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RESEARCH

Effects of rosmarinic acid and quercetin on methotrexate-induced liver and small intestine damage in rats

Rosmarinik asit ve kuersetinin sıçanlarda metotreksat kaynaklı karaciğer ve ince bağırsak hasarı üzerine etkileri

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Abstract

Purpose: Rosmarinic acid (RA) is a natural phenolic compound with antioxidant and anti-inflammatory effects. Quercetin (QCT) is a powerful antioxidant that prevents oxidative damage and cell death by clearing oxygen radicals. It also has anti-inflammatory effects. In this study, it was aimed to compare the effects of RA and QCT against liver and small bowel damage that may occur due to methotrexate (MTX) use.

Materials and Methods: The study was conducted on a model of MTX-induced liver and small intestine damage in 40 Spraque Dawley male rats. RA and QCT were administered separately and in combination prophylactically (MTX+QCT group, MTX+RA group, MTX+QCT+RA group respectively). At the end of the study, liver and small intestine tissue were removed. Histopathological evaluations were performed using scoring. Malondialdehyde level, superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were examined in the tissues.

Results: In the liver tissue, pathological findings of all prophylaxis groups decreased considerably. When compared to the control group, MDA level increased significantly in the MTX, MTX+RA and MTX+RA+QCT groups The SOD and GPX activities of the MTX group decreased significantly when compared to the control group. It was found that GPX activity increased in the MTX+QCT group and SOD activity increased in the MTX+QCT+RA group when compared to the MTX group. In addition, SOD activity was significantly increased in the MTX+QCT+RA group when compared to the MTX+RA and MTX+QCT groups. In the small intestine tissue, pathological findings decreased significantly in the MTX+QCT group. Pathological

Öz

Amaç: Rosmarinik asit (RA), antioksidan ve antiinflamatuar etkileri olan doğal bir fenolik bileşiktir. Kuersetin (QCT), oksijen radikallerini temizleyerek oksidatif hasarı ve hücre ölümünü önleyen güçlü bir antioksidandır. Aynı zamanda anti-inflamatuar etkileri de vardır. Bu çalışmada metotreksat (MTX) kullanımına bağlı oluşabilecek karaciğer ve ince bağırsak hasarına karşı RA ve QCT'nin etkilerinin karşılaştırılması amaçlandı.

Gereç ve Yöntem: Çalışma, 40 Spraque Dawley erkek sıçanda MTX'in neden olduğu karaciğer ve ince bağırsak hasarı modeli üzerinde gerçekleştirildi. RA ve QCT profilaktik olarak ayrı ayrı ve kombinasyon halinde uygulandı (sırasıyla MTX+QCT grubu, MTX+RA grubu, MTX+QCT+RA grubu). Çalışmanın sonunda karaciğer ve ince bağırsak dokusu çıkarıldı. Histopatolojik değerlendirmeler skorlama kullanılarak yapıldı. Dokularda malondialdehit düzeyi, süperoksit dismutaz (SOD) ve glutatyon peroksidaz (GPX) aktiviteleri incelendi.

Bulgular: Karaciğer dokusunda tüm profilaksi gruplarının patolojik bulgularında belirgin azalma görüldü. MTX, MTX+RA ve MTX+RA+QCT gruplarında MDA düzeyi kontrol grubuna göre arttı. MTX grubunun SOD ve GPX aktiviteleri kontrol grubuna göre azaldı. MTX grubuna göre MTX+QCT grubunda GPX aktivitesinin, MTX+QCT+RA grubunda ise SOD aktivitesinin arttığı tespit edildi. MTX+QCT+RA grubunda SOD aktivitesi, MTX+RA ve MTX+QCT gruplarına göre arttı. İnce bağırsak dokusunda patolojik bulgular MTX+QCT grubunda azaldı. MTX+RA, MTX+QCT+RA gruplarında patolojik bulgular bir miktar azaldı. MTX ve MTX+RA+QCT gruplarında MDA düzeyleri kontrol grubuna göre yüksekti. MTX grubunun SOD ve GPX aktivitelerinde kontrol grubuna göre azalma görüldü. GPX

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findings decreased slightly in MTX+RA, MTX+QCT+RA groups. MDA levels were significantly higher in the MTX and MTX+RA+QCT groups when compared to the control group. The SOD and GPX activities of the MTX group decreased significantly compared to the control group. GPX activity decreased significantly in the MTX+QCT and MTX+RA groups when compared to the control group. SOD activity increased significantly in MTX+RA+QCT group when compared to MTX group, GPX activity increased significantly in MTX+RA+QCT group compared to MTX+QCT group.

Conclusion: RA and QCT may be effective in preventing liver damage caused by MTX. It was concluded that QCT may be more effective than RA in preventing small bowel injury caused by MTX.

Keywords: Rosmarinic acid, quercetin, methotrexate, liver, small intestine tissue

aktivitesi MTX+QCT ve MTX+RA gruplarında kontrol grubuna göre azaldı. MTX+QCT+RA grubunda MTX grubuna göre SOD aktivitesinin arttığı tespit edildi. GPX aktivitesi MTX+RA+QCT grubunda MTX+QCT grubuna göre arttı.

Sonuç: MTX'in neden olduğu karaciğer hasarını önlemede RA ve QCT'nin etkili olabileceği sonucuna varıldı. MTX'in neden olduğu ince bağırsak hasarını önlemede QCT'nin RA'dan daha etkili olabileceği sonucuna varıldı.

Anahtar kelimeler: Rosmarinik asit, kuersetin, metotreksat, karaciğer, ince bağırsak

INTRODUCTION

Methotrexate (MTX) is a dihydrofolate reductase inhibitor used in the treatment of many diseases including malignant, inflammatory and autoimmune diseases[1,2]. In addition to its success in treatment, there are also side effects[3]. Due to its side effects in many systems such as the gastrointestinal system, central nervous system, hematological system, its usage is restricted and the quality of life of patients is negatively affected[4]. The toxic effects of MTX may vary on a patient-by-patient basis. Therefore, methotrexate is used in a wide dose range[5]. Studies have shown that MTX treatment increases reactive oxygen species and oxidative stress[6,7]. Since it suppresses growth and proliferation in cells, it may cause toxic effects especially on rapidly dividing cells[3]. Although the toxicity mechanisms of MTX have not been fully elucidated, various hypotheses related to oxidative stress have been put forward[7,8]. For this reason, antioxidant substances have been the focus of interest of scientists in the prevention of MTX toxicity.

Rosmarinic acid (RA); [(R) -O- (3,4-Dihydroxycinnamoyl) -3- (3,4-dihydroxyphenyl) lactic acid, 3,4-Dihydroxycinnamic acid (R) -1-carboxy-2- (3,4-dihydroxyphenyl) ethyl ester] is a natural phenolic compound found in the Lamiaceae plant family[9]. It attracted the attention of researchers due to its antioxidant, anti-inflammatory antimutagen, antibacterial and antiviral effects and has been the subject of many studies[10,11]. With its antioxidant properties, it has been used in traditional European herbal medicine to treat various diseases such as cancer, rheumatoid arthritis, bronchial asthma, cataract, peptic ulcer[12]. Quercetin (QCT) (3,5,7,3 ',4' -pentahydroxy flavone) is a substance with a strong antioxidant effect common in many fruits and vegetables, especially oranges, apple, onion, broccoli, cabbage, blueberries and tea. It has been the subject of many studies due to its antioxidant effects. Oxygen radicals prevent oxidative damage and cell death by cleaning[13]. QCT chelates metal ions and inhibits the activities of lipoxygenase, xanthine oxidase and cyclooxygenase enzymes[14]. It has an anti-inflammatory and anticarcinogenic effect[15]. There are publications indicating that QCT is protective against acute liver injury and may be a new hepatoprotective and therapeutic agent for patients with liver diseases[16].

The hypothesis of our study is that single and combined administration of quercetin and rosmarinic acid, which have been shown to have therapeutic properties in different organs, may also show healing effects in mtx-related ib and kc injury. There is no study in the literature that comparatively examines these antioxidants or investigates the effect of their combined use. The aim of our study is to biochemically and histopathologically reveal the effects of the simultaneous use of these two antioxidants in treatment as well as their combined use in different organs.

MATERIALS AND METHODS

Animals and procedure

The study was started with the approval of the Animal Experiments Local Ethics Committee of Karadeniz Technical University (31.05.2022, Ethical

Committee File No: 2022/17). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. In the study, 40 Spraque Dawley male rats weighing 200-250 grams from the Karadeniz Technical University Experimental Animals Surgical Research and Application Center, Türkiye (KTUESRAC) were used.

Power analysis was calculated with the G power program. The analysis was performed with the power of α error probability = 0.05, effect size f = 0.6 and power (1-β error probability) = 0.80. It was planned that each group would have n=8.

The animals were randomly divided into five equal groups: In the control group (n=8); no application was performed. In MTX group (n=8); on the 5th day of the experiment, 30 mg/kg MTX (Koçak Farma, Tekirdağ, Türkiye) was administered intraperitoneally (ip). In MTX+QCT group (n=8); on the 5th day of the experiment, 30 mg/kg MTX was administered intraperitoneally (ip) and 30 mg/kg QCT was administered daily for 7 days (Sigma-Aldrich, Co, St. Louis, USA) by gavage. MTX+RA group (n=8); on the 5th day of the experiment, 30 mg/kg MTX was administered intraperitoneally (ip) and 30 mg/kg RA (Sigma-Aldrich Chemie) was administered daily for7 days by gavage. MTX+QCT+RA group (n=8); on the 5th day of the experiment, 30 mg/kg MTX was administered intraperitoneally (ip) and 15 mg/kg QCT and 15 mg/kg RA was administered daily for 7 days by gavage.

Animals were kept in a 12 h light:12 h dark cycle with no dietary restrictions. Tap water was provided for drinking, together with standard rat chow (Bayramoğlu Feed and Flour Industry Trading Corp. Erzurum / Türkiye) Temperature was set at 22 ± 2ºC and humidity at 50 ± 5%. Rats were housed in type I rat cages throughout the study. All experimental procedures and rat maintenance and sacrifice took place in the KTUESRAC.

Tissue preparation

At the end of the experiment period (day 8), rats were anesthetized by using 90 mg/kg Ketalar® (Eczacıbaşı Co., Istanbul, Turkey) and a midline abdominal incision was made by using a scalpel. The peritoneum was exposed, and liver was removed with small intestine. Half of the right lobe of the liver and part of the small intestine (from the jejunum) were used for histopathological analysis following routine histological tissue preparations (Bancroft et al., 1994). The remaining halves were placed in 1.5 ml tubes (Eppendorf, Hamburg, Germany) for biochemical analysis and kept in a deep freeze (U410 Premium; Ultra-Low Temperature Laboratory Freezers, New Brunswick Scientific Co. Inc., St. Albans, England) at -80 ºC. Finally, the rats were sacrificed by exsanguination.

Histopathological examination

After the liver and small intestinal tissues taken from the rats were detected in 10% formalin solution, they were embedded in paraffin blocks by routine tissue monitoring. Sections were cut at 5µm by using a rotary microtome (RM 2255; Leica Instruments, Nussloch, Germany). The sections selected by systematic and random sampling were stained with Hematoxylin & Eosin (H&E) and Masson's trichrome[17]. Histopathological evaluation of the liver and small intestine tissue stained with H&E was scored (0: none-mild, 1: moderate, 2: intense). Inflammatory cell infiltration, necrotic cell, edema, and increased bleeding in liver tissue were evaluated by scoring[18]. Surface epithelial degeneration, villous fusion, inflammatory cell infiltration in mucosa, and increased bleeding in mucosa in small intestine tissue were evaluated by scoring[19]. Collagen fiber density in connective tissue of small intestine was evaluated with Masson's trichrome staining. Histological evaluation of the tissues was performed using Analysis 5 Research (Olympus Soft Imaging Solution, Germany) program in a digital camera (Olympus, DP 71, Japan) with attachment light microscope (Olympus BX 51, Japan). Photos were taken with the help of the same light microscope and digital camera and transferred to the digital environment.

Immunohistochemical procedures (TUNEL assay and AI%)

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay was used to evaluate apoptosis in liver and small intestine tissues. Sections were stained with the in situ cell death detection POD kit (Roche, 11684817910, Berlin, Germany) as recommended by the manufacturer. Cells whose nuclei were stained brown using a light microscope were defined as TUNEL (+). Analysis 5 Research program (Olympus Soft Imaging Solutions, Münster, Germany) was used for counting. 100 cells were counted in 5 different

fields for each animal. The percentage of apoptotic cells was calculated as the apoptotic index (AI) according to the formula below[20].

AI = apoptotic cell nuclei number/total cell nuclei number counted x100

Biochemical analysis

The levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPX) were investigated as oxidative stress markers in liver and small intestine tissue. MDA measurement was performed according to Mihara and Uchiyama method. The basis of this method is based on the measurement of the absorbance of the colour formed by MDA with thiobarbituric acid in an acidic environment at 532 nm. The results were expressed as nmol/g protein[21]. (Mihara M et al. 1978).

SOD enzyme activity was performed by modifying the method developed by Sun and Oberley. This method is based on the principle of preventing the formation of purple formazan dye from nitroblue tetrazolium by using the superoxides formed by xanthine-xanthine oxidase by SOD. Since the higher the SOD activity in the environment, the more O_2^- will be used, after the reaction of a decrease in the intensity of the colour formed is observed. The absorbance of the coloured compound formed as a result of the reaction was measured spectrophotometrically at 560 nm. The results were expressed as U/mg protein[22].

GPX activity in supernatants obtained from liver and small intestinal tissues was determined in accordance with the recommendations of the manufacturer using the commercial ELISA kit (CT LAB, Zhejiang, China) with the catalogue number E1242Ra. The results were expressed as ng/mg protein.

Protein determination in the tissue homogenates obtained was made by Bradford method. The principle of this method is based on the principle of Coomassie Brillant Blue G250, an organic dye, binds to proteins in a phosphoric acid environment and the blue complex formed shows maximum absorbance at 600 nm[23]. (Bradford MM 1976).

Statistical analysis

Statistical analysis was performed by using Stata 14.0. Data are expressed as the mean ± standard deviation (SD). Kruskal-Wallis variance analysis (the Mann-Whitney U test with Bonferroni correction as post hoc) was used to compare the groups of histopathological scoring and biochemical results. P-values lower than 0.05, were statistically significant.

RESULTS

Histopathological analysis

In the control group, the liver tissue was normal (Figure 1A). In the MTX group, an increase in necrotic cells, as well as intense edema, bleeding and inflammatory cell infiltration, especially in the portal area, were observed. (Figure 1B). It was observed that pathological findings increased significantly in the MTX group compared to the control group ($p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$ respectively). It was observed that pathological findings decreased significantly in MTX+QCT ($p<0.001$, $p=0.001$, $p=0.001$, $p<0.001$ respectively), MTX+RA ($p=0.024$, $p=0.001$, $p=0.001$, $p<0.001$ respectively) and MTX+QCT+RA ($p<0.001$, $p<0.001$, $p<0.001$, $p=0.001$ respectively) groups compared to MTX group (Figure 1C, 1D, 1E).

Table 1. Histopathological analysis results of liver tissue and apoptotic index values

	Control group mean±SD	MTX group mean±SD	MTX+QCT group mean±SD	MTX+RA group mean±SD	MTX+RA+QCT group mean±SD
Necrotic cells	0.13±0.35	1.75±0.46 *	0.13±0.35†	0.75±0.71†	0.13±0.35†
Edema	0±0	1.75±0.46 *	0.25±0.46†	0.25±0.46†	0.13±0.35†
Bleeding	0±0	1.75±0.46 *	0.25±0.46†	0.25±0.46†	0.13±0.35†
Inflammatory cell infiltration	0.13±0.35	1.75±0.46 *	0.13±0.35†	0.25±0.46†	0.13±0.35†
AI (%)	19.26±2.46	58.75±5.28 *	21.62±3.24 †	31.03±2.52 *†‡	37.12±2.31 *†‡§

Data are mean ± SD; n = 8 for each group. MTX: methotrexate; QCT: quercetin; RA: rosmarinic acid; AI: apoptotic index.
* $p<0.05$ compared to the control group, † $p<0.05$ compared to MTX group, ‡ $p<0.05$ compared to MTX+QCT,
§ $p<0.05$ compared to MTX+RA group

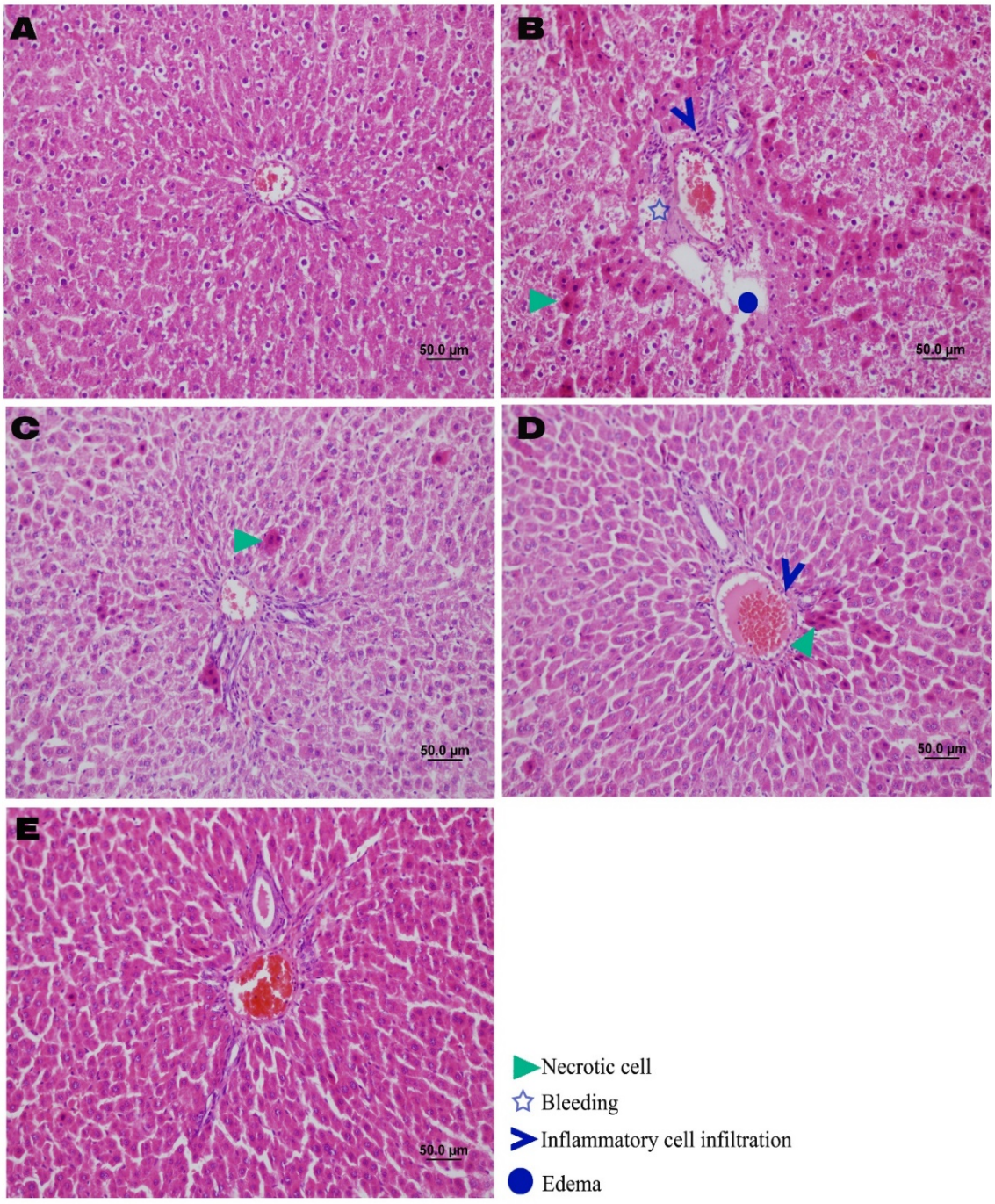

Figure 1. Liver tissue sections stained with H&E.

Control group (A) had normal structure. In the MTX group (B), intense edema (circle), bleeding (star), severe necrotic cell (arrow) and inflammatory cell infiltration (notched arrowhead) were observed especially in the portal area. In the MTX+QCT group (C), MTX+RA group (D), MTX+QCT+RA group (E) pathological findings were considerably reduced. All panels × 200.

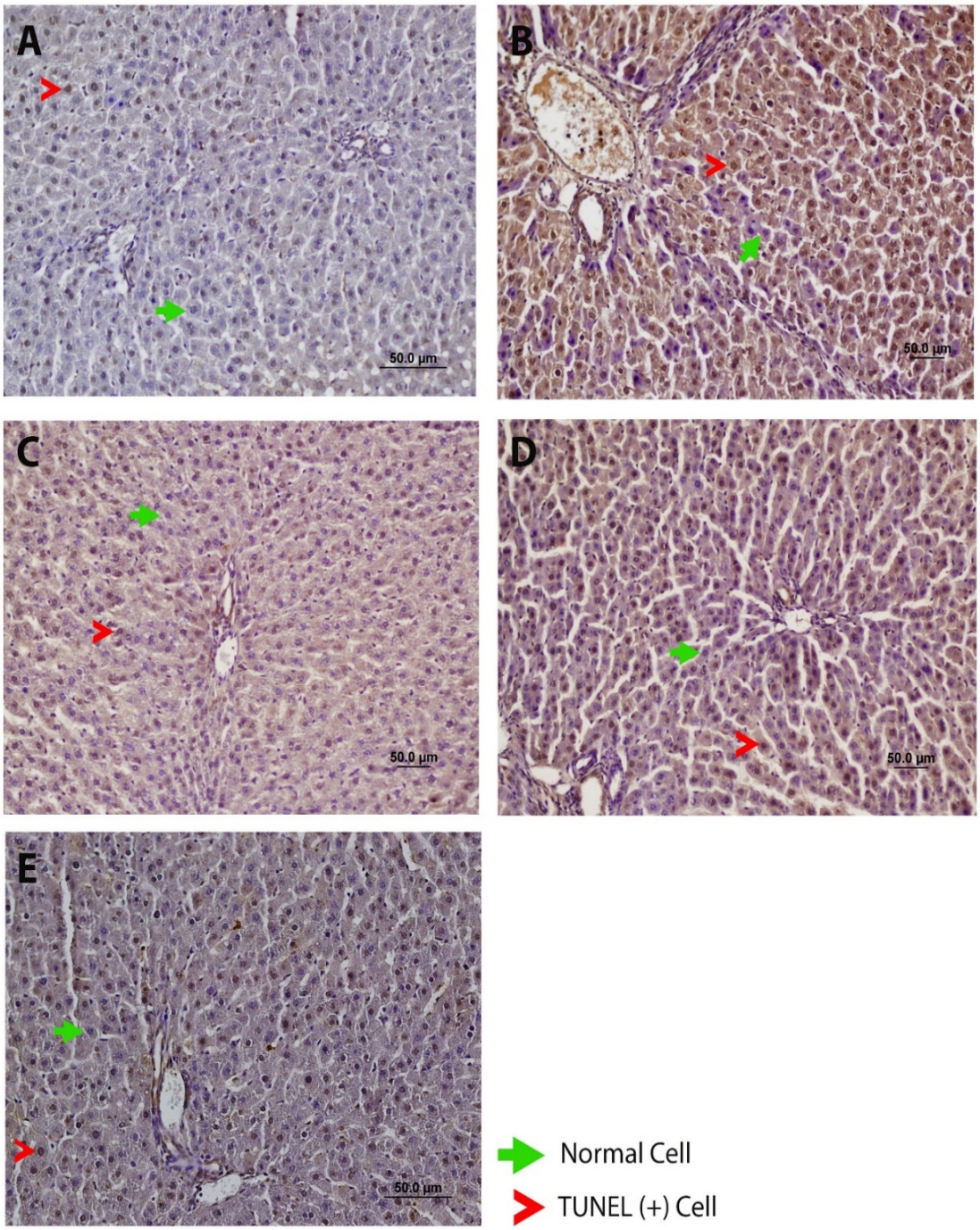

Figure 2. Liver tissue sections stained with TUNEL. Normal cell (arrow), TUNEL (+) cell (notched arrowhead). Control group (A), MTX group (B), MTX+QCT group (C), MTX+RA group (D), MTX+RA+QCT group (E). All panels × 200.

When compared to control group (p<0.001), AI in hepatocytes in liver tissue increased significantly in MTX groups. When compared to MTX group (p<0.001, p<0.001, p<0.001 respectively), AI was decreased in MTX+QCT, MTX+RA and MTX+RA+QCT groups. The reduction in AI was greater in the MTX+QCT group than in the MTX+RA and MTX+RA+QCT groups (p<0.001, p<0.001 respectively). In addition, the reduction in AI was greater in the MTX+RA group than in the MTX+RA+QCT group (p=0.002) (Figure 2) (Table 1)

In the control group, villus epithelium, villus structure, lamina propria and submucosa were normal in the small intestine. (Figure 3A). In the MTX group, villus surface epithelial degeneration, fusion in the villi, inflammatory cell infiltration and bleeding areas in the mucosa were observed (Figure 3B). In the MTX group, surface epithelial degeneration, villi fusion and inflammatory cell infiltration were significantly increased compared to the control group (p=0.004, p=0.001, p=0.018 respectively). In the MTX+QCT group, surface epithelial degeneration, villi fusion and inflammatory cell infiltration decreased significantly compared to the MTX group (p=0.029, p=0.008, p=0.018 respectively) (Figure 3C). Pathological findings decreased slightly in the MTX+RA, MTX+QCT+RA groups (Figure 3D, 3E). In the scoring, surface epithelial degeneration was found to be significantly higher in MTX+RA and MTX+QCT+RA groups compared to the control group (p=0.001, p=0.010 respectively). In addition, surface epithelial degeneration was significantly increased in the MTX+RA group compared to the MTX+QCT group (p=0.019). In the MTX+RA+QCT group, the fusion of the villi was decreased compared to the MTX group (p=0.050). There was no significant difference between the groups in terms of edema and bleeding findings. Submucosal thickness and collagen fibers density were similar in all groups (Figure 4). AI in the small intestine tissue increased significantly in MTX groups compared to the control group (p<0.001). AI was decreased in MTX+QCT, MTX+RA and MTX+RA+QCT groups compared to MTX group (p<0.001, p<0.001, p<0.001 respectively), but they were elevated compared to the control group (p<0.001, p<0.001, p<0.001 respectively). The reduction in AI was greater in the MTX+QCT group than in the MTX+RA and MTX+RA+QCT groups (p<0.001, p<0.001 respectively) (Figure 5) (Table 2).

Biochemical analysis

In the liver tissue, MDA level increased in all drug-applied groups. While MDA increased significantly in the MTX, MTX+RA and MTX+RA+QCT groups were compared to the control group (p =0.015, p=0.004, p=0.037 respectively), it was close to the control in the MTX+QCT group. The SOD and GPX activities of the MTX group decreased significantly compared to the control group (p=0.015, p=0.041 respectively). In the MTX+QCT group, GPX level increased significantly compared to the MTX group (p=0.028) and was similar to the control group. It was found that SOD activity increased significantly in the MTX+RA+QCT group compared to the MTX group (p=0.002) and was similar to the control group. In addition, SOD activity was significantly increased in the MTX+QCT+RA group compared to the MTX+RA and MTX+QCT groups (p=0.045, p=0.045 respectively) (Figure 6).

Table 2. Histopathological analysis results of small intestine tissue and apoptotic index values

	Control group mean±SD	MTX group mean±SD	MTX+QCT group mean±SD	MTX+RA group mean±SD	MTX+RA+QCT group mean±SD
Surface epithelium degeneration	0.13±0.35	1.50±0.76 *	0.38±0.52 †	1.38±0.52 *‡	1.38±0.74 *
Villous fusion	0.13±0.35	1.38±0.52 *	0.25±0.46 †	1.13±0.83	0.8±0.99 †
Inflammatory cell infiltration	0.13±0.35	1.25±0.89 *	0.13±0.35 †	0.5±0.76	0.5±0.76
Edema	0.25±0.46	0.25±0.71	0.13±0.35	0.25±0.46	0.25±0.71
Bleeding	0.13±0.35	0.75±1.03	0.13±0.35	0.13±0.35	0.50±0.93
AI (%)	13.21±1.54	42.89±1.64 *	18.60±1.38 *†	30.58±2.78 *†‡	30.99±2.07 *†‡

Data are mean ± SD; n = 8 for each group. MTX: methotrexate; QCT: quercetin; RA: rosmarinic acid; AI: apoptotic index.
* p<0.05 compared to the control group, † p<0.05 compared to MTX group, ‡p<0.05 compared to MTX+QCT
There is no difference between the groups in edema and bleeding scoring.

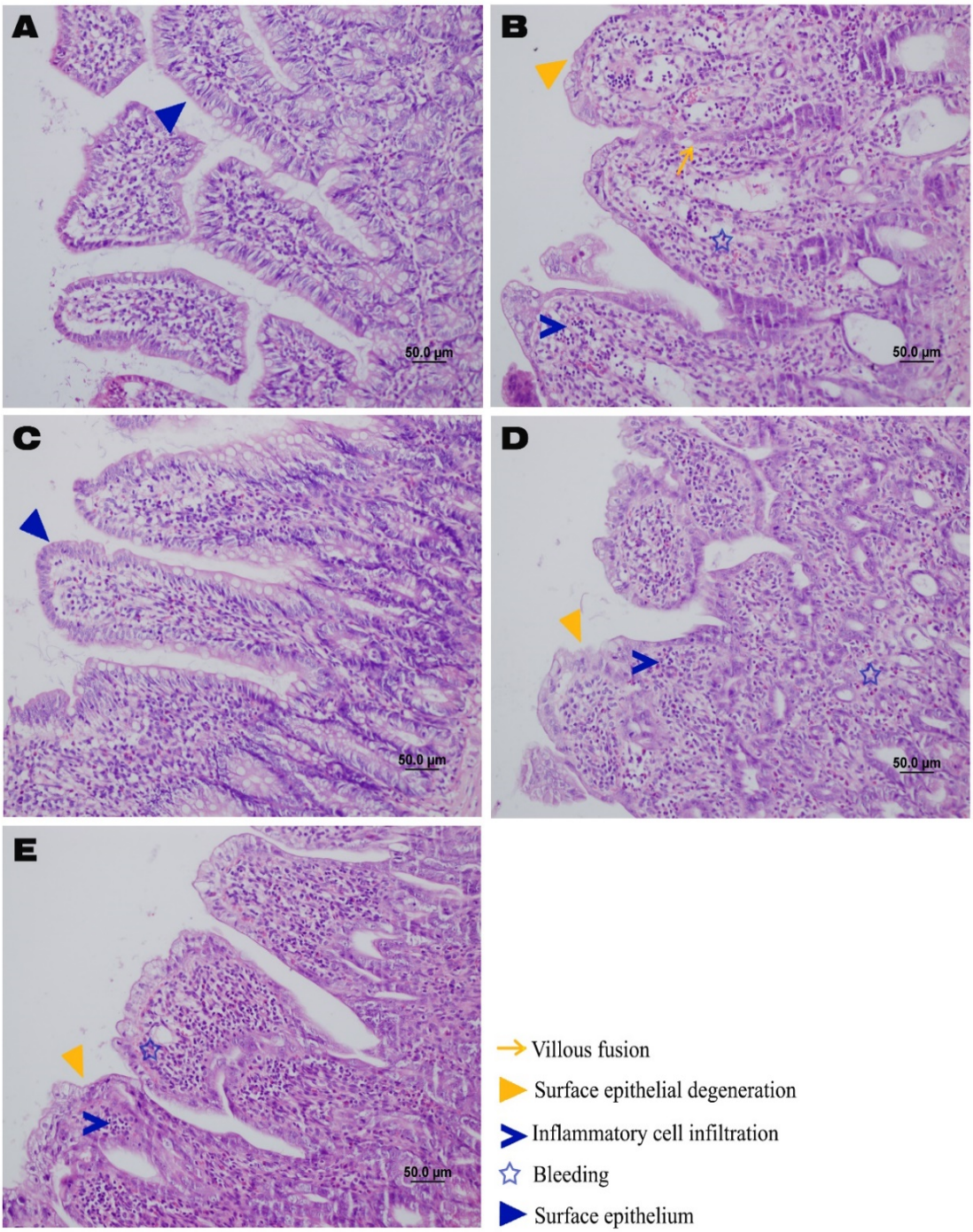

Figure 3. Small intestine tissue sections stained with H&E. Normal morphology was observed in the Control Group (A). In the MTX group (B), surface epithelium degeneration (yellow arrowhead), villous fusion (arrow), inflammatory cell infiltration (notched arrowhead) and areas of bleeding (star) in the mucosa were observed.

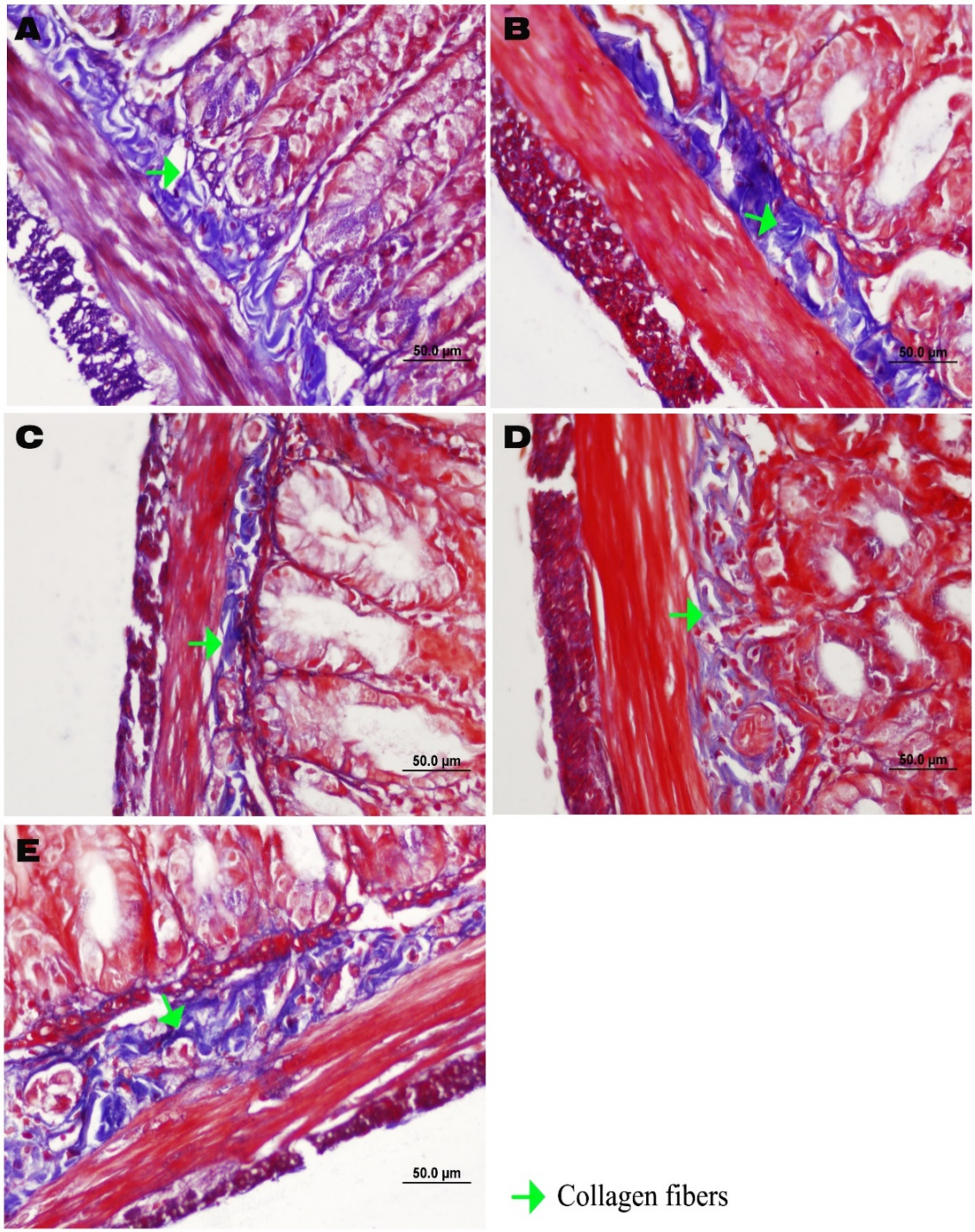

Figure 4. Small intestine tissue sections stained with Masson's trichrome. Collagen fibers (arrow) density was similar in all groups. Control group (A), MTX group (B), MTX+QCT group (C), MTX+RA group (D), MTX+RA+QCT group (E). All panels × 400.

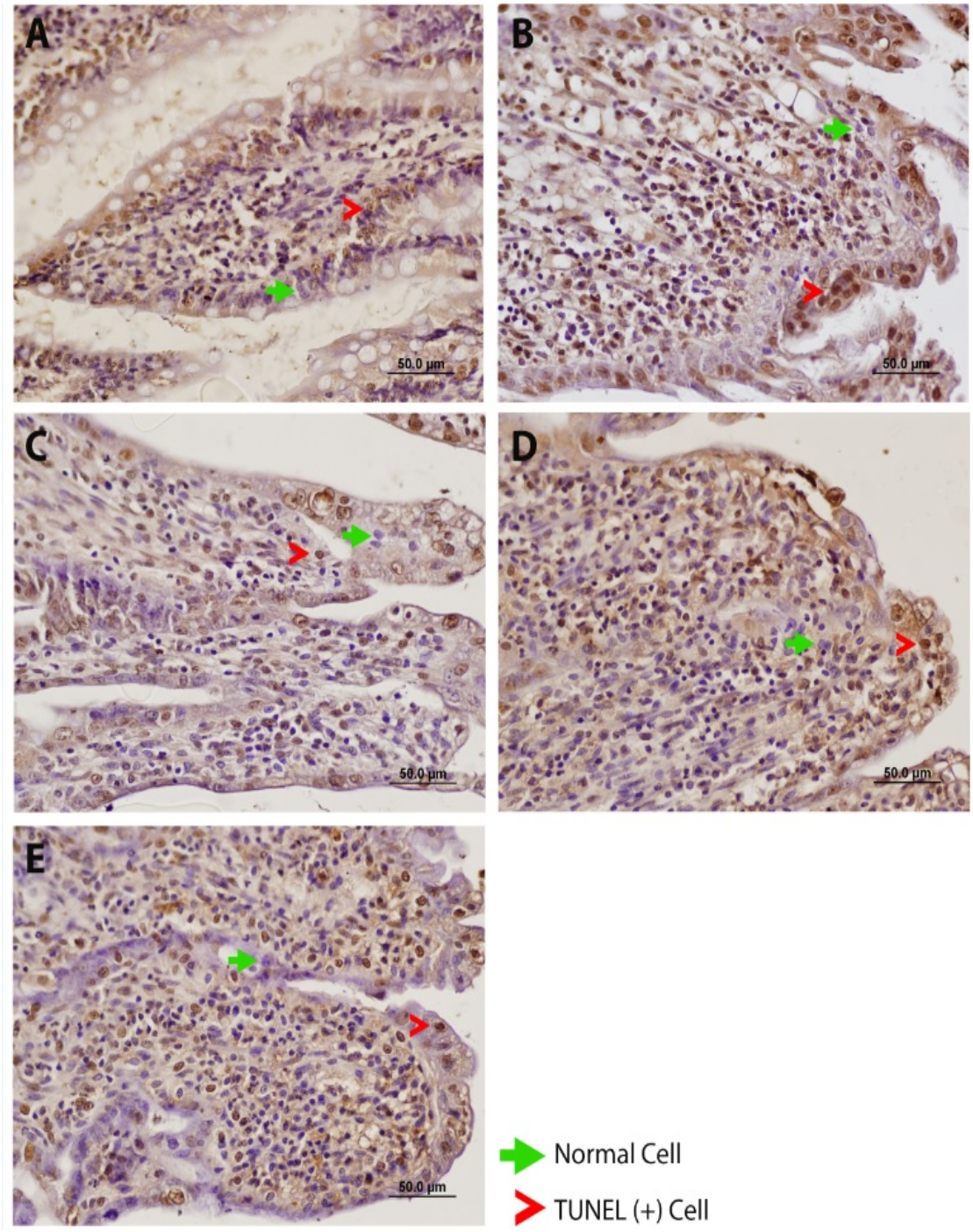

Figure 5. Small intestine tissue sections stained with TUNEL. Normal cell (arrow), TUNEL (+) cell (notched arrowhead). Control group (A), MTX group (B), MTX+QCT group (C), MTX+RA group (D), MTX+RA+QCT group (E). All panels × 200.

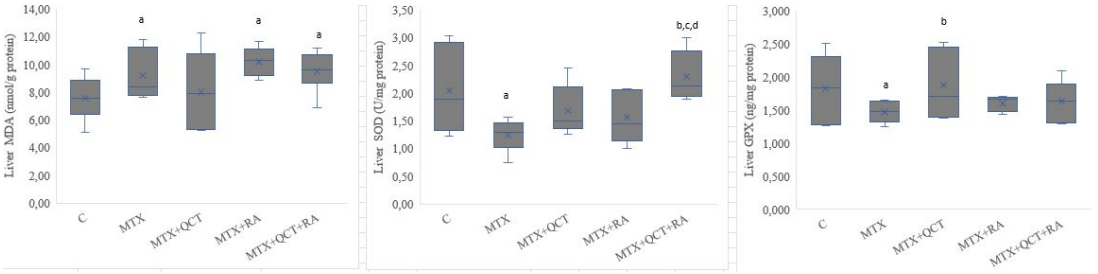

Figure 6. MDA, SOD, GPX levels of liver tissue

[a] p<0.05 compare control group, [b]p<0.05 compare MTX group. [c] p<0.05 compare MTX+QCT group, [d] p<0.05 compare MTX+RA group.

In the small intestine tissue, MDA level increased in all drug-applied groups. MDA levels were significantly higher in the MTX and MTX+RA+QCT groups compared to the control group (p=0.009, p=0.01 respectively). The SOD and GPX activities of the MTX group decreased significantly compared to the control group (p=0.026, p=0.037 respectively). GPX activity decreased significantly in the MTX+QCT and MTX+RA groups compared to the control group (p=0.018, p=0.017 respectively). SOD activity increased significantly in MTX+RA+QCT group compared to MTX group (p=0,016), GPX activity increased significantly in MTX+RA+QCT group compared to MTX+QCT group (p=0.045) (Figure7).

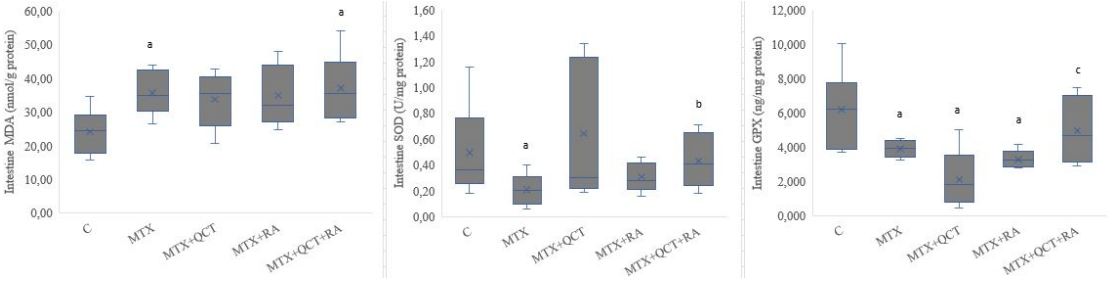

Figure 7. MDA, SOD, GPX levels of small intestine tissue

[a] p < 0.05 compare control group, [b] p < 0.05 compare MTX group, [c] p<0.05 compare MTX+QCT group.

DISCUSSION

MTX is widely used in the treatment of many diseases[2]. However, the drug also causes side effects in organs belonging to many systems[4]. There are studies in the literature showing that MTX causes damage to the liver, small intestine and many other tissues[24,25,26,27,28,29]. In our study, it was observed that IP MTX injection in rats caused an increase in oxidative stress in both the liver and small intestine, decreased antioxidant enzyme activities and histopathological damage to the tissue. The increase in MDA level in the liver and small intestine tissue and the decrease in GPX and SOD enzyme activity in all groups to which we applied MTX showed us that MTX caused oxidative stress in the liver and small intestine tissue.

There are studies showing that QCT and RA reduce hepatotoxicity from different pathways such as antioxidant and anti-inflammatory mechanisms[30,31,32] (Ma JQ et al., 2015; Ince E., 2020; Hasanein P et al., 2018). For example, Ma JQ et al. 2015 showed in their study that QCT has strong protective effects against inflammation by partially suppressing the activation of TLR2/4 and MAPK/NF-ϰB and inhibiting iNOS, COX-2, IL-1β and NO levels[30]. Again, there are possible mechanisms in the literature where QCT

reduces the hepatotoxicity of different substances due to antioxidant, free radical scavenger, antiapoptotic, anti-inflammatory and CYP2E1 inhibitor activities[33]. In our study, although GPX and SOD activity increased in the MTX+QCT group in liver tissue, the increase in GPX activity was statistically significant and similar to the control group compared to the MTX group. In addition, histopathological damage was improved, and AI decreased in the MTX+QCT group[34]. This suggests that QCT increases the activity of antioxidant enzymes, especially GPX, and prevents liver damage caused by MTX. Again, in our study, although GPX and SOD activity increased in the MTX+QCT+RA group in liver tissue, only the increase in SOD activity was statistically significant compared to the MTX group and was similar to the control group. Histopathological damage was also improved, and AI decreased in the MTX+QCT+RA group. This suggests that QCT-RA combined prophylaxis prevents damage by increasing the activity of antioxidant enzymes, especially SOD. In our study, there was no significant increase in GPX and SOD activities in the MTX+RA group in liver tissue. However, histopathological damage was improved, and AI decreased in the MTX+RA group. It is thought that this improvement may have occurred due to the positive effects of QCT and RA on parameters in other antioxidant enzymes or anti-inflammatory mechanisms that we did not measure in our study [33,35,36].

In our study, although there was no significant increase in antioxidant enzyme activities in the MTX+QCT group in the small intestinal tissue, it was observed that the histopathological damage caused by MTX decreased similarly to the literature[37]. It is thought that this improvement may have occurred due to the positive effects of QCT on the parameters in different antioxidant enzymes or anti-inflammatory mechanisms that we did not measure, as it is mentioned before. In other prophylaxis groups, only a slight improvement was observed in histopathological findings. AI was decreased in all prophylaxis groups in small bowel tissue. Jafaripour L et al. (2021) reported in their study that RA was effective in preventing nephrotoxicity and hepatotoxicity caused by MTX[36]. In our study, while there was a significant improvement in hepatotoxicity in the RA groups, a very slight improvement was observed in the pathological findings in the small intestine tissue. It is thought that this incompatibility is due to the different application doses or tissues studied. As a matter of fact, in our study; even though AI in the small intestine was significantly reduced in all prophylaxis groups compared to the MTX group, there were still differences between the groups. Although QCT and RA were administered at the same doses, the reduction in the MTX+QCT group was greater than in the MTX+RA group. In the MTX+QCT+RA group, which was administered by halving the dose of antioxidants, the reduction in AI was less than in the other prophylaxis groups.

In their study, Zhao P et al. 2020 applied 5-50-100 mg/kg QCT in rats with hyperthyroidism model[38]. They observed a decrease in liver function tests, an increase in antioxidant enzymes in the liver and an improvement in histopathological findings in all dose groups. It is stated that treatment with high dose QCT had more protective effects against liver damage. Gheshlaghi-Ghadim A et al. 2022 used QCT at 2.5-5-10mg/kg doses to prevent liver injury. It is stated that 10 mg/kg QCT provided a moderate improvement in hepatic histopathological damage, while QCT was less effective at lower doses[39]. In addition to the type of antioxidant used, administration doses and combined uses may also cause differences in the protective effects of the antioxidant.

In our study, there was an improvement in histopathological findings in all groups undergoing prophylaxis in the liver, while only MTX+QCT group showed an improvement in histopathological findings in the small intestine. It is thought that these differences may be due to different biochemical, anatomical, functional, and physiological properties of the tissues examined[40]. For example, Oğuz A et al. 2020 investigated the protective effects of hepatic ischemia reperfusion RA in their study[41]. They stated that RA reduces oxidative stress, increases antioxidant capacity, and leads to significant histopathological improvement in the liver after hepatic ischemia reperfusion injury, and is an effective hepatoprotective agent. However, they reported that although RA reduced oxidative stress in the lung and increased antioxidant capacity, no difference was observed in histopathology, and RA had no beneficial effect on kidney damage. This shows us that antioxidant substances may have a protective or therapeutic effect in a different dose range for each organ. Therefore, it is thought that studies showing the efficacy of an agent used in different organs at the same time may be useful in

determining the effective dose range of the agent used.

Although the number of subjects in our study groups was determined by taking similar studies as an example and in accordance with the 3R rule, the number of subjects is one of the limitations of our study. In addition, the fact that histopathologically demonstrated antiapoptotic and anti-inflammatory effects are not demonstrated through biochemical markers (such as caspaz3 and TNF alpha), it is one of the limitations of our study. Again, the fact that the effects we showed with staining were not supported may be another limiting factor in our study. Therefore, it would be appropriate to increase the number of subjects, to increase statistical significance in future studies and to use biochemical markers in addition to the evaluation of antiapoptotic and anti-inflammatory effects.

It was concluded that QCT and RA may be effective in preventing liver damage caused by MTX. QCT was thought to be more effective than RA in preventing small bowel damage caused by MTX. However, we recommend doing other studies to better understand the mechanisms of the protective effects of QCT and RA on the liver and small intestine and to determine the dose range required for prophylaxis in different organs.

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