RESEARCH ARTICLE

Evaluation of the hepatoprotective effect of curcumin alone or in combination with vitamin C in methotrexate-induced hepatotoxicity in mice

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Abstract

Objective: To assess the hepatoprotective effect of curcumin and/or vitamin C in methotrexate-induced hepatotoxicity.

Method: The experimental study was conducted at the Department of Pharmacology, College of Medicine, and the Iraqi Centre for Cancer and Medical Genetic Research, Mustansiriya University, Baghdad, Iraq, from Nov 12, 2020, to June 1, 2021, and comprised Swiss albino female mice aged 3-4 months and weighing 30-40g each. The mice were divided into 5 groups and treated for 10 days. Group 1 received distilled water, group 2 received single-dose methotrexate on the 10th day of the trial, group 3 was treated with curcumin plus methotrexate, group 4 was treated with vitamin C plus methotrexate, and group 5 was treated with curcumin and vitamin C plus methotrexate. Parameters measured were serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase, as well as hepatic tissue malondialdehyde, superoxide dismutase and glutathione. Data was analysed using SPSS 16.

Results: There were 35 mice; 7(20%) in each of the 5 groups. Hepatoprotection produced by curcumin as reflected by a decrease in lactate dehydrogenase and malondialdehyde levels was significant ($p<0.05$). Vitamin C also produced a significant hepatoprotection, demonstrated by a decrease in alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase and malondialdehyde levels. The combination of curcumin and vitamin C reflected an additive effect demonstrated by a significant decrease in malondialdehyde ($p<0.05$).

Conclusion: Curcumin and/or vitamin C provided hepatoprotection against methotrexate-induced hepatotoxicity through modulation of oxidative stress biomarkers.

Key Words: Phosphatase, Transaminase, Curcumin, Methotrexate, Oxidative Stress, Ascorbic, Glutathione, Liver, Dismutase, Aminotransferases, Dehydrogenases, Malondialdehyde

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Introduction

Methotrexate (MTX), an anti-neoplastic and immunosuppressive drug, is a folate analogue and is widely used in the treatment of different types of cancers and the management of chronic inflammatory diseases [1,2]. About 20-30% of patients stop MTX treatment during the first year of therapy due to intolerable side effects, including gastrointestinal (GI) issues ranging from nausea, vomiting, diarrhoea, and abdominal upset to mucocutaneous ulcer, hepatotoxicity, pulmonary toxicity, haematological toxicity, carcinogenicity and infections[3]. MTX is supposed to induce acute liver injury by oxidative stress (OS), nitrosative stress, inflammation, apoptosis and necrosis4. MTX produces a negative effect on the mitochondrial mechanism, and, as a result, induces supernumerary production of reactive oxygen species (ROS), which can damage cellular macromolecules and induce lipid peroxidation by interacting with polyunsaturated fatty acids and membrane lipids, which leads to membrane and cellular damage with subsequent cell death[5]. Therefore, anti-oxidant agents may produce hepatoprotective effects and reduce hepatotoxicity through OS and tissue-damage reduction[6]. Curcumin is a natural compound extracted from the rhizome of turmeric, or Curcuma (C.) longa[7]. Curcumin is a polyphenolic compound that exists in 2 tautomeric forms; keto and enol. The enol form is the most stable one in both solid and soluble phases[8]. Curcumin exhibits potent biological and pharmacological effects, including anti-inflammatory, antioxidant, antimicrobial, and anti-cancer effects, along with hepatic and nephroprotective effects [9]. Vitamin C is a water-soluble molecule that consists of 6 carbon atoms. The reduced form, which is called ascorbic acid or ascorbate, has biological activity. Vitamin C is considered a powerful antioxidant agent because it can act as a reducing agent, thus preventing oxidation of other compounds[10,11].

The current study was planned to assess the hepatoprotective effect of curcumin, vitamin C and their

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combination in MTX-induced hepatotoxicity in mice.

Materials and Methods

The experimental study was conducted at the Department of Pharmacology, College of Medicine, and the Iraqi Centre for Cancer and Medical Genetic Research, Mustansiriya University, Baghdad, Iraq, from Nov 12, 2020, to June 1, 2021. Approval was obtained from the institutional ethics committee as well as the animal care committee.

The sample comprised Swiss albino female mice aged 3-4 months and weighing 30-40g each. The animals were kept in cages at suitable room temperature with an artificial 12/12hr light-dark cycle. The mice were left for one week for acclimatisation without any intervention and with access to water and chow pellets. Hepatotoxicity was induced in mice[12], and the dose and route of administration for curcumin and vitamin C were in line with literature[13,14].

The mice were divided into 5 groups and treated for 10 days. Group 1 received distilled water, group 2 received single-dose intraperitoneal injection of MTX 20mg/kg on the 10th day of the trial, group 3 was treated with curcumin 10mg/kg for 10 days plus MTX 20mg/kg on the 10th day, group 4 was treated with vitamin C 100mg/kg for 10 days plus MTX 20mg/kg on the 10th day, and group 5 was treated with curcumin 10mg/kg and vitamin C 100mg/kg for 10 days plus MTX 20mg/kg on the 10th day.

Curcumin 500mg capsule (Curcumin95, 21st Century, United States) was opened in 250ml freshly prepared distilled water. Vitamin C powder (Witamina C 1000 forte, Uniphar, EC European Commission) was dissolved in 50ml freshly-prepared distilled water. Each drug was given intra-gastrically via mouse oral gavage. MTX vial (Methotrexate, Kocak Farm, Turkey) was given to groups 2, 3, 4 and 5 on the 10th day.

On the 13th day of the experiment, chloroform was used to anaesthetise the mice in a closed plastic container. A 5cc syringe was used to collect a blood sample from the heart of the mice. The sample was allowed to drain into a sterile gel tube, and refrigerated at 4°C for the night, then centrifuged for 5 minutes at 3,000 rounds per minute (rpm) at room temperature. The serum was isolated in an Eppendorf tube and frozen at -20°C to be assessed later.

After collecting the blood sample, the animal was sacrificed and the liver was separated, washed with distilled water, tissue slice taken and isolated in a plain tube and washed out in 0.01 monophosphate buffer solution. The tissue protein extraction reagent was then added at 1g:5-10ml ration and mixed in ice water. After being blended, the mixture was centrifuged for 10 minutes at 5,000rpm, and the resulted supernatant was frozen at -20°C to be assessed later[12-14]. The remainder of the whole tissue was stored in formalin 10% for histopathological analysis which classified tissue lesion severity according to the grade of the changes: (-) = no changes, (+\-) = changes in <5% of fields, (+) = changes in <20% of fields, (++) = changes in 20-60% of fields, (+++) = changes in >60% of fields[15]. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were assessed in the serum using an automated device (Flexor-EL80, Vitalab, South Africa). Further, 500µl of serum was added and incubated for 30 minutes, and, then in International Units Per Liter (i.u./l) terms, lactate dehydrogenase (LDH) was estimated in serum, while superoxid dismutase (SOD), glutathione (GSH) and malondaldehyde (MDA) were measured in a liver tissue sample using enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, US) as per the manfacturer's directions.

Data was analysed using SPSS 16. Data was presented as mean ± standard deviation or frequencies and percentages, as appropriate. One-way analysis of variance (ANOVA) test with post-hoc multiple comparisons was used to investigate the significance of differences among the study groups. P<0.05 was considered significant.

Results

There were 35 mice; 7(20%) in each of the 5 groups. Serum ALT, ALP, LDH and tissue MDA increased significantly, while tissue SOD and GSH decreased significantly in all intervention groups compared to the control group (p<0.05).

Serum LDH decreased from 38.483±3.662ng/ml in methotrexate group to 12.794±3.496 in curcumin group (p=0.001), while serum ALT, AST and ALP slightly increased compared to methotrexate group but the increase was not significant (p>0.05). MDA in liver tissue significantly decreased from 4.851±0.217nmol/ml in methotrexate group to 2.139±847 in curcumin group (p=0.001). Tissue SOD and GSH increased in curcumin group compared to methotrexate group; 84.643±33.006i.u./ml compared to 65.771±34.571i.u./ml and 48.436±8.282µg/ml compared to 43.098±9.192µg/ml, respectively.

Treatment with 100mg/kg vitamin C resulted in a significant decrease in serum ALT, LDH and ALP compared to methotrexate group (p<0.005), while tissue MDA insignificantly decreased from 4.581±0.217nmol/ml in methotrexate group to 4.005±0.657nmol/ml in vitamin C group (p=0.076). SOD tissue level significantly increased

Table-1: Effect of treatment with curcumin, vitamin C and their combination on oxidative stress biomarkers and hepatic enzymes during methotrexate-induced hepatotoxicity.

Variable	Control (n=7)	Methotrexate (n=7)	Curcumin C (n=7)	Vitamin (n=7)	Curcumin & Vitamin C (n=7)
MDA (nmol/ml)	1.077±0.227	4.581±0.217	2.139±.847*	4.005±0.657	1.908±0.880*
SOD (i.u./ml)	476.920±33.529	65.771±34.571	84.643±33.006	294.970±205.825*	117.230±98.101
GSH(µg/ml)	67.612±7.228	43.098±9.192	48.436±8.282	34.312±13.611	40.170±16.273
ALT (i.u./l)	33.428±4.928	50.714±7.674	52.571±4.429	40.285±10.160*	44.714±7.432
AST (i.u./l)	27.428±4.157	34.714±8.788	40.857±6.039	32.857±6.517	38.857±7.151
ALP (i.u./l)	267.28±65.632	458.000±74.917	466.280±80.167	209.140±141.019*	459.570±45.825
LDH (ng/ml)	21.838±5.200	38.483±3.622	12.794±3.496*	22.897±4.536*	14.898±3.055*

*=p<0.05, one-way ANOVA test

SD: Standard deviation, n= Number of animals in each group, MD: Malonedialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, ALP: Alkaline phosphatase.

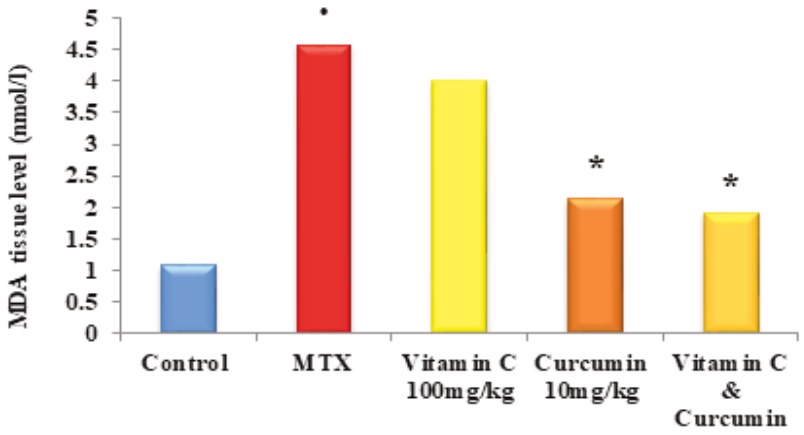

Figure-1: Malondialdehyde (MDA) level among different treatment groups compared to control and methotrexate groups.
• = Significant difference compared to controls, * = Significant difference compared to methotrexate group (p<0.05).

in vitamin C group compared to methotrexate group (p=0.001).

Treatment with 10mg/kg curcumin and 100mg/kg vitamin C resulted in a decrease in serum ALT from 50.714±7.674i.u./l in methotrexate group to 44.714±7.432i.u./l in combination group. LDH decreased in combination group (p=0.001). MDA tissue level decreased from 4.851±0.217nmol/ml in methotrexate group to 1.908±0.880nmol/ml in the combination group. SOD increased in the combination group (p=0.374) (Table, Figure 1).

Histological liver sections in all the study group showed varying characteristics (Figure 2).

Discussion

Methotrexate-induced liver injury is proposed to be related to OS due to the generation of ROS and deregulation of cellular defence mechanisms, and is related to its negative effect on mitochondrial machinery result in mitochondrial dysfunction. Besides, inflammation, apoptosis and necrosis are also associated with methotrexate-induced hepatotoxicity[4,5,16]. It has been reported that methotrexate induces a decline in messenger ribonucleic acid (mRNA) of nuclear factor erythroid 2-related factor 2 (NRF2) and a decrease in NRF2 binding capability. This could partially be attributed to the decline of the antioxidant status of the liver. Generation of ROS along with the depletion of cellular antioxidant defence mechanism resulted in increased lipid peroxidation, demonstrated by the significant increase in MDA tissue level in methotrexate-treated mice compared to the control group in the present study. ALT, AST, ALP and LDH are cytosolic enzymes, and elevation of their level in the serum indicates leakage in the membrane[12].

In the present study, treatment of mice with curcumin resulted in attenuation of hepatotoxicity produced by methotrexate, represented by a significant decrease in tissue MDA level along with increased SOD and GSH tissue level. Curcumin can increase the concentration of GSH and activity of GSH-peroxidase and SOD enzymes through upregulation of NRF2 genes[17]. Curcumin has free radical scavenging property, which means it can neutralise ROS and reactive nitrogen species (RNS) produced by methotrexate in hepatocyte. Curcumin also exhibits down-regulation of inducible nitric oxide synthase (iNOS) which means decreased intracellular production of RNS[18], The decreased MDA level in curcumin group was an indication of decreased lipid peroxidation produced by free radicals[19].

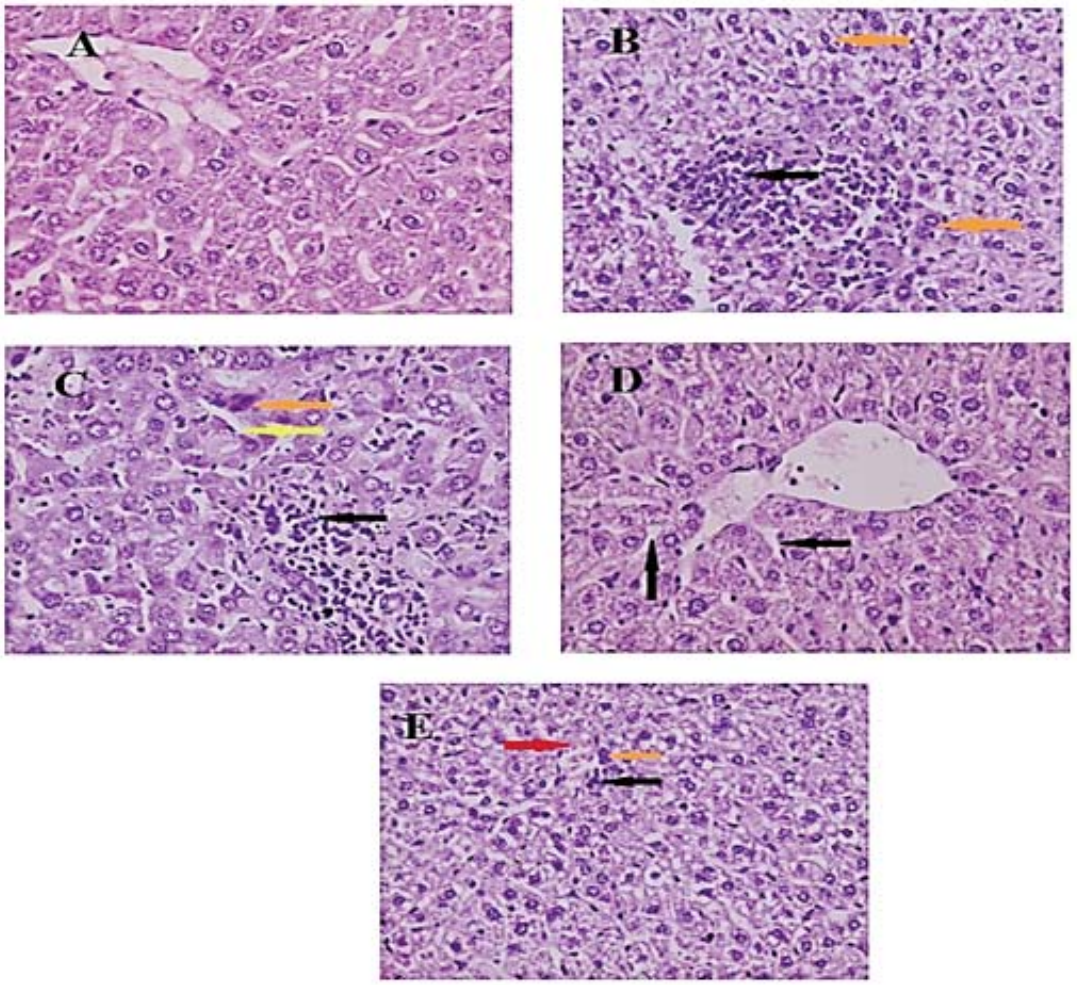

Figure-2: Histological section of the liver in the 5 study groups. A: Liver section of control group showing normal hepatocyte with a mild cellular degeneration (Haematoxylin and Eosin [H&E], x40). B: Methotrexate group showing necrosis of hepatocyte (brown arrow), infiltration of the inflammatory cell (black arrow) and prominent cellular degeneration, scored +++ (H&E, x40). C: Curcumin group showing hepatocyte necrosis (brown arrow), sinusoidal dilation (yellow arrow) and inflammatory cells infiltration (black arrow), scored ++ (H&E, x40). D: Vitamin C group showing slight sinusoidal dilation (arrow) with mild cellular degeneration, scored +/-, (H&E, x40). E: The curcumin-vitamin C combination group showing inflammatory cell infiltration (black arrow), necrotic cells (brown arrow) and slight congestion (red arrow), scored +, (H&E, x40).

Curcumin might mediate its hepatoprotective effect through several mechanisms, so it might modulate the hepatocyte's apoptosis process. The elevated level of AST and ALP along with a significant decrease in lipid peroxidation reflected by MDA might be attributed to the anti-apoptotic effect of curcumin on hepatocyte. Curcumin may increase the survival of hepatocyte partially damaged by methotrexate, and, as a result, its enzymes are released to the extracellular compartment. This effect needs further investigations.

Several studies have reported that vitamin C produces a protective effect against drugs and chemical agents that induce hepatotoxicity[20]. The target of vitamin C is the mitochondria, preventing mitochondrial swelling, mitochondrial membrane potential dissipation, and ROS burst, thereby preventing hepatic apoptosis[21]. These effects might oppose the mitochondrial negative action of methotrexate. Vitamin C supports the action of SOD in scavenging superoxide through a donation of electrons to free radicals, like hydroxyl and superoxide radicals, and switch off their activity[22]. This explains the increase in tissue SOD in vitamin C group compared to the methotrexate group in the present study.

The level of MDA was significantly decreased to less than that produced by either curcumin or vitamin C alone. This reflected an additive effect in the prevention of lipid peroxidation produced when curcumin and vitamin C were used together. The present study showed

amelioration of liver injury induced by methotrexate via treatment with vitamin C and curcumin. This fact was demonstrated by a decrease in ALT along with a significant decrease in LDH serum levels. Attenuated OS results in less damage to the hepatocyte's organelles and membrane, which means less leakage of the cytosolic enzyme to the extracellular part and subsequently less detection in serum[12]. This explains the decrease in ALT and LDH. Histopathological changes reflected severe injury produced by methotrexate-included necrosis of hepatocytes, inflammatory cells infiltration and glycoprotein depletion, while treatment with curcumin and/or vitamin C produced restoration in the histological profile of hepatocytes.

Conclusion

Curcumin and/or vitamin C produced a hepatoprotective effect against methotrexate-induced hepatotoxicity through modulation of oxidative/antioxidant pathways.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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