

Does alfa lipoic acid prevent liver from methotrexate induced oxidative injury in rats?¹

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ABSTRACT

PURPOSE: To determine the antioxidant and anti-inflammatory effects of alfa lipoic acid (ALA) on the liver injury induced by methotrexate (MTX) in rats.

METHODS: Thirty two rats were randomly assigned into four equal groups; control, ALA, MTX and MTX with ALA groups. Liver injury was performed with a single dose of MTX (20 mg/kg) to groups 3 and 4. The ALA was administered intraperitonealy for five days in groups 2 and 4. The other rats received saline injection. At the sixth day the rats decapitated, blood and liver tissue samples were removed for TNF- α , IL-1 β , malondialdehyde, glutathione, myeloperoxidase and sodium potassium-adenosine triphosphatase levels measurement and histological examination.

RESULTS: MTX administration caused a significant decrease in tissue GSH, and tissue Na⁺, K⁺ ATPase activity and which was accompanied with significant increases in tissue MDA and MPO activity. Moreover the pro-inflammatory cytokines (TNF- α , IL- β) were significantly increased in the MTX group. On the other hand, ALA treatment reversed all these biochemical indices as well as histopathological alterations induced by MTX.

CONCLUSION: Alfa lipoic acid ameliorates methotrexate induced oxidative damage of liver in rats with its anti-inflammatory and antioxidant effects.

Key words: Thioctic Acid. Methotrexate. Liver. Rats.

Introduction

Methotrexate (MTX) is an effective cytotoxic drug and has been widely used in chemotherapeutic based treatments for malignancies primarily in leukaemias^{1,2} as well as inflammatory diseases including psoriasis and rheumatoid arthritis³⁻⁶. Long-term methotrexate use, or its use in high doses, may cause hepatic steatosis, cholestasis, fibrosis and cirrhosis⁷. Accordingly the dose of methotrexate should be lowered or the drug should be discontinued in case of hepatic toxicity which causes delay in the treatment of the disease. On the other hand much attention is now being paid to factors that may enhance the effectiveness of existing drugs while reducing their unwanted side effects.

Alfa-lipoic acid (ALA) is described as a therapeutic agent in a number of conditions related to liver disease, including alcohol-induced damage, mushroom poisoning, metal intoxication, CCl₄ poisoning, and hyperdynamic circulation in biliary cirrhosis⁸⁻¹⁰. The effect of ALA against methotrexate toxicity on the liver is not yet clearly.

Furthermore the effect of ALA on MTX induced liver injury has not been studied before. Thus, in this present study we aimed to investigate whether the ALA has any effect on treatment against MTX induced oxidative injury on the liver in rats.

Methods

The experimental protocols were approved by the animal care and use committee of İnönü University Faculty of Medicine.

Thirty two Wistar albino rats of both sexes of 200–250 g were used in this experiment. The rats maintained at a constant temperature (22°C) with a 12-h light–dark cycle and randomly divided into four groups. Group 1 (control group): rats in this group received only physiological saline. Group 2 (α -lipoic acid group): rats in this group received α -lipoic acid (Sigma, St Louis, USA) for five days intraperitoneally (60 mmol/kg). Group 3 (Methotrexate group): rats received a single dose of MTX (Onco-Tain; Faulding Pharmaceuticals Plc, Leamington Spa, UK) intraperitoneally (20 mg/ kg). Group 4 (Methotrexate group- α -lipoic acid group): rats received a single dose of MTX and also received α -lipoic acid for five days. Alfa-lipoic acid was dissolved in 0.1% dimethyl sulfoxide (DMSO). At the end of the experiment rats were decapitated and blood samples were obtained for the measurement of tumour necrosis factor-alpha (TNF- α) and interleukin-1-beta (IL-1 β). The levels of malondialdehyde (MDA) and glutathione (GSH), as well as myeloperoxidase (MPO) and sodiumpotassium adenosine triphosphatase (Na⁺/K⁺-ATPase)

activity were also analysed in the liver tissues. Furthermore the degree of inflammation and histopathologic damage (necrosis, inflammation, vacuolization and vascular congestion) were evaluated via histological examination under a light microscope.

Measurement of malondialdehyde and glutathione levels

To determine the MDA and GSH levels, liver tissue samples were homogenized in ice cold 150mm KCl. The MDA levels (nmol MDA/g tissue) were assayed for the products of lipid peroxidation¹¹. The GSH levels (mg GSH/g tissue) were measured by spectrophotometric method using Ellman's reagent¹².

Measurement of myeloperoxidase activity

Tissue-associated MPO (U/g tissue) activity was measured according to the procedure reported by Hillegas *et al.*¹³ Liver tissue samples were homogenized in 50mm potassium phosphate buffer (PB, pH 6.0) and homogenates were centrifuged at 41 400g for 10 min; pellets were suspended in 50mm PB containing 0.5% hexadecyltrimethylammonium bromide. After three cycles of freezing and thawing, with sonication between the cycles, the samples were centrifuged at 41 400g for 10 min. Volumes of 0.3 ml were added to 2.3 ml of reaction mixture containing 50mm PB, o-dianisidine, and 20mm H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 min.

Measurement of Na⁺/K⁺-ATPase activity

The measurement of Na⁺/K⁺-ATPase activity was based on the measurement of inorganic phosphate produced from 3mm disodium adenosine triphosphate added to the incubation medium¹⁴. The medium (containing in mm: 100 NaCl, 5 KCL, 6 MgCl₂, 0.1 EDTA and 30 Tris HCL (pH 7.4)) was incubated at 37°C in water bath for 5 min. Following this preincubation period, Na₂ATP, at a final concentration of 3mm, was added into each tube and incubated at 37°C for 30 min. After the incubation, the tubes were placed in an ice bath to stop the reaction. The mixture was then centrifuged at 3500g, and Pi in the supernatant fraction was determined by the method of Fiske and Subarow¹⁵. The specific activity of the enzyme was expressed as nmol Pi mg⁻¹ protein h⁻¹. The protein concentration of the supernatant was measured by the Lowry method¹⁶.

Biochemical analysis

The plasma TNF- α and IL-1 β were analysed using the enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium). These kits were particularly selected because of their high degree of sensitivity, specificity and inter-assay and intra-assay precision, and due to requiring a small amount of plasma sample.

Histological evaluation

Each liver samples were processed for light microscopic examination. The samples were placed in 10% neutral formalin for 48 h and prepared for routine parafin embedding. Tissue samples were cut into 5 μ m thick sections, mounted on slides and stained with hematoxylin– eosin (H&E).

The degree of inflammation and histopathologic damage (necrosis, inflammation, vacuolization and vascular congestion) was expressed within each liver section (Table 1), classified on a scale of 0–3 (0, absent; 1, mild; 2, moderate; 3, severe) with a maximum score of 12¹⁷.

TABLE 1 - The degree of inflammation and histopathologic damage (necrosis, inflammation, vacuolization and vascular congestion) was expressed as means within each liver section of groups was shown.

Parameters	Control Group	ALA	MTX	MTX-ALA
Necrosis	0	0	1.5	0.2
Inflammation	0	0	1.6	0.3
Vacuolization	0	0	2.1	0.7
Vascular congestion	0	0	1.1	0.7
Total score	0	0	6.3	2.1

Statistical analysis

Statistical analysis was performed by GraphPad Prism 3.0 (GraphPad Software, San Diego, USA). The data were expressed as mean \pm standard error of the mean (SEM). Group comparisons were performed with the analysis of variance followed by Tukey’s tests. The p<0.05 was considered as statistically significant.

Results

In the MTX group, TNF- α levels were significantly increased (p<0.001) when compared to control group, while this MTX-induced rise in serum TNF- α level was abolished (p<0.001) with α -lipoic

acid treatment. Similarly IL-1 β proinflammatory cytokine, was also increased in the MTX group (p<0.001), however when rats were treated with α -lipoic acid following MTX administration, these cytokines were back to control levels (Table 2).

TABLE 2- Effect of α -lipoic acid (ALA) treatment on some biochemical parameters in the serum of control, methotrexate (MTX), MTX-ALA (α -lipoic acid) groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

Parameters	Control Group	MTX	MTX-ALA
TNF- α (pg/ml)	9.7 \pm 1.3	33.1 \pm 3.5 ***	13.9 \pm 2.8 ***
IL-1 β (pg/ml)	10.5 \pm 1.3	31.6 \pm 3.1 ***	16.3 \pm 2.7 **

Data are mean \pm s.d. ** p <0.01, *** p <0.001 compared to control group. ** p <0.01, *** p <0.001, compared to MTX group.

In accordance with these findings, levels of the major cellular antioxidant GSH of liver samples in MTX group were significantly lower than those of the group (p<0.001). On the other hand, α -lipoic acid treatment to MTX group restored the GSH levels in all tissues (p<0.01, Figure 1).

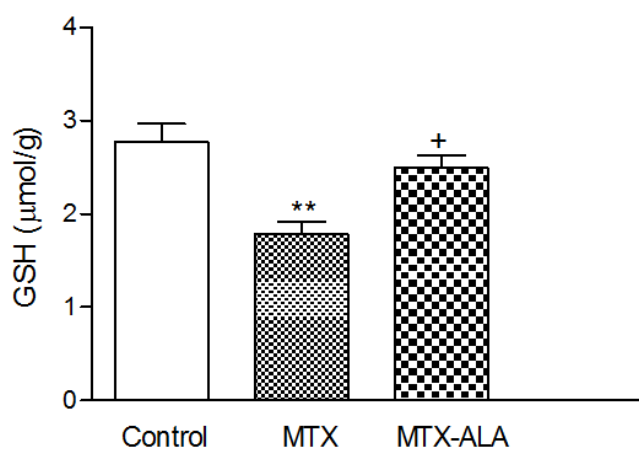


FIGURE 1 - Glutathione (GSH) levels in the liver tissues of control, methotrexate (MTX), MTX-ALA (α -lipoic acid) groups. Each group consists of eight animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. *** p<0.001; compared to control group. **p<0.01; compared to MTX group.

The mean level of MDA, which is a major degradation product of lipid peroxidation, was increased in all tissues after MTX administration when compared with the control group (p<0.001), while α -lipoic acid treatment to the MTX group caused a marked decrease in MDA levels (p<0.01, Figure 2).

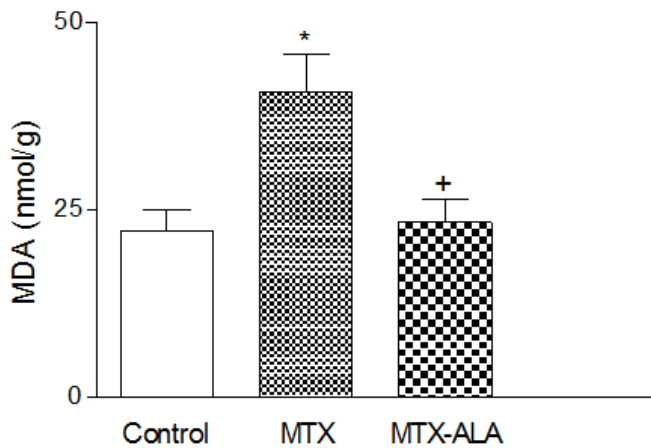


FIGURE 2 - Malondialdehyde (MDA) levels in the liver tissues of control, methotrexate (MTX), MTX-ALA (α -lipoic acid) groups. Each group consists of eight animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p < 0.001$; compared to control group. ** $p < 0.01$; compared to MTX group.

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the liver tissues of the MTX group when compared to control group ($p < 0.001$). On the other hand, α -lipoic acid treatment in MTX group significantly decreased all tissues MPO level ($p < 0.001$, Figure 3), which was found to be not different than that of the control group.

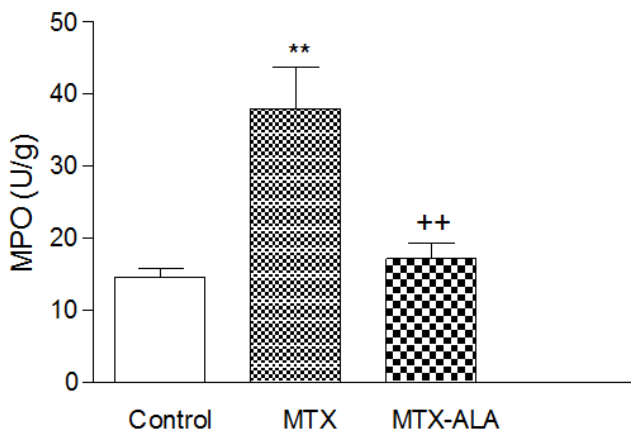


FIGURE 3 - Myeloperoxidase (MPO) activity in the liver tissues of control, methotrexate (MTX), MTX-ALA (α -lipoic acid) groups. Each group consists of eight animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p < 0.001$; compared to control group. ** $p < 0.01$, *** $p < 0.001$; compared to MTX group.

The activity of $\text{Na}^+\text{-K}^+$ ATPase was shown to be significantly decreased in the liver tissue of saline treated MTX group compared with control group; however, α -lipoic acid treatment in MTX group significantly increased all tissues $\text{Na}^+\text{-K}^+$ ATPase activity ($p < 0.001$, Figure 4).

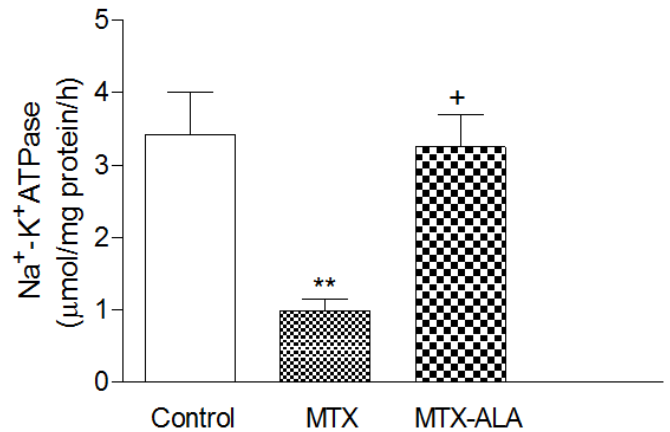


FIGURE 4 - $\text{Na}^+\text{-K}^+$ ATPase activity in the liver tissues of control, methotrexate (MTX), MTX-ALA (α -lipoic acid) groups. Each group consists of eight animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. ** $p < 0.01$, *** $p < 0.001$; compared to control group. + $p < 0.01$, *** $p < 0.001$; compared to MTX group.

Liver sections from the control (Figure 5A-B) and LA (Figure 6A-B) groups were normal in histological appearance.

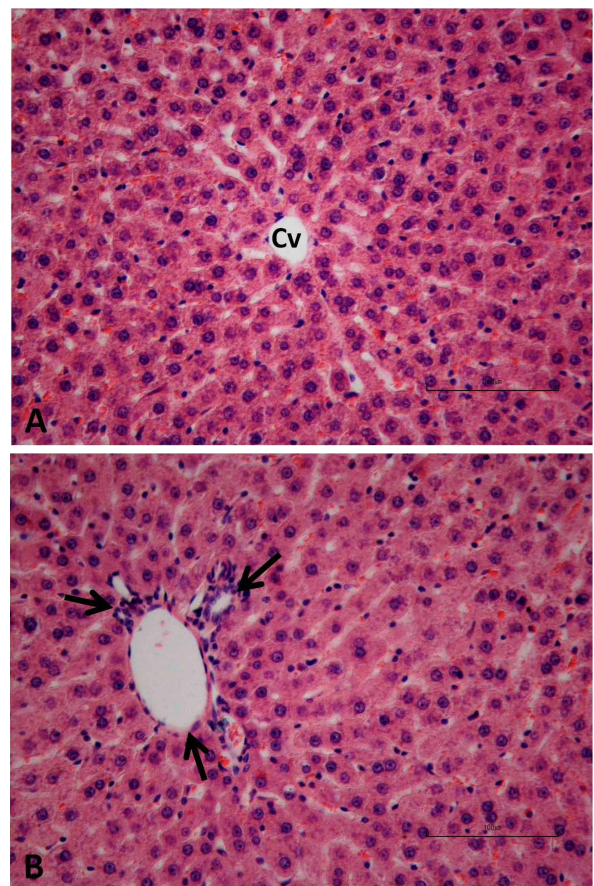


FIGURE 5 - Control group; Normal histological appearance of liver tissues, (A) central vein (Cv), (B) portal area (arrows). H&E, Scale bar = 100 μm .

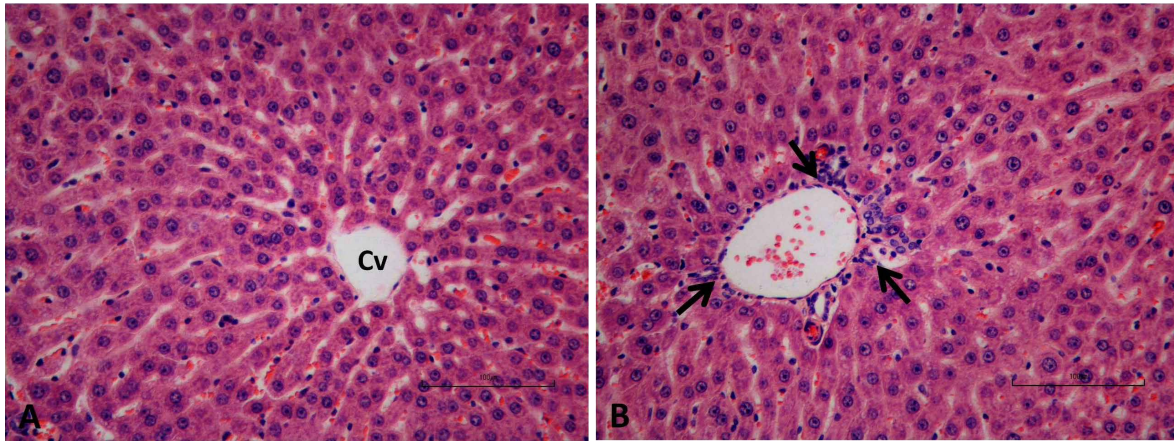


FIGURE 6 - ALA group; Normal histological appearance of liver tissues, (A) central vein (Cv), (B) portal area (arrows). H&E, Scale bar = 100 µm.

The liver sections from the MTX treated group showed some histopathological changes such as hepatic necrosis, inflammatory cell infiltration especially in the periportal area and widespread

intracellular vacuolization in hepatocytes and dark eosinophilic cytoplasm and heterochromatic, fragmented nuclei in hepatocytes and apoptotic bodies and vascular-sinusoidal congestion (Figure 7A-D).

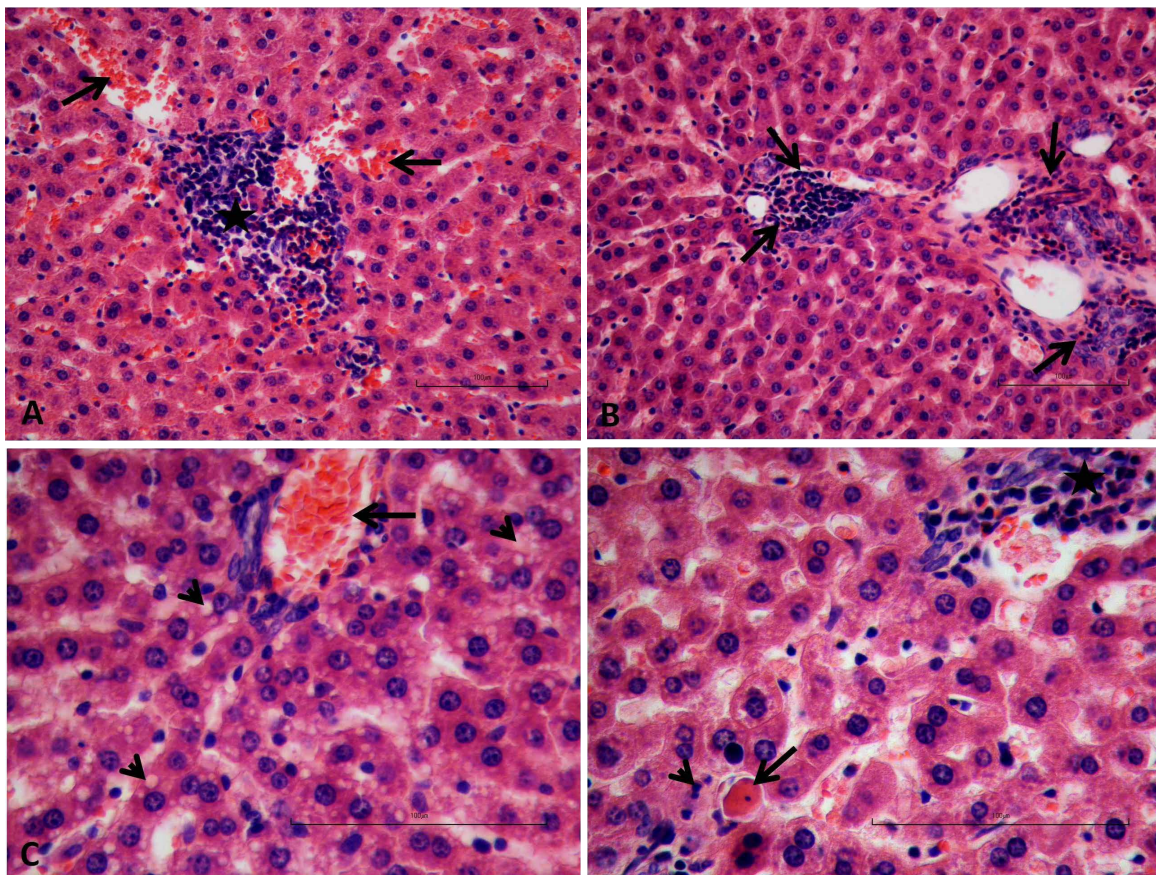


FIGURE 7 - MTX group; (A) necrosis and inflammatory cell infiltration (aster), sinusoidal congestion (arrows), (B) inflammatory cell infiltration in the periportal area (arrows), (C) widespread intracellular vacuolization in hepatocytes (arrow heads) and vascular congestion (arrow), (D) inflammatory cell infiltration (aster), dark eosinophilic cytoplasm and heterochromatic nuclei in hepatocyte (thick arrow), apoptotic body (thin arrow) and fragmented nuclei in hepatocyte (arrow head). H&E, Scale bar = 100 µm.

However, administration of LA reduced the histopathological damage score significantly in the Mtx+LA group in comparison to the Mtx group. In the Mtx+LA group, histopathological evidence of hepatic damage was markedly reduced. In this group, liver sections

showed rare dark eosinophilic cytoplasm and heterochromatic nuclei in hepatocytes, mild intracellular vacuolization in hepatocytes and inflammation was limited in localized areas and mild sinusoidal congestion (Figure 8A-D).

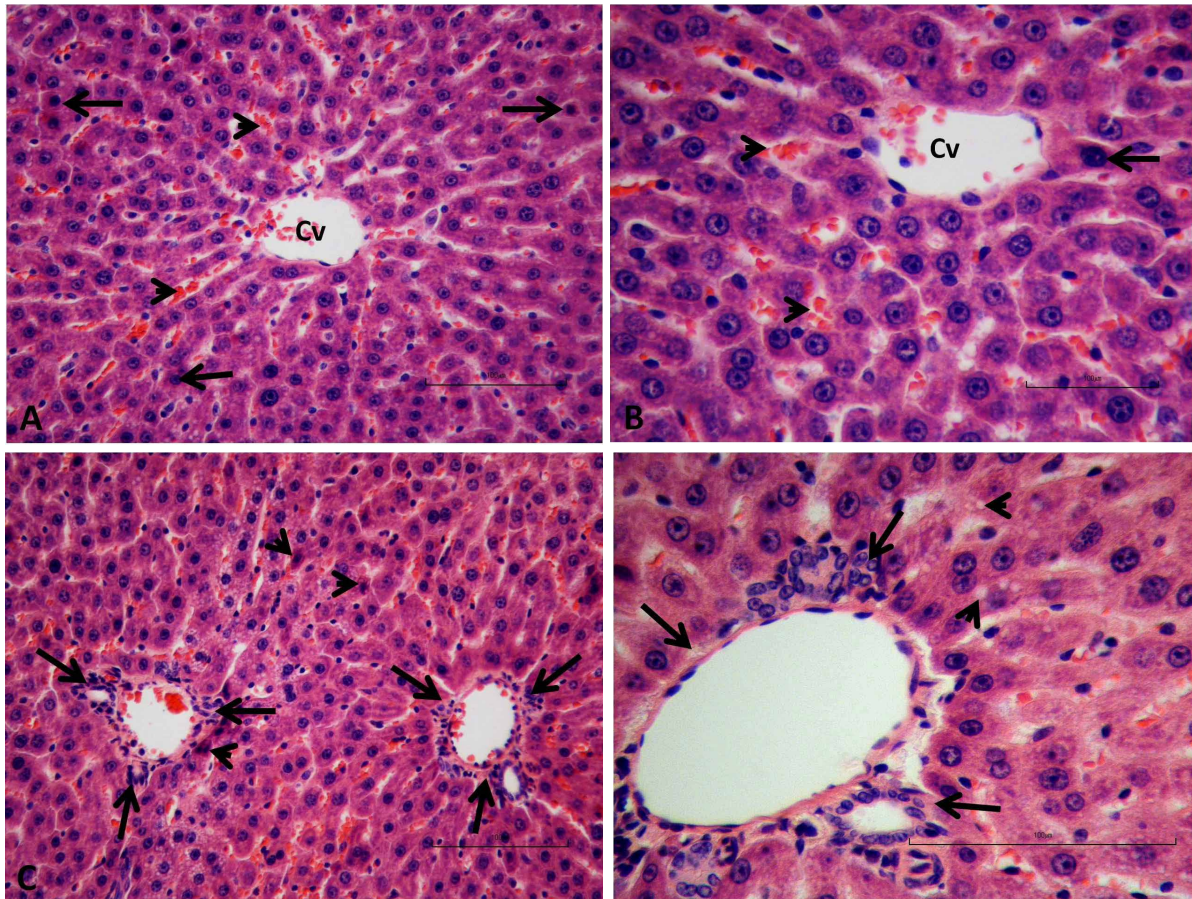


FIGURE 8 - MTX+ALA group; (A, B) central vein (Cv), eosinophilic cytoplasm and heterochromatic nuclei in hepatocyte (arrow), sinusoidal congestion (arrow heads), (C) portal area (arrows) and eosinophilic cytoplasm and heterochromatic nuclei in hepatocyte (arrow heads), (D) portal area (arrows), mild intracellular vacuolization in hepatocytes (arrow heads). H&E, Scale bar = 100 μ m.

Discussion

Findings from our study revealed that MTX administration causes oxidative tissue damage, while with its free radical scavenging effect the ALA prevented lipid peroxidation and neutrophil infiltration of the rat liver tissues. Furthermore, ALA treatment decreased the plasma cytokines and improved the liver tissue morphological changes caused by methotrexate.

Methotrexate is an antimetabolite that competitively inhibits the folic acid metabolism thus impairs the DNA synthesis. 7-hydroxymethotrexate (7-OH-MTX) is the major extracellular metabolite of MTX that is metabolized in the liver by an enzymatic system¹⁸. In the cell MTX stores in a polyglutamate form. With the use of MTX intracellular amount of polyglutamate increases on

the other hand folic acid levels decreased, that leads to necrosis of hepatocyte¹⁹. Hepatotoxic effect of methotrexate was caused by an increase of its polyglutamate form intracellularly. The hepatotoxic effects of MTX have been reported in many studies^{1,20,21}.

ALA is found in mitochondria as cofactor of pyruvate dehydrogenase and α -ketoglutarate dehydrogenase^{22,23} and is an effective free radical scavenger²⁴.

Lipid peroxidation by free oxygen radicals is an important cause of destruction and oxidative damage to cell membranes these containing unsaturated fatty acids, nucleic acids and proteins²⁵. It has contributed to develop methotrexate associated tissue damage^{10,11,18,21}.

With the attack of free oxygen radicals lipid peroxidation increases and fails the Na⁺/K⁺-ATPase activity²⁶. Na⁺/K⁺-ATPase

is the other target of cellular oxidative tissue damage²⁵. In this present study, MTX administration caused to a significant liver tissue damage since MDA which was the end product of lipid peroxidation is increased while Na⁺/K⁺-ATPase activity is depressed due to damage of cell membrane.

Liver tissue injury was also observed microscopically. On the other hand, following MTX administration, treatment with ALA was significantly reduce the MDA levels and increased the Na⁺/K⁺-ATPase enzyme activity, while normal histological appearance was observed in liver tissue.

Glutathione (GSH) plays a particularly important role in the maintenance and regulation of the thiol-redox status of the cell²⁷. Tissue GSH depletion is one of the primary factors permitting liver tissue damage is associated with oxidative stress caused by MTX in our study.

It was expected that free radicals plays an important role in MTX induced liver toxicity^{28,29}. The reactive oxygen metabolites play a role in mediating liver toxicity of some xenobiotics and pathogenesis of organ failure^{10,11,18,21,27}. It was reported that ALA protect the nuclear DNA, cell membrane lipids and intracellular proteins from oxidative tissue damage³⁰.

Free oxygen radicals trigger the leukocytes accumulation in tissue and activate the enzyme (including MPO, elastase and protease) secretion of neutrophils thus leads to further tissue damage. Therefore, MPO plays role in oxidant production by neutrophils^{31,32}. In our study MPO level which is an index of polimorphonuclear leukocyte infiltration was increased. Systemic inflammatory response indicators; TNF- α and IL-1 β levels were also found increased. Increased levels of MPO indicate that neutrophil accumulation contributes to MTX induced oxidative injury in liver tissues. Treatment with ALA decreased the MPO activity and plasma TNF- α and IL-1 β .

Conclusion

Alfa lipoic acid can be capable of reducing the methotrexate induced liver oxidative injury through its anti inflammatory and antioxidative effects.

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