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Elucidation of the role of α-lipoic acid and vitamin C in methotrexate-induced hepatoxicity in mice

H.M. AL-KURAISHY[1], R.R. ABD HASAN[1], A.I.A. AL-GAREEB[1], S.S. AGGAD[2], A.S.M. ALJOHANI[3], I.M. EL-ASHMAWY[3,4], E. ELEKHNAWY[5], S.A. MOSTAFA[6], W.A. NEGM[7], G.E. BATIHA[3]

[1]Department of Clinical Pharmacology and Medicine, College of Medicine, University of Al-Mustansiriyah, Baghdad, Iraq
[2]Department of Food and Nutrition, Faculty of Human Sciences and Design, King Abdulaziz University, Jeddah, Saudi Arabia
[3]Department of Veterinary Medicine, College of Agriculture and Veterinary Medicine, Qassim University, Buraydah, Saudi Arabia
[4]Department of Pharmacology, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt
[5]Pharmaceutical Microbiology Department, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[6]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[7]Department of Pharmacognosy, Faculty of Pharmacy, Tanta University, Tanta, Egypt
[8]Department of Pharmacology and Therapeutics, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt

Abstract. – OBJECTIVE: Although methotrexate (MTX) is used to treat several malignancies and chronic inflammatory diseases, its clinical use is constrained because of its negative side effects, the most prevalent of which are hepatotoxicity and nephrotoxicity. So, this study aims to determine whether α-lipoic acid (ALA) and vitamin C can protect mice against the liver damage that methotrexate causes.

MATERIALS AND METHODS: A total of 49 male mice were divided into seven groups at random. Group I received sodium bicarbonate, while groups II to VII received an intraperitoneal injection of MTX (20 mg/kg) on the tenth day, following ten days of pretreatment with ALA (60 mg/Kg), ALA (120 mg/Kg), vitamin C (100 mg/Kg), vitamin C (200 mg/Kg), ALA (60 mg/Kg), and vitamin C (100 mg/kg).

RESULTS: When compared to mice in group I, mice in group II (the control group) had significantly higher levels of the enzymes malondialdehyde (MDA), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) and significantly lower (p <0.05) levels of the enzymes superoxide dismutase (SOD) and glutathione (GSH). As compared to the control group, pretreatment groups with ALA and vitamin C showed a dose-dependent substantial rise (p <0.05) in GSH and SOD levels, a dose-dependent notable decrease (p <0.05) in MDA, ALT, ALP, and LDH levels, and better liver histological architecture. In order to increase the antioxidant capacity, pretreatment with ALA and vitamin C may be able to prevent MTX-induced hepatotoxicity.

CONCLUSIONS: These results imply that ALA and vitamin C are useful in the treatment of MTX-induced liver damage.

Key Words:
Liver enzymes, Oxidative stress, Antioxidant, Histological assessment, α-lipoic.

Introduction

The most frequent cause of acute liver failure in the United Kingdom and the United States is drug-induced liver injury (DILI), which accounts for 50% of liver failure[1]. The low prediction of DILI is due to several variables, including small sample sizes during clinical testing, species differences between preclinical *in vivo* animals and people, and a lack of knowledge of the pathways that can cause toxicity[2]. There is speculation that the increasing prevalence of DILI may be related to the interactions between the drug, host, and en-

Corresponding Authors: Walaa A. Negm, MD; e-mail: walaa.negm@pharm.tanta.edu.eg;
Gaber El-Saber Batiha, MD; e-mail: gaberbatiha@gmail.com;
dr_gaber_batiha@vetmed.dmu.edu.eg;
A.S.M. Aljohani, MD; e-mail: jhny@qu.edu.sa

vironmental variables. DILI has also been linked to non-genetic host traits such underlying liver disease, gender, and age[3].

Children who had DILI may have experienced its effects because of mitochondrial dysfunction. However, the increased lipophilicity and biliary excretion of the medications may have contributed to the cholestatic DILI found in the elderly[4]. Regarding gender, females are more likely to get acute liver failure and hepatic DILI[5]. Five medications, including diclofenac, antibiotics, and macrolides, increase this risk in females. Contrarily, children who take valproate and other antiepileptic medications experience liver damage[4]. Due to changes in drug absorption, distribution, metabolism, and excretion that lead to drug accumulation with age, there is also a high risk of developing liver damage[6].

An immunosuppressant medication called methotrexate (MTX) is prescribed for autoimmune inflammatory diseases like psoriasis and rheumatoid arthritis[7]. MTX is also used to treat multiple sclerosis, Crohn's disease, and organ transplants including allogenic bone marrow to prevent graft-versus-host disease[7]. Acute liver injury is one of MTX's significant side effects, which is characterized by elevated levels of liver enzymes and oxidative stress indicators and decreased levels of glutathione and superoxide dismutase[8].

The primary defense against oxidative stress is provided by antioxidant characteristics, which include glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA)[9]. In addition, key liver damage biomarkers include the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP). This enzyme's infiltration into the bloodstream is a crucial quantitative marker of acute liver injury[10]. A mitochondrial fatty acid called α-lipoic acid (ALA), also known as thioctic acid, has a variety of functions in several disorders, including cancer, fibrosis, ischemic/reperfusion injury, diabetes mellitus, liver injury, atherosclerosis, Alzheimer's disease, and autoimmune diseases[11,12,13]. ALA is a potent antioxidant dietary supplement with anti-inflammatory properties that may work as an antioxidant enzyme cofactor[13].

Additionally, vitamin C is a dietary supplement antioxidant that is successful in treating many oxidative stress illnesses[14,15,16]. Antioxidants like ALA and vitamin C may therefore be useful in treating acute liver damage brought on by MTX due to their antioxidant effects. The current study aim was to ascertain if ALA or vitamin C, alone or in combination, can prevent acute liver injury caused by MTX.

Materials and Methods

Drugs and Chemicals

MTX was acquired from Kocak Pharma (Istanbul, Turkey), ALA from America Medic & Science (Woodinville, WA, USA), and vitamin C from UNIPHAR (Dublin, Ireland). The remaining chemicals and solvents were all bought from Merck (Rahway, NJ, USA).

Animals

The Center of Medical Genetic Research provided adult, healthy male albino Swiss mice. They were set up with an artificial 12:12 h light-dark cycle and at a suitable temperature of 22-25°C. They were allowed free access to water and regular meal pellets during their one-week acclimatization period. The study was approved by Ethical Committee of Tanta, Faculty of pharmacy (Approval code # TU/ RE/ 10/ 22P-0047).

Study Design

Figure 1 displays the study design. In a nutshell, 49 Swiss albino mice, each weighing 30-40 g and aged 3-4 months, were divided into 7 groups, with 7 mice in each group, as follows:

Group I: Using oral gavage, mice were given distilled water and sodium bicarbonate treatments for 10 days; on the tenth day of the experiment, they were only given an intraperitoneal injection of normal saline.

Group II: Mice were gavaged with distilled water and sodium bicarbonate for 10 days; on the tenth day of the experiment, they only received a single intraperitoneal injection of MTX (20 mg/kg).

Group III: Mice received intraperitoneal injections of MTX (20 mg/kg) on the tenth day after receiving ALA 60 mg/kg orally for ten days via oral gavage.

Group IV: Mice received intraperitoneal injections of MTX (20 mg/kg) on the tenth day after receiving ALA 120 mg/kg orally for ten days.

Group V: Mice received intraperitoneal injections of MTX (20 mg/kg) and 100 mg/kg vitamin C orally over the course of ten days using oral gavage.

Group VI: Mice received intraperitoneal injections of MTX (20 mg/kg) on the tenth day

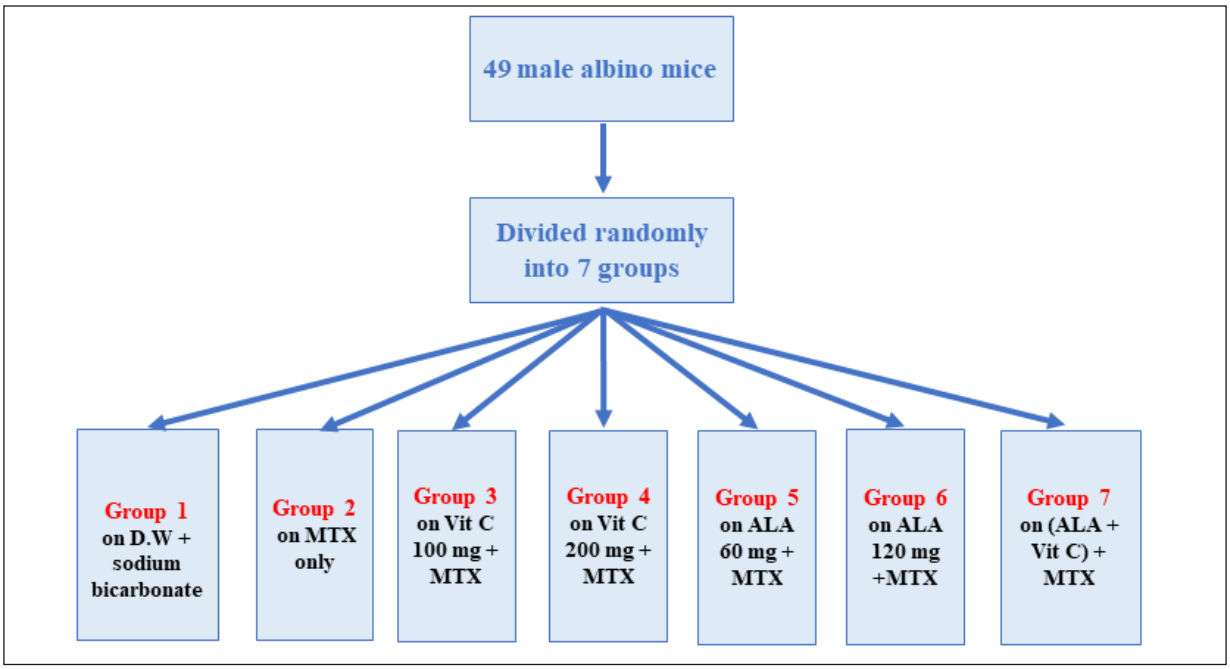

Figure 1. Flow diagram of the utilized study design (mg: mg/Kg body weight).

after receiving vitamin C 200 mg/kg orally for ten days by oral gavage.

Group VII: Mice were administered ALA 60 mg/kg and vitamin C 100 mg/kg orally for ten days by oral gavage, and on the tenth day, MTX 20 mg/kg was administered intraperitoneally (Figure 1). According to a prior study[17,18], the amount of MTX was employed.

Measurement of Liver Enzymes and Oxidative Stress Biomarkers

Mice were sedated using sodium pentobarbital, and blood samples were taken for biochemical evaluation of liver enzymes (ALT, AST, ALP) using Flexor-EL80 automated device reader (Vita lab, Sandton, South Africa), and LDH using ELISA kit (MyBioSource, San Diego, CA, USA) by ELISA reader, at the conclusion of the experiment (i.e., after 48 hours of the last treatment) (Huma reader, Wiesbaden, Germany). The liver samples from the sacrificed mice were then conserved, fixed in 10% formalin, and kept until they were needed for histopathological analysis. Small pieces of the dissected liver were then chopped, weighed, and cleaned in distilled water. Phosphate-buffered saline was then added, and it was combined using a homogenizer before being centrifuged for 15 minutes at 4,000 rpm.

Histological Assessment

The liver tissues were rinsed with phosphate buffer saline (PBS) until all of the fixative agent was gone after 48 hours of being fixed in 10% formalin. Using ethanol and xylene, the automated tissue processor (LEICA TP1020, Wetzlar, Germany) processed the liver tissues. The tissues were processed, imbedded in freshly made liquid paraffin by a paraffin dispenser, and then kept on a cold plate at 4°C for preservation. The manual rotary microtome (LEICA RM2245, Wetzlar, Germany) was used to cut the paraffin-embedded tissue block into 5 m sections, which were then mounted on glass slides and allowed to air dry for 12-24 hours at 25°C. The formalin-fixed paraffin-embedded tissue sections were then rehydrated, deparaffinized, hematoxylin and eosin (H&E) stained, and seen under a light microscope (Olympus, Japan)[19].

Scoring System of Histopathological Changes

According to the quantity and degree of the changes, the injury score for altered liver histology was graded according to a severity scale that varied from 0 to 3[20]:

1. Pathological alterations are denoted by (-).
2. Very little changes in less than 5% of the field are indicated by (+/-).

3. (+) means changes within 20% of the field or less (mild change).
4. (20-60%) means changes in the field as a whole (++) (moderate change).
5. Changes in more than 60% of the field are indicated by (+++) (severe change).

Changes include:

- Congestion in the sinusoids and hepato-portals.
- Hydropic degeneration and cloudy enlargement.
- Necrosis in cells (nuclear pyknosis, karyorrhexis, and karyolysis).
- Cellular infiltration of inflammation.

Statistical Analysis

The analysis was done with SPSS version 16 (SPSS Inc., Chicago IL, USA). The data for this investigation were displayed as mean SD. To compare the control and methotrexate groups, an unpaired *t*-test was used. The significance of differences between the groups was investigated using a one-way ANOVA and a post hoc test. When the probability (*p*-value) was less than 0.05, it was deemed significant.

Results

Effect of the Different Treatments on the Liver Enzymes

MTX considerably increased the levels of ALT, ALP, and LDH (p-value <0.05) when compared to the control group. Additionally, the AST level rose but failed to achieve significance.

Furthermore, pretreatment with vitamin C at low and high dosages considerably (p-value <0.05) reduced the ALT level in comparison to MTX group. Comparing the vitamin C to the MTX group, the ALP level dropped considerably (p-value <0.05) in the vitamin C group. In addition, compared to the MTX group, vitamin C considerably (p-value <0.05) reduced LDH levels. Additionally, pretreatment with low dosages of vitamin C raised ALT, ALP, and LDH levels, although the difference from the control group was not statistically significant. Additionally, high dose vitamin C increased ALT and LDH levels while having no statistically significant difference from the control.

Compared to the MTX group, pretreatment with low and high dosages of ALA resulted in a marked decrease (p-value <0.05) in ALT activity. ALP activity was considerably reduced (p-value < 0.05) in the high dose (120 mg/kg) of the ALA pretreatment group. Compared to the MTX group, the LDH activity in the ALA pretreatment group dropped substantially (p-value <0.05). Additionally, pretreatment with ALA at a low dose (60 mg/kg) improved ALT and LDH activities with no discernible differences from the control group. Improved ALT, ALP, and LDH levels as a result of pretreatment with a high dose of ALA led to non-significant changes from the control group.

Pretreatment with the combination of ALA and vitamin C reduced LDH levels substantially (p-value <0.05) when compared to the MTX group. Additionally, ALT, AST, and ALP levels significantly differed from the control after pretreatment with the combined drugs. Figure 2 depicts how several therapies affected the liver enzymes.

Effect of the Different Treatments on the Oxidative Stress Biomarkers

In comparison to the control, MTX induced a marked decrease (p-value <0.05) in SOD and GSH levels and an increase (p-value <0.05) in MDA. When compared to the MTX group, pretreatment with vitamin C at a low dose (100 mg/Kg) significantly boosted SOD (p-value <0.05). Relative to the MTX group, the MDA level significantly decreased following vitamin C administration (p-value <0.05). Additionally, there was a noticeable difference between the pretreatment effects of low and high vitamin C on the levels of SOD, MDA, and GSH relative to the control group.

When compared to the MTX group, pretreatment with ALA resulted in a notably higher (p-value <0.05) level of hepatic SOD. When compared to the MTX group (p-value <0.05), the ALA pretreatment group's hepatic MDA concentration was considerably lower (p-value <0.05). Additionally, in relation to the control group, pretreatment with a low dose of ALA produced a significant change in SOD, MDA, and GSH levels. MDA concentration was reduced, and concentration was increased as a result of the high dose of ALA. The modifications, however, did not differ significantly from the control group. In comparison to the MTX group, pretreatment with ALA and vitamin C led to a significantly lower MDA concentration (p-value < 0.05).

Additionally, in relation to the control, the combined pretreatment significantly altered the levels of SOD, MDA, and GSH. Figure 3 depicts how the various treatments affected the oxidative stress indicators.

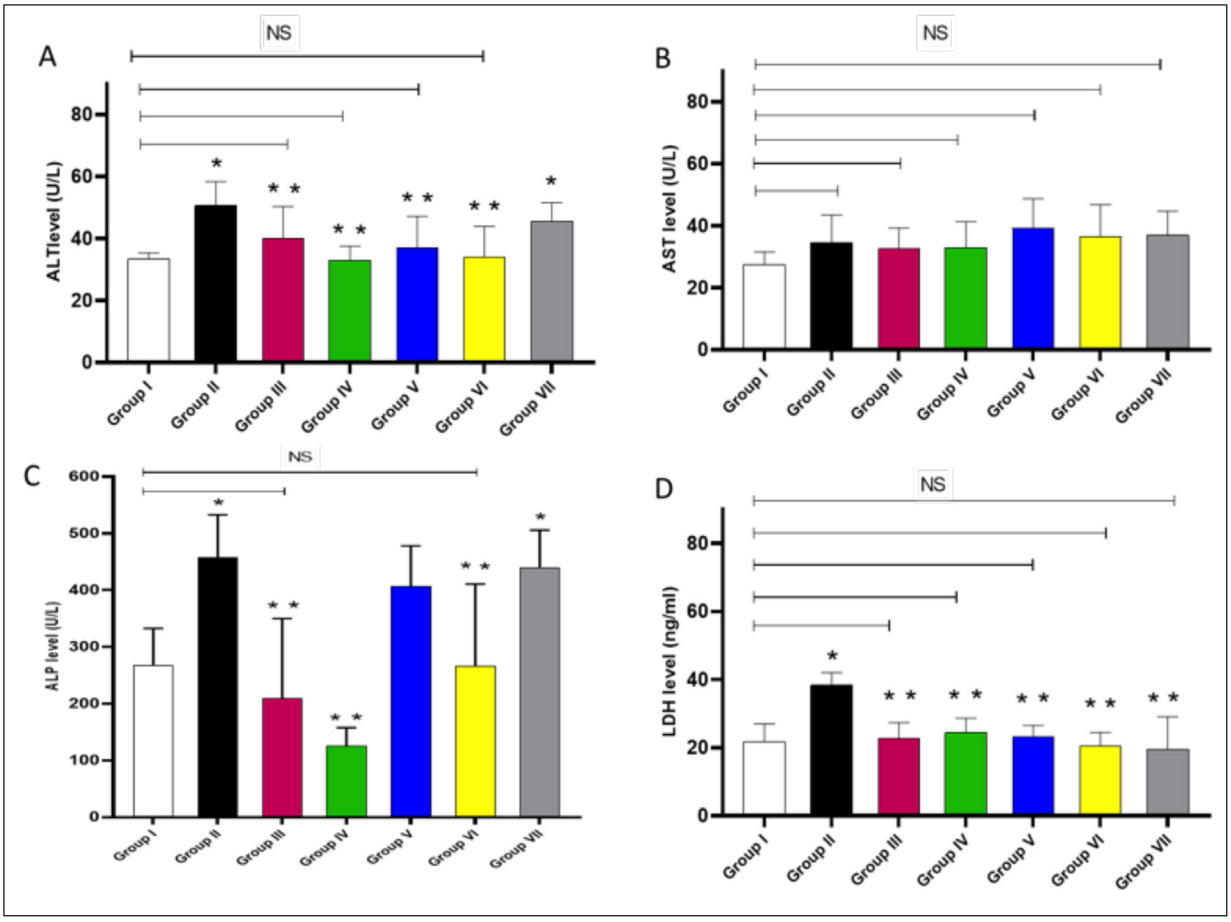

Figure 2. **A**, Alanine aminotransferase (ALT), (**B**) aspartate aminotransferase (AST), (**C**) alkaline phosphatase (ALP), and (**D**) lactate dehydrogenase levels (LDH) after the various treatments. The data are displayed using an unpaired *t*-test and the mean and standard deviation. If a biomarker in the MTX group has a superscript (*), it means that there is a significant difference (p-value <0.05) between it and group I. A significant difference (p-value <0.05) from group II is denoted by the superscript (**). The designation (NS) stands for a non-significant deviation from group I.

Liver Histopathological Changes

Figure 4 shows the histological analysis of liver sections from the control (H&E; 10×) which revealed normal hepatic lobules with a central vein (yellow arrow) surrounded by normal hepatocytes (red arrow) and normal sinusoid (blue arrow), scoring zero; the control group (H&E; 40×) revealed normal hepatic lobules with a central vein (yellow arrow) surrounded by normal hepatocyte (blue arrow), score of injury was moderate (++) 40%, MTX group (H&E; 40×) displayed multifocal cellular necrosis (pyknosis and karyolysis), hydropic degenerative hepatocyte and vacuolation, sinusoidal dilatation, and severe inflammatory reaction, and score of injury was severe (+++) > 70% (Figure 4).

Furthermore, the histological liver section of the vitamin C (100 mg/Kg) group (H&E; 40×) showed scattered inflammatory cells (black arrows), mild sinusoidal dilatation (blue arrows), and mild hepatocyte degeneration (red arrows). The score of injury was mild (+) 12% in each case. The vitamin C (200 mg/Kg) group (H&E; 40×) showed mild central vein congestion (brown arrows), scattered degenerative (black arrow), score of injury was very mild change (+/-) less than 5%, ALA (60 mg/Kg) and ALA (120 mg/Kg) group (H&E; 40×) showed scattered hepatocyte hydropic degeneration (red arrow), mild sinusoidal dilatation (blue arrow), and very mild inflammatory reaction (black arrow), and combined (ALA and vitamin C) group (H&E; 40×) showed central vein congestion (brown arrow), mild hepatocyte (Figure 5).

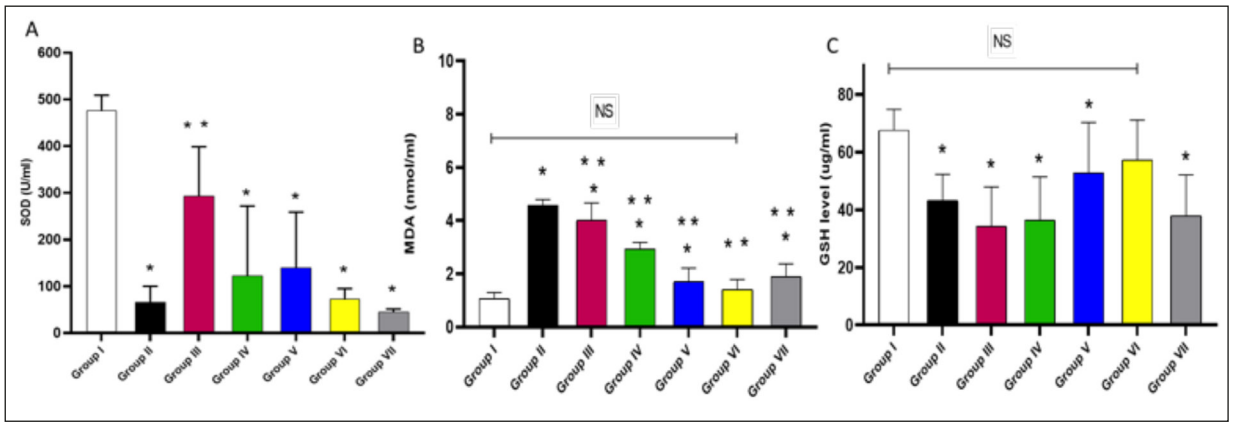

Figure 3. **A**, Superoxide dismutase (SOD), (**B**) Malondialdehyde (MDA), and (**C**) Glutathione levels as a result of various treatments (GSH). The data are displayed using an unpaired *t*-test and the mean and standard deviation. If a biomarker in the MTX group has a superscript (*), it means that there is a significant difference (p-value < 0.05) between it and group I. A significant difference (p-value <0.05) from group II is denoted by the superscript (**). (NS) stands for a non-significant difference in comparison to group I.

Discussion

MTX-induced hepatic injury has been related to several variables, including treatment duration, risk factors, and genetic and molecular elements[21]. In this study, MTX caused a substantial reduction (p<0.05) in SOD level and increased MDA concentration as compared to the control, this finding is comparable to a previous study[22]. In animals with MTX-induced hepatotoxicity, the elevated level of MDA in the liver may be due to membrane lipid peroxidation caused by free radical generation and the inability of antioxidant defense systems to prevent the creation of excessive free radicals[23]. SOD is the first line of defense against the damaging effects of reactive

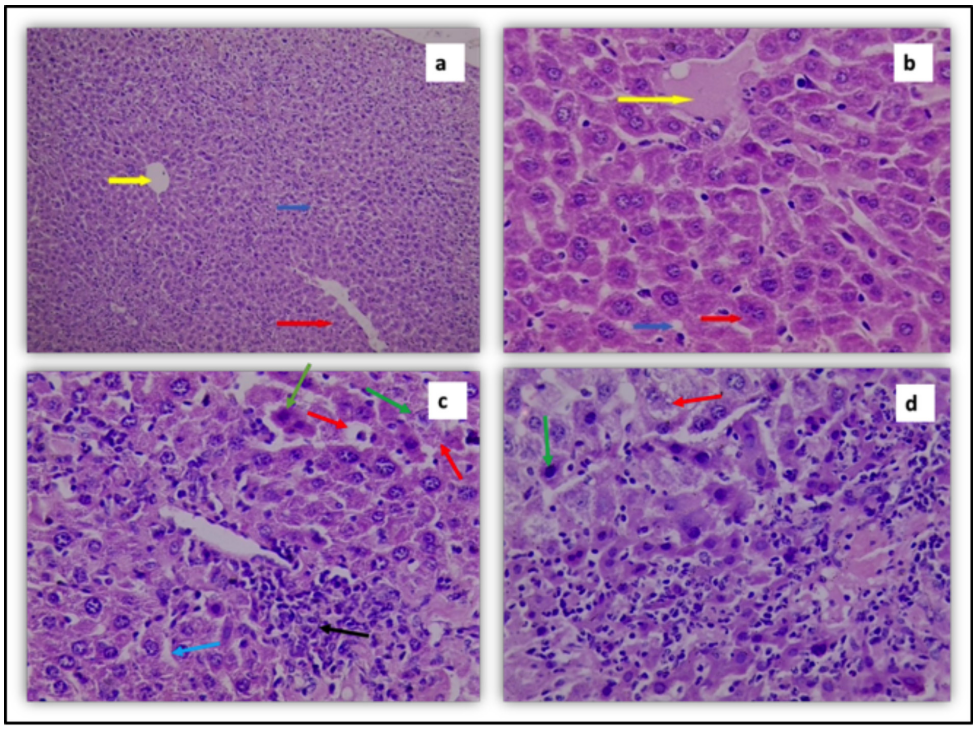

Figure 4. Liver sections examined histologically of (**a**) control group (H&E; 10×), (**b**) control group (H&E; 40×), (**c**) methotrexate (MTX) group (H&E; 10×), and (**d**) methotrexate (MTX) group (H&E; 40×).

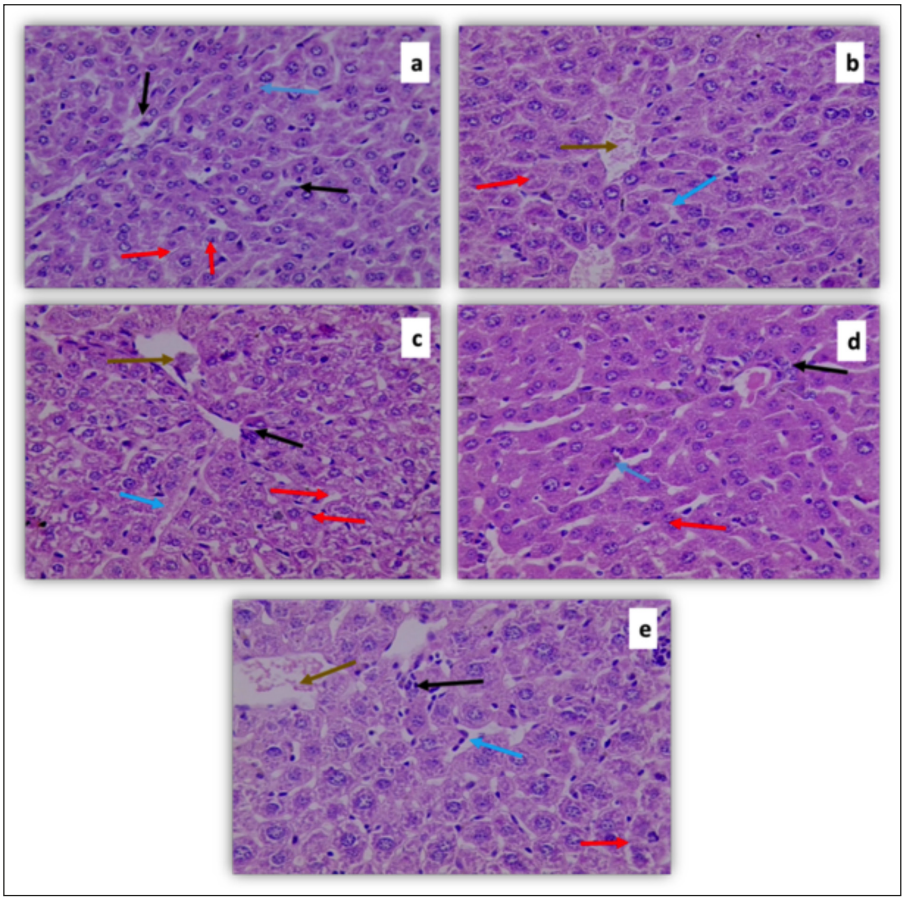

Figure 5. Histological liver sections of the following groups: (**a**) vitamin C (100 mg/Kg); (**b**) vitamin C (200 mg/Kg); (**c**) ALA (60 mg/Kg); (**d**) ALA (120 mg/Kg); and (**e**) combined (ALA and vitamin C) group (H&E; 40×).

oxygen species; by speeding up the dismutation of superoxide anion-free radicals into molecular oxygen and hydrogen peroxide (H_2O_2), it lowers the level of O_{2-}, which is detrimental to cells at high concentrations[24,25]. It is well known[26,27] that GSH protects membrane lipids from oxidative attack by providing them with protons. As one of the byproducts of polyunsaturated fatty acid peroxidation, MDA is a useful indicator of the degree of lipid peroxidation[24,28,29]. When compared to the control group, the ALT level in the current study considerably rose (p <0.05) in the MTX group. This might be connected to oxidative stress and lipid peroxidation impairing the liver structural integrity. The cytoplasmic enzymes are released into the bloodstream as a result of necrosis, which is caused by lipid peroxidation, which also affects the membrane bilayer and stability of the cell[30]. Other liver enzymes like AST are less accurate indicators of hepatocellular cell damage than ALT[31].

ALT levels are frequently higher than AST levels when there is an acute hepatic injury[32,33]. Comparing the MTX to the control group, the level of ALP increased considerably (p-value <0.05), which may be related to bile duct obstruction and increased blood ALP emission from canalicular spilling into the hepatic sinusoid[34,35].

Increased LDH activity indicates cell death and lysis and can be utilized as a marker for cellular damage and chemical cytotoxicity[36,37].

After six months of treatment, MTX is efficient in controlling psoriatic arthritis (PsA) and rheumatoid arthritis (RA)[38]. Additionally, after MTX therapy, well-known gastrointestinal problems have been recorded[38]. These symptoms were also present in RA and PsA patients prior to MTX administration. The Methotrexate Intolerance Severity Score should be used to regularly monitor RA and PsA patients using MTX in order to discover MTX intolerance early and take appropriate action to prevent stopping a beneficial treatment[39]. The emergence of MTX-induced hepatotoxicity in RA and PsA patients is associated with prolonged MTX use[40].

Patients in a prospective analysis[40] with 550 RA and 69 PsA patients receiving MTX treatment showed signs and symptoms of he-

patotoxicity. A single weekly dose of folic acid decreased the incidence of MTX-induced hepatotoxicity, according to a study by Garcia et al[41] that involved RA and PsA patients receiving MTX treatment. These findings imply that long-term MXT use is associated with the development of hepatotoxicity, and that folic acid taken after MTX therapy may help prevent MTX-induced hepatotoxicity.

The use of ALA or vitamin C alone or in conjunction with other treatments in mice with MTX-induced hepatotoxicity was found to be useful in this condition by reducing oxidative stress in this experimental study.

A low dose of vitamin C induced a non-significant drop in MDA levels in the current investigation. However, the high dose of vitamin C markedly decreased the MDA content (p <0.05), indicating that the impact of vitamin C as an antioxidant is dose dependent. This is in line with a prior study[42]. The observed decrease in liver enzyme levels in the pretreatment groups with vitamin C may be attributed to its capacity to protect the hepatocyte membrane from oxidative damage and reduce cellular content leakage[10].

The experimental work by Akbulut et al[43] showed that the antioxidants N-acetyl cysteine and amifostine were superior to vitamin C in reducing the MTX-induced hepatotoxicity in mice.

In accordance with a prior study[44], there was a considerable reduction in the MDA concentration in the pretreatment groups which received low and high dosages of ALA. The observed decrease in ALT, ALP, and LDH in groups who received the low and high dosages of ALA prior to treatment in the MTX group may be explained by ALA's ability to reduce enzyme leakage.

Therefore, the current study validated that ALA may have hepatoprotective effects against MTX-induced liver damage in mice. It is interesting to note that ALA reduced the hepatotoxicity caused by MTX by preventing mitochondrial toxicity and free radical scavenging[45,46]. It was proven[47] that using ALA might stop MTX-induced hepatotoxicity just as well as folic acid. Additionally, ALA is beneficial in treating a variety of inflammatory and oxidative stress diseases that are common in RA patients[48]. When compared to folic acid's effectiveness in preventing MTX-induced hepatotoxicity, ALA decreased liver injury indicators in a fashion comparable to folic acid[49].

In groups pretreated with the combined regimen (ALA and vitamin C), there was a decrease in MDA concentrations and a corresponding rise in the concentration of GSH. This could have been due to the protective effect of both substances against lipid peroxidation brought on by MTX-induced toxicity.

In earlier studies[14,50], giving rats vitamin C, ALA, and silymarin lowered the MDA level with acetaminophen-induced liver damage by a negligible amount. In a different study[51], silymarin and 200 mg/Kg vitamin C significantly boosted SOD levels in acetaminophen-induced liver injury.

The findings of this study demonstrated that there was no significant rise in the levels of ALT, ALP, and LDH in groups treated with the combined regimen of ALA and vitamin C as compared to groups pretreated with vitamin C to ALA alone. This means that, compared to utilizing ALA or vitamin C alone, the liver enzyme that was investigated in this study will not be considerably increased by the addition of vitamin C to ALA. According to the statistical analysis of the current investigation, ALA and vitamin C together had a less significant hepatoprotective effect on MXT-induced hepatotoxicity than ALA or vitamin C alone.

In contrast to mice in the group treated with ALA low dose, those in the group pretreated with a high dose of ALA showed superior histological characteristics that presented with very mild inflammatory infiltration, scattered hepatocyte degeneration, and no cellular necrosis. Our result is in line with a prior study[52] that claimed the antioxidant and anti-inflammatory properties of ALA were responsible for its protective effects.

Both high and low vitamin C pretreatment groups exhibit the same histological traits as the ALA pretreatment group. These results may be attributed to vitamin C's capacity to reduce MDA and boost SOD, which protects against superoxide anion radicals in addition to its anti-inflammatory effects, which have improved liver cell damage and may stimulate the renewal of hepatocytes[52]. Similar research[53] shows that vitamin C reduces triptolide-induced acute liver damage *via* indicating oxidative stress.

The liver portion exhibited mild changes in the group pretreated with an ALA and vitamin C mixture. These changes are characterized by sporadic degenerative hepatocytes without cellular necrosis, mild central venous congestion, and mild inflammatory reaction. According to the histology findings, vitamin C alone or ALA alone do not provide as much hepatoprotection against MTX-induced liver injury.

Conclusions

The current study demonstrated that hepatotoxicity caused by MTX is characterized by increased liver enzymes, lipid peroxidation, oxidative stress, and decreased antioxidant capacity. Anti-MTX-induced hepatotoxicity pretreatment with either ALA or vitamin C resulted in greater hepatoprotective benefits than their combination. Therefore, ALA or vitamin C use may be advantageous in reducing the risk of MTX-induced hepatotoxicity in patients with chronic MTX use, such as those with psoriasis and rheumatoid arthritis. To confirm the protective effects of ALA, vitamin C, and their combination in reducing MTX-induced hepatotoxicity, large-scale experimental, preclinical, and prospective studies are necessary.

Ethics Approval

The study was approved by Ethical Committee of Tanta, Faculty of pharmacy (Approval code # TU/ RE/ 10/ 22P-0047).

Availability of Data and Materials

All data are contained within the article.

Conflict of Interest

The authors declare no conflict of interest.

Informed Consent

Not applicable.

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ORCID ID

Walaa A. Negm: 0000-0003-0463-8047
Gaber El-Saber Batiha: 0000-0002-7817-425X

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