

# A potential hepatoprotective effect of ranolazine against methotrexate-induced liver injury in rats

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## Abstract

Methotrexate (MTX) is an important drug for rheumatic and non-rheumatic disease therapy. MTX has been associated with many adverse effects ranging from asymptomatic transaminase elevation to fibrotic tissue formation and fatal hepatic necrosis due to oxidative stress. Concerns regarding potential liver toxicity have led to the avoidance of medication, termination, or advice for inquiries in clinical care. The protective and therapeutic effects of a new generation anti-angina drug, ranolazine (RAN), on MTX-induced liver damage were investigated by evaluating its antioxidant mechanism in rats. Thirty-two female Wistar Albino rats were randomly assigned to Control, RAN, prophylaxis, and treatment groups (n=8/group). Liver function enzymes, histopathological assessment and serum biochemical parameters were examined. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were also measured in liver tissue. MTX administration caused mononuclear inflammation, vascular congestion, ductal proliferation, vacuolization, and fibrosis as evaluated using Roening grading and increases in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase levels ( $p < 0.05$ ) in the blood, which is compatible with hepatocyte damage in the blood and increased MDA levels in the tissue. Histopathologically, vascular congestion and ductal proliferation, and biochemically, MDA and SOD levels and serum biochemical parameters, significantly decreased in the RAN+MTX and MTX+RAN groups ( $p < 0.001$ ) when compared with the MTX group. No significant changes were observed in terms of SOD and GSH levels and fibrosis scores in RAN-administered groups ( $p > 0.05$ ). According to our results, RAN may be a potential hepatoprotective agent against MTX-induced liver injury.

## INTRODUCTION

Methotrexate (MTX) is an antifolate drug used to treat rheumatoid arthritis and psoriasis. After conversion to its metabolite 7-hydroxymethotrexate in the liver (Chladek et al., 1997). MTX attaches to hepatocytes as polyglutamate, which leads to a decrease in folic acid levels and an increase in the amount of MTX within the cell (Kamen et al., 1981). However, the clinical use of MTX is often limited by its side effects on various organ systems, particularly the liver.

Long-term use of MTX can activate pathophysiological pathways that cause elevation in liver enzymes, hepatotoxicity, liver fibrosis, and cirrhosis. These pathways include increased oxidative stress, disruption of intracellular ion balance, activation of the immune system, apoptosis, hepatocyte necrosis, and mitochondrial dysfunction. MTX inhibits pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and cytosolic nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenase. NADP and glutathione reductase enzyme are used to produce reduced glutathione (GSH), which is a protective antioxidant against reactive oxygen species (ROS) (Babiak et al., 1998). It has been reported to cause hepatocyte damage by lowering NADP levels due to MTX use and glutathione levels, which sensitize hepatocytes to reactive oxygen radicals (Uraz et al., 2008).

Ranolazine (RAN) is a drug that has anti-anginal and anti-arrhythmic effects (Chaitman et al., 2004). It regulates intracellular  $\text{Ca}^{2+}$  by inhibiting late Na channels in cardiac cells, which may have an antioxidant effect by suppressing the formation of SOR (Song et al., 2006). Recent studies have also shown that RAN has anti-inflammatory effects, inhibiting markers such as C-reactive protein, IL-1, IL-6, and TNF- $\alpha$  (Likozar and Å ebeÅ, 2021). RAN has been shown to inhibit fatty acid uptake and oxidation in the liver by inhibiting cell membrane permeability and mitochondrial electron transfer with pyridine nucleotides (Mito et al., 2010). Moreover, RAN has been found to be effective not only in the heart but also in liver mitochondria, suggesting that it may act on many cells with aerobic metabolism (Wyatt et al., 1995).

As the liver is a metabolic organ where RAN undergoes biotransformation, it is important to suppress oxidative stress mechanisms that cause hepatotoxicity, particularly with the wide use of MTX. Therefore, the aim of this study was to investigate the therapeutic effects of RAN on MTX-induced liver toxicity in terms of oxidative stress, antioxidant capacity, and liver fibrosis.

## MATERIALS and METHODS

The study was conducted with the approval of the Ethics Committee of İnönü University Experimental Animals (protocol No: 2019/A-20), and all experimental procedures adhered to the ARRIVE guidelines (Colak et al., 2012). A total of 32 female Wistar Albino breed rats aged 4-6 months were obtained from İnönü University Experimental Animal Production and Research Center and were housed in rooms maintained at 60-65% humidity, constant heat of  $25\pm 2^\circ\text{C}$ , and ventilation. The rats were exposed to 12 hours of light and 12 hours of darkness and were provided with standard commercial rat feed (pellets) and tap water *ad libitum*.

The rats were randomly assigned to the following groups:

- \* Control group: the experiment was terminated on the 5th day after administering 0.5 ml intraperitoneal (i.p.) saline.
- \* MTX group: the experiment was terminated on the 5th day after administering 20 mg/kg MTX i.p.
- \* RAN+MTX group: RAN 100 mg/kg/day per oral (p.o.) was administered for 7 days, and a single dose of MTX 20 mg/kg i.p. was administered on the 2nd day after RAN administration. The trial was terminated on the 7th day.
- \* MTX+RAN group: RAN 100 mg/kg/day p.o. was administered for 5 days starting from the 5th day after administering a single dose of MTX 20 mg/kg i.p. The trial was terminated on the 10th day.

Prior to the experiment's termination, the rats were weighed using a highly sensitive weighing instrument. At the end of the experiment, the rats were anesthetized with i.p. 5 mg/kg xylazine and 75 mg/kg ketamine before being sacrificed. To conduct serum biochemical analysis [alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH)], 4-6 mL of blood was taken from the heart using a sterile syringe, centrifuged for 10 minutes at 3000 rpm, and stored at  $-80^\circ\text{C}$ . The midline was cut, and weighing was performed after total hepatic resection. For histopathological examinations, the lobus hepatis dexter was separated and fixed with 10% formaldehyde-containing tubes. The lobus hepatis sinister was wrapped in aluminum paper and held at  $-80^\circ\text{C}$  for the measurement of tissue biochemical parameters [superoxide dismutase (SOD), catalase (CAT), reduced glutathione (tGSH), and malonyldialdehyde (MDA)].

The biochemical analysis involved determining AST, ALT, ALP, and LDH levels from the serum samples obtained after centrifugation, using the spectrophotometric method in an Architect C16000 device (Abbott, Chicago, IL, USA) based on the manufacturer's instructions (Vardi et al., 2010).

## Materials

The study employed MTX (50 mg/ml, Kocak Farma, Istanbul) and RAN (Latixa, 375 mg extended-release tablet, Menarini, Istanbul) preparations.

## **Tissue Biochemistry**

### **Homogenization of tissues**

Liver samples weighing 0.1 g were collected in plastic tubes and supplemented with 1 mL of pH 7.4 PBS buffer. The tissue samples were homogenized using a homogenizer under ice isolation until complete disintegration. The resulting homogenates were sonicated four times at 30-second intervals using a sonificiper under ice insulation. Subsequently, the homogenates were centrifuged for 15 minutes at 14,000 rpm and +4degC using a cooled centrifuge. Finally, the obtained supernatants were stored at +4degC until further analyses.

### **Determination of total protein**

The total protein concentration in the samples was determined using the Bradford method (Bradford, 1976), a rapid and straightforward technique. This involved adding the Bradford reagent to the homogenate samples and measuring the absorbance change at 550 nm. Bovine serum albumin was employed as the standard protein.

### **Determination of catalase activity**

The activity of the CAT enzyme was analyzed using Aebi method (Aebi, 1974), which is based on the absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm. Initially, the absorbance was adjusted to 0.7-0.9 by adding concentrated H<sub>2</sub>O<sub>2</sub> to the Na-K-phosphate buffer solution (pH: 7). Subsequently, 1 mL of the prepared mixture was placed in a quartz bath, and the final supernatant was added. After gentle stirring, the absorbance of H<sub>2</sub>O<sub>2</sub> ( $\epsilon = 0.0396 \text{ cm}^2/\mu\text{mol}$ ) was monitored using the Shimadzu 1601-UV visible spectrophotometer for 60 sec at 240 nm. The results were expressed as U/mg protein.

### **Determination of superoxide dismutase activity**

The activity of the SOD enzyme was determined using the method previously reported by McCord and Fridovich (McCord and Fridovich, 1969). This method involves preventing the SOD enzyme from reducing cytochrome-C by superoxide radicals formed in the xanthine/xanthine oxidase system. The activity of the SOD enzyme is based on measuring this inhibition. The amount of SOD that inhibited this reaction by 50% was considered to be 1 Unit (U). SOD enzyme activity was measured at 550 nm by kinetic monitoring, and the results were expressed as U/mg protein.

### **Reduced Glutathione Measurement**

GSH levels were measured using the method reported by Akerboom and Sies (Akerboom and Sies, 1981). This measurement method involves spectrophotometric measurement at 412 nm of the increase in 5-tio-2-nitrobenzoate (TNB) formation as a result of the reaction of DTNB, NADPH, and glutathione reductase. The absorbance change was monitored by UV-vis pectrophotometry at 412 nm for 15 min. The results were presented in nmol/mg protein.

### **Malondialdehyde Measurement**

Measurement of MDA was determined using the method previously reported by Placer et al (Placer et al., 1966). This method is based on the measurement of the pink-colored product formed at 532 nm as a result of the reaction between MDA, the breakdown product of peroxidized lipids, and thiobarbituric acid (TBA). The standard used in this method was 1,1,3,3-tetraethoxypropane. The MDA content of the samples was expressed as nmol/g protein.

### **Histopathological examinations**

Liver sections (4  $\mu\text{m}$ ) was performed using hematoxylin-eosin (H&E) staining for general morphology and Masson's trichrome staining for hepatic fibrosis. The sections were stained on poly-L-lysine coated slides using a Bcl-2 antibody (Novus Biologicals, LLC 8100, catalog number: NB100-56101, Southpark Way, A-8 Littleton, CO 80120, USA) and a Ventana Benchmark XT immunohistochemical staining device (Ventana

Medical Systems, Roche Group, Tucson, AZ, USA). All sections were examined using a light microscope (Olympus BX53, Tokyo, Japan).

MTX-induced liver injury was evaluated histomorphologically according to the Roenigk classification as follows:

Grade 1: Normal tissue, no fat change/mild, no nuclear pleomorphism/no mild nuclear pleomorphism, no fibrosis, and the presence of mild portal inflammation.

Grade 2: Moderate/severe fat changes, moderate/severe nuclear pleomorphism, no fibrosis, or the presence of moderate/severe portal inflammation.

Grade 3a: Mild fibrosis, portal fibrotic septa extending to the lobules and portal dilation.

Grade 3b: Presence of moderate/severe fibrosis.

Grade 4: Cirrhosis, regenerated nodules and bridging of portal paths.

Sections stained with the Bcl-2 antibody were scored semi-quantitatively and evaluated according to the Rappaport model (Samdanci et al., 2019). In this model, three regions were defined according to blood flow: zone 1 (periportal area), zone 2 (midzonal area), and zone 3 (centrilobular area). The Bcl-2 antibody staining was scored as follows:

Score 0: No staining with the Bcl-2 antibody.

Score 1: Positive staining of 1-2 rows of hepatocytes with the Bcl-2 antibody in zone 3.

Score 2: Positive staining of hepatocytes diffuse with the Bcl-2 antibody in addition to score 1.

Score 3: Positive staining of hepatocytes in zone 1 and zone 2 with the Bcl-2 antibody.

### Statistical Analysis

The data obtained from the study were evaluated using the SPSS (Statistical Package for Social Sciences) version 23.0 program. The Shapiro-Wilk test was used to check the normal distribution of the data. Parametric tests were used for normally distributed data, and non-parametric tests were used for non-normally distributed data. The results were expressed as mean  $\pm$  standard deviation (SD). The statistical significance level was considered as  $p < 0.05$ . One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons, and the Kruskal-Wallis test followed by Dunn's post-hoc test was used for non-parametric tests. The correlation between the variables was evaluated by Pearson's or Spearman's correlation test, depending on the distribution of the data. The statistical significance level was considered as  $p < 0.05$ .

### RESULTS

This study involved 32 rats divided into Control, MTX, MTX+RAN, and RAN+MTX groups. The experiment was terminated in the Control and MTX+RAN groups without mortality, while 1 rat from the MTX group and 2 rats from the RAN+MTX group died during the experiment. The same number of rats was added to these groups to maintain equal numbers. Before the end of the experiment, the weight of rats and liver was measured and found to be comparable among the groups ( $p > 0.05$ ) (Figure 1).

The study analyzed serum biochemical findings, tissue biochemistry, and histopathological results. The results showed a statistically significant increase in serum AST, ALT, ALP, and LDH levels in the MTX group compared to the Control group. However, the MTX+RAN and RAN+MTX groups resulted in significant improvement compared to the MTX group. The biochemical results were presented in Figure 2.

MDA values were significantly increased in the MTX group, while CAT value decreased compared to the Control group. In the MTX+RAN group, the SOD value showed a significant decrease compared to the Control group, whereas SOD and the MDA value showed a significant decrease compared to the MTX group. When the RAN+MTX group was examined, MDA value significantly decreased compared to MTX

and MTX+RAN groups. However, no significant change was observed for tGSH levels among the groups. The results were presented in Figure 3.

### Histopathological Results

Microscopic livers of rats belonging to the control group were observed with normal morphological structure. Histopathological results showed a significant increase in mononuclear inflammation, vascular congestion, ductal proliferation, and vacuolization consistent with hepatocyte damage in the MTX group compared to the Control group ( $p < 0.001$ ). The RAN+MTX and MTX+RAN groups showed a significant reduction in vascular congestion and ductal proliferation compared to the MTX-treated group alone ( $p < 0.001$ ). There was no significant difference in Roening grades observed when the RAN+MTX and MTX+RAN groups were compared with the MTX group (Figure 4). The administration of RAN was found to be promising in preventing and alleviating histopathological damage. Overall, the study suggests that RAN administration can prevent hepatocyte damage caused by MTX.

### DISCUSSION

Methotrexate (MTX) is commonly used for the treatment of cancer and autoimmune diseases (Pivovarov and Zipursky, 2019). However, its adverse effects on the liver, kidney, small intestine, and central nervous system remain a significant concern (Koroglu et al., 2021; Pannu, 2019; Samdanci et al., 2019; Vardi et al., 2012; Vardi et al., 2013). Various pathways, such as free oxygen radicals, intracellular ion imbalance, mitochondrial dysfunction, and apoptosis, play a crucial role in MTX-induced hepatotoxicity (El-Sheikh et al., 2015; Pivovarov and Zipursky, 2019). Suppression of these pathways and prevention of ROS formation are essential for reducing the severity of MTX-related organ damage (Jaeschke et al., 2002; Song et al., 2006). Recent studies have investigated the mechanism of action of ranitidine (RAN) in this regard (Naveena et al., 2018).

Our study demonstrated that increased levels of biochemical markers such as AST, ALT, ALP, and LDH in the blood of rats indicate hepatocyte damage caused by MTX (McGill, 2016). The elevation of malondialdehyde (MDA), an oxidant marker, suggests that oxidative stress plays a significant role in MTX-induced hepatotoxicity. In the RAN+MTX and MTX+RAN groups, we observed a decrease in liver function parameters and MDA levels, indicating that RAN's mechanism of action in our hepatotoxicity model reduces oxidative stress. Our histopathological data also supports the potential of RAN as a treatment for MTX-induced hepatotoxicity.

In a study by Ghoneum et al. reported that ALT, AST, and ALP values significantly increased with MTX application, consistent with our findings (Ghoneum and El-Gerbed, 2021). We also observed an improvement in ALT, AST, ALP, and LDH values in RAN-treated groups. Additionally, Esenboğa et al. reported that RAN significantly reduced ALT and AST levels in rats with coronary artery disease and non-alcoholic fatty liver disease (Esenboga et al., 2022), further supporting the potential of RAN in preventing hepatocyte damage (Han et al., 2018; Imprialos et al., 2022).

MDA is a marker that increases in various conditions such as liver toxicity, cardiovascular diseases, diabetes, and cancers and is associated with oxidative stress (Aslankoç et al., 2019; Zhang et al., 2006). Our study revealed a significant increase in MDA levels in rats given MTX, suggesting that oxidative stress is one of the mechanisms by which MTX generates liver damage. However, the significant decrease in MDA levels in the MTX+RAN group indicates that RAN administration provides inhibition in oxidative stress and may be used in the treatment of hepatocyte damage. Notably, we observed a decrease in MDA levels in the RAN+MTX group compared to the MTX and MTX+RAN groups, suggesting that RAN may be more beneficial in prophylactic use than in treatment. Other studies in the literature corroborate our findings (Krishna et al., 2016; Matsumura et al., 1998; Ramakrishna et al., 2014; ullah Baig et al., 2020).

Jahovic and colleagues observed that MTX increased levels of MDA and myeloperoxidase in the blood, while decreasing tissue levels of GSH (Jahovic et al., 2003). In another study, Arinno et al. investigated the therapeutic effects of RAN, melatonin, and metformin on doxorubicin-induced cardiotoxicity. They demonstrated

that RAN administration resulted in decreased MDA levels and improved left ventricular function (Arinno et al., 2021).

The balance between antioxidants and free oxygen radicals is crucial for maintaining cellular homeostasis. The disruption of this balance, favoring reactive oxygen species (ROS), can lead to various forms of cellular damage. Antioxidant mechanisms are characterized by markers such as superoxide dismutase (SOD), catalase (CAT), and GSH. MTX has been shown to activate the oxidative stress response (SOR) mechanism. Miyazono and colleagues investigated the role of oxidative stress in small intestine damage induced by MTX in rats. They found that MTX administration reduced levels of antioxidant markers such as SOD, CAT, and GSH, while increasing MDA levels as an indicator of oxidative stress. In another study, Had and colleagues evaluated the histological and biochemical effects of MTX on liver tissue in Wistar Albino rats. The biochemical parameters examined revealed increased MDA values, decreased SOD, CAT, and GSH levels, and elevated AST and ALT values by a factor of 7 (Vardi et al., 2012).

In our study, we observed a significant reduction in CAT levels in the MTX group compared to the control group, while levels increased in the RAN-treated groups. These results suggest that RAN acts through an antioxidant mechanism to prevent MTX-induced damage. Interestingly, there were no significant changes in GSH levels among the groups, and SOD levels decreased in the treatment and prophylaxis groups compared to the control and MTX groups. This may suggest that the antioxidant mechanism and the adaptation process against oxidative stress were not yet established, and the markers did not reach the desired level during the chronic phase. Alternatively, the study may be more effective by increasing the number of rats (Kisaoglu et al., 2013; Spolarics, 1998).

Our histopathological results showed that MTX caused damage to inflammatory lesions and hepatocytes, while RAN alleviated hepatocyte degeneration. The livers of rats in the control group showed normal morphological structures under microscopic observation, but the livers of rats in the MTX group exhibited statistically significant increases in mononuclear inflammation, vascular congestion, ductal profibrosis, and vacuolization findings consistent with hepatocyte damage. Upon examination of the Roening grade (Samsanci et al., 2019), a significant increase in fibrosis was observed in the MTX group as compared to the control group, providing evidence of the damaging effect of MTX on fibrosis. However, in the RAN+MTX and MTX+RAN groups, significant reductions in vascular congestion and ductal proliferation were noted as compared to the MTX group. These results suggest that RAN administration has the potential to prevent hepatocyte damage and facilitate liver tissue regeneration. Similar findings have been reported in other studies. For instance, Dalaklioglu et al. (2013) showed that the MTX group exhibited histopathological alterations such as degenerate hepatocytes, vascular occlusion in sinusoids, dilatation of sinusoids, and increased activation of Kupffer cells (Dalaklioglu et al., 2013).

## Conclusion

Our study demonstrated that the use of MTX resulted in liver tissue damage, leading to both biochemical and histopathological changes. However, RAN may be a promising alternative agent by activating the antioxidant mechanism and suppressing the ROS mechanism in the liver. RAN is a new antianginal agent with an antioxidant mechanism that warrants further investigation. To our knowledge, this is the first study to investigate the role of RAN in preventing and treating MTX-induced liver damage. Our findings suggest that RAN may be a potential hepatoprotective agent for future clinical applications.

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## Contributions

MEP, ES, LHT, MG and HP: Designed the experiments; MEP, ES, LHT, and MG: Performed experiments and collected data; LHT, MG, MA, AFS, BA and HP: Discussed the results and strategy; MA, AFS, BA

and HP: Supervised, directed and managed the study; MEP, ES, LHT, MG, MA, AFS, BA and HP: All authors have seen the final version of the paper.

### Declaration of Interest Statement

The authors report there are no competing interests to declare.

### Data Availability Statement

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

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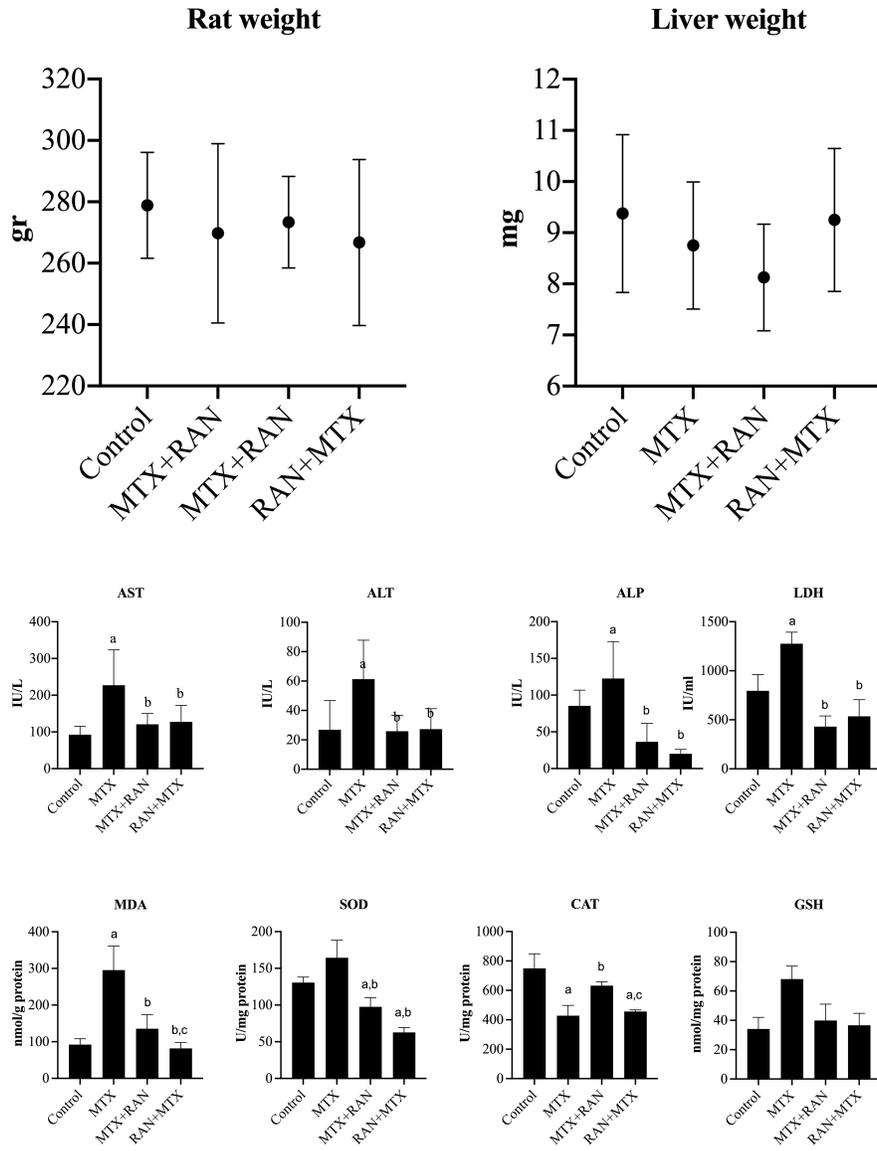
## Figure Legends

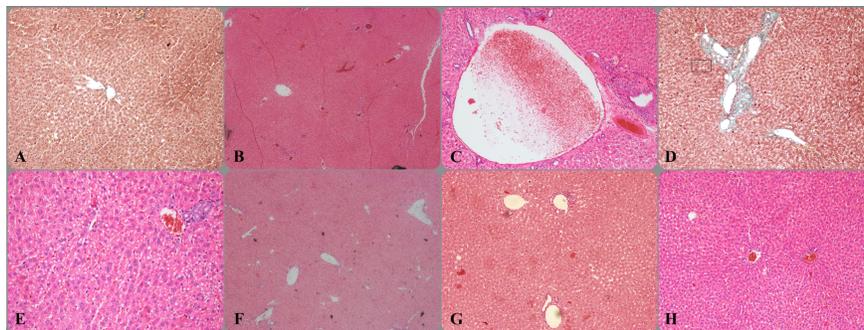
**Figure 1.** Rat and liver weights in the study.

**Figure 2.** Liver function parameters among the groups.

**Figure 3.** Tissue oxidant and antioxidant parameters in the study.

**Figure 4.** Histopathological alterations and scores in the study. **A:** Inflammation and fibrosis in the Control group (10X, Masson trichrom staining), **B:** Inflammation, congestion, and vacuolization in the MTX group (4X, H&E staining), **C:** Ductal proliferation and congestion in the MTX group (10X, H&E staining), **D:** Ductal proliferation and fibrosis observed at 10X magnification with Masson trichrom staining in the MTX group (10X, Masson trichrom staining), **E:** Vacuolization and congestion detected in the MTX group (20X, H&E staining), **F:** Inflammation observed in the MTX+RAN group (4X, H&E staining), **G:** Inflammation in the RAN+MTX group (4X, H&E staining), **H:** Congestion and minimal vacuolization in the RAN+MTX group (4X, H&E staining).





Groups	Mononuclear inflammation	Vascular congestion	Ductal proliferation	Vacuolisation	Fibroblastic activity	Roening Grade
Control	0 (0-1)	0,5 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)	1 (1-1)
MTX	1 (1-2) <sup>a</sup>	2 (1-3) <sup>a</sup>	3 (2-3) <sup>a</sup>	1,5 (1-2) <sup>a</sup>	0 (0-0)	2 (2-2) <sup>a</sup>
MTX+RAN	1,5 (0-2)	1 (0-1) <sup>b</sup>	1 (0-2) <sup>b</sup>	1 (0-2)	0 (0-0)	2 (2-2)
RAN+MTX	1 (1-2)	1 (1-2) <sup>b</sup>	1 (1-2) <sup>b</sup>	1 (0-2)	0 (0-0)	2 (2-2)

Histopathological scores are expressed as median (min-max). n=8 for each group.

<sup>a</sup>p<0.001 vs. Control group.

<sup>b</sup>p<0.001 vs. MTX group.