Original Article

Hepatoprotective Effects of Alpha-Lipoic Acid, Vitamin C Alone, or in Combination on Methotrexate-Induced Liver Injury

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Abstract

Background: Different mechanisms have been attributed to methotrexate (MTX)-induced liver injury, one of which is related to MTX generation of free radicals and inducing the disturbance of oxidative stress biomarker. Alpha-lipoic acid (ALA) is a strong antioxidant dietary supplement and has an anti-inflammatory effect. Vitamin C is an antioxidant and might produce an anti-inflammatory action through its antioxidant effect. **The Aim of the Current Study:** To evaluate the effect of ALA and Vitamin C single or concurrent use of them on MTX-induced liver injury. **Materials and Methods:** Thirty-five adult male albino mice were divided into five groups: Group 1 were administered distilled water and sodium bicarbonate orally by oral gavage for 10 days and injected normal saline intraperitoneally (ip) in the 10th day, Group 2 were injected MTX ip on the 10th day only, Group 3 were administered 100 mg/kg Vitamin C orally for 10 days and injected MTX ip on the 10th day, Group 4 were administered ALA 60 mg/kg orally for 10 days and injected MTX ip on the 10th day, Group 5 were administered both ALA and Vitamin C orally and injected MTX ip on the 10th day. After 2 days of the last treatment, the animal was anesthesized and the blood was withdrawn to be used for biochemical assessment of liver functions and liver tissue was dissected out to be used for oxidative stress biomarker determination and histopathological study. **Results:** MTX group showed an increase in serum level of alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase, an increase liver tissue malondialdehyde (MDA), and a reduction of tissue glutathione and superoxide dismutase (SOD), while the treatment groups showed an improvement of liver enzyme elevation and decreased lipid peroxidation MDA and increased SOD. **Conclusion:** Pretreatment by using ALA alone or vitamin alone provides comparable improvement of liver histology and liver injury while combined regimen did not provide more significant improvement of liver injury than using each one alone.

Keywords: Alpha-lipoic acid, liver injury, methotrexate, reactive oxygen species, Vitamin C

Introduction

Methotrexate (MTX) is greatly utilized as a primer option to treat rheumatoid arthritis and psoriatic disease and various autoimmune diseases. It is also used as a traditional systemic disease-modifying antirheumatic drug.[1] Liver injury might be produced as a result of different drugs, one of which is chemotherapeutic drug, as the liver is responsible for the removal of toxic material to inhibit its toxic effect, and it may predispose to liver damage that could appear in different forms. MTX could induce liver injury through blocking metabolism of folate, decrease level of folate in the liver lead to improper DNA replication, and cause toxin aggregation in the liver and increase transaminase level.[2] As Methotrexate enters the cell through reduced folate carrier 1, it is polyglutamated through folypolyglutamate synthase, MTX polyglutamate aggregate intracellularly and by this way methotrexate has the ability to prevent different enzymatic reaction.[3] Liver toxicity due to MTX might occur due to the prevention of tetrahydrofolate synthesis by interaction with dihydrofolate reductase, resulting in apoptosis mediated by DNA damage.[4] Moreover, MTX may decreases the synthesis of methionine and antioxidant enzyme (superoxide dismutase [SOD], catalase, and glutathione [GSH] peroxidase) which leads to increase reactive oxygen species (ROS) production. These were associated with harmful effect on proteins, lipid, DNA, and different cell organelles and promotes apoptosis in the

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liver. .[5] MTX suppresses polyamine-producing enzymes in an indirect manner. According to the postulated mechanism of action, MTX indirectly inhibits the enzymes that produce polyamines. Reduced polyamine synthesis thus results in higher intracellular ROS levels.[7] MMP breakdown, mitochondrial enlargement, decreased mitochondrial ATP and GSH levels, and cytochrome C release are all symptoms of an increase in ROS.[7]

Ascorbic acid is a water-soluble (hydrophilic) antioxidant present in fruits such as strawberries and lemon and in green vegetables such as tomatoes and spinach.[8] Ascorbic acid is used as a cofactor by eight human enzymes;[9] it is a powerful antioxidant that works by donating and transferring electrons to neutralize oxidative damage. Vitamin C can diminish unstable oxygen, nitrogen, and sulfur radicals in the body while also renewing other antioxidants such as alpha-tocopherol. Vitamin C has also been demonstrated to be beneficial in reducing lipid peroxidation caused by peroxide radicals in the human plasma.[10]

Alpha-lipoic acid (ALA) or thioctic acid which is 1,2-dithiolane-3-pentanoic acid[11] is a mitochondrial fatty acid involved in metabolism of energy and has various actions in different diseases such as cancer, fibrosis, ischemic/reperfusion injury, and diabetes mellitus.[12] ALA is named antioxidant of antioxidant as it undergoes recycling of various cellular antioxidants including Vitamin C, coenzyme Q10, GSH, and Vitamin E (alpha-tocopherol);[13] it is synthesized in the mitochondria of plants and animals through reaction stimulated by lipoic acid synthase from octanoic acid and cysteine through donation of sulfur; ALA has hydrophilic and hydrophobic proprieties.[14]

Table 1: Scoring System of Histopathological Changes

Score	Meaning
–	Mean no pathological change
+/–	Mean very mild change
+	Mean change in <20% of field
++	Mean change in 20%-60% of field
+++	Mean change in more than 60% of field

The current study was aimed to evaluate hepatoprotective effects of ALA, Vitamin C alone, or in combination on MTX-induced liver injury.

Materials and Methods

Thirty-five male adult Swiss albino mice were provided from Iraqi Center of Cancer Research, their weight ranged from 30 to 40 g, mice were put for 1 week without intervention to be acclimated and supplied with water and normal chow pellets, and the care of animal was according to the international guidelines for care and use of laboratory animal.

MTX was provided from Kocak Pharma (Turkey), Vitamin C was supplied from UNIPHAR (EC), ALA was supplied from America Medic and Science (USA), serum level assay of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was done through using automated device analyzer flexor-EL80 (Vita Lab, South Africa), while kits used for the assay of tissue live of malondialdehyde (MDA), SOD, and GSH and serum level of lactate dehydrogenase (LDH) were purchased from Mybiosource (USA).

Experimental design

the animals were divided into five groups, and in each group, 7 mice were included in the study; Group 1 (control group) mice were treated with distilled water and sodium bicarbonate orally for 10 days and injected normal saline on the 10th day only, Group 2 (MTX group) mice were injected MTX 20 mg/kg intraperitoneally (ip) (single injection) on the 10th day of the study only, Group 3 (ALA group) mice were treated with oral dose 60 mg/kg of ALA by utilizing oral gavage for 10 days and injected MTX ip 20 mg/kg on the 10th day, Group 4 (Vitamin C group) mice were treated with Vitamin C 100 mg/kg for 10 days orally by oral gavage and injected MTX ip 20 mg/kg on the 10th day, and Group 5 (combination) mice were treated with Vitamin C 100 mg/kg and ALA 60 mg/kg for 10 days and injected MTX ip 20 mg/kg on the 10th day.

After 2 days of the last treatment, the mice were anesthetized through using chloroform, and blood sample was withdrawn

Table 2: Effect of Alfa lipoic acid, Vitamin C and combination on Liver enzyme and Oxidative stress Biomarkers in Methotrexate Induced Liver toxicity as compare to Methotrexate and Control group

Variable	Control	Methotrexate	Vitamin C	ALA	Combination
ALT (U/L)	33.4±4.92	50.7±7.67$^{\#}$	40.2±10.1^{¶,a}	37.2±9.95*^{,a}	45.5±6.05$^{\#}$
ALP (U/L)	267.2±65.6	458±74.9$^{\#}$	209±141*^{,a}	407±70.9	439±66.8$^{\#}$
LDH (ng/ml)	21.8±5.20	38.4±3.62$^{\#}$	22.8±4.53*^{,a}	23.2±3.73*	19.6±9.52*^{,a}
MDA (nmol/ml)	1.07±0.22	4.58±0.21$^{\#}$	4.01±0.65$^{\P,\#}$	1.70±0.51*	1.90±0.47*$^{,\#}$
GSH (ug/ml)	67.6±7.22	43.1±9.19$^{\#}$	34.3±13.6$^{\#}$	52.8±17.4$^{\#}$	37.9±14.2$^{\#}$
SOD (U/ml)	476±33.5	65.7±34.5$^{\#}$	294±205*$^{,\#}$	140±119$^{\#}$	68.7±26.4$^{\#}$

$^{\#}$mean highly significant difference from control group, *mean highly significant difference from methotrexate group, ^{a}mean non significantly different from control group, ¶mean significant different from methotrexate group. The data were presented as mean±SD, Combination: Alpha-lipoic acid and Vitamin C, $P<0.05$, $^{\#}P<0.01$, Highly significant difference from control group, $^{\P}P<0.05$, Significant difference from MTX; *$P<0.01$, Highly significant difference from MTX, $^{a}P\geq0.05$, Nonsignificantly different from control. ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, MDA: Malondialdehyde, GSH: Reduced glutathione, SOD: Superoxide dismutase, ALA: Alpha-lipoic acid, SD: Standard deviation

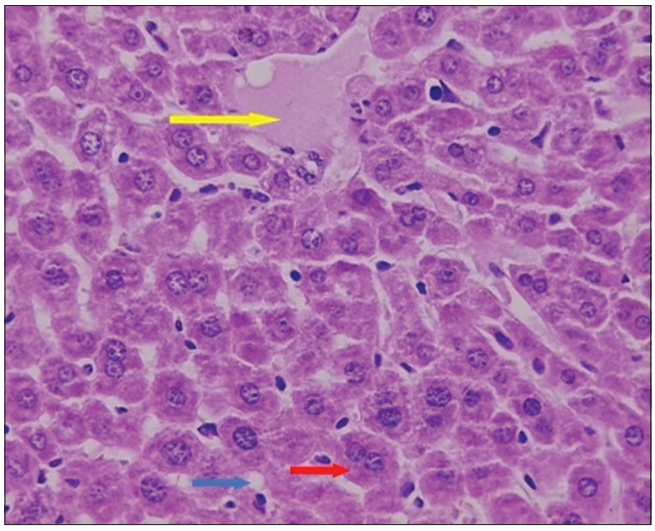

Figure 1: Histological liver segment of control group (H and E, ×40) showing normal liver architecture with a central vein (yellow arrow) surrounded by normal hepatocyte (red arrow) and normal sinusoid (blue arrow). Score is zero

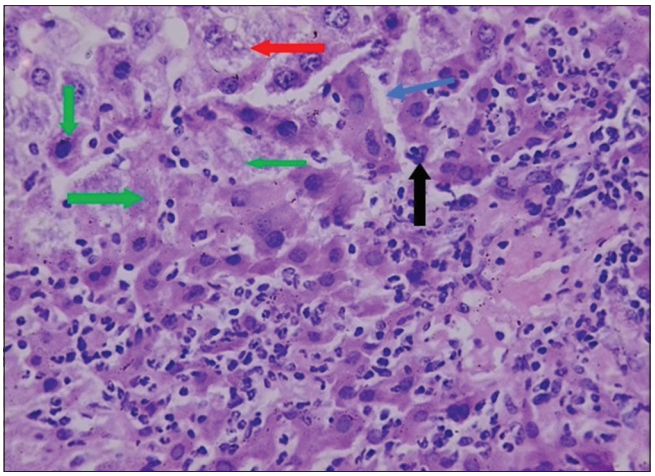

Figure 2: Histological liver segment of methotrexate group (H and E, ×40) showing multifocal cellular necrosis (pyknosis and karyolysis, green arrow), hydropic degenerative hepatocyte and vacuolation (red arrow), sinusoidal dilatation (blue arrow), severe inflammatory reaction (black arrow). Score of injury was sever (+++) >70%

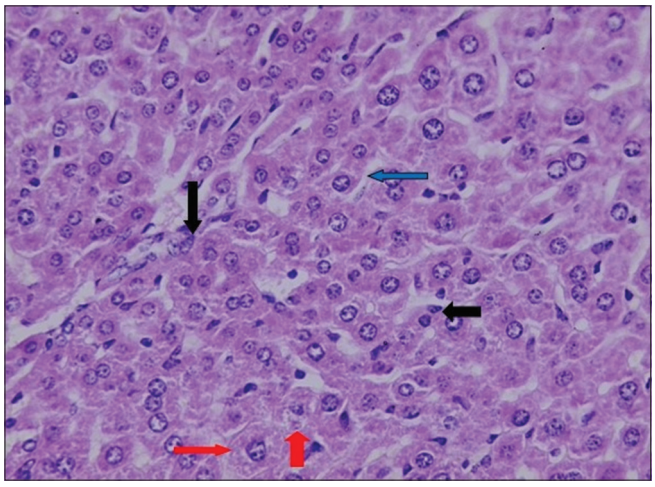

Figure 3: Histological liver segment of Vitamin C 100 mg/kg group (H and E; ×40) showing mild hepatocyte degeneration (red arrow), scattered inflammatory cell (black arrow), sinusoidal dilatation (blue arrow). Score of injury was mild (+) 12%

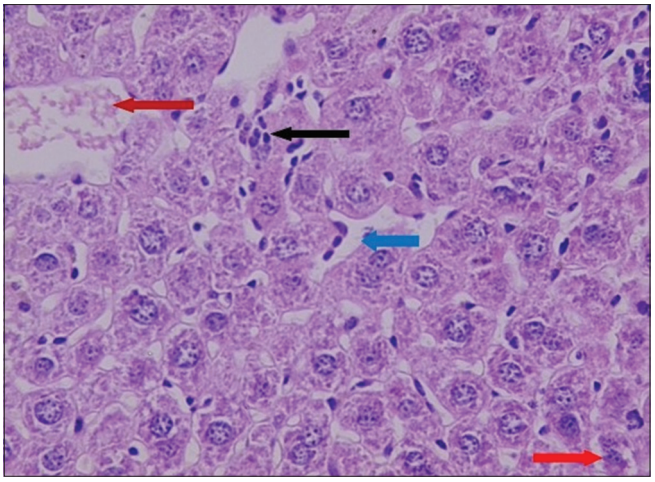

Figure 4: Histological liver section of combined (Alpha-lipoic acid and Vitamin C) mice group (H and E; ×40) showing central vein congestion (brown arrow), mild hepatocyte hydropic degenerative change (red arrow), mild inflammatory reaction (black arrow), and sinusoidal dilatation (blue arrow). Score of injury was mild (+) 10%

by direct needle puncture of heart, was allowed to be settled in a sterile gel tube, and was centrifuged at 4000 rpm for 10 min at room temperature. The mice were sacrificed when blood sample withdrawn was completed and the abdomen was cut by using a sharp scissors and the liver was dissected out and then cut it into two parts. The smallest one was used for oxidative stress assay of tissue and the largest part was converted to plane tube and added 10% formalin to preserve the tissue structure from autolysis till use for histopathological study.

The assay of liver tissue MDA was made through using enzyme-linked immunoassay (ELISA) kit that based on double antibody sandwich technique according to the manufacturers' instructions, liver tissue GSH was determined by using ELISA kit which depends on double antibody sandwich method according to the manufacturers' instructions, and determination of liver tissue SOD was made through using ELISA kit which was based on quantitative sandwich method, and serum level of LDH was determined through using ELISA kit according to the manufacturers' instruction.

The assay of serum level of ALT and ALP was made through utilizing automated device analyzer flexor – EL80.

Histopathological study

Liver tissue was dehydrated in different alcohol concentrations and paraffin-embedded; the paraffin-embedded tissue block was cut into 7 μm segment and using hematoxylin and eosin stain for tissue staining. Liver injury score can be classified

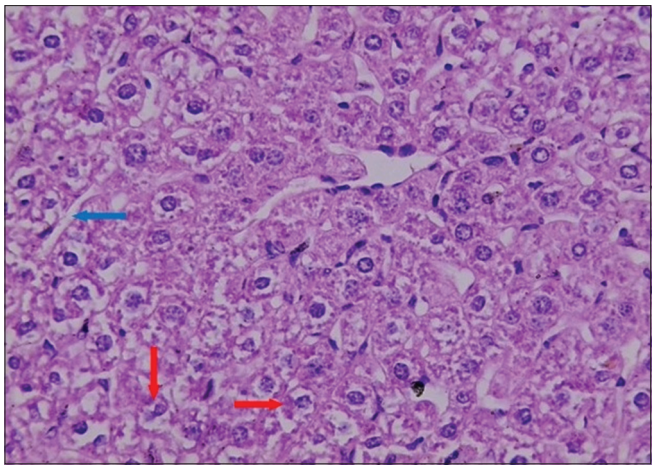

Figure 5: Histological liver segment of alpha-lipoic acid 60 mg/kg group (H and E; ×40) showing hepatocyte degenerative change (red arrow) and sinusoidal dilatation (blue arrow). Score of injury was mild (+) <15%

according to grade of severity depending on the extent and degree of change as follows.[15]

Results

Effect of methotrexate, Vitamin C, and alpha-lipoic acid on liver enzyme

The serum level of ALT, AST, and LDH in Methotrexate Group was increased significantly (highly significant) when compared to control group ($P < 0.01$), in group pretreated with Vitamin C was showed decrease ALT level significantly ($P < 0.05$) in addition to decrease level of ALP and LDH significantly (highly significant) ($P < 0.01$) when compared to MTX group, serum level of ALT and LDH were decrease significantly and ALP level was decrease but not significant in group pretreated with ALA when compared to MTX group, group pretreated with combined regimen showed decrease level of LDH significantly (highly significant) ($P < 0.01$) and decrease ALP and ALT level but not significant.

Effect of methotrexate, Vitamin C and alfa-lipoic acid on oxidative stress

MTX group showed an increase in MDA level significantly (highly significant) ($P < 0.01$) when compared to control group, SOD decreased significantly (highly significant) ($P < 0.01$) in the MTX group when compared to control group as shown in Table 2, GSH decreased significantly (highly significant) in MTX group when compared to control group. Group pretreated with Vitamin C was result in decreased MDA significantly ($P < 0.05$) when compared to MTX group, SOD increased significantly (highly significant) ($P < 0.01$) when compared to methotrexate group, but level of GSH was decreased to a level lower than MTX group shown in Table 2. ALA pretreatment group led to decrease MDA level significantly (highly significant) ($P < 0.01$), SOD and GSH increased but not significantly when compared to MTX as shown in Table 2. Group pretreated with combined regimen showed decreased MDA level significantly (highly significant) ($P < 0.01$), SOD increased but not significantly and GSH decreased to level lower than MTX group as shown in Table 2.

Histopathological change

The histopathological changes of the control group in the present study was show normal liver architecture with a central vein surrounded by normal hepatocyte and normal sinusoid. Score of injury is zero as shown in Figure 1, while in methotrexate group was show multifocal cellular necrosis (pyknosis and karyolysis), hydropic degenerative hepatocyte and vacuolation, sinusoidal dilatation, severe inflammatory reaction. Score of injury was sever (+++) >70% as shown in Figure 2 in group pretreated with Vitamin C 100 mg/kg was show mild hepatocyte degeneration, scattered inflammatory cell, sinusoidal dilatation. Score of injury was mild (+) 12% as shown in Figure 3 in group pretreated by using combination of (Alpha-lipoic acid and Vitamin C) was show central vein congestion , mild hepatocyte hydropic degenerative change , mild inflammatory reaction , and sinusoidal dilatation . Score of injury was mild (+) 10% as shown in Figure 4 in group pretreated with alpha-lipoic acid 60 mg/kg was hepatocyte degenerative change and sinusoidal dilatation. Score of injury was mild (+) <15% as shown in Figure 5.

Discussion

The present study found that MTX injected IP induced significant reduction of SOD level as comparable to control group, these findings are consistent with previous studies.[16-18] SOD is the first line of defense against damage produced by ROS which accelerate the dismutation of superoxide anion free radical into molecular oxygen and hydrogen peroxide (H_2O_2), lowering the level of O2−, which damages the cell at high level.[19] The level of superoxide Dismutase (SOD) was reduce in the current study could beto raise superoxide anion levels, which are recognized to inactivate GSH peroxidase, which responsible for reduce H_2O_2 to water and oxygen utilizing GSH, when GSH peroxidase fails to eject H_2O_2 from the cell, the accumulating H_2O_2 has been demonstrated to trigger SOD deactivation.[20] Level of reduced GSH was reduced significantly in the current study in MTX group when compared to control group; these results are in the same line with previous studies.[16-18] GSH level is essential to preserve the redox balance. H_2O_2 is reduced to H_2O through GSH peroxidase oxidation of GSH to mean glutathione disulfide (GSSG) that could mitigate H_2O_2 released by manganese SOD.[21] GSH level reduction caused by MTX in the current study could be attributed to MTX-induced reduction of intracellular nicotinamide adenine dinucleotide phosphate (NADPH). Under normal situation, NADPH is essential to preserve the reduced GSH storage, which defends against ROS; as MTX reduces NADPH level, the reduction of NADPH will lead to deplete GSH levels and elevate the vulnerability of liver cells to the produced radical, resulting in liver damage.[22,23] MDA level in the current study increased significantly in the MTX group when compared to control

group; these findings are in consistent with previous studies.[16-18] MDA it is one of the final products of polyunsaturated fatty acid peroxidation, and it is useful indication of the extent of lipid peroxidation.[18] Increased level of MDA in the present study in group injected MTX could be related to ROS generation such as superoxide anion radical.[24] Lipid peroxidation and cell injury are initiated when free radicals interact with membrane lipids and polyunsaturated fatty acids.[25] The increased level of ALT in the present study caused by MTX might be attributed to the disruption of certain amino and nucleic acid synthesis caused by MTX by blocking the enzyme tetrahydrofolate reductase, which transforms folic acid to tetrahydrofolate. The blocking of amino acid synthesis could induce injury to hepatic parenchymal cells' organelles and cell membranes, impairing their function and permitting enzyme leakage[26] . Moreover, hepatocellular necrosis leads to elevate permeability of the cell membrane, triggering the release transaminase into the bloodstream.[27] Level of ALT in the current study increased significantly in the MTX group as comparable to control group; these findings agreed with previous studies.[28] Level of LDH in the current study increased significantly in MTX group as comparable to control group; these findings are consistent with previous studies.[16,17] LDH level increased after acute liver injury might be related to enzyme spillage through the injured hepatocyte membranes; increased LDH production could also be due to anerobic circumstances. In anerobic conditions, hepatocytes produce more LDH until they become necrotic,[29] disruption to the plasma membrane of hepatocytes can cause LDH to seep into the bloodstream,[8] and increase level of ALP in MTX group in the present study could be related to bile duct occlusion because of increased translation of ALP mRNA (interceded by increasing bile acid concentration) and increased emission of ALP into serum through canalicular spillage into the hepatic sinusoid.[30] Level of SOD in the present study was increased when pretreated by using Vitamin C when compared to methotrexate group; these findings are consistent with previous study.[31] Increase level of SOD in the present study when pretreated by utilizing Vitamin C might be attributed to ability of ascorbate to interact with biologically produced radicals such as superoxide, tocopheroxyl radicals, and alkoxyl/peroxyl radicals. Ascorbate could thus assist SOD for elimination of superoxide *in vivo*[32] as Vitamin C has the ability to conserve mitochondria from oxidative stress induced by increasing leak of electron from dysfunctional electron transport chain.[33] It is interesting to found that the level of reduced glutathione (GSH) in the present study decreased in the group pretreated with Vitamin C which disagreed with previous study[34] that found using Vitamin C against liver injury by lead acetate toxicity result in an increase of GSH level, depletion of GSH in the present study in group pretreated with Vitamin C might be attributed to metabolism of Vitamin C inside the cell, as Vitamin C has two isoforms: ascorbic acid and dehydroascorbic acid and the interchange between these two isoforms needs GSH as a cofactor[35] as dehydro-ascorbic acid reduction and conversion to ascorbic acid is expected to be ensured by NADPH and GSH. The conversion of significant amounts of DHA to AA through NADPH and GSH-dependent processes might decrease the amount of NADPH and GSH inside the cell;[36] in addition, great exposure of hepatic cell to high level of free radical that generated during MTX treatment induces reduction of GSH level, adding to that Vitamin C needs GSH as cofactor which might contribute to depletion of GSH**.** The increase level of malondialdehyde (MDA) in the present study decreased when used Vitamin C, this might be attributed to free radicals can be effectively scavenged by Vitamin C before they can cause lipid peroxidation,[37] this result is consistent with previous study.[31] Decreased level of ALT, ALP, and LDH in the present study by using Vitamin C could be related to the ability of Vitamin C to defend the hepatocyte membrane against oxidative injury, minimizing cellular content leakage.[38] Increase level of SOD in the present study in group pretreated with ALA could be due to capacity of ALA to elevate SOD gene expression that is a factor in the synthesis of SOD.[39] Increase level of GSH in the current study when pretreated with ALA might be related to ability of ALA to donate electrons to the GSSG for reduction; by this method, ALA can reestablish the reduced/oxidized GSH ratio (GSH/GSSG) [14] that is essential to protect against oxidative stress. In addition, ALA raises Nrf2-dependent transcriptional activity by creating lipoyl-cysteinyl mixed disulfides on Keap1, and as such, activation of Nrf2 will cause GSH replenishment[40] as Nrf2 modulates antioxidant reactions by promoting the production of cytoprotective genes. Cell survival is improved when the expression of Nrf2 was increased[40] decrease MDA level in the present study by utilizing ALA is agree with previous study[41]which might be related to ability of ALA to repair oxidized damage and replenishes endogenous antioxidants such as GSH and Vitamins C and E,[42] and the disulfide bond of ALA might be interact immediately with ROS;[43] furthermore, free radicals such as superoxide, H_2O_2, peroxyl, singlet oxygen, peroxyl, and hydroxyl radicals might be successfully scavenged by LA, avoiding cellular damage induced by free radicals; all these mechanisms might lead to decreased MDA by using ALA;[44] the decreased level of ALT and LDH significantly in group pretreated with ALA could be related to ALA ability to decrease lipid peroxidation (MDA) and increase antioxidant biomarker (SOD and GSH) due to its antioxidant capacity and anti-inflammatory action. While in group pretreated with combination of ALA and Vitamin C was showed decrease MDA significantly but GSH level was decreased which might be related to Vitamin C recycling lead to GSH consumption, SOD was increased but not significant might be due to low sample size or small dose, decrease level of LDH was significantly. Liver histology of MTX group showed cellular necrosis (nuclear pyknosis, karyolysis), severe inflammatory reaction, hydropic degenerative hepatocyte and vacuolation as shown in Table 1, score of injury was sever (+++) >70% which is consistent with,[17] while in treatment group was showed improvement in liver injury and decrease score of hepatic injury from (+++) severe

to (+) mild characterized by decrease inflammatory reaction and disappear of necrosis and improvement of degenerative hepatocyte as shown in Table 1. As a result, we found that pretreatment by using ALA alone or Vitamin C alone is better than using combination because, in the present study, the combination of ALA and Vitamin C was not produce more improvement of liver injury, this might be related to small sample size or low dose and low duration of pretreatment, and if we increase the dose and sample size, it might produce further protection.

Conclusion

Prophylaxis against MTX-induced liver injury can be provided by using either ALA alone or Vitamin C alone because their combination not result in further significant improvement in liver injury when compared to use ALA alone or Vitamin C alone.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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