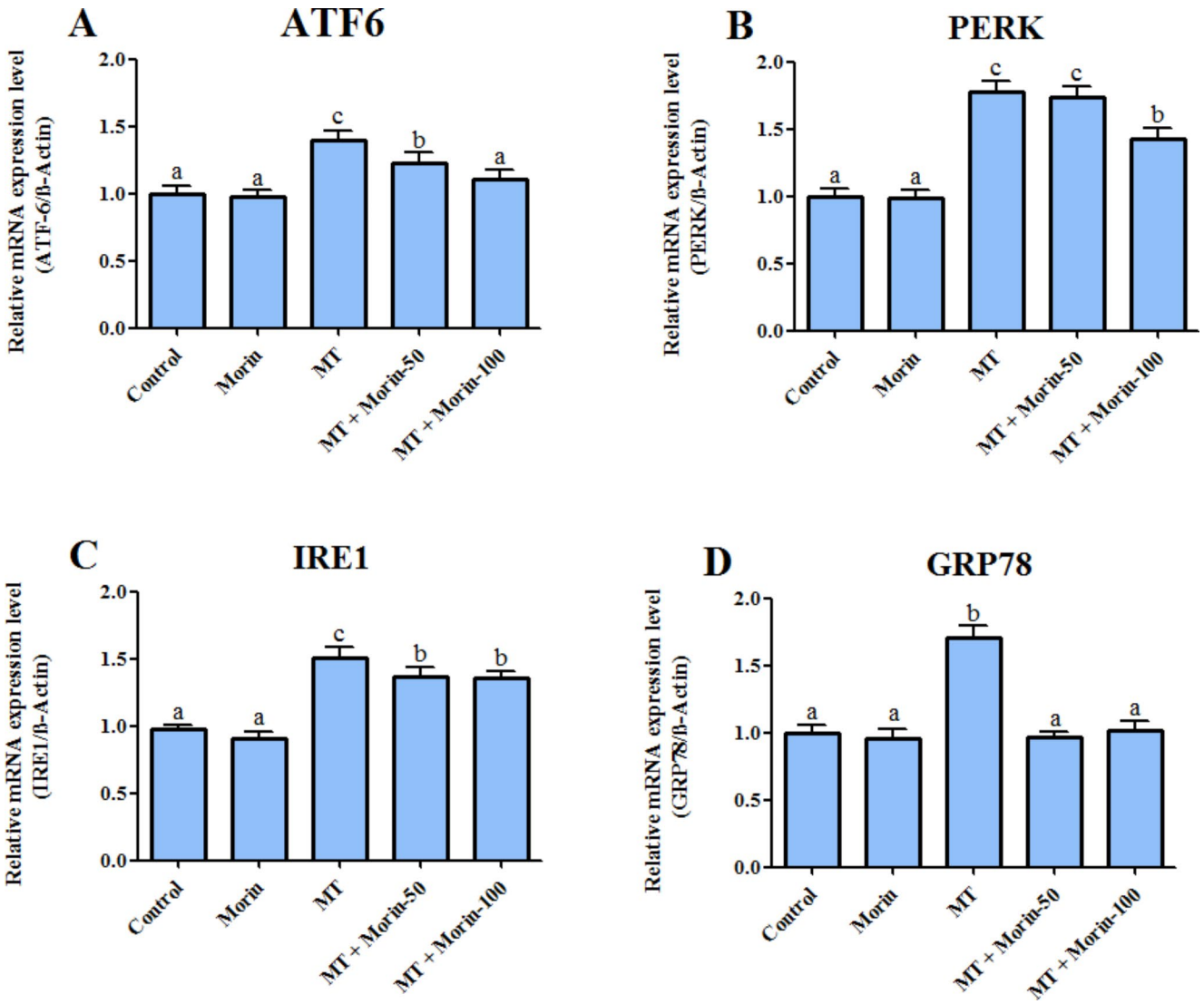

Fig. 4 Ameliorative effects of morin and MT treatments on ATF-6, PERK, IRE1 and GRP78 mRNA transcript levels in testis tissue. (A) ATF-6 mRNA transcript levels, (B) PERK mRNA transcript levels, (C) IRE1 mRNA transcript levels, (D) GRP78 mRNA transcript levels. Values are expressed as mean ± SD. Different letters (a–d) on the columns show a statistical difference ($p < 0.05$)

doses [26]. In the current study, we examined the ameliorative effects of morin on MT-induced testis toxicity through investigation of anti-oxidative, anti-inflammatory, anti-apoptotic and anti-endoplasmic reticulum stress effects.

Prooxidant/antioxidant equilibrium is obstructed by external stimuli, thereby increasing the level of reactive oxygen species (ROS) [27]. Generation of excess amount of ROS may result in oxidative damage, alteration of membrane structure and function, and lipid peroxidation [28]. As plasma membrane of sperm comprises extraordinary amount of polyunsaturated fatty acids and cytosol of sperm possess low levels of antioxidants (enzymatic or non-enzymatic), excess amount of ROS may be harmful to sperm functions. Nonetheless, testes tissue comprises a wide array of antioxidant enzymes and free radical scavengers that defend it from the detrimental impacts of ROS [29]. However, MT is reported to cause an imbalance in pro-oxidant/antioxidant ratio, produce a state of oxidative damage and thus, plays a crucial role in the pathogenesis of MT prompted tissue damage [2]. In the present study, MT treatment increased MDA levels while decreasing GSH levels and the activities of SOD, CAT, and GPx in the testis tissues while morin treatment mitigated these effects. A study carried out by Heidari Khoei, Fakhri [30] demonstrated that MT causes oxidative damage through reducing the activities of SOD and CAT in the testis tissue. In another study, Felemban, Aldubayan [31] suggested that, MT treatment significantly decreases the levels of GSH, CAT and SOD compared with control group. Previous studies have also shown that morin

could have ameliorative effects in mitigating drug induced testicular oxidative stress [32, 33].

As well as oxidative stress, inflammation plays a key role in the pathogenesis of MT-induced toxicities [34]. The inflammatory cytokines NFκB, TNF-α, and IL-1β play an important role in the pathogenesis of MT-induced testicular damages. NFκB and TNF-α prompts oxidative injury, the release of other inflammatory mediators, leading to impairment of spermatogenesis [7, 35]. In our study, NFκB, TNF-α and IL-1β mRNA transcript levels increased due to MTX toxicity. In parallel with the current study, former study revealed similar outcomes as regards the capability of morin to ameliorate the testicular increase of acrylamide-induced NFκB, TNF-α and IL-1β levels [10].

MAPK signals have been reported to be upstream of several stress stimulated cascade reactions [36]. Oxidative stress might causes activation of MAPK14 phosphorylation, which consecutively stimulates the separation of Nrf2 from Keap1 [37]. Afterwards, Nrf2 is translocated into the nucleus and attaches to the antioxidant response components in order to activate several associated genes, such as *HO-1* and *NQO1* [38]. In our study, MT treatment reduced the mRNA transcript levels of HO-1 and Nrf2, and up-regulated MAPK14 while their levels were markedly mitigated after the treatment with morin in comparison to the cells treated with MT alone. In agreement with current study, previous studies have also shown that MT treatment down-regulated expression of *HO-1* and *Nrf2* [39, 40].

Several independent studies reported that excess ROS generation further than the antioxidant machineries of the cells is among the mechanisms causing apoptosis linked with mitochondrial damage. In the stimulation of ROS-triggered apoptosis, ROS overgeneration leads to attack of free radicals to the membrane phospholipid that consecutively results in mitochondrial membrane depolarization and the loss of mitochondrial membrane permeabilization [41–44]. Afterward, the apoptotic factors will release into the cytosol from the mitochondrial inter-membrane space and activate the caspase cascade that might prompt apoptosis [45]. This is thought to be the commencement of the intrinsic pathway of apoptosis. The current study showed that MT-induces apoptosis as pointed to by up-regulation of Bax and caspase-3 and down-regulation of Bcl-2 while morin treatment reversed these effects. In this study, apoptosis is likely prompted by MT-mediated excessive ROS generation that simultaneously result in the generation of mitochondrial lipid peroxidation products, leading to loss of mitochondrial membrane potential and cytochrome c release which ultimately causes to testis apoptosis through caspase-3 activation. For that motivation, destruction of MT-triggered ROS protection by morin treatment could mitigate apoptosis.

Endoplasmic reticulum is a vital organelle in charge of synthesis, folding and maturation of several types of proteins in eukaryotes [46, 47]. In normal circumstances, the cellular requisite for protein synthesis in endoplasmic reticulum is coordinated through its folding capacity. Nonetheless, the physiological burdens or anomalies in folding could possibly lead to an imbalance that might result in the accumulation of the misfolded protein documented as endoplasmic reticulum stress [48, 49]. This subsequently activates an unfolded protein response (UPR) for restoration of endoplasmic reticulum function. The major endoplasmic reticulum-proximal controllers of the UPR comprises IRE1, PERK and ATF-6 which are generally bound to and separated by a fundamental ER chaperone GRP78 [50]. According to the results of our study, it was observed that MT treatment caused up-regulation of *ATF-6, PERK, IRE1* and *GRP78* in testicular tissue and triggered endoplasmic reticulum stress in agreement to literature [3]. However, after morin administration, the expressions of the corresponding genes were suppressed signifying ameliorative effects of morin in MT-induced testicular damage.

Conclusion

This study demonstrated that morin treatment ameliorated the testis toxicity induced by MT through antioxidant, anti-inflammatory and anti-apoptotic mechanisms and by preventing endoplasmic reticulum stress. The data of the current study show that higher dose of morin (100 mg/kg) is more effective in attenuating the toxic effects of MT on testicular tissue.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07873-5.

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Authors' contributions All authors contributed equally to this work.

Data availability The data that support the findings of this study are available from the corresponding author, [Cuneyt Caglayan], upon reasonable request.

Declarations

Conflict of interest There are no conflicts of interest to declare.

Ethical approval Experimental and animal-care protocols were approved by the Animal Experimentation Ethics Committee of Bingol University (Protocol No: 2022-E.53634).

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