

Original Article



Hepatoprotective Potential of Micronutrients Against Methotrexate-Induced Hepatotoxicity in Experimental Mice Model

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¹Conception and design, Collection and assembly of data, Final approval and guarantor of the article, ²Analysis and interpretation of the data, ^{3,4}Drafting of the article, ⁴Critical revision of the article for important intellectual content,

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ABSTRACT

Objective: To evaluate the hepatoprotective role of micronutrients, ascorbic acid and alpha-tocopherol, against methotrexate (MTX)-induced hepatic damage in mice.

Methodology: A laboratory-based experimental study was carried out at Foundation University Medical College in collaboration with the National Institute of Health, Islamabad, from October to December 2021. Twenty-four male BALB/c mice were randomly divided into four groups (n=6). Group I received intraperitoneal normal saline. Group II received a single intraperitoneal injection of MTX at a dose of 20 mg/kg. Groups III and IV were given oral ascorbic acid (200 mg/kg) and oral alpha-tocopherol (100 mg/kg) respectively, both for 7 days, with MTX administered on day 4. Blood and liver samples were collected 24 hours after the last dose for evaluation of hepatic serum biomarkers and histological analysis, respectively.

Results: Ascorbic acid demonstrated a protective effect against MTX-induced hepatic damage. However, alpha-tocopherol did not show hepatoprotection on liver histopathology.

Conclusion: Ascorbic acid exhibits protective potential when administered concomitantly with MTX. However, alpha-tocopherol does not provide complete hepatoprotection against MTX-induced liver injury.

Keywords: Alpha-tocopherol, Ascorbic acid, Hepatotoxicity, Methotrexate, Micronutrients

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Introduction

Methotrexate (MTX) is a well-known, first-line clinically effective disease-modifying drug. It is used in wide range of autoimmune diseases like rheumatoid arthritis, psoriasis, vasculitis, sarcoidosis, myasthenia gravis and inflammatory bowel syndrome attributed to the anti-inflammatory and immunosuppressant properties^{1,2}.

However, its beneficial effects are limited by its dose-related liver injury³. Acute liver damage with MTX as manifested by elevated serum liver enzymes, is usually self-limiting and recovers on its own after sometime. With chronic use, MTX causes nonalcoholic fatty liver disease, fibrosis and hepatic cirrhosis making it mandatory to

withdraw the drug². However, discontinuation of stable MTX therapy can result in rapid and severe flare of RA.⁴

Ascorbic acid is a six carbon compound which closely resembles glucose. It is a water-soluble antioxidant that is present naturally in citrus fruits and green leafy vegetables. It is also found in liver and kidneys of various animals and birds respectively.⁵ However, humans are dependent on exogenous sources of ascorbic acid as the terminal enzyme, gluconolactone oxidase, that is required for its synthesis is deficient in humans⁵. It is involved in the production of cholesterol, catecholamines, amino acids and some peptide hormones⁶. Moreover, it has got antiaging, antioxidant, anti-inflammatory properties as well. Ascorbic acid has been reported to attenuate hepatotoxic effects of acetaminophen, cyclosporine,

cocaine, carbon tetra chloride, cypermethrin, deltamethrin, methidathion, organophosphate insecticides and heavy metals in experimental rodents.⁷

Alpha tocopherol belongs to vitamin E family and is present naturally in green leaves. It is a fat-soluble vitamin that remains confined to biological lipid membranes because of its lipophilic nature. It stabilizes biological membranes by preventing lipid peroxidation. In addition to its anti-oxidant potential, alpha tocopherol also possesses anti-inflammatory, anti-platelet aggregation, and immunomodulatory properties.⁸ The deficiency of this important vitamin can cause free radical induced hemolytic anemia, muscle weakness, infertility and neurological symptoms like ataxia, peripheral neuropathy, myopathy, pigmented retinopathy^{8,9}. Its preventive role has been established in cardiovascular diseases, Alzheimer's disease and various types of cancers as well.⁹

In the light of limited available studies related to shielding effect of ascorbic acid and alpha tocopherol against liver damage caused by MTX, our current study aimed at investigation and establishment of the role of these micronutrients in MTX induced liver damage in experimental mice model.

Methodology

The standardized drugs and chemicals of analytical grade were used in current study. The animals used in this experiment were 8-12 weeks old BALB/C male mice, weighing 30-40g. The animals were kept under controlled and standard laboratory conditions (temperature: 20-25°C; humidity 70 ± 15 percent; light/dark cycle 12hr/12hr) in National Institute of Health (NIH), Islamabad. Mice were given free access to their diet and water for the entire experiment. Ethical Review Committee of Foundation University, Islamabad, had granted approval of the current study.

Non-probability convenience sampling method was used for animal's selection. Twenty-four (24) mice were then split into 4 groups ($n = 6$) randomly. Group-I was labeled as control group and was given normal saline intraperitoneally. Group-II was labeled as toxic group and

was given single dose of methotrexate injection 20 mg/kg intraperitoneally¹⁰. Group-III and Group-IV was given ascorbic acid 200 mg/kg per oral¹¹ and Alpha tocopherol 100 mg/kg per oral¹² respectively. In Group-III and Group-IV treatment was continued for 7 days, however, methotrexate injection was given at day 4. Blood samples were taken after 24 hours of respective treatment for evaluating serum biomarkers. Livers were also removed, processed and the slides (stained with Eosin and Haematoxylin) were then analyzed meticulously for histopathological changes.

Data was assessed by using Statistical Package for the Social Sciences 23. Results were stated as mean ± S.E.M. Multiple comparisons of biochemical markers between the groups were conducted by employing one way ANOVA followed by Post Hoc Tukey Test. Histopathological changes in liver were graded using Ishak modified Histological activity index, which were then explored by Chi square test. In all comparisons, p value was < 0.05 was taken as significant.

Results

Mice treated with MTX (group- II) showed significant elevation in all hepatic serum biomarkers. In protective group-III (MTX + Ascorbic acid) there was remarkable reduction of all serum biomarkers. However, in protective group-IV (MTX + Alpha tocopherol) there was remarkable reduction of only serum ALT and ALP. (Table I, Figures 1,2,3).

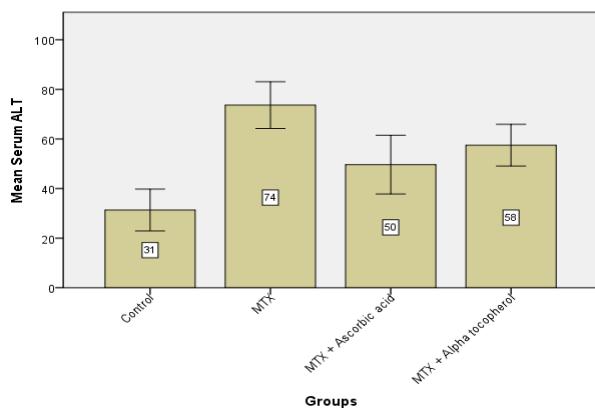


Figure 1. Graphical representation of Mean Serum ALT (IU/L) Levels of all groups.

Table I: Serum Liver Enzymes of all groups*

| Parameters | ALT (U/L) | AST (U/L) | ALP (U/L) |
|------------------------|--------------------|---------------------|----------------------|
| Control | 31.33 ± 3.28 | 87.83 ± 3.57 | 94.67 ± 4.9 |
| MTX | 73.67 ± 3.66^x | 128.50 ± 7.77^x | 315.33 ± 12.44^x |
| MTX + Ascorbic acid | 49.67 ± 4.60^y | 100.00 ± 3.21^y | 199.83 ± 10.27^y |
| MTX + Alpha tocopherol | 57.50 ± 3.27^y | 111.00 ± 4.81 | 219.83 ± 12.66^y |

*Data is stated as mean ± SEM, $^x p < 0.05$ in comparison with control group, $^y p < 0.05$ in comparison with MTX group

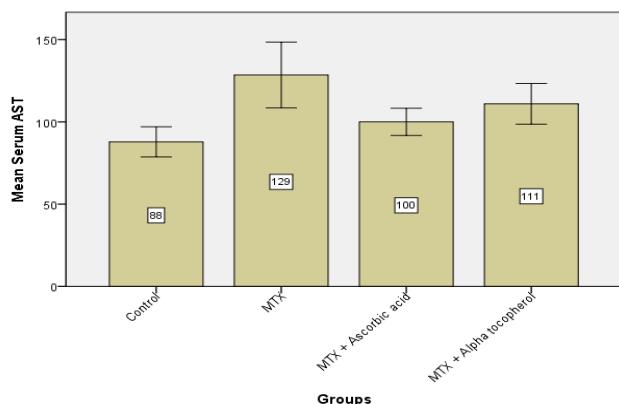


Figure 2: Graphical representation of Mean Serum AST (IU/L) Levels of all groups

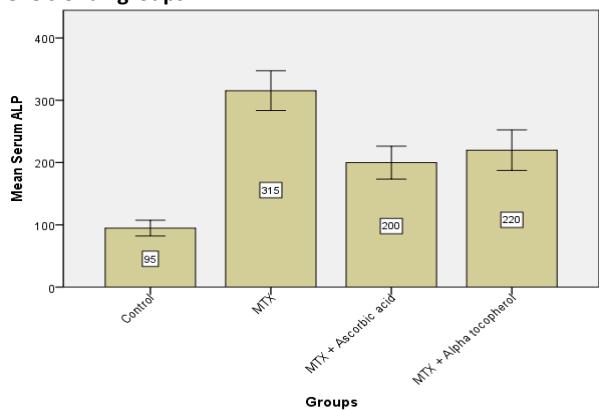


Figure 3: Graphical representation of Mean Serum ALP (IU/L) Levels of all groups

The histopathological findings in group-II (MTX) showed mild steatosis and focal necrosis (figure 4). However, in group-III (MTX + Ascorbic acid) reparative and regenerative changes were found, though minimal inflammation was also seen in few slides (figures 5,6). Mice liver of group-IV (MTX + Alpha tocopherol), however, showed pleomorphism and inflammation (figure 7).

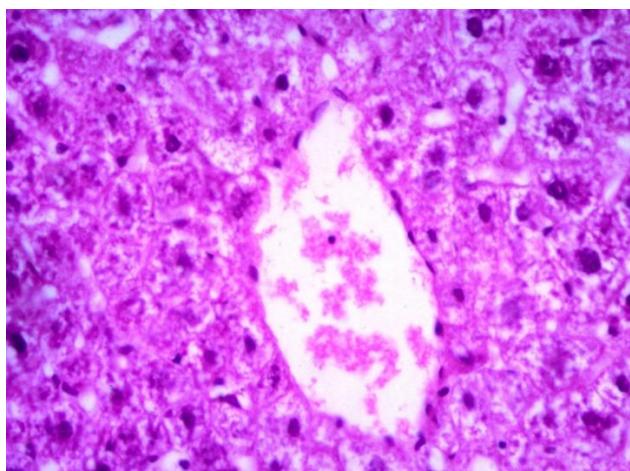


Figure 4: Hepatic tissue photomicrograph in Group-II (MTX) showing focal necrosis around portal vein (H&E 40X)

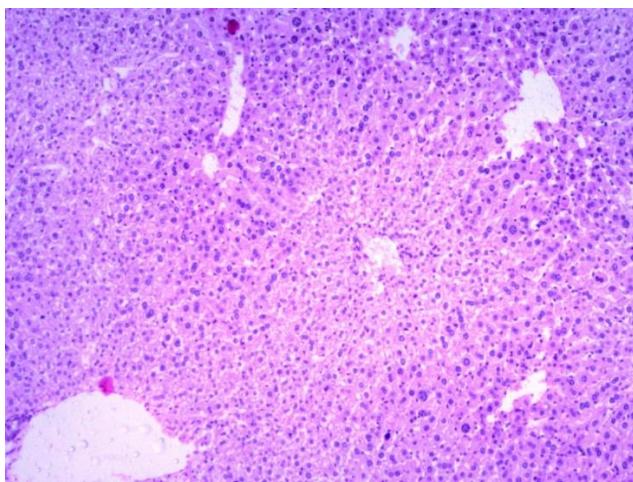


Figure 5: Hepatic tissue photomicrograph in Group-III (MTX + Ascorbic acid) showing reparative / regenerative changes (H&E 10X)

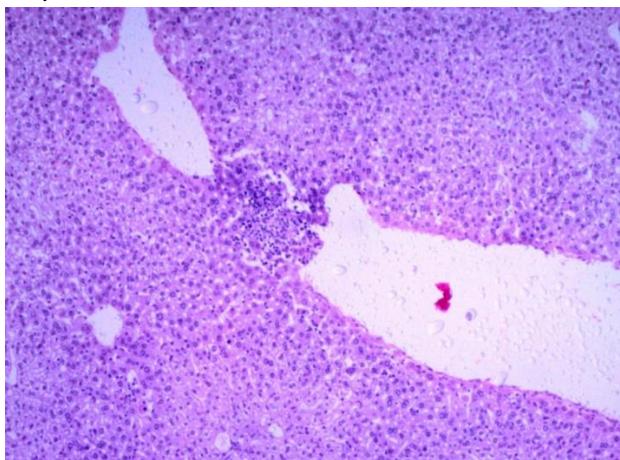


Figure 6: Hepatic tissue photomicrograph in Group-III (MTX + Ascorbic acid) showing minimal inflammation (H&E 10X)

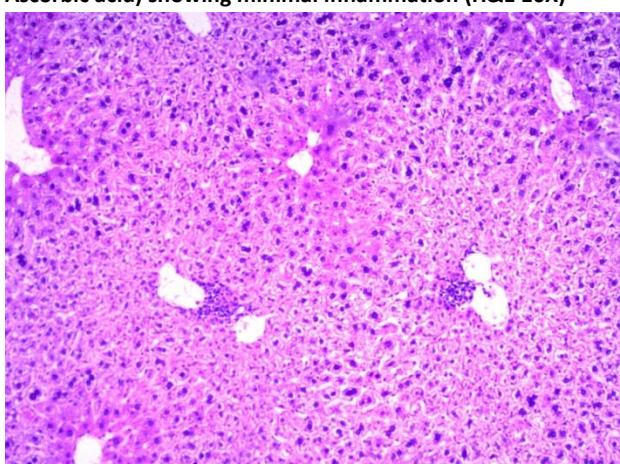


Figure 7: Hepatic tissue photomicrograph in Group-IV (MTX + Alpha tocopherol) showing inflammation and pleomorphism (H&E 10X)

Discussion

Drug induced steatohepatitis is one of the forms of drug induced hepatic damage that is characterized by fat deposition in the liver, hepatic inflammation, hepatocellular damage, along with underlying mitochondrial dysfunction, surge in reactive oxygen and nitrogen species formation, lipid peroxidation and oxidative stress. The pathology of drug induced fatty liver is almost similar to non-alcoholic fatty liver disease and alcoholic steatohepatitis.¹³ Literature showed association of non-alcoholic steatohepatitis with reduce intake of dietary micronutrients having antioxidant capability especially ascorbic acid and alpha tocopherol, though high doses of vitamins especially ascorbic acid can have opposite effect primarily due to obesity.^{14,15}

MTX build up within the cells especially hepatocytes in the form of MTX polyglutamates (MTX-PGs) results in rupture and lipid peroxidation of hepatocyte plasma membrane causing oxidative stress. Moreover, this is boosted by enhanced levels homocysteine which sensitizes the cells to reactive oxygen and nitrogen species.^{1,16} Sustained high levels of homocysteine in plasma dysregulates cholesterol metabolism encouraging hepatic steatosis.¹⁷ MTX-PGs also trigger cascade of inflammatory processes, deplete mitochondrial folate necessary for DNA and RNA synthesis, deposit adenosine intracellularly resulting in hepatocyte apoptosis and hepatic fibrosis.^{1,16}

MTX induced liver damage ranges from varying degree of macro-vesicular steatosis, portal inflammation, nuclear pleomorphism, hepatocellular necrosis to development of fibrosis and cirrhosis.^{1,16}

In present study, ascorbic acid was observed to produce hepatoprotective effects against MTX as depicted by remarkable mitigation of all hepatic biomarkers. These findings are in consistent with other *in vivo* studies¹⁸⁻²⁰ which also showed dose dependent attenuation of MTX induced oxidative stress with resultant hepatotoxicity by both biochemical markers and histopathological findings by using ascorbic acid. Ascorbic acid is an essential antioxidant which scavenges free radicals especially hydroxyl and super oxide radicals by electron donation. It boosts the activity of endogenous antioxidant enzymes facilitating disposal of reactive oxygen species. Ascorbic acid prevents reactive oxygen species induced lipid peroxidation and thereby protects liver. It also stabilizes mitochondrial electron transport chain and thereby

inhibiting glutathione loss from mitochondria and subsequent apoptosis.⁷

Alpha tocopherol is a potent antioxidant that prevents lipid peroxidation by binding to oxygen radicals in biological lipid membranes.²¹ This antioxidant effect of alpha tocopherol is evident from the results of our study as it significantly reduces ALT and ALP when co administered with MTX. However, the histopathological findings of mice liver showed marked inflammation and pleomorphism. A study by Bordbar et al²² showed no improvement of mild hepatic fibrosis with concomitant use of alpha tocopherol in leukemic children receiving low dose MTX along with other chemotherapeutic agents. Whereas, in a preclinical study vitamin E report to mitigate MTX induced liver injury²¹. This hepatoprotective effect of alpha tocopherol against MTX is also supported by a clinical study in patients of rheumatoid arthritis.²³ This is also supported by a clinical study. This controversy between the results of different studies may be resolved by conducting similar studies in large number of patients and dose adjustment of antioxidant such as alpha tocopherol.

Conclusion

The results of our study showed that ascorbic acid exerts a hepatoprotective effect against methotrexate-induced hepatotoxicity. In contrast, alpha-tocopherol did not provide complete protection against hepatic oxidative damage caused by methotrexate. The hepatoprotective potential of ascorbic acid may be attributed to its antioxidant properties. However, further studies are recommended to explore whether higher doses and different routes of administration of both ascorbic acid and alpha-tocopherol have greater hepatoprotective effects against methotrexate-induced liver damage.

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