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RESEARCH

Therapeutic potential of morin hydrate in methotrexate-induced liver in experimental rats: regulation of organ function and alleviation of oxidative stress

Deneysel sıçanlarda metotreksat ile indüklenmiş karaciğerde morin hidratın terapötik potansiyeli: organ fonksiyonlarının düzenlenmesi ve oksidatif stresin azaltılması

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Abstract

Purpose: Methotrexate (MTX) is an antineoplastic agent with a wide clinical use. However, its complications in tissues cause limitations. The effects of many compounds have been studied in reducing the toxicity of MTX in liver tissue. There are limited studies on the elimination of this damage with morin hydrate (MH) flavonoid. In this study, we investigated the effect of MH in MTX-induced hepatoxicity with a focus on oxidative stress.

Materials and Methods: In the experiment, 32 female rats were grouped as Control, MTX, MH, MTX+MH (n=8). On the first day of the study, MTX 20 mg/kg single dose was administered ip. MH was administered 100 mg/kg by gavage for 10 days. On the 11th day, biochemical and histopathological analyses were performed on liver tissues from rats. All data were presented with statistical comparison.

Results: Serum AST, ALT and LDH levels were highest in the MTX group and lower in the MTX+MH group. MH increased glutathione peroxidase (GPX), reduced glutathione (GSH) and total antioxidant capacity (TAC) levels, but had no regulatory effect on superoxide dismutase (SOD) level. Additionally, MH significantly lowered malondialdehyde (MDA) levels and reduced total oxidant capacity (TOC). Histopathological findings included inflammation, congestion, degeneration, mononuclear cell clusters and dead cells. Tissue damage was most severe in the MTX group, whereas these effects were attenuated in the MTX+MH group.

Öz

Amaç: Metotreksat (MTX) geniş klinik kullanımı olan antineoplastik bir ajandır. Bununla birlikte, dokulardaki komplikasyonları sınırlamalara neden olmaktadır. MTX'in karaciğer dokusundaki toksisitesini azaltmada birçok bileşikle çalışılmıştır. Morin hidrat (MH) flavonoidi ile bu hasarın giderilmesi konusunda sınırlı sayıda çalışma bulunmaktadır. Bu çalışmada MTX ile indüklenen hepatoksisitede oksidatif strese odaklanılarak MH'nin etkisi araştırıldı.

Gereç ve Yöntem: Deneyde 32 dişi sıçan Kontrol, MTX, MH, MTX+MH olarak gruplandırıldı (n=8). Çalışmanın ilk gününde MTX 20 mg/kg tek doz ip olarak uygulandı. MH 10 gün boyunca 100 mg/kg gavaj yoluyla uygulandı. 11. günde sıçanlardan alınan karaciğer dokuları üzerinde biyokimyasal ve histopatolojik analizler yapıldı. Tüm veriler istatistiksel karşılaştırma ile sunulmuştur.

Bulgular: Serum AST, ALT ve LDH düzeyleri MTX grubunda en yüksek, MTX+MH grubunda ise daha düşüktü. MH glutatyon peroksidaz (GPX), indirgenmiş glutatyon (GSH) ve toplam antioksidan kapasite (TAC) düzeylerini artırırken, süperoksit dismutaz (SOD) düzeyi üzerinde düzenleyici bir etkisi olmamıştır. Ayrıca, MH malondialdehit (MDA) seviyelerini önemli ölçüde düşürmüş ve toplam oksidan kapasiteyi (TOC) azaltmıştır. Histopatolojik bulgular arasında enflamasyon, konjesyon, dejenerasyon, mononükleer hücre kümeleri ve ölü hücreler yer almıştır. Doku hasarı en şiddetli MTX grubunda görülürken, MTX+MH grubunda bu etkiler hafiflemiştir.

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Conclusion: Our results indicated that MH may be a potential therapeutic agent in MTX-induced acute liver injury.
Keywords:. Antioxidant, hepatoxicity, liver function test, methotrexate, morin hydrate, oxidative stress

Sonuç: Sonuçlarımız MH'nin MTX ile indüklenen akut karaciğer hasarında potansiyel bir terapötik ajan olabileceğini göstermiştir.
Anahtar kelimeler: Antioksidan, hepatoksisite, karaciğer fonksiyon testi, metotreksat, morin hidrat, oksidatif stres

INTRODUCTION

Methotrexate (MTX) is an antimetabolite from the subgroup of neoplastic drugs developed by Faber and Subbarrow in the 1940s to cope with childhood leukaemia. During that time, the idea of depriving tumor cells of folic acid to reduce cell proliferation was introduced. Antifolate molecules, aminopterin and amethopterin, were synthesized, and eventually, the expected effects were observed[1,2]. Later, these compounds were developed and marketed as MTX (N-10-methylaminopterin), which is the form used today. MTX, which was noticed to have a steroid-like effect, has been used in the treatment of many diseases such as rheumatoid arthritis, ectopic pregnancy, inflammatory bowel diseases, psoriasis, systemic lupus erythematosus and alleviation of complications of organ transplants[3]. Due to its wide treatment network in malignancy and non-malignancy, it has been recognized by the WHO as one of the essential drugs with a high successrate. Therefore, it is a drug utilized by millions of individuals[4].

MTX enters into competitive inhibition with folate and inhibits the MTX dihydrofolate reductase enzyme, which has a lower Km (affinity of the enzyme to the substrate). Folate deficiency within the cell disrupts RNA and, DNA synthesis, as well as DNA repairall of which rely on folate leading to apoptosis and impaired cellular replication[4]. At the same time, MTX shows anti-inflammatory and immunomodulatory effects by inhibiting the Jak-stat pathway and releasing adenosine into the extracellular space[5,6]. Additionally, complications arise either form dosage and duration of the drug or from underlying conditions. It causes myelosuppression and toxicity in many other tissues (kidney, liver, pulmonary, gasrointestine, cutaneous) [5]. MTX is metabolized by the liver's enzymatic system, and 90% of it is eliminated through the kidneys. MTX leads to a reduction in nucleotide levels, which in turn decreases the availability of reducing forces (NADPH, NADH and FADH) whitin the cell, imparing the fucntion of enzymes that rely on them[4]. Toxicity arises when reactive oxygen species, generated by the accumulation of intracellular MTX derivatives in hepatocytes, cannot be neutralized due to a deficiecny in reducing agents[7].

Smart drugs, herbal products and nanoparticles are utilised prevent complications caused by many drugs used for treatment[5,8]. Among these agents, morin hydrate (MH) is a yellow crystalline flavonol found in white mulberry, guava, osega orange, apple peel, almond, sweet chestnut and many other plants. MH, which is an isomeric form of quarsetin, is characterised by the presence of hydroxyl groups at the 3' position, which emphasises its radical scavenging effect[7,9,10]. Indeed, many effects of MH has such as antioxidant, antilipidemic, antidiabetic, anti-inflammatory and neuroprotective effect which are included in the literature[11,12].

In this study, we tested the hypothesis that MH would have a therapeutic effect on MTX-induced liver damage, especially by prioritising its antioxidant and anti-inflammatory properties, by oxidative stress biomarkers, liver function tests and microscopic examination. MH regulated liver function, its antioxidant properties were clearly demonstrated and this feature was revealed by many damage scores in tissue microscopy. The findings of the study provide a focal point for further studies at the molecular level, suggesting that it may be a potential therapeutic candidate.

MATERIALS AND METHODS

Chemicals and kits

MTX 50 mg/ 5 ml was supplied by Koçak Farma (Istanbul), MH (2',3,4',5,7-Pentahydroxy-flavone) was supplied by Acros Organics (CAS Number: 654055-01-3, Germany). Total antioxidant capacity (TAC) commercial kit (Lot. No: EL2213A, Gaziantep), total oxidative level (TOC) commercial kit (Lot. No: EL22145O, Gaziantep) were obtained from Rel Assay, superoxide dismutase (SOD, Cat. No: E0168Ra, Chine), glutathione peroxidase (GPX, Cat.No: E1172Ra, Chine) were obtained from Bt Lab and all chemicals/reagents were obtained from Merck, Sigma Aldrich or other brands.

Experimental animals

In this study, 32 female Wistar albino rats, weighing 200-300 g, 12-13 weeks old, were used. The rats were obtained from Bolu Abant Izzet Baysal Experimental Animals Application and Research Centre (Bolu, Turkey). The rats were kept under conventional laboratory conditions throughout the study (22 ± 1 °C, 12-h day/night cycle, free access to tap water and food). The study was implemented with the approval of the Bolu Abant Izzet Baysal University Animal Research Local Ethics Committee (Bolu, Turkey) (Decision number: 2020/12/A-1) and in accordance with the decisions of the Universal Declaration of Animal Rights (October 15, 1978) adopted by the institution. In addition, all applications were carried out by TK, SS, IT, SHWW, who are in the study team, under the supervision of the responsible veterinarian of the institution.

Experimental design

The number of samples was determined based on similar animal models and parameters in the literature [13]. Thirty-two rats whose adaptation process was completed were divided into four groups with eight animals in each group:

Control group: Each rat in the group was given a single dose 0,9% NaCl (ip, the first day) of and tris-HCl by gavage every day.

MTX group: On the first day, a single dose of MTX (20 mg/kg, i.p.) diluted with 0.9% NaCl was administered to each rat in the group [5].

MH group: MH (100 mg/kg) dissolved in tris-HCl was administered to each rat in the group by gavage [14].

MTX+MH group: Each rat in the group was treated with a single dose of MTX (20 mg/kg, ip) on the first day and MH 100 mg/kg by gavage every day.

Sample collection and preparation

On the 11th day of the experiment, rats were anaesthetised with ketamine (90 mg/kg) and xylazine (10 mg/kg) by ip injection, and blood samples were were taken directly by cardiac puncture. The blood samples were transferred into gel tubes and centrifuged at 4000 rpm for 10 minute and the serums obtained were transferred into eppendorf tubes and stored at -80 °C until the time of analysis. Then, the liver tissues of the rats were removed and washed in cold phosphate buffered saline (PBS). Some of the liver tissue was embedded in 10% formaldehyde for histopathological analyses. Some of it was frozen in liquid nitrogen and stored at -80 °C until analysis. On the day of analysis, the liver tissues brought to room temperature were homogenised by adding phosphate buffer 1/10 (w/v, pH 7.5). The homogenates obtained were centrifuged at 4000 rpm for 15 minutes at +4 °C. The supernatants in the tubes were prepared for biochemical analyses.

Evaluation of biochemical parameters

Liver function test biomarkers aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels in serum samples were analyzed using commercial kits on a Siemens brand autoanalyzer. MDA was analysed by spectrophotometric reading at 532 nm wavelength using a modified version of the thiobarbituric acid reaction method developed by Ohkawa et al.[15]. Reduced glutathione level (GSH) was obtained by spectrophotometric measurement of the colour formed by thiol groups of 5.5'-dithiobis-(2-nitrobenzoic acid) reagent developed by Beutler et al.[16] at a wavelength of 412 nm. SOD, GPX analyses were performed by ELISA method based on ready-made commercial kits sandwich method. TAC, TOC measurements were performed with ready-to-use kits developed by Erel[17,18]. TAC was measured by reading the colour reduction at 660 nm as a result of reduction of 2,2"-azinobis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{+}$) by antioxidants present in the sample and the results were expressed as mmol Trolox Equiv /L. As for TOC, oxidants in the sample caused the conversion of iron (Fe^{+2}) ion to ferric (Fe^{+3}) ion and the colour intensity of these ions in acidic medium was determined spectrophotometrically (at 560 nm) and the results calibrated with H_2O_2 were expressed as μmol H_2O_2 Equiv /L.

Histopathological analysis

Liver tissues were fixed with 10% buffered formalin and processed and embedded in paraffin. Sections (5 μm) taken from paraffin blocks were stained with haematoxylin-eosin (H&E). The sections were analysed blindly by a pathologist. Passive/severe congestion, hyperaemia, periportal oedema, marked hydropic deneration, necrobiotic changes in hepatocytes, as well as mononuclear cell clusters in the sinusoids and/or periportal areas of the liver to

various degrees were recorded under light microscopy (Nikon Eclipse Ci connected to a Hayear® digital camera); the findings were scored as no lesions (0), mild lesions (1), moderate lesions (2) and severe lesions (3) and numerical data were obtained.

Statistical analysis

All statistical analyses were performed using Minitab (version 21) and IBM SPSS Statistic 27 Homogeneity of variances was evaluated by Shapiro Wilk test. Variables with homogeneous distribution (GSH, MDA, GPX, TAC, TOC and all histopathological damage scores) were evaluated using one-way ANOVA and post hoc Tukey test and Dunnet T3. Results were expressed as mean ± standard deviation. Variables not showing homogeneous distribution (ALT, AST, LDH, SOD) were analysed by Kruskal-Wallis H test and post hoc; Pairwise Comparison multiple comparisons. Results were presented as median (min-max). Statistical significance between groups was accepted as $p<0.05$.

RESULTS

Effect of MH on liver function tests of MTX-treated rats

Transaminases and LDH are enzymes used in the clinical evaluation of liver function. The findings showed that serum concentrations of ALT, AST and LDH increased significantly in the MTX group compared to the control group ($p <0.001$; Figure 1). Serum AST, ALT and LDH enzyme concentrations were significantly decreased in the group given MH together with MTX; MH showed a curative effect.

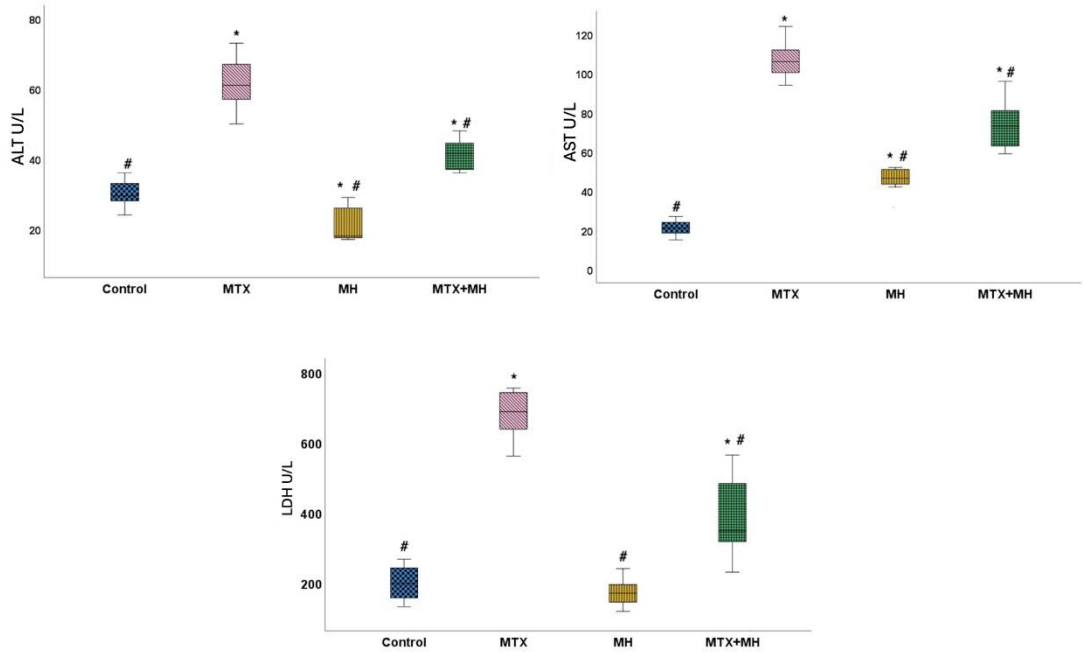

Figure 1. Liver function biomarkers according to groups.

Effect of MH on liver function biomarkers in MTX induction. Alanine transaminase (ALT), Aspartate transaminase (AST), Lactate dehydrogenase (LDH). All data are given as mean ± standard deviation (n=8). * $p <0.001$ indicates significance compared to control group, #$p <0.001$ indicates significance compared to MTX group. MTX: Methotrexate, MH: Morin hydrate

Effect of MH on oxidative stress parameters of MTX-treated rats

The results of biomarkers related to antioxidant status and oxidative stress of possible damage to liver tissue as a result of MTX induction are given in Figures 2, 3, 4. Compared with MTX group, MH treatment (MTX+MH group) substantially increased the intracellular antioxidant GSH ($p < 0.001$). In addition, GSH was distinctly decreased in the MTX group and increased in the MH-treated group compared to the control group ($p < 0.05$). There was a significant increase in MDA level in the MTX group compared to the control group ($p < 0.05$). This value, which reveals the presence of lipid peroxidation, was found to be considerably decreased with MH treatment ($p < 0.001$) (Figure 2).

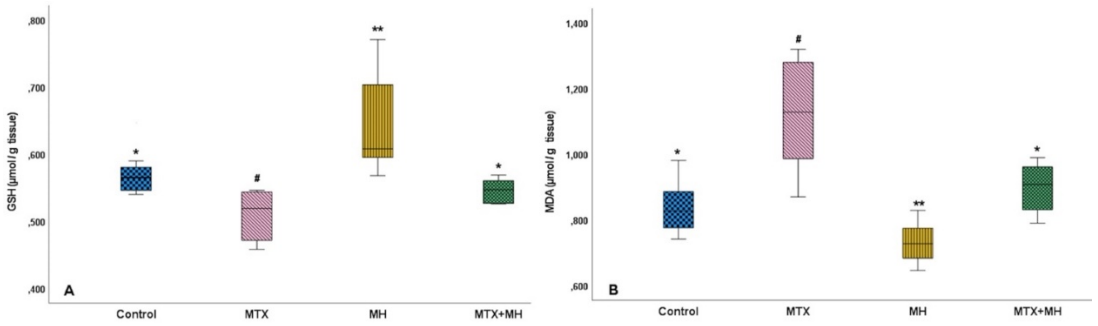

Figure 2. Intracellular antioxidant molecule and lipid peroxidation levels in liver tissue.

Reduced glutathione (GSH) and malondialdehyde (MDA) levels in liver tissue. All data are presented as mean ± standard deviation (n=8). *p <0.05 indicates significance according to MTX group, **p <0.001 indicates significance according to MTX group, #p <0.05 indicates significance according to control group. MTX: Methotrexate, MH: Morin hydrate

Antioxidant enzymes of the cell, GPX, SOD decreased in the MTX group ($p < 0.05$); GPX increased significantly in the MH-treated group ($p < 0.05$) (Figure 3). However, no significant result was obtained in the SOD, MTX+MH group ($p > 0.05$)

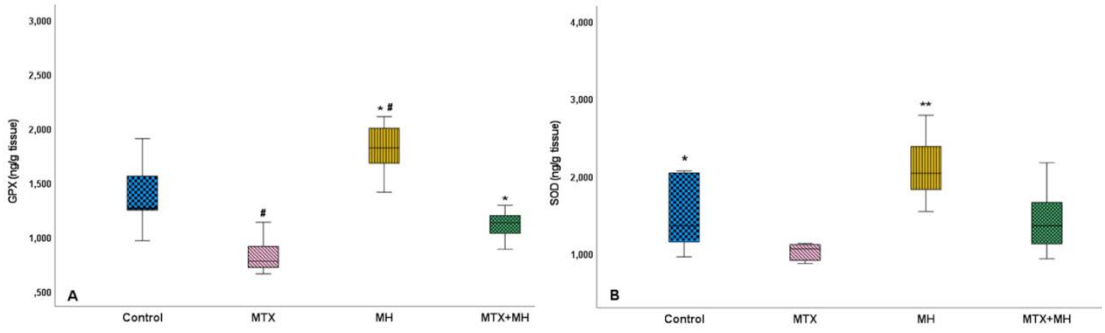

Figure 3. Intracellular antioxidant enzyme levels in liver tissue.

Glutathione peroxidase (GPX), superoxide dismutase (SOD) levels in liver tissue. All data were given as mean ± standard deviation (n=8). *p <0.05 indicates significance according to MTX group, **p <0.001 indicates significance according to MTX group, #p <0.05 indicates significance according to control group. MTX: Methotrexate, MH: Morin hydrate

The results of TAC and TOC analyses revealed that the highest TOC was in the MTX group and the lowest TAC was in the MTX group. While a essentially increase in TAC level was observed in MTX+MH group ($p < 0.05$), no important decrease in TOC level was observed ($p > 0.05$) (Figure 4.)

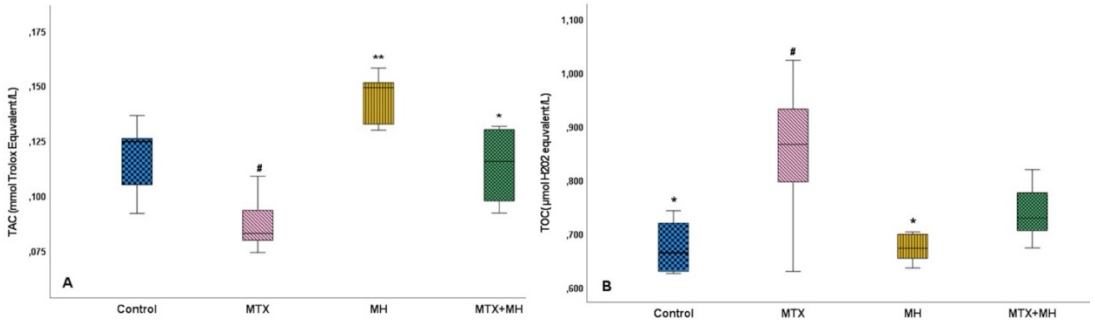

Figure 4. Total antioxidant and oxidant levels in the cell.

Total antioxidant capacity (TAC), total oxidant capacity (TOC) levels in liver tissue. All data were given as mean ± standard deviation (n=8). *p <0.05 indicates significance according to MTX group, **p <0.001 indicates significance according to MTX group, #p <0.05 indicates significance according to control group. MTX: Methotrexate, MH: Morin hydrate

Effect of MH on histopathological findings in hepatocytes of MTX-treated rats

H&E staining revealed MTX-induced damage in liver tissue. Severe congestion, marked hydropic degeneration, numerous dying cells and focal mononuclear cells were observed in the MTX group. In the group in which MTX induction was treated with MH, no important reduction was observed in the findings of Kupfer cell activation and fibrous connective tissue infiltration ($p>0.05$), whereas all other detected pathologies were considerably reduced ($p < 0.05$).

Table 1. Damage scores of histopathological findings in liver tissue

Group	Control	MTX	MH	MTX+MH
Congestion/hyperemia/ hemorrhage	0.13±0.35b*	2.17±0.75d*	0.13±0.35	0.63±0.74b*
Mononuclear cells infiltration	0.25±0.46db*	2±0.63da*	0.50±0.53b*	1.13±0.64ba*
Hydropic degeneration	0.38±0.58b*	2.33±0.51acd*	1±0.53b *	1.13±0.83b*
Fibrous connective tissues infiltration	0±0b*	1.33±0.51ac*	0.13±0.35b*	0.50±0.53
Kupffer cells activation	0.13±0.35b*	1.17±0,.40c*	0±0b*	0.50±0.53
Bile duct hyperplasia	0±0b*	1.33±0.51cd*	0.38±0.51b*	0.40±0.62b*

The significance of the differences between the groups is shown with the letters a, b, c and d for control, MTX, MH and MTX+MH groups, respectively. All data were given as mean ± standard deviation (n=8). * p values <0.001 were considered statistically significant. MTX: methotrexate, MH: morin hydrate, MTX+MH: methotrexate+ morin hydrate

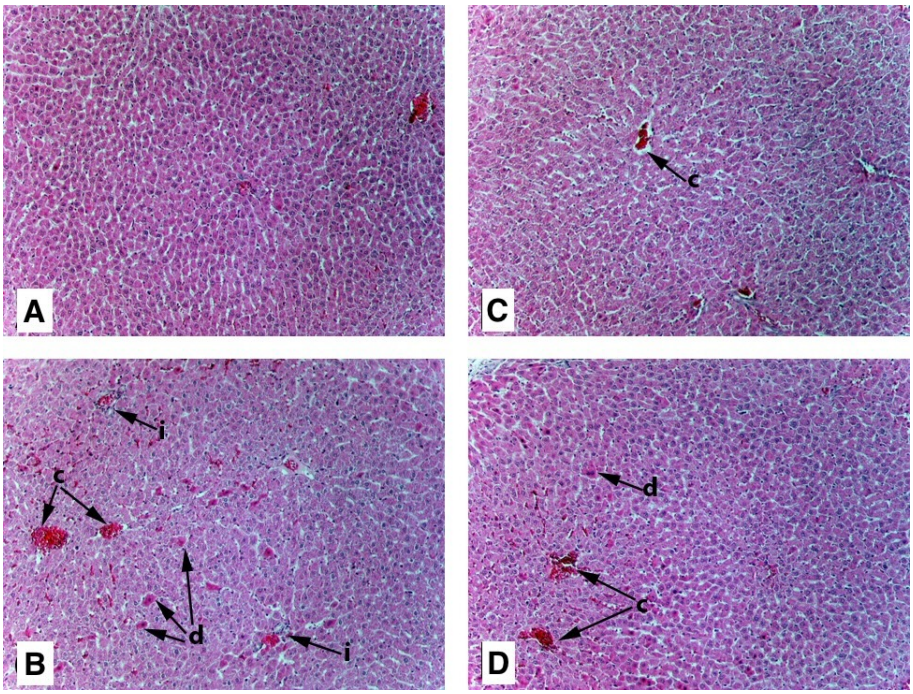

Figure 5. Histopathological findings in liver tissue.

Histopathological sections were examined by haematoxylin-eosin (H & E) staining method to observe the general histological structure of the kidney tissue. Histopathological findings using 10X magnification; A-Control group: Normal liver tissue, B-MTX group: Severe congestion, marked hydropic degeneration, many dead cells and focal mononuclear cells, C- MH group: Mild passive congestion, D-MTX+MH group: Significant reduction in severity of lesions compared to MTX group. Arrows pointed events, c: congestion, d: dying cells, i: inflammation.

DISCUSSION

MTX is a versatile anti-metabolite used in the indication of cancer, non-cancer; rheumatological diseases, inflammatory diseases and immunological diseases. However, it may cause side effects in many organs of the body. These side effects may occur in the presence of another accompanying disease or acutely dose-dependent[1,4]. The accumulation of downstream metabolites of MTX metabolized in the liver in hepatocytes causes many pathologies, including oxidative stress, inflammation and cell death[3]. As a result of the inability of the organism to remove such cytotoxic agents taken into the body, hydroxyl radicals, superoxide anions and hydrogen peroxides are released. These free radicals may lead to liver tissue damage[19]. Increased tissue exposure to MTX, which is also an inhibitor of dihydrofolate reductase enzyme, leads to a decrease in tetrahydrobiopterin, a cofactor of NO synthase (involved in nitric oxide (NO) synthesis) in the cell, which mediates an increase in reactive oxygen species[20]. In the clinic, folinic acid, glucarpidase and alkalisation treatments are used to prevent MTX complications[6]. Apart from these drugs developed for combined use, studies are being conducted with many herbal agents aiming to reduce complications[3,9,21]. MH is a flavonoid with various biological properties and low toxicity rate[10]. The therapeutic feature of MH in hepatoxicity caused by many agents is that it shows an antioxidant and anti-inflammatory effect [22,23]. In this study, the probable positive effects of MH compound in MTX-induced hepatoxicity were determined by serum liver function test, tissue antioxidant and oxidative stress parameters and histopathological examination results.

AST and ALT enzymes involved in amino acid transamination are mostly found in liver tissue. Serum levels of these transaminase enzymes, LDH and ALP increase in conditions such as cell

membrane damage and cell death of hepatocytes. These biomarkers have been reported to increase in hepatic damage caused by many agents[3,23,24]. In the MTX induction study of Kizil et al., serum ALT, AST and ALP levels were measured at the highest level in the MTX group and were measured at a lower level in the groups treated with morin[25]. In the present study, AST, ALT and LDH levels were measured at the highest level in the MTX group and downward improvement with MH confirmed the finding of the previous study. Here, we demonstrated that MH is an agent that regulates liver function.

Increased oxidative stress in the cell causing lipid peroxidation is an important condition that causes impairment of tissue function. The inability of reactive oxygen species (ROS) to be rendered harmless as a result of decreased antioxidant levels in the cell leads to the attack of polyunsaturated fatty acids (PUFA) in the cell membrane. MDA, the lipid peroxidation product released as a result of this event, is an endogenous genotoxic product[26]. Additionally, it is important for the cell to maintain its own GSH amount in terms of lipid peroxidation. Reduced GSH causes MDA formation by attacking PUFAs in the cell membrane as a result of decreased amount of NADPH reducing power in the cell cytoplasm and inability to neutralise the ROS[27]. In this study, GSH levels measured to evaluate the status of GSH-dependent lipid peroxidation repair system in liver tissue were found to be significantly lowered in the MTX group compared to the control group, whereas GSH concentration was found to be less decreased in the MH-treated group. In addition, while MDA levels were significantly higher in the MTX group compared to the control group, it was reported that MDA was considerably decreased in the MTX+MH group. Similar to this study, Samdanci et al. reported an increase in MDA levels and a decline in GSH levels in MTX-induced hepatoxicity[13].

Intracellular antioxidant enzyme SOD, which requires the presence of copper or manganese for its catalytic activity, is involved in rendering superoxide radicals more harmless. SOD converts superoxides to hydrogen peroxide and other intracellular antioxidant enzymes like GPX and CAT protect the intracellular antioxidant-oxidative balance by reducing them to water[13]. In the SOD analyses performed in liver tissues in this study, the lowest concentration obtained in the MTX group was significant compared to the control ($p < 0.05$). However, the SOD level in the MTX group treated with MH was not significantly higher than in the MTX group alone. Therefore, since no significant results were obtained on the SOD enzyme level of MH in MTX hepatoxicity, it is clear that further studies are needed to understand the mechanisms. The situation was slightly different in another parameter, GPX. While GPX was found to be very low in the MTX group compared to the control, higher GPX concentration was found in the MTX+MH group, suggesting that MH may improve oxidative damage by increasing the amount of this selenoprotein enzyme.

Previous studies have shown that oxidative damage caused by many agents in the liver leads to a decrease in antioxidant capacity[23]. In this study, MTX toxicity in hepatocytes was analysed by tissue TAC and TOC analysis. In the literature, few studies were found in which TAC and TOC levels of liver tissue were measured in MTX induction and results supporting our findings were obtained[19,28]. The number of studies on the effect of MH on total antioxidant/oxidant capacity in liver tissue was insufficient. MH increased the level of antioxidant molecules and enzymes, which were evaluated by concentration measurements or enzyme activity of substances such as GSH, GPX, SOD, CAT in the cell[22–24]. In the present study, TAC levels were the lowest in the MTX group, while it was slightly higher in the MTX+MH group, indicating that it may be due to the antioxidant property of MH. In TOC analyses, while the highest concentration was in the MTX group, oxidative capacity was not significantly decreased in the MH-treated group compared to MTX. However, the fact that there were significant results in TAC level in MTX+MH group but not in TOC suggested that more detailed studies on the effects of MH on intracellular oxidative stress and antioxidant levels could be performed.

Especially antineoplastic drugs used for treatment have many toxic effects. With the idea of how to reduce these toxicities, the effects of many drugs and many agents are investigated by combining them[13,23,24]. In this study, we investigated the presumed therapeutic effect of MH on the liver by histopathological examination to increase the accuracy of our biochemical analyses. In MTX-induced hepatoxicity, pathological findings in the study by Gurler et al. were necrotic, apoptotic cells, cell infiltration and congestion[19]. In the present study, microscopic examination of the damage in the liver tissue revealed the presence of congestion, hydropic degenerations and dead cells probably caused by their

progression. Additionally, the presence of mononuclear cells indicated inflammation in the tissue. The present study corroborated previous data on cellular damage in MTX-induced hepatoxicity. In addition, in the present study, these cellular damages were reported to be attenuated in the MTX+MH group, indicating that MH showed hepatoprotective properties (Table 1. and Figure 5 B, D).

The important limitations of this study are the lack of studies examining the molecular effects of MH in liver tissue in MTX induction. Another deficiency is the involvement of immune response in the hepatoxicity induced and it is thought that in vitro experiments should be performed simultaneously with in vivo due to the complexity of the mechanisms. In addition, since MTX is a drug with long-term use in the clinic, its effects can be examined by creating chronic models in experimental studies. However, in addition to oxidative stress and liver function tests, inflammation markers (TNF-α, IL-6, IL-1β, etc.), apoptosis (BAX, BCL-2, caspase-3, etc.) and necrosis parameters could have been analysed due to budgetary constraints.

In this study, it was revealed that MH alleviated the complication of MTX in the liver with its antioxidant and anti-inflammatory properties. Considering the antioxidant and anti-inflammatory effects of MH, it is necessary to evaluate the systemic effects of MH at a more molecular level or with more specific biomarkers. Additionally, while examining its effects in conditions such as inflammation and cell death, it is necessary to focus on certain pathways and to investigate through which pathways it acts. These results should be a target for future studies.

Author Contributions: Concept/Design : SS, TK, HK, IT; Data acquisition: SS, TK, MNB, MFB, HK, SHWW, IT; Data analysis and interpretation: SS, TK, MNB, MFB, HK, SHWW, IT; Drafting manuscript: SS, TK, HK; Critical revision of manuscript: SS, TK, HK; Final approval and accountability: TK, SS, MNB, MFB, HK, SHWW, İT; Technical or material support: SS, MNB, MFB, HK, İT; Supervision: TK; Securing funding (if available): n/a.
Ethical Approval: The study was carried out with the decision of the local ethics committee of Bolu Abant Izzet Baysal Animal experiments dated 01/08/2022 and decision numbered 2020/12/A-1. The rats used in the study were obtained from of Bolu Abant Izzet Baysal University Experimental Animals Application and Research Centre.
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