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Original article

Crocin ameliorates methotrexate-induced liver injury *via* inhibition of oxidative stress and inflammation in rats

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ABSTRACT

Background: Methotrexate (MTX) is used commonly in the treatment of various cancers and inflammatory diseases; nevertheless, the associated hepatotoxicity has limited its clinical application. Crocin (CRO) is described as a natural carotenoid with analgesic, antioxidant, and antiinflammatory properties. This study aimed to determine the effects of CRO on MTX-induced hepatotoxicity.
Methods: For pretreatment, CRO at doses of 25 and 50 mg/kg (*po*), as well as 20 mg/kg (*ip*) of MTX, was injected in rats.
Results: MTX led to hepatotoxicity, as confirmed by the significant increase in liver markers, histopathological changes, decreased GSH content, and reduced antioxidant enzyme activity (i.e., CAT, SOD, and GPx). It increased TNF-α, IL-1β, lipid peroxidation, and nitric oxide levels. Nevertheless, by increasing antioxidant defense in hepatic tissues and reducing oxidative stress and proinflammatory mediators, pretreatment with CRO could alleviate hepatotoxicity.
Conclusion: CRO can inhibit MTX-induced hepatotoxicity through improving antioxidant defense and reducing oxidative stress and inflammation.

Introduction

Methotrexate (MTX) is described as an anticancer antifolate agent used against multiple disorders, including osteosarcoma, head and neck tumors, and acute lymphoblastic leukemia. According to the literature, this chemotherapeutic drug not only affects cancer cells, but also involves normal cells with a high proliferation rate. Hepatotoxicity is an important side effect of MTX administration that limits the clinical application of this agent [1,2].

There is inadequate information about the mechanisms of MTX-mediated hepatotoxicity. As indicated in several studies, the formation of reactive oxygen species (ROS), besides nitric oxide (NO) synthesis and lipoperoxidation, increases in the liver as a result of MTX administration [3]. Conversely, MTX results in the depletion of protective antioxidants, such as glutathione (GSH), and inhibits free radical scavenging enzymes, including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [4].

The antioxidant and prooxidant systems become imbalanced due to excess ROS production (e.g., superoxide, hydrogen peroxide, and hydroxyl radicals), which, in turn, promotes oxidative stress. Oxidative damage to cells cannot be prevented by the defense mechanisms of endogenous antioxidants [5]. However, according to several studies, MTX-induced toxicity decreases through supplementation with natural antioxidant compounds [6,7]. Therefore, the use of complementary drugs, along with MTX treatment, can decrease the incidence of MTX-related hepatotoxicity [8].

MTX-mediated hepatotoxicity is a complex phenomenon, associated with a noticeable increase in the serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) [9]. By ameliorating the effects of free radicals on the cell components, antioxidant agents are able to prevent oxidative damage [10–12]. Overall, MTX-induced liver toxicity can be prevented using antioxidant compounds, including berberine, chlorogenic acid, thiamine, and vitamin C [4,9,13,14].

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Saffron (*Crocus sativus* L.) is a plant from the *Iridaceae* family, which is commonly used for flavoring and coloring food preparations. Stimulant, antispasmodic, eupeptic, sedative, anti-catarrhal, diaphoretic, and stomachic activities have been attributed to saffron in traditional medicine [15]. In addition, it has been shown to have anticonvulsant, antimicrobial, antitumor, antidepressant, anti-inflammatory, and chemopreventive effects [16–21], along with protective effects on nephrotoxicity due to cisplatin in rats [21].

Crocin (CRO) is recognized as a water-soluble carotenoid compound, producing the red color of saffron [22]. Substantial evidence suggests that CRO has a variety of pharmacological effects, including anti-inflammatory, antioxidant, antiarthritic, anticancer, antiatherosclerotic, radical scavenging, neuroprotective, hypolipidemic, and memory-improving effects [23–28].

The present study examined the hepatoprotective effects of CRO on MTX with respect to inflammation, oxidative stress status, serum biochemical parameters, and histopathological changes.

Materials and methods

Animals

The Animal House affiliated to Ahvaz Jundishapur University of Medical Sciences provided 35 male Wistar rats for this study. The animals (200–220 g), which had free access to water and food, were kept in a facility (24 ± 2 °C) in a 12:12 h light/dark cycle. The study was approved by Ethical Committee Acts of Ahvaz Jundishapur University of Medical Science for care and use of laboratory animals (IR.AJUMS.ABHC.REC.1397.003).

Materials

Sigma (St. Louis, MO, USA) provided MTX, CRO, and all other chemicals in the study.

Experimental design

Following one week of adaptation, we divided the animals into five groups of seven rats. Group I (control) was administered 0.9% saline (CRO vehicle, *po*) for nine consecutive days, as well as 0.9% saline (MTX vehicle, *ip*) on the seventh day. Group II (MTX) was injected 0.9% saline for nine consecutive days, besides 20 mg/kg of MTX (*ip*) on the seventh day. Groups III and IV (CRO + MTX) received 25 and 50 mg/kg of CRO (*po*), respectively for nine consecutive days, along with 20 mg/kg of MTX on day seven. Finally, group V (CRO) received 50 mg/kg of CRO (*po*) for nine consecutive days, as well as 0.9% saline on day seven.

Ketamine and xylazine (Alfasan Co, Woerden, the Netherlands) (100/10 mg/kg, *ip*) were combined for inducing anesthesia on day 10 of the experiment. Following that, blood samples from the jugular vein were collected. The serum was removed *via* 10 min of centrifugation at 3000 *g* and stored at −20 °C until further analysis. Following that, the rats were decapitated, and for examination, liver samples were collected. The animal's liver was divided into two sections. For histological assessments, one liver section was fixed in 10% phosphate-buffered formalin, while for the analysis of oxidative stress and inflammation, the other section was homogenized in ice-cold tris−HCl buffer (0.1 M; pH, 7.4) and kept at −20 °C.

Protein measurements

Using the Bradford assay, protein content was measured in the homogenates [29], with bovine serum albumin (BSA) used as the standard.

Biochemical analysis

Serum measurements

A spectrophotometer (Model 1200, UNICO Instruments Co., USA) was used to determine serum ALT (Pars Azmoon Co,Tehran, Iran), AST (Pars Azmoon Co,Tehran, Iran), and ALP (Pars Azmoon Co,Tehran, Iran), based on the colorimetric kit instructions.

Assessment of antioxidant enzymes

A method proposed by Aebi et al. [30] was used to measure CAT activity, while SOD kit (IBL Labs, Crumlin, UK) was applied to determine SOD activity. In addition, the method proposed by Ellman et al. [31], was used to measure GSH activity, while a GSH peroxidase kit (IBL Labs, Crumlin, UK) was employed to determine GPx activity.

Assessment of oxidative stress parameters

The method proposed by Buege and colleagues was applied to evaluate lipid peroxidation by measuring the level of malondialdehyde (MDA) [32]. In addition, based on Griess diazotization [33] reaction, the liver NO metabolites was measured following nitrate-to-nitrite conversion using nitrate reductase in the supernatant.

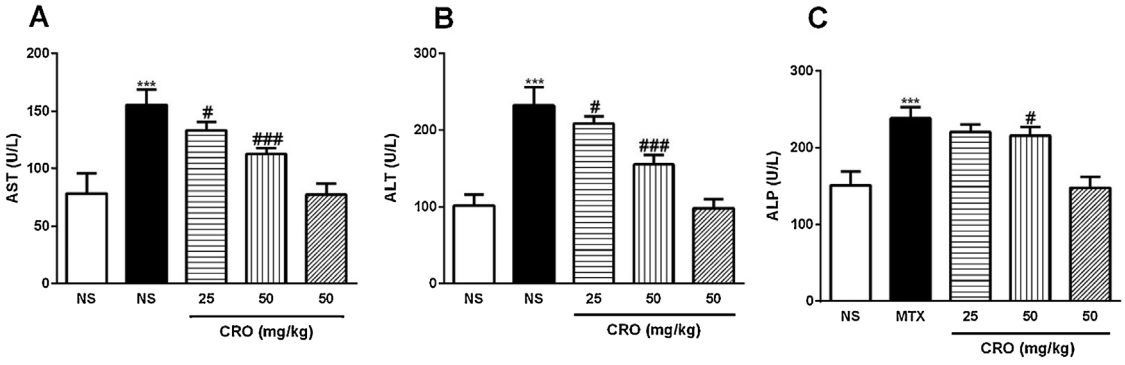

Fig. 1. Effect of pretreatment with CRO on of AST (A), ALT (B) and ALP (C) levels in MTX-induced hepatotoxicity. Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. *Significant difference in comparison with the control group (*** $p < 0.001$). #Significant difference in comparison with the MTX group (### $p < 0.01$), (# $p < 0.05$).

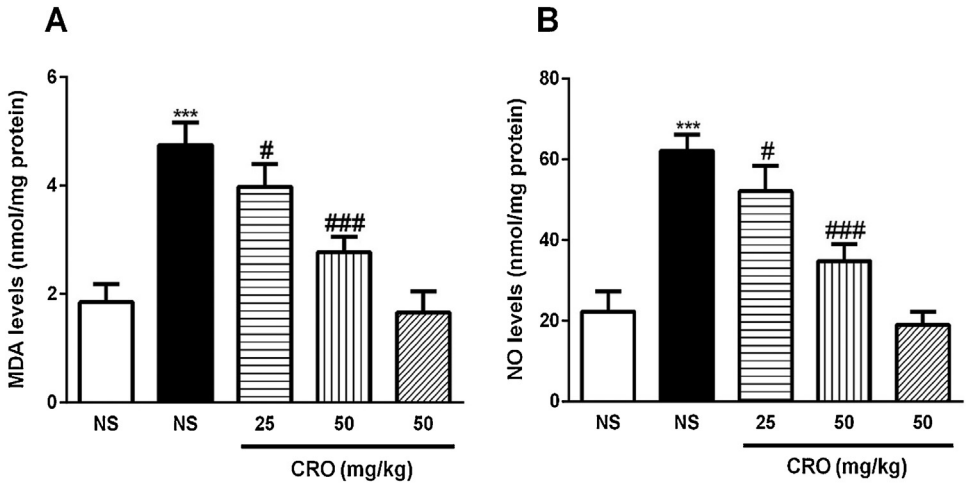

Fig. 2. Effect of pretreatment with CRO on MDA (A) and NO (B) levels in MTX-induced hepatotoxicity. Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. *significant difference in comparison with the control group (*** $p < 0.001$). #significant difference in comparison with the MTX group (### $p < 0.001$ and # $p < 0.05$).

Assessment of proinflammatory parameters

Using an ELISA kit, IL-1β (Ransel kit, Randox Laboratories Ltd, Crumlin, UK) and TNF-α (Ransel kit, Randox Laboratories Ltd, Crumlin, UK) were measured in the liver tissues, based on the manufacture protocols.

Histopathological analysis

Histological analyses were carried out after fixing the samples in 10% formalin for at least one day. The liver tissues were paraffin-embedded and cut into sections (5 μm) after they were dehydrated in ethanol solution; then, H&E staining was performed. A light microscope (Nikon Labophot, Japan) was used to observe the slides. Indices including inflammatory cell infiltration, RBC congestion, and nuclear pyknosis were also examined. In addition, a semiquantitative analysis was carried out using the following scoring system: zero, normal; one, mild; two, moderate; and three, severe. The average score for each group was measured to determine the total score.

Statistical analysis

Data are presented as mean ± SD. For data analysis, one-way ANOVA and Tukey's *post-hoc* test were performed, and the value of $p < 0.05$ was considered to be statistically significant.

Results

Effects of CRO on serum factors

Compared to the control group, MTX significantly increased serum AST, ALT, and ALP levels ($p < 0.001$). However, nine days of CRO pretreatment (25 and 50 mg/kg) reduced the ALT and AST levels dose-dependently ($p < 0.05$ and $p < 0.001$, respectively). Moreover, the MTX-mediated ALP increase was prevented by CRO administration at 50 mg/kg ($p < 0.05$). CRO pretreatment at 50 mg/kg did not change AST, ALT, and ALP levels in normal rats in comparison with the controls (Fig. 1A–C).

Effect of CRO on MTX-mediated changes in liver oxidative stress biomarkers

MTX significantly increased the levels of MDA and NO in the liver compared to the controls ($p < 0.001$). The MDA and NO levels reduced dose-dependently in the liver of rats, which respectively received 25 and 50 mg/kg of CRO ($p < 0.05$ and $p < 0.001$, respectively). Also, these antioxidant indices did not significantly change due to CRO administration, compared to the control group. The effects of CRO on oxidative stress markers are illustrated in Fig. 2A–B.

Effect of CRO on MTX-induced changes in liver antioxidant enzymes and GSH content

Compared to the controls, GSH content, as well as GPx, CAT, and SOD activities, significantly reduced in the MTX group ($p < 0.001$). On the other hand, CRO pretreatment at 25 and 50 mg/kg for nine consecutive days caused a dose-dependent increase in the GSH content, besides GPx activity, compared to the MTX group ($p < 0.05$ and $p < 0.001$, respectively). Additionally, nine days of pretreatment with 50 mg/kg of CRO increased the SOD activity, compared to the MTX group ($p < 0.01$). Nine consecutive days of pretreatment with 25 and 50 mg/kg of CRO increased the activity of CAT versus the MTX group, although the difference was insignificant. Moreover, oxidative stress parameters did not change in normal rats after CRO administration, compared to the controls. The effects of CRO on GPx, CAT, SOD, and GSH activities are illustrated in Fig. 3A—D.

Effects of CRO on MTX-induced changes in liver proinflammatory cytokines

IL-1β and TNF-α were measured to assess the protective effects of CRO on MTX-induced inflammation in the liver of rats. IL-1β and TNF-α significantly increased in rats receiving MTX, compared with the controls ($p < 0.001$). Nine days of pretreatment with 50 mg/kg of CRO markedly reduced IL-1β, compared with the MTX group ($p < 0.01$). Additionally, pretreatment with CRO (25 and 50 mg/kg) for nine consecutive days reduced IL-1β and TNF-α dose-

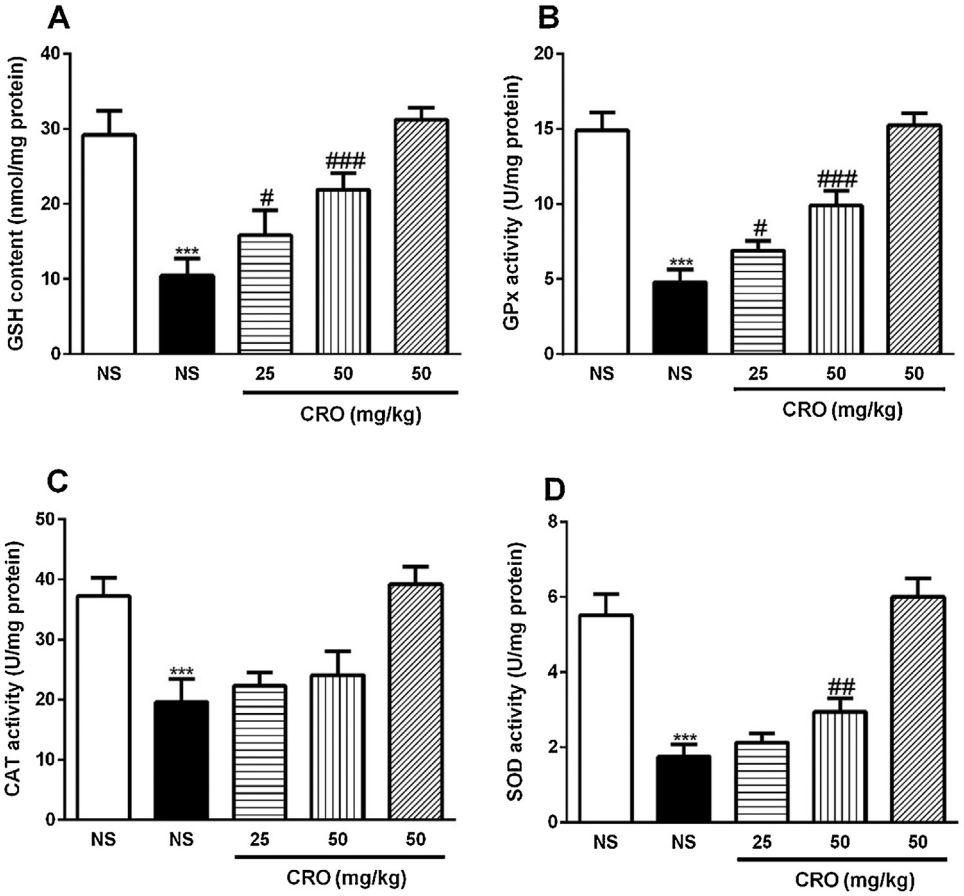

Fig. 3. Effect of pretreatment with CRO on GSH content (A) and GPx (B), CAT (C) and SOD (D) activity in MTX-induced hepatotoxicity. Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. *Significant difference in comparison with the control group (***$p < 0.001$). #significant difference in comparison with the MTX group (### $p < 0.001$, ## $p < 0.01$ and # $p < 0.05$).

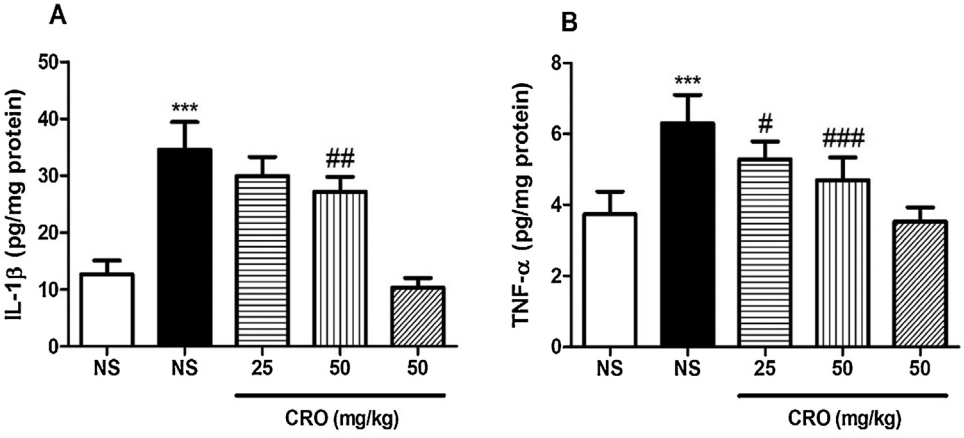

Fig. 4. Effect of pretreatment with CRO on IL-1β (A) and TNF-α (B) in MTX-induced hepatotoxicity. Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. *Significant difference in comparison with the control group (*** $p < 0.001$). #Significant difference in comparison with the MTX group (### $p < 0.001$, ## $p < 0.01$ and # $p < 0.05$).

dependently ($p < 0.05$ and $p < 0.001$, respectively). Compared to the controls, these proinflammatory cytokines did not change in rats receiving CRO. The effects of CRO on IL-1β and TNF-α are illustrated in Fig. 4A–B.

Effects of CRO on liver histopathology

The protective effects of CRO on physiological damage were determined *via* H&E staining. The normal structure of liver cells

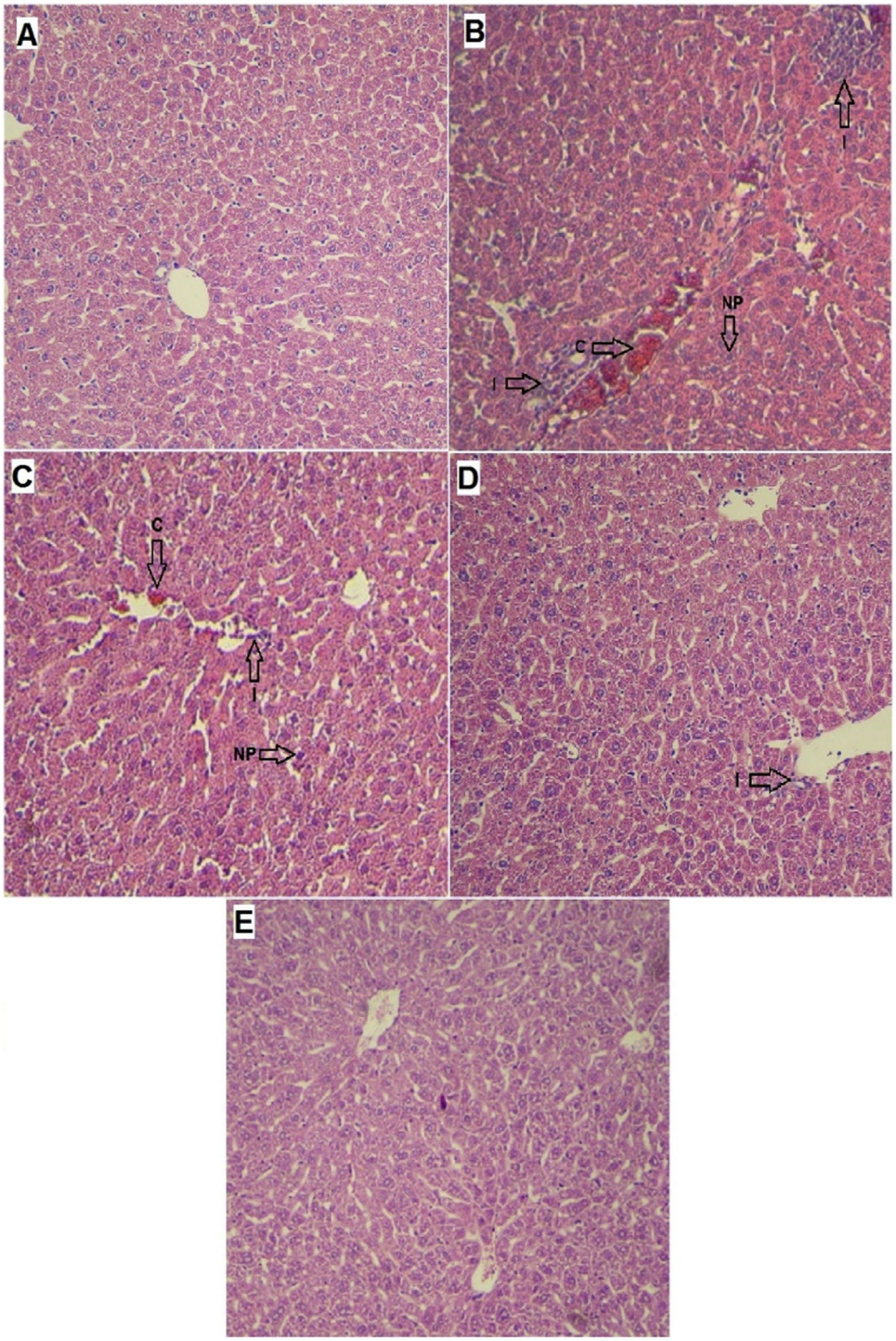

Fig. 5. Histopathological observations (liver sections stained with Hematoxylin & Eosin, magnification X 40) showing effects of CRO on MTX-induced hepatotoxicity changes in liver. (A) Control group; (B) MTX group; (C) MTX + CRO (25 mg/kg) group; (D) MTX + CRO (50 mg/kg) group; E: CRO group. C: Congestion of RBC, I: Infiltration of inflammatory cells, P: Nuclear pyknosis.

was confirmed in the control group (Fig. 5A and Table 1). Liver tissues showed extensive injuries in the MTX group (i.e., pyknosis, RBC congestion, and inflammatory cell infiltration) (Table 1 and Fig. 5B). The severity and incidence of lesions were attenuated compared with the MTX group; it should be noted that similar results were reported in the MTX + CRO group (Table 1 and Fig. 5C-D). Moreover, the liver structure did not significantly change in normal rats due to the administration of CRO (Table 1 and Fig. 5E).

Discussion

Chemotherapeutic agents are commonly used against various cancers. These agents may demolish physiological homeostasis in different organs. Radical formation, along with oxidant damage, triggers physiological side effects in non-tumor cells [33]. In this study, we investigated the effects of CRO on MTX hepatotoxicity in male Wistar rats. Based on the findings, MTX injection led to major

Table 1
Effect of pretreatment with CRO on injury scores in liver tissues following MTX-induced hepatotoxicity.

Histological criteria	Groups				
	Control	MTX	MTX+ CRO 25 mg/kg	MTX+ CRO 50 mg/kg	CRO 50 mg/kg
Congestion of RBC	0.02 ± 0.00	2.1 ± 0.32***	1.1 ± 0.31#	0.3 ± 0.04###	0.01 ± 0.00 ###
Infiltration of inflammatory cells	0.05 ± 0.00	1.8 ± 0.27***	0.7 ± 0.35#	0.2 ± 0.08###	0.04 ± 0.00 ###
Nuclear Pyknosis (%)	0.03 ± 0.00	12.3 ± 2.3***	7.9 ± 1.6##	1.3 ± 1.9###	0.04 ± 0.00 ###

Values are means ± SD (n = 7). Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons.* significant with control group. # significant with MTX group.

hepatotoxicity, as indicated by the increased serum levels of ALT, AST, and ALP, oxidative stress, and inflammation parameters in the liver of rats; the histopathological data confirmed this finding.

Additionally, in MTX-induced rats, CRO exerted hepatoprotective effects by improving the antioxidant defense, inhibiting oxidative stress, and reducing inflammation in the liver. Multiple studies have described MTX-induced hepatotoxicity [13,34]. Based on the literature [35,36], 20 mg/kg of MTX was used in our study to induce MTX-related tissue damage. Serum toxicity markers (e.g., ALT and AST) are recognized as standard indices of liver function. Based on the present results, MTX increased AST, ALT, and ALP in the serum, leading to hepatotoxicity and liver dysfunction; our findings are consistent with previous results [36,37]. In the present study, CRO pretreatment caused a major reduction in AST, ALT, and ALP in the rat liver.

Oxidative stress seems to be involved in MTX-related tissue damage [4,38–41]. MTX is known to increase the markers of oxidative stress, including lipid peroxidation and NO content in the liver [9,42]. Lipid peroxidation indicates a remarkable increase in MDA level; a lipid peroxidation product was detected after MTX treatment, attenuated markedly by natural antioxidants [41,43–45].

NO is described as a molecular mediator of various pathological and physiological processes, such as vasodilatation and inflammation. NO overproduction contributes to tissue damage, in addition to oxidative stress, through interactions with superoxide to form peroxynitrite as a potent cytotoxic agent [46]. In line with previous studies, MTX significantly increased MDA and NO content in the rat liver, while CRO pretreatment reduced MDA and NO dose-dependently.

The GSH redox cycle is recognized as an important intracellular antioxidant system. GSH has a major contribution to the maintenance of cell integrity because of its involvement in cell metabolism and reducing properties. The depletion of liver GSH has been reported in response to MTX-mediated oxidative stress in rats [44]. The present findings showed that MTX depleted GSH reservoirs, while CRO pretreatment significantly restored GSH in a dose-dependent manner.

SOD is described as an intracellular antioxidant enzyme, which protects tissues against oxidative damage and reduces superoxide anion to hydrogen peroxide. On the other hand, GPx and CAT detoxify hydrogen peroxide to water [47]. According to earlier studies, MTX-induced hepatotoxicity reduces the level of antioxidant enzymes in the liver [47]. The present results are in accordance with the literature. Moreover, nine consecutive days of pretreatment with CRO increased the levels of SOD and GPx.

With respect to MTX-mediated changes in biochemical and oxidative stress, accumulating evidence suggests that MTX triggers inflammatory reactions in the liver and increases the level of proinflammatory cytokines [48]. TNF-α and IL-1β are recognized as proinflammatory cytokines [49]. The high level of these cytokines can result in liver damage. In line with previous research [50], the MTX group had significantly high levels of IL-1β and TNF-α in our study. CRO pretreatment significantly decreased the levels of IL-1β and TNF-α.

In the present study, histopathological data were in line with biochemical findings, indicating structural changes in the liver tissues of rats receiving MTX. MTX produced histopathological lesions in the liver (e.g., pyknosis, RBC congestion, and inflammatory cell infiltration); several other studies have confirmed these findings [50,51]. Additionally, a histopathological analysis was carried out to evaluate the protective effects of CRO (25 and 50 mg/kg) on the liver.

The lack of analysis of apoptosis pathways was a limitation of our study. Further research is necessary to determine the cellular mechanisms contributing to the therapeutic significance of CRO.

Conclusion

The amelioration of liver damage with CRO pretreatment could be mediated through multiple mechanisms: (1) reduction of MTX-mediated oxidative stress; (2) prevention of MTX-induced reduction in antioxidant activity; and (3) obstruction of MTX-induced increase in proinflammatory cytokines.

Authors' contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

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