RESEARCH ARTICLE

Evaluation the effects of Omega-3 and vitamin C alone or in combination on Methotrexate-Induced hepatotoxicity (in mice)

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Abstract

Objective: To assess the effect of pretreatment with omega-3 alone or in combination with vitamin C on hepatotoxicity raised by methotrexate.

Method: The experimental study was conducted at the animal house of the Iraqi Centre of Cancer Research and Medical Genetics and the Department of Pharmacology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq, from November 2020 to July 2021, and comprised Swiss albino mice who were divided into 5 groups. Group A was treated with normal saline, group B was injected with methotrexate 20mg/kg intraperitoneally, group C was pretreated with omega 3 100mg/kg, group D was pretreated with vitamin C 100mg/kg, and group E was pretreated with concomitant vitamin C and omega 3 100mg/kg. The intervention lasted 9 days in each group, and were injected with methotrexate on day 10. The mice were sacrificed under chloroform anaesthesia after 48 hours. Blood sample was taken for biochemical examination. One part of liver was preserved in formalin 10% for histopathological study.

Results: Of the 35 mice, there were 7(20%) in each of the 5 groups. There was a significant increase in malondialdehyde and lactate dehydrogenase levels and a decrease in the superoxide dismutase and glutathione ($p<0.05$).

Conclusion: Pretreatment with omega-3 and vitamin C ameliorated the hepatotoxicity induced by methotrexate due to strong antioxidant, anti-inflammatory and anti-apoptotic effects.

Key Words: Antioxidants, Methotrexate, Chloroform, Saline, Glutathione, Liver, Malondialdehyde, Superoxide, Lactate Dehydrogenases, Ascorbic Acid, Formaldehyde

(JPMA 74: S414 (Supple-8); 2024) **DOI: https://doi.org/10.47391/JPMA-BAGH-16-94**

Introduction

The fundamental role of methotrexate (MTX) is reducing the stimulation of purine and pyrimidine required for production of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the rapid division of cytotoxic cells, with irreversible competitive dihydrofolate reductase (DHFR) inhibitor leading to its effects.[1] MTX is metabolised to polyglutamate and stored in the hepatic cell for a long time. Excess metabolites accumulation can generate oxidative stress (OS) and stimulation of hepatic satellite cells (HSCs), which result in the accumulation of leukocytes, neutrophils secretion, and activate pro-inflammatory cytokines, like nuclear factor-κB (NF-κB) and tumour necrosis factor-alpha (TNF-α), that leads to fatty liver disease and fibrosis.[2]

Since MTX provokes the reactive oxygen species (ROS) in the hepatocyte that causes damage to mitochondrial proteins, structural change, deprived functions, complexes II, III and IV activation, and electron transport chain (ETC) depletion, the synthesis of adenosine triphosphate (ATP) reduces with propagation of superoxide anions that collect in response to diminishing of superoxide dismutase (SOD) and reduced glutathione (GSH). Likewise, MTX increases OS generation and inflammation due to lipid peroxidation collecting, leading to decreased energy and increased malondialdehyde (MDA) with more cellular damage.[3] The apoptotic effect of MTX is illustrated by activation of p21 and p53 protein expression, and the production of OS.[4,5]

Omega 3 fatty acids are present as phospholipids. Omega 3 can scavenge ROS / reactive nitrogen species (ROS\RNS) by enhancing total antioxidant capacity (TAC).[6] The anti-inflammatory effect of omega 3 against hepatotoxicity happens by inhibiting of prostaglandin, leukotriene synthesis by 5-lipoxygenase, reduction of pro-inflammatory cytokines, and dampening the hepatic gene expression of interleukin-1 (IL-1), interferon (IFN)-IL-6 and TNF-α. Besides, NF-KB suppression and enhancement of kupffer cell activities are also seen.[7] Vitamin C (L-ascorbic acid) is a robust hydrophilic antioxidant agent that oxidises to dehydroascorbic acid (DHA).[7,8] Vitamin C can prevent hepatotoxicity by its functional antioxidant capacity, scavenges the free

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radicals ROS, and maintains the integrity of cellular membrane against OS and lipid peroxidation. It also prevents lipid peroxidation prompted by hydrogen peroxide (H2O2).[9,10]

The current study was planned to assess the effect of pretreatment with omega-3 alone or in combination with vitamin C on hepatotoxicity raised by MTX.

Materials and Methods

The experimental study was conducted at the animal house of the Iraqi Centre of Cancer Research and Medical Genetics and the Department of Pharmacology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq, from November 2020 to July 2021, and comprised Swiss albino mice who were divided into 5 groups.

Group A was treated with normal saline via oral gavage, then injected with normal saline on day 10, group B was injected with single-dose MTX 20mg/kg intraperitoneally (IP), group C was pretreated with omega 3 100mg/kg, group D was pretreated with vitamin C 100mg/kg, and group E was pretreated with concomitant vitamin C and omega 3 100mg/kg. The intervention lasted 9 days, and groups B-E were injected with MTX on day 10. The mice were sacrificed under chloroform anaesthesia after 48 hours.

The liver tissue was isolated for scanning. The serum level of oxidative stress markers MDA, SOD and reduced GSH, as well as lactate dehydrogenase (LDH) were measured using double antibody sandwich technique,[11] which depends on countenances of the tested antigen with two valances and more that can realise the coated antibody and find out antibody. The enzyme-linked immunosorbent assay (ELISA) microplate wells were covered with the serum of OS biomarkers. From the tissue sample collected for histopathological study, portion of liver was placed in a tube with 10ml of formalin 10%. Liver tissue sectioning was done using the paraffin-embedded method,[12] embedding and staining with haematoxylin and eosin (H&E) for microscopic estimation.

Results

Of the 35 mice, there were 7(20%) in each of the 5 groups. Compared to the control group, the MTX group showed a significant increase in the level of LDH and MDA, while the level of SOD and GSH decreased significantly ($p<0.05$).

Pretreatment with combination led to raised level of GSH and SOD significantly compared to the MTX group, while MDA reduced significantly (1.53±0.72nmol/ml) compared to the MTX group (4.58±0.21nmol/ml). The effect of the combination on the level of LDH showed a significant decrease compared to the MTX group ($p=0.030$) (Table, Figure 1).

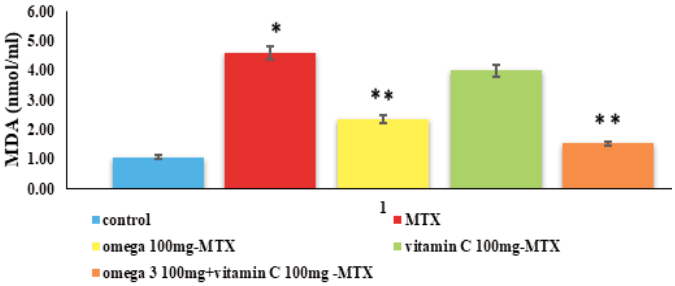

Figure-1: Mean malondialdehyde (MDA) level in the group pretreated with concomitant omega 3 + vitamin C in suppression of methotrexate-induced liver injury. * $p<0.05$ compared to control group, **$p<0.05$ compared to methotrexate (MTX).

The liver section of the control group appeared normal in structure (Figure 2A); the MTX group showed severe tissue necrosis, severe inflammatory cells infiltration, and severe depletion of glycoprotein (Figure 2B); the omega 3 group showed a small area of tissue necrosis with few areas of sinusoidal dilatation (Figure 2C); the vitamin C group showed fat droplet accumulation inside the liver cells with slight sinusoidal dilatation (Figure 2D); and the combination group showed very mild glycoprotein

Table-1: The effect of pretreatment with Omega 3 100mg or vitamin C 100mg alone or in combination on the oxidative stress markers and liver enzyme in methotrexate-induced liver injury.

Parameters of the liver	Control groups (n=7)	Methotrexate 20mg/kg (I.P.) groups (n=7)	Omega -3 (100mg) groups (n=7)	Vitamin C (100mg) groups (n=7)	Omega -3 (100mg) + Vitamin C (100mg) (n=7)
MDA (nmol/ml)	1.07±0.22	4.58±0.21*	2.35±0.28**	4.00±0.65	1.53±0.72**
SOD (ng/ml)	476.92±33.52	65.77±34.57*	236.57±112.23**	294.97±205.2**	361.46±187.15**
GSH (µg/ml)	67.61±7.22	43.09±9.19*	49.46±12.37	46.16±5.35	63.88±13.08**
LDH(ng/ml)	21.83±5.20	38.48±3.62*	19.40±7.81**	22.89±4.53**	18.51±8.55**

MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, LDH: Lactate dehydrogenase. *$P<0.05$ significant compared to control group, **$P<0.05$ significant compared to methotrexate (MTX).

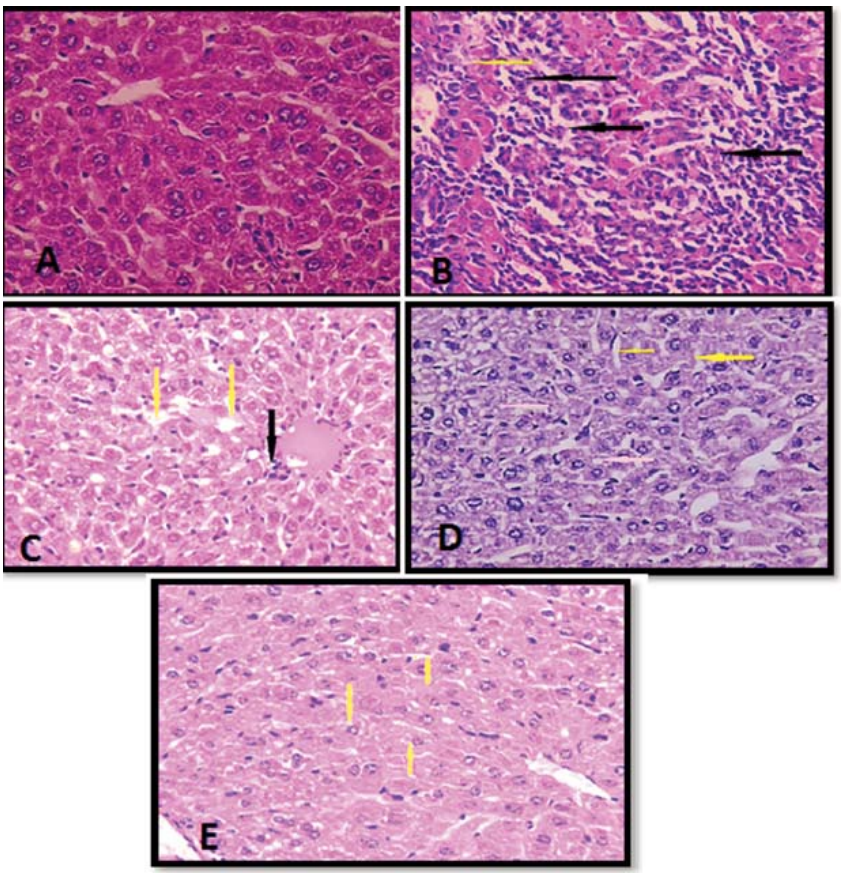

Figure-2: Histopathological findings. (A) Liver histological section of control group appeared normal in structure stained with Haemotoxylin and Eosin (H&E) (x40) (score 0), (B) Liver section of methotrexate (MTX) with severe tissue necrosis and severe inflammatory cells infiltration (black arrow) also severe depletion of glycoprotein (green arrow) (Severe score +++ >60%). (C) Liver section of omega 3 showing area of mild tissue necrosis (black arrow) with area of mild sinusoidal dilatation (yellow arrow) (mild score + 18%). D. Liver section of vitamin C showing accumulation of fat droplet inside the hepatocyte cells (pink arrow) with slight dilatation of sinusoid (yellow arrow) (mild score + 15%). E. Liver histological section of combination showing very mild depletion of glycoprotein inside the hepatocyte cells (green arrow) (very mild score +/- <5%).

depletion inside the hepatic cells (Figure 2E).

Discussion

Several mechanisms of MTX-augmented hepatotoxicity involving cellular and molecular antioxidant defence dysregulation, raised ROS, generation of free radicals with the reinforcement of lipid peroxidation and biomarkers of tissue OS, liberation of cytokines and depletion of anti-inflammatory cytokine have been mentioned in literature as elements leading to intracellular liver enzymes dysfunction.[13] The current study clarified significant rise in the level of MDA among MTX-treated group in line with a previous study.[14]

MTX-enhanced mitochondrial damage led to the release of calcium ion from the endoplasmic reticulum (ER), reversing mitochondrial membrane potential and potentiating releasing of cytochrome C, which would enhance caspase 3 activation and downstream apoptotic events. Also, cardiolipin oxidation gor enhanced as a target of ROS and lowering of the electron transport chain ETC. It boosted the FR (free radicals or oxidative stress used for determination of hepatic cells damage) generation and ROS formation in response to the accumulation of lipid peroxidation, leading to lower ATP and raised MDA level with more hepatic cells damage.[15] .

Additionally, the current study illustrated a significant decrease in SOD and GSH in groups treated with MTX compared to the control group, which was in accordance with earlier studies.[16,17] The decrease in SOD levels is attributed to the effect of MTX that increases the count of ROS and impairs the endogenous antioxidant enzymes capacity with reinforcement of the production of H_2O_2.[18]

MTX causes dysbiosis between the pro-oxidant and antioxidant systems and decreases GSH, which is the first line of prevention against oxidation of cellular damages and scavengers of reactive electrophiles of the drugs. In the current study, MTX raised FR production, causing hepatotoxicity, and produced H_2O_2 by impairing the endogenous antioxidant defence enzyme GSH, and this turned H_2O_2 to water (H_2O). SOD promoted oxidative phosphorylation reaction and removed the superoxide anion that generated H_2O_2. By glutathione peroxidase that uses GSH as a cofactor, H_2O was formed.[17] MTX induces liver injury since its metabolism to an active form hold in the liver cells as MTX-polyglutamate for a long time. This leads to an increase in the intracellular liver enzyme LDH.[7,19,20] Pretreatment with omega 3 revealed a significant decrease in MDA level, enhanced SOD level, and improvement in GSH level with amelioration of the hepatic function via reducing the serum level of LDH.[7,21-23] These findings are further promoted by the histopathological report, which illustrated reduction in the severity of necrosis induced by MTX to a mild score [17]. The significant depletion in the level of MDA occurs due to boosted antioxidant and anti-apoptotic roles of omega 3 to inhibit LPO, (lipid peroxidation for determination of

malondialdehyde parameters), alleviate cellular inflammation induced by MTX, promote cellular integrity and exert hepato-protective actions[7,23]. Minimized MDA in the group pretreated with vitamin C matched a previous study[24]. Vitamin C exerts strong antioxidant actions by compensating ROS and RNS storage with other FR generation that causes liver necrosis in MTX-intoxicated mice. This can be characterised by the cyto-protective effect of vitamin C by reducing MDA and LPO to upgrade hepatic necrosis and oxidative tissue damage[24,25].

Vitamin C could suppress superoxide radical anion that protonated to H2O2 by its free radical scavenger's effect. Vitamin C also upgrades SOD levels by revoking of superoxide oxygen free radicals and preventing hepatic injury[26]. The antioxidant activities of vitamin C catalysed the ROS, converted H_2O_2 to H_2O, and ameliorated the hepatic injury by improving GSH. This finding agrees with histopathological results that showed significant improvement of inflamed tissue necrosis induced by MTX, and the score changed from severe to mild for the group pretreated with vitamin C[26].

The antioxidant effect of vitamin C also showed reduction in the level of LDH. This is consistent with a study that showed alleviation in the liver's venous congestion due to the anti-inflammatory effect of the vitamin C by decline in the release of cytokines mediators and diminishing the endothelial dysfunction[26].

The combination of omega three and vitamin C, to our knowledge, has not been used previously, which makes the current study the first for using this summation. The study showed a strong defence against oxygen radicals' hepatotoxicity since omega 3 acts as a strong antioxidant, anti-inflammatory product with its ability to eliminate the FR, and upgrade mitochondrial liver injury induced by MTX[22]. Both omega 3 and vitamin C could reduce ROS and LPO that caused leakage in the mitochondrial membrane potential and huge free radicals formed, which increased hepatotoxicity in response to their anti-apoptotic, anti-inflammatory and excess antioxidant action[7,27].

The combination produced a more potential hepato-protective effect, which explains the significant increase in the level of GSH and SOD, and significant lowering of MDA level[26].

Conclusion

Concomitant omega 3 and vitamin C as pretreatment could exert more preserving effect to the liver cell and tissue against oxidative damage induced by MTX by improving the liver functions and reducing OS depending on the strong antioxidant, anti-inflammatory, and anti-apoptotic effects of these supplements.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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