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Original Article

Effects of Omega-3 and Vitamin C on Methotrexate-Induced Liver Injury

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Abstract

Context: Methotrexate (MTX)-induced liver injury is a serious side effect characterized by the increased level of hepatic biomarkers and resulted in acute liver failure. Omega 3 and Vitamin C act as antioxidant that participate in the fighting of free radicals generation during the inflammatory process. **Aims:** To evaluate the effect of omega 3 and Vitamin C on hepatotoxicity induced by MTX. **Settings and Design:** 42 (Swiss albino mice) used and divided into six groups (7 mice each): First: Maintained with normal saline, second: Received a single dose injection of MTX (20 mg/kg, intraperitoneally), third: Pretreated with omega 3 100 mg/kg, fourth: Pretreated with omega 3 200 mg/kg, fifth: Pretreated with Vitamin C 100 mg/kg, sixth: Pretreated with Vitamin C 200 mg/kg, then these group injected with MTX on day 10. **Subjects and Methods:** MTX as 50 mg injection. Omega 3 as capsule 1000 mg. Vitamin C as powder 1000 mg. Assessment of liver enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST], and alkaline phosphatase [ALP]) made using automated computering device (Flexor–EL80) provider by Vitalab (South Africa). Assessment of oxidative stress markers (malondialdehyde [MDA], superoxide dismutase [SOD], reduced glutathione [GSH]) and lactate dehydrogenase (LDH) made by using competitive ELISA kits using (ELISA microplate Humareader). **Results:** This study showed a significant increase in the liver enzymes (ALT, AST, ALP, and LDH) as well oxidative stress markers (MDA, SOD, and GSH) with severe changes in the histopathological findings (severe inflammatory cell necrosis) among group injected with MTX as compared with control group and illustrated improvement in serum level of ALT, ALP, LDH, MDA, SOD and reduced GSH; besides improved histopathological findings (mild and moderate changes) for a group of mice pretreated with omega 3 and Vitamin C. **Conclusions:** This study concluded that pretreatment with omega 3 (which was strong antioxidant supplement) and Vitamin C (which was dose-dependent manner with beneficial antioxidant action) exert more hepatoprotective effect against oxidative tissue damage induced by MTX.

Keywords: Hepatotoxicity, methotrexate, omega 3, Vitamin C

Introduction

Drug-induced liver injury (DILI) is remarkable and challenging, it makes about 3%–5% of person admit to the hospital due to acute liver injury and jaundice in most Western countries, causing drug withdrawal, liver transplantation, and acute liver failure.[1] DILI perform different clinical manifestation including liver enzymes increased, hepatitis, hepatocellular necrosis, cholestasis, fatty liver, and liver cirrhosis.[2] In general, DILI is divided into intrinsic, idiosyncratic, and indirect injury types; intrinsic DILI commonly is predictable and dose-dependent (e.g., acetaminophen toxicity), while Idiosyncratic DILI is unpredictable and does not affected by the dose immediately (e.g., methotrexate [MTX]-induced liver injury).[2,3] MTX is a folate antagonist, which recognized as the anti-metabolite, chemotherapeutic agent that has immunomodulatory and anti-inflammatory activities. MTX act as the first-line disease-modifying anti-rheumatic drug in the treatment of rheumatoid arthritis, psoriasis, and juvenile idiopathic.[4,5] Fundamental action of MTX is competitive irreversible dihydrofolate reductase inhibitor and deplete stimulation of purine and pyrimidine required for DNA and RNA production in rapidly malignant cells division resulting in its cytotoxic effects.[6] MTX metabolized to polyglutamate

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and store in the hepatocyte for long time. Excess metabolites accumulation can generate oxidative stress and stimulation of the immune system starting with hepatic satellite cells (HSCs) which result in leukocyte accumulation, neutrophils secretion, and activate proinflammatory cytokines like nuclear factor-kappa B (NF-κB) and tumor necrosis factor-α (TNF-α) that leads to fatty infiltration of the live and fibrosis.[7]

Since MTX increases the production of reactive oxygen species (ROS) in the hepatocyte. That cause damage to mitochondrial lipids, proteins, and DNA resulted in structural alteration, loss of function, inhibition of the electron transport chain ETC, activities of Complexes II, III, and IV, decreases ATP synthesis as well increases the generation of superoxide anions, which accumulate due to decreased activity of superoxide dismutase (SOD) and deplete glutathione (GSH). Furthermore, MTX increases the inflammation and generation of oxidative stress due to the accumulation of lipid peroxidation leading to decrease energy formation and increase the level of malondialdehyde (MDA) with more cellular damage.[8] The apoptotic effect of MTX is applied by upregulation of p21 and p53 protein expression and production of oxidative stress.[9,10]

Omega 3 fatty acid present as phospholipids when two fatty acids bind to the phosphate group via glycerol and as triglycerides when three fatty acids connected to glycerol. It is characterized by anti-inflammatory, antioxidant, immunomodulation activities, omega 3 long-chain fatty acid divided into 3 important types as α-linolenic acid (18:3), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6) depending on the location of double bound and carbon atoms.[11] omega 3 had the ability to scavenger ROS/reactive nitrogen species (ROS\RNS) by enhanced total antioxidant capacity.[12] The anti-inflammatory effect of omega 3 to protect the liver against progressive damage produce by inhibit the biosynthesis of leukotriene by 5-lipoxygenase, reduced prostaglandin synthesis, dampening pro-inflammatory cytokines and decrease hepatic gene expression of TNF-α interleukin (IL-1), interferon- and IL-6 also inhibition of NF-κB with the regulation of Kupffer cell activities.[13] Vitamin C (L-ascorbic acid) is a more potent hydrophilic antioxidant agent that oxidized to dehydroascorbic acid (DHA).[14] Vitamin C reacts with oxygen or with free ions to provide of superoxide, H_2O_2 and highly reactive oxidants, such as the hydroxyl radical also provide a role in the inhibition of cytokines, NF-κB, TNF-α, IL-1, and IL-6 in plasma by its anti-inflammatory and anti-apoptotic effects.[15] Vitamin C can prevent liver damage by its efficient antioxidant capacity and scavenges the free radicals ROS and maintain the cellular membrane integrity against lipid peroxidation and oxidative stress. It also prevents H_2O_2 induced lipid peroxidation.[16]

Aim of the study

This study aimed to evaluate the hepatoprotective effect of omega 3 and Vitamin C on liver injury induced by MTX.

Subjects and Methods

The experimental study was implemented in the animal's house at the Department of Pharmacology–College of MedicineAL-Mustansiriyah University and the Iraqi Center of Cancer Research and Medical Genetics (ICCMGR), AL-Mustansiriyah University, Baghdad, Iraq. The period of study from November 2020 to February 2021 (the real interval to allow the drugs was twelve days for each groups of study). A 42 (Swiss albino mice) their aged about (2–3) months and their body weight ranged from (20 to 40) g were collected from (ICCMGR). These animals divide into six groups; the first group was maintained with normal saline via oral gavage, the second group received a single dose injection of MTX (20 mg/kg, intraperitoneally, third group pretreated with omega 3 in dose 100 mg/kg, fourth group pretreated with omega 3 in dose 200 mg/kg, fifth group pretreated with Vitamin C in dose 100 mg/kg and sixth group pretreated with Vitamin C in dose 200 mg/kg for 9 days then each group inject with MTX on day 10th. MTX was purchased from a local pharmacy as 50 mg/5 ml injectable solution (KOÇAK Pharma, Ístanbul, Turkey). Omega 3 was purchased as soft gelatin capsule 1000 mg (NOW FOODS, USA). Vitamin C was purchased as powder sachet 1000 mg (UNIPHAR, EC). The estimation of serum of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) was done using automated computering device called (Flexor–EL80) provider by Vitalab (South Africa). Serum level of MDA, SOD, reduced GSH (oxidative stress markers) and lactate dehydrogenase (LDH) were measured by competitive ELISA kits using (ELISA microplate Humareader) to read the optical density that measured the spectrophotometry at the wavelength (450 nm) within 10 min. Tissue sample collected for histopathological study by taken part of the liver settled in the tube with 10 ml of formalin 10%. Liver sectioning and embedding were done according to (paraffin-embedded method) to prepare liver tissue for microscope evaluation, then the tissue was stained with hematoxylin and eosin. The scoring system of histopathological examination was made according to Semi-quantitative scoring that ranked from 0 to 3 depending on the degree and extent of the alteration.[17] The histopathological changes described below: (−) no pathological lesions, (±) very mild changes in <5% of fields, (+) histopathology changes in <20% of fields, (++) histopathology changes in 20%–60% of fields and (+++) histopathology changes in >60% of fields. The statistical analysis was performed by SPSS (IBM) SPSS version 16, package for windows 8, New York ,USA. A one-way ANOVA test was used to estimate the significance of differences among different groups (control group and another group). The level of significance was considered when $P < 0.05$.

Results

In comparison with the control group, MTX treated group showed a significant increase in the level of hepatocellular enzymes (ALT, ALP, and LDH) ($P = 0.003$). While AST level showed an insignificant increase in MTX treated group compared with the control group. Furthermore, the result demonstrated a significant elevation in MDA level among

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the group treated with MTX in comparison with the control group: MDA level (4.58 ± 0.21 nmol\l vs. 1.07 ± 0.22 nmol\l) (P = 0.000). While the level of (SOD and GSH) for MTX treated group was decreased significantly (P = 0.000), compared with the control group as shown in Table 1.

Pretreatment with omega 3 in dose 100 mg/kg showed significant reduction in MDA level (2.35 ± 0.28 nmol/ml) compared with MTX treated group (4.58 ± 0.21 nmol/ml) (P = 0.001). With significant increase in the level of SOD and insignificant increase in the level of GSH. Moreover, this result showed a significant reduction in the serum level of hepatocellular enzymes (ALT and ALP and LDH) among the group of omega 3 pretreatment compared with MTX treated group (P = 0.006) while, AST level showed insignificant reduction. When the dose of omega 3 increased to 200 mg/kg the result showed significant decrease in the serum level of MDA as compared with MTX treated group (3.41 ± 1.10 nmol/ml vs. 4.58 ± 0.21 nmol/ml) (P = 0.013). The comparison between pretreated omega 3 in graded dose showed statistically significant reduction in level of MDA among group pretreated with omega 3 dose 100 mg compared with 200 mg omega 3 (P = 0.001). Moreover, pretreatment with omega 3 in dose 200 mg/kg showed significant increase in the level of SOD and insignificant increase in the level of GSH. The liver enzymes (ALT, AST and ALP) showed insignificant decrease among group pretreated with 200 mg omega 3 in comparison with MTX group, while LDH levels decrease significantly compared with MTX-treated group. The level of ALT among group pretreated with 100 mg/kg Omega 3 revealed significant reduction compared with group of pretreated omega 3 in dose 200 mg/kg (P = 0.0512) as showed in Table 2 and Figures 1, 2 and histopathological studies of liver section in Figure 5c and d. Pretreatment with Vitamin C 100 mg/kg showed insignificant reduction in the level of MDA (4.00 ± 0.65 nmol/ml) compared with MTX treated group (4.58 ± 0.21 nmol/l) (P = 0.205). While showed a significant reduction in the level of MDA among group pretreated with dose 200 mg Vitamin C (2.93 ± 0.24 nmol/l) compared with MTX treated group (4.58 ± 0.21 nmol/l) (P = 0.001). As well, The result represented significant reduction in the level of MDA among group pretreated with 200 mg Vitamin C (2.93 ± 0.24 nmol/l) compared with the dose 100 mg Vitamin C pretreated group (P = 0.022). The level of SOD was significant increase among group pretreated with Vitamin C in dose 100 mg (P = 0.004) and the level of GSH in the graded dose of Vitamin C showed insignificant increase compared with the MTX group (P = 0.267), the effect of pretreated Vitamin C on the liver enzymes showed insignificant decrease in the serum level of ALT and AST compared with MTX treated group. While ALP and LDH were statistically significant deplete in group pretreated with Vitamin C in dose 100 mg compared with MTX (P = 0.000), while the level of liver enzymes among group pretreated with Vitamin C in dose 200 mg showed a statistically significant reduction in the level of ALT, ALP and LDH and insignificant decrease in the level of AST in comparison with MTX treated group, as showed in Table 3 and Figures 3, 4 and 5e and f. The Histopathological investigations of experimental livers mice are represented in Figure 5.

Table 1: The effect of methotrexate on the liver enzyme and oxidative stress markers in methotrexate-induced liver injury

Parameters of the liver	Control groups (n=7)	MTX 20 mg/kg (I.P.) groups (n=7)
ALT (ng/ml)	33.42±4.92	50.71±7.67*
AST (ng/ml)	27.42±4.15	34.71±8.78
ALP (ng/ml)	267.28±65.63	458.00±74.91*
LDH (ng/ml)	21.83±5.20	38.48±3.62*
MDA (nmol/ml)	1.07±0.22	4.58±0.21*
SOD (ng/ml)	476.92±33.52	65.77±34.57*
GSH (µg/ml)	67.61±7.22	43.09±9.19*

*P<0.05: Unpaired t-test compared with control group. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, MTX: Methotrexate, I.P.: Intraperitoneally

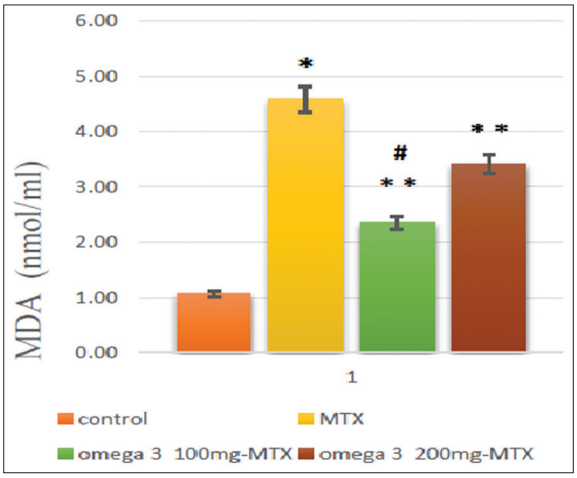

Figure 1: Mean of MDA for group pretreated with omega 3 in methotrexate induced liver injury. *P < 0.05 compared with control group, **P < 0.05 compared with MTX, #P < 0.05 omega 100 mg versus omega 200 mg. MDA: Malondialdehyde, MTX: Methotrexate

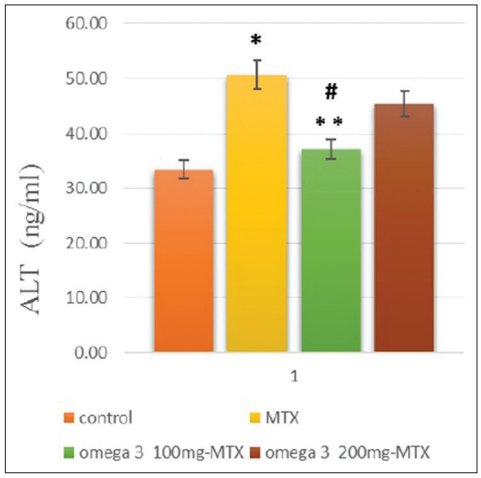

Figure 2: Mean of ALT for group pretreated with omega 3 in MTX induced liver injury. *P < 0.05 compared with control group, **P < 0.05 compared with MTX, #P < 0.05 omega 100 mg versus omega 200 mg. ALT: Alanine aminotransferase, MTX: Methotrexate

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Discussion

MTX-induced liver injury is related to its effect on the synthesis of folate, inflammation, hepatic drug metabolism and accumulation, oxidative stress, and apoptosis. There are several mechanisms of MTX that augmenting liver toxicity involved molecular and cellular antioxidant defense dysregulation and increase ROS, free radicals generation with the enhancement of lipid peroxidation and the tissue oxidative stress biomarkers, the liberation of pro-inflammatory cytokines these leading to intracellular liver enzymes dysfunction.[18-20] This study clarified significant elevation in

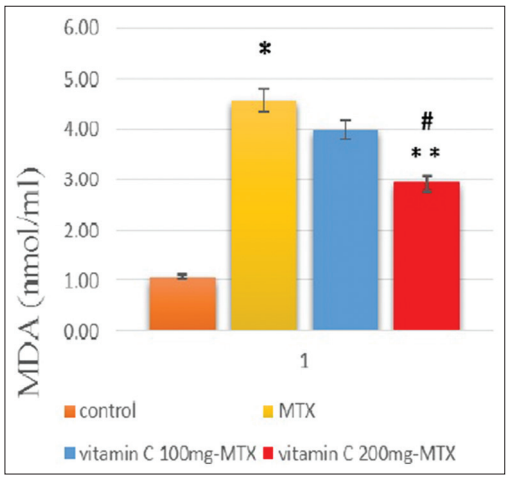

Figure 3: Mean of MDA for group pretreated with Vitamin C in methotrexate induced liver injury. *$P < 0.05$ compared with control group, **$P < 0.05$ compared with MTX, #$P < 0.05$ Vitamin C 100 mg versus Vitamin C 200 mg. MDA: Malondialdehyde, MTX: Methotrexate

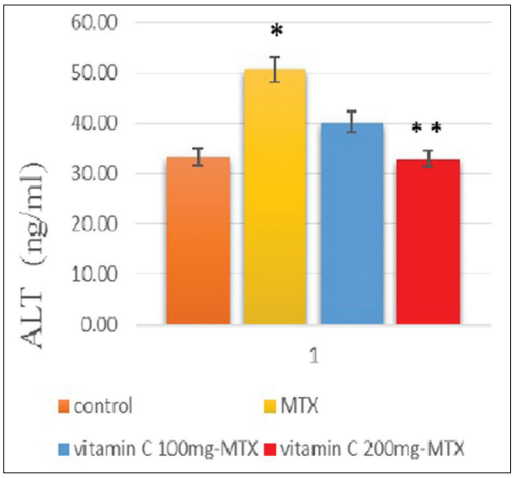

Figure 4: Mean of ALT for group pretreated with Vitamin C in methotrexate induced liver injury. *$P < 0.05$ compared with control group, **$P < 0.05$ compared with MTX. ALT: Alanine aminotransferase, MTX: Methotrexate

Table 2: The effect omega 3 pretreatment on the oxidative stress biomarkers and liver enzymes in methotrexate-induced liver injury

Parameters of the liver	Control groups ($n=7$)	MTX 20 mg/kg (I.P.) groups ($n=7$)	Omega-3 (100 mg) groups ($n=7$)	Omega-3 (200 mg) groups ($n=7$)
MDA (nmol/ml)	1.07±0.22	4.58±0.21*	2.35±0.28**,#	3.41±1.10**
SOD (ng/ml)	476.92±33.52	65.77±34.57*	236.57±112.23**	222.18±130.20**
GSH (µg/ml)	67.61±7.22	43.09±9.19*	49.46±12.37	44.27±10.42
ALT (ng/ml)	33.42±4.92	50.71±7.67*	37.14±7.15**,#	45.42±15.16
AST (ng/ml)	27.42±4.15	34.71±8.78	32.24±9.75	44.00±19.79
ALP (ng/ml)	267.28±65.63	458.00±74.91*	374.57±101.72**,#	438.00±72.90
LDH (ng/ml)	21.83±5.20	38.48±3.62*	19.40±7.81**	19.64±7.32**

*$P<0.05$ significant compared with the control group, **$P<0.05$ significant compared with MTX, #$P<0.05$ significant omega 3 100 mg versus omega 3 200 mg. MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, I.P.: Intraperitoneally, MTX: Methotrexate

Table 3: The effect Vitamin C pretreatment on the oxidative stress markers and liver enzymes in methotrexate-induced liver injury

Parameters of the liver	Control groups ($n=7$)	MTX 20 mg/kg (I.P.) groups ($n=7$)	Vitamin C (100 mg) groups ($n=7$)	Vitamin C (200 mg) groups ($n=7$)
MDA (nmol/ml)	1.07±0.22	4.58±0.21*	4.00±0.65	2.93±0.24**,#
SOD (ng/ml)	476.92±33.52	65.77±34.57*	294.97±205.82**,#	122.61±150.10
GSH (µg/ml)	67.61±7.22	43.09±9.19*	46.16±5.35	48.42±13.61
ALT (ng/ml)	33.42±4.92	50.71±7.67*	40.28±10.16	33.00±4.54**
AST (ng/ml)	27.42±4.15	34.71±8.78	32.85±6.51	33.14±8.27
ALP (ng/ml)	267.28±65.63	458.00±74.91*	209.14±141.01**	126.00±31.85**
LDH (ng/ml)	21.83±5.20	38.48±3.62*	22.89±4.53**	24.40±4.52**

*$P<0.05$ significant compared with the control group, **$P<0.05$ significant compared with MTX, #$P<0.05$ significant Vitamin C 100 mg versus Vitamin C 200 mg. MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, I.P.: Intraperitoneally, MTX: Methotrexate

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the level of MDA among MTX treated group as a result of increased production of lipid peroxidation and free radical generation due to mitochondrial dysfunction and progress the oxidative damage of hepatic tissue, in accordance with the previous study.[21] MTX enhanced mitochondrial damage results in the release of Ca^{2+} ion from endoplasmic reticulum (ER) altered mitochondrial membrane potential and trigger cytochrome c (cyt. c) release, which would activate caspase 3 also cardiolipin oxidation enhanced as a target of ROS, as well increases the generation of oxidative stress due to accumulation of lipid peroxidation leading to decrease energy formation and increase the level of MDA with more hepatocellular damage.[22] In addition, this study illustrated significant decrease in the level of endogenous antioxidant defense enzyme SOD and GSH among the group treated with MTX compared with the control group, in accordance with the previous study.[23,24] this reflected the action of MTX that increase the generation of ROS and impaired in the capacity of the endogenous antioxidant enzyme with enhancement in the production of H_2O_2.[25] MTX cause an imbalance between prooxidant and antioxidant system and decrease GSH which is the first line of defense against oxidation cellular damages and scavengers of highly reactive electrophiles of the drugs by detoxification of H_2O_2 convert it to H_2O.[24] This leads to hepatocellular damage and elevation of intracellular liver enzyme ALT, AST, LDH, and ALP, in accordance with the previous study.[13,22,26] In agreement with the previous study, pretreatment with omega 3 revealed significant decrease in MDA level, enhanced in SOD level and improvement in GSH level with amelioration the hepatic function via reduce the serum level of ALT, ALP, AST, and LDH (especially in dose 100 mg/kg Omega 3).[13,27-30] these findings further promoted by the histopathological report which illustrate reduce in the severity of necrosis induced by MTX to mild score.[22,30] The significant lowering in the level of MDA occurs due to boosted antioxidant, anti-inflammatory and anti-apoptotic actions of omega 3 to inhibited lipid peroxidation, alleviated cellular congestion and inflammation induced by MTX, maintain cellular integrity and exert hepatoprotective actions.[13,30] The lowering in the level of MDA among group pretreated with 200 mg Vitamin C matched with the previous study.[31] Vitamin C exerts strong antioxidant actions and have eligibility to neutralize the accumulation of ROS and/or RNS, with other free radicals generation that causes oxidative damage to the liver tissue in MTX intoxicated mice. This can be attributed to the cytoprotective effect of Vitamin C through reduction the level of MDA and reduce the lipid peroxidation with ameliorate the hepatic necrosis and oxidative tissue damage.[31,32] This study illustrated that the high dose of pretreated Vitamin C showed more cytoprotective effect through significant reduction in the level of MDA than the Vitamin C in low dose.[32] Moreover, the current study revealed the significant elevation in the serum level of SOD and GSH among group pretreated with Vitamin C in graded dose.[33] Vitamin C act as a radical scavenger and it have ability to deplete superoxide radical anion that can then be protonated to yield H_2O_2. As well, Vitamin C up-regulates

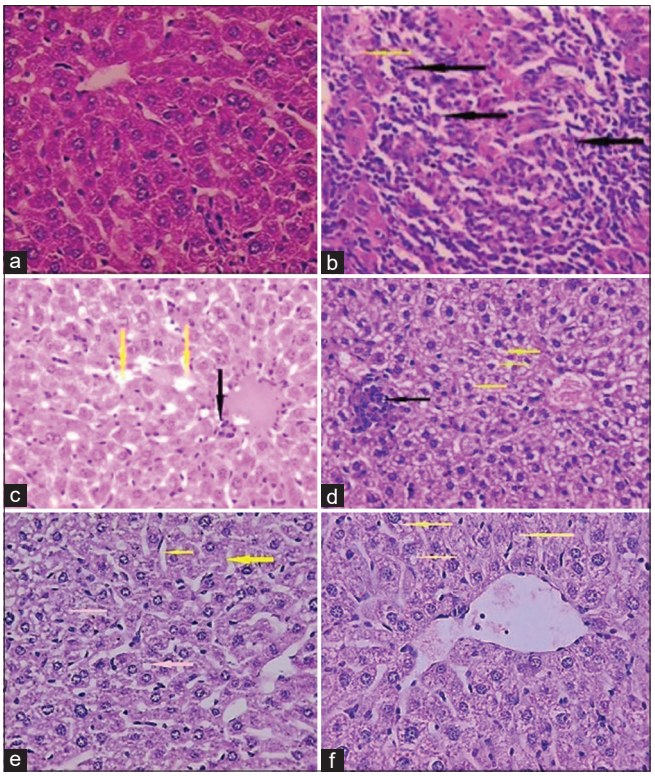

Figure 5: (a) Liver section of control group, it look like normal in structure appearance of the liver tissue lobules Score (0) no changes, (b) liver section of MTX with sever tissue necrosis and sever inflammatory cells infiltration (black arrow) also sever depletion of glycoprotein (green arrow). Sever Score (+++) >60%, (c) liver section of omega 3 in dose 100 mg/kg showing area of mild tissue necrosis (black arrow) with area of mild sinusoidal dilatation (yellow arrow). Mild Score (+) 18%, (d) liver section of omega 3 in dose 200 mg/kg show depletion of glycoprotein inside the hepatocyte cells (green arrow). With necrotic tissue appearance and inflammatory cells infiltration (black arrow). Moderate score (++) 30%, (e) liver section of Vitamin C in dose 100 mg/kg showing accumulation of fat droplet inside the hepatocyte cells (pink arrow) with slight dilatation of sinusoid (yellow arrow). Mild score (+) 15%, (f) liver section of Vitamin C in dose 200 mg/kg showing area of mild depletion of hepatic glycoprotein (yellow arrow). Very mild score (±) <5%. MTX: Methotrexate

SOD level by elimination of superoxide oxygen free radicals and protect hepatic tissue against oxidative damage.[33] The potential antioxidant activities of Vitamin C pretreated group in different doses catalyzed the ROS and convert H_2O_2 to H_2O and ameliorate the hepatic oxidative damage by improved the antioxidant defense enzyme (SOD and GSH). This finding agrees with the histopathological result that showed significant improvement of inflamed tissue necrosis induced by MTX and the score changed from severe-to-mild score for group pretreated with graded dose of vitamin C.[33] The antioxidant, anti-inflammatory, and antiapoptotic effect of pretreatment with Vitamin C in different dose (especially in dose 200 mg Vitamin C) showed improvement in the hepatic function via the reduction in the level of liver enzymes ALT, AST, ALP, and LDH. This is consistent with a previous study that showed alleviation in venous congestion of the liver due to

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anti-inflammatory effect of the Vitamin C through reducing the release of inflammatory mediators and vascular permeability or lowering endothelial dysfunction.[33,34]

Conclusions

This study demonstrated that omega 3 provides hepatoprotective effect and amelioration in the histopathological alterations (especially in low dose) while Vitamin C provides cytoprotective effect by decrease the oxidative tissue damage as dose-dependent manner since both of these agents present with strong antioxidant and anti-inflammatory effects against MTX induced hepatotoxicity.

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Conflicts of interest

There are no conflicts of interest.

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