Original Article

Evaluation of the Hepatoprotective effect of Different Doses of Curcumin and Vitamin C in Methotrexate-Induced Hepatotoxicity in Mice

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Abstract

Background: Methotrexate, the antineoplastic and immunosuppressive drug, is used in the treatment of different types of cancers and the management of chronic inflammatory diseases. Hepatotoxicity is one of its major side effects. **Objectives:** The present study assesses the hepatoprotective effect of different doses of curcumin and Vitamin C in methotrexate-induced hepatotoxicity. **Materials and Methods:** The prospective experimental study was conducted at the College of Medicine, Mustansiriyah University, Baghdad, Iraq, and in the animal's house of the Iraqi Center for Cancer Research, Baghdad-Iraq, from November 2020 to June 2021, and comprised Swiss albino female mice aged 3–4 months and weighing 30–40 g each. The mice were divided into 6 groups, the first group was considered as control which received only distilled water, the second group was considered as methotrexate group, third and fourth groups orally supplemented with 10 mg/kg and 20 mg/kg curcumin, respectively, fifth and sixth groups orally supplemented with 100 mg/kg and 200 mg/kg Vitamin C, respectively, The experiment continued for 10 days, and on the 10th day all groups, except the control one, received 20 mg/kg methotrexate intraperitoneally to induce hepatotoxicity. Parameters measured were serum alanine aminotransferase (ALT), aspartate aminotransferase, alkaline phosphatase (ALP), and lactate dehydrogenase (LDH), and liver tissue malondialdehyde (MDA), superoxide dismutase, and glutathione. SPSS 16 was used for data analysis. **Results:** The results show significant hepatoprotection produced by curcumin reflected by a decrease in LDH and MDA. Vitamin C also produced a significant hepatoprotection demonstrated by a decrease in ALT, ALP, LDH, and MDA. **Conclusion:** Curcumin and Vitamin C were found to provide hepatoprotection against methotrexate-induced hepatotoxicity through the modulation of oxidative stress biomarkers in a dose-dependent manner.

Keywords: Curcumin, hepatoprotective, hepatotoxicity, methotrexate, Vitamin C

Introduction

Methotrexate, the antineoplastic and immunosuppressive drug, is a folate analog and is widely used in the treatment of different types of cancers and the management of chronic inflammatory diseases.[1,2] Methotrexate depletes tetrahydrofolate intracellular stores through inhibition of dihydrofolate reductase enzyme, tetrahydrofolate plays an important role in purines and thymidylate synthesis, which play a vital role in DNA synthesis and cell division.[3] Anticancer activity of methotrexate is mainly related to DNA synthesis inhibition thus blocking cell proliferation.[4] It has been reported that 20%–30% of patients stopped methotrexate treatment during the 1st year of therapy due to intolerable side effects, including gastrointestinal side effects ranging from nausea, vomiting, diarrhea, and abdominal upset to mucocutaneous ulcer, hepatotoxicity, pulmonary toxicity, hematologic toxicity, carcinogenicity, infections.[5] Accumulation of methotrexate polyglutamated metabolite inside the hepatic cells leads to folate depletion

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with a subsequent decrease in DNA and ribonucleic acid (RNA) synthesis that leads to hepatic cells death.[6] Moreover, methotrexate is supposed to induce acute liver injury by oxidative stress, nitrosative stress, inflammation, apoptosis, and necrosis.[7] MTX produces a negative effect on the mitochondrial machinery, and as a result, induces supernumerary production of ROS, which can damage cellular macromolecules and induce lipid peroxidation by interacting with polyunsaturated fatty acids and membrane lipids, which lead to membrane and cellular damage with subsequent cell death.[8] Therefore, antioxidant agents carry hepatoprotective effects and reduce hepatotoxicity through the reduction of tissue damage and oxidative stress.[9] Curcumin is a natural compound extracted from the rhizome of turmeric (Curcuma longa).[10] Curcumin is a polyphenolic compound that exists in two tautomeric forms, keto and enol forms. The enol form is the most stable one in both solid and soluble phases.[11] Curcumin exhibits potent biological and pharmacological effects including anti-inflammatory, antioxidant, antimicrobial, and anticancer effects along with hepatic and nephroprotective effects.[12] Vitamin C is a water-soluble molecule, consists of six carbon atoms, the reduced form, which is called ascorbic acid or ascorbate, has biological activity. Vitamin C is a powerful antioxidant agent because it can act as a reducing agent preventing other compounds from being oxidized.[13,14] The current study was planned to evaluate the hepatoprotective effect of each curcumin and Vitamin C alone and in combination in methotrexate-induced hepatotoxicity in mice.

Materials and Methods

The prospective experimental study was conducted at the College of Medicine, Al-Mustansiriya University, Baghdad, Iraq, and in the animal's house of the Iraqi Center for Cancer Research, Baghdad-Iraq, from November 12, 2020, to June 1, 2021, and comprised Swiss albino female mice aged 3–4 months and weighing 30–40 g each. The animals were obtained from the Iraqi Center for Cancer Research, Serum and Vaccine Institute, and the National Center for Drug Control and Research, Baghdad-Iraq. The work started following the approval of the ethical committee of the pharmacology department, college of medicine, Al-Mustansiriya University. The animals were isolated as 7 mice in each cage and placed at suitable room temperature under an artificial 12/12 h light-dark cycle. They were left for 1 week for acclimatization without any intervention and with free access to normal chow pellets and water. Hepatotoxicity was induced in mice according to the procedure described by Abo-Haded *et al*.[15] The dose and route of administration of curcumin and Vitamin C were according to Vasanthkumar *et al.* and Sabiu *et al.*,[16,17] respectively.

- Group 1 (n = 7): Control group, mice received distilled water until the termination of the experiment
- Group 2 (n = 7): Methotrexate group, mice received a single intraperitoneal injection of methotrexate (20 mg/kg, i.p.) on the 10th day of the experiment
- Group 3 (n = 7): Mice were orally supplemented with 10 mg/kg curcumin and they received methotrexate at the same dose given to Group 2 on the 10th day of the experiment
- Group 4 (n = 7): Mice were orally supplemented with 20 mg/kg curcumin and they received methotrexate at the same dose given to Group 2 on the 10th day of the experiment
- Group 5 (n = 7): Mice were orally supplemented with 100 mg/kg Vitamin C, then, on the 10th day of the experiment, they received methotrexate at the same dose given to Group 2
- Group 6 (n = 7): Mice were orally supplemented with 200 mg/kg Vitamin C, then, on the 10th day of the experiment, they received methotrexate at the same dose given to Group 2.

Curcumin 500 mg capsule (Curcumin 95, 21st century, USA) was opened in 250 ml freshly prepared distilled water. Vitamin C powder (witamina C 1000 forte, UNIPHAR, EC) was dissolved in 50 ml freshly prepared distilled water. Each drug was given intragastrically through mouse oral gavage. Methotrexate vial (Methotrexate, Kocak Farma, Turkey) was given to all groups, except the control one, on the 10th day at a dose of 20 mg/kg i.p.

On the 13th day of the experiment, chloroform was used to anesthetize the mouse in a closed plastic container, when the mouse was anesthetized, a 5cc syringe was used to collect a blood sample from the heart of the mouse. The blood sample allowed to drain into a sterile gel tube, and refrigerated at 4°C for the night, then centrifuged for 5 min at 3000 rounds/min at room temperature. The supernatant layer (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 and washed with distilled water, tissue slice was taken and isolated in a plain tube and washed out in 0.01 monophosphate buffer solution, then tissue protein extraction reagent was added according to a proportion of 1 g: 5–10 ml and mixed in ice water. After being blended, the mixture was centrifuged for 10 min at 5000 rpm, and the resulted supernatant was frozen at −20°C to be assessed later. The reminder whole tissue was stored in formalin 10%, to strengthen and preserve the tissue structure from autolysis that resulted from tissue lysosomal enzymes, for histopathological study.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were assessed in serum using the automated device Flexor-EL80, Vitalab, South Africa. At which 500 µl of serum was added and incubated for 30 min and then read (i.u./l). Lactate dehydrogenase (LDH) was estimated in serum while superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) were measured in a liver tissue sample, using enzyme-linked immunosorbent assay according to the instructions given on the kit by the manufacturer (MyBioSource, USA).

The Statistical Package for the Social Science Software (IBM, SPSS version 16, package for windows 8, New York, USA) was

used for data analysis. Data of this study were presented by mean ± standard deviation (mean ± SD). An unpaired *t*-test was used to compare the control and the methotrexate group. One-way ANOVA (analysis of variance) test with *post hoc* multiple comparisons was used to investigate the significance of differences among different groups. The probability value (p) was considered significant when the value is <(0.05).

In this study, liver histopathological examination was evaluated by ranking tissue lesion severity. Ranking from 0 to 3 depending on the degree of the changes as follows: (–) no pathological changes, (+\–) very mild changes in <5% of fields, (+) histopathological changes in <20% of fields, (++) histopathological changes in 20%–60% of fields, (+++) histopathological changes in more than 60% of fields.[18]

Results

Throughout methotrexate-induced hepatotoxicity, serum ALT, ALP, LDH, and tissue MDA increased significantly while tissue SOD and GSH were decreased significantly when compared to the control group ($P < 0.05$) [Table 1].

Table 1: Effect of methotrexate on hepatic enzyme and oxidative stress biomarkers during methotrexate-induced hepatotoxicity

Variable	Groups (mean±SD)	
	Control (*n*=7)	Methotrexate (*n*=7)
MDA (nmol/ml)	1.077±0.227	4.581±0.217*
SOD (i.u./ml)	476.920±33.529	65.771±34.571*
GSH (µg/ml)	67.612±7.228	43.098±9.192*
ALT (i.u./l)	33.428±4.928	50.714±7.674*
AST (i.u./l)	27.428±4.157	34.714±8.788
ALP (i.u./l)	267.28±65.632	458.00±74.917*
LDH (ng/ml)	21.838±5.200	38.483±3.622*

*P<0.05, unpaired *t*-test. SD: Standard deviation, *n*: Number of animals in each group, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

Serum LDH decreased from 38.483 ± 3.662 ng/ml in methotrexate group to 12.794 ± 3.496 and 11.661 ± 3.218 in 10 mg/kg and 20 mg/kg of curcumin pretreated groups, respectively ($P = 0.000$), while serum ALT, AST, and ALP slightly increased from that of methotrexate group but this increase was statistically insignificant. MDA in liver tissue was significantly decreased from 4.851 ± 0.217 nmol/ml in methotrexate group to 2.139 ± 847 and 2.551 ± 0.608 in curcumin pretreated groups ($P = 0.000$). Tissue SOD was increased to 84.643 ± 33.006i.u./ml in 10 mg/kg dose and to 84.422 ± 49.144 i.u./ml in 20 mg/kg dose of curcumin pretreated groups compared to 65.771 ± 34.571 i.u./ml in methotrexate group.

Pretreatment with 100 mg/kg and 200 mg/kg Vitamin C resulted in a significant decrease in serum ALT, ALP, and LDH when compared to methotrexate group ($P < 0.005$), while tissue MDA was insignificantly decreased from 4.581 ± 0.217 nmol/ml in methotrexate group to 4.005 ± 0.657 nmol/ml in 100 mg/kg Vitamin C pretreated group ($P = 0.076$), while pretreatment with 200 mg/kg Vitamin C significantly decreased MDA to 2.938 ± 0.247 nmol/ml ($P = 0.000$). SOD tissue level significantly increased in 100 mg/kg Vitamin C pretreated group when compared to methotrexate group ($P = 0.000$), while pretreatment with 200 mg/kg Vitamin C increased SOD to 122.610 ± 150.104 i.u./ml [Table 2 and Figures 1, 2].

Histopathological study of liver tissue supported the biochemical results. Control group shows normal hepatocyte with a mild depletion of glycoprotein, while the liver of methotrexate treated group shows severe injury represented by hepatocytes necrosis, infiltration of inflammatory cells, and prominent depletion of glycoprotein, the injury represents about (65%) of the field which scored (+++). Mice pretreated with 19 mg/kg curcumin showed a moderate injury (45%) which scored (++), while pretreatment with 20 mg/kg curcumin resulted in only (30%) injury that scored (++). The injury in Vitamin C pretreated group was very mild (<5%) of the field scored as (+/–) [Figure 3].

Table 2: Effect of pretreatment with different doses of curcumin and Vitamin C on oxidative stress biomarkers and serum liver enzymes during methotrexate-induced hepatotoxicity

Variable	Groups (mean±SD)				
	Methotrexate (*n*=7)	Curcumin 10 mg/kg (*n*=7)	Curcumin 20 mg/kg (*n*=7)	Vitamin C 100 mg/kg (*n*=7)	Vitamin C 200 mg/kg (*n*=7)
MDA (nmol/ml)	4.581±0.217	2.139±0.847*	2.551±0.608*	4.005±0.657	2.938±0.247*
SOD (i.u./ml)	65.771±34.571	84.643±33.006	84.422±49.144	294.970±205.825*	122.610±150.104
GSH (µg/ml)	43.098±9.192	48.436±8.282	50.320±11.036	34.312±13.611	36.427±15.025
ALT (i.u./l)	50.714±7.674	52.571±4.429	43.000±11.445	40.285±10.160*	33.00±4.546*
AST (i.u./l)	34.714±8.788	40.857±6.039	50.142±9.459*	32.857±6.517	33.142±8.275*
ALP (i.u./l)	458.000±74.917	466.280±80.167	490.710±39.343	209.140±141.019*	126.000±31.859
LDH (ng/ml)	38.483±3.622	12.794±3.496*	11.661±3.218*	22.897±4.536*	24.406±4.528*

*P<0.05, one-way ANOVA test. SD: Standard deviation, *n*: Number of animal in each group, MDA: Malone dialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, ANOVA: Analysis of variance

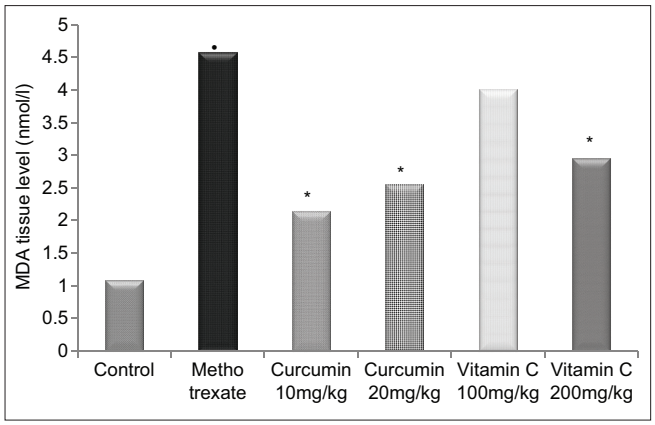

Figure 1: Malondialdehyde level among different pretreatment groups compared to control and methotrexate group, •: Significant difference from control, *: Significant difference from methotrexate group

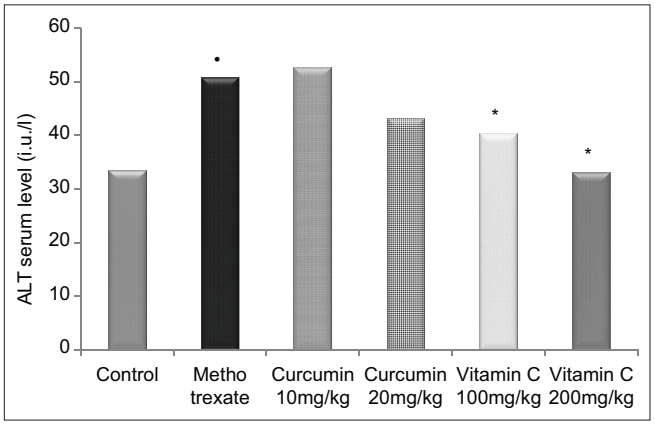

Figure 2: Alanine aminotransferase level among different pretreatment groups compared to control and methotrexate group, •: Significant difference from control, *: Significant difference from methotrexate group

Discussion

Methotrexate-induced liver injury is proposed to be related to oxidative stress due to the generation of ROS and deregulation of cellular defense mechanisms and related to its negative effect on mitochondrial machinery result in mitochondrial dysfunction. Besides that, inflammation, apoptosis and necrosis are also associated with methotrexate-induced hepatotoxicity.[7,8,19] It has been reported that methotrexate induces a decrease in mRNA of Nrf2 and Nrf2 binding capacity. This could partially be attributed to depression of the antioxidant status of the liver. Generation of ROS along with the depletion of cellular antioxidant defense mechanism resulted in increased lipid peroxidation demonstrated by the significant increase in MDA tissue level in methotrexate treated mice when 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 cell membrane.[15] Depletion of folate that leads to a decrease in DNA and RNA synthesis may also lead to hepatic cell death.[6]

In the present study, pretreatment of mice with different doses of curcumin resulted in an attenuation of hepatotoxicity produced by methotrexate, represented by a significant decrease in tissue MDA level along with increase SOD and GSH tissue level, these results are in agreement with Aljebori and Abady[4] which studied the hepatoprotective effect of curcumin on Iraqi White Domestic Rabbits in methotrexate-induced hepatotoxicity. Curcumin dose-dependently decreased ALT and increased GSH. Curcumin can increase the concentration of GSH and the activity of GSH-peroxidase and SOD enzymes through the upregulation of Nrf2 genes.[20] Curcumin has free radical scavenging property that means it can neutralize ROS/RNS produced by methotrexate in hepatocyte. Curcumin also exhibits downregulation of inducible nitric oxide synthase which means decreased intracellular production of RNS.[21] The decreased MDA level in the curcumin pretreated group is an indication of decreased lipid peroxidation produced by free radicals.[22]

Curcumin might mediate its hepatoprotective effect through more than one mechanism, 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 antiapoptotic effect of curcumin on hepatocytes, at which curcumin may increase the survival of hepatocytes that are partially damaged by methotrexate and as a result, its enzymes released to the extracellular compartment, this effect should be farther investigated in future studies.

In the present study, pretreatment with Vitamin C resulted in attenuation of liver injury produced by methotrexate. This attenuation was demonstrated by a significantly decreased level of serum ALP, and LDH, along with lowering ALT and AST. Pretreatment with Vitamin C also showed a significant increase in tissue level of SOD and decreased level of MDA.

Several studies reported that Vitamin C produces a protective effect against drugs and chemical agents that induced hepatotoxicity.[23] The target of Vitamin C is the mitochondria, preventing mitochondrial swelling, mitochondrial membrane potential dissipation, and ROS burst, thereby preventing hepatic apoptosis.[24] These effects might oppose the mitochondrial negative action of methotrexate which mentioned above. Vitamin C supporting 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,[25] this explains the increase in tissue SOD in Vitamin C pretreated mice when compared to the mice that received methotrexate alone in the present study.

Vitamin C was used in two doses 100 mg/kg and 200 mg/kg. The biochemical results show a dose-dependent effect of Vitamin C in producing its hepatoprotective effect against MTX-induced hepatotoxicity represented by a dose-dependent decreasing of MDA tissue level, which means less lipid peroxidation, and a dose-dependent decreasing serum ALT and ALP level, which reflect less damage of hepatocytes and bile duct membrane or bile duct epithelial cell.

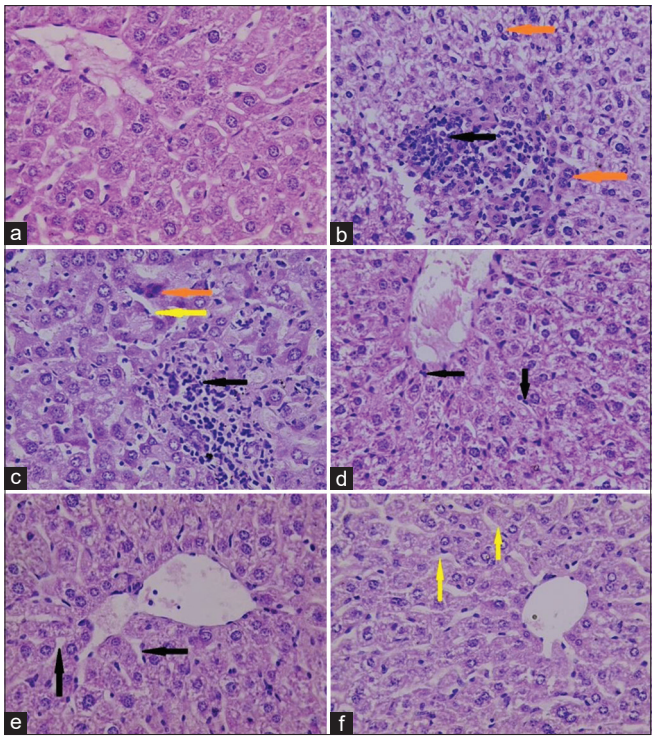

Figure 3: (a) Histological liver section of control group shows normal hepatocyte with a mild depletion of glycoprotein (H and E, ×40). (b) Histological liver section of methotrexate treated group shows necrosis of hepatocyte (brown arrow), infiltration of the inflammatory cell (black arrow), and prominent depletion of glycoprotein, score (+++) (H and E, ×40). (c) Histological liver section of 10 mg/kg curcumin pretreated group shows hepatocyte necrosis (brown arrow), sinusoidal dilation (yellow arrow), and inflammatory cells infiltration (black arrow), score (++) (H and E, ×40). (d) Histological liver section of 20 mg/kg curcumin pretreated group shows depletion of glycoprotein with mild infiltration of inflammatory cells (black arrow) (H and E, ×40). (e) Histological liver section of mice pretreated with100 mg/kg Vitamin C shows slight sinusoidal dilation (arrow) with mild depletion of glycoprotein, score (+/–) (H and E, ×40). (f) Histological liver section of 200 mg/kg Vitamin C pretreated group shows slight sinusoidal dilation (yellow arrow) with fat droplets accumulation inside hepatocyte (H and E, ×40)

Depending on MDA level in liver tissue, a comparison between pretreatment with curcumin and Vitamin C reflected that curcumin even in its low dose provided better protection than Vitamin C in its two doses.

Conclusion

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

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Conflicts of interest

There are no conflicts of interest.

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