RESEARCH ARTICLE

Evaluation of concurrent use of Vitamin C and Niclosamide against methotrexate-induced liver injury in mice

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Abstract

Objective: To examine the effect of using vitamin C and niclosamide together on liver damage caused by methotrexate.

Method: The study was conducted at the Pharmacology Department and the Iraqi Center for Cancer and Medical Genetics Research, College of Medicine, Mustansiriya University, Baghdad, Iraq, from November 2020 to July 2021, and comprised albino mice who were randomly assigned to 5 groups. Group 1 comprised controls, groups 2 to 5 was received methotrexate, niclosamide 70mg/kg/day, vitamin C 100mg/kg/day, and a combination of niclosamide and vitamin C, respectively. Mice in groups 3, 4 and 5 also received an intraperitoneal injection of methotrexate 20mg/kg to induce hepatotoxicity. After 48 hours of the injection, the mice were sacrificed under chloroform anaesthesia. Cardiac blood samples were drawn for biochemical examination. The liver, after being washed, was divided into two parts; one part was taken for histological examination, and the other was preserved in formalin 10% for histopathological analyses. Data was analysed using SPSS 16.

Results: Of the 35 mice, there were 7(20%) in each of the 5 groups. The overall age ranged between 9-12 weeks and weight between 18-38gm. The results show significant hepatoprotection (p-value <0.05) produced by both niclosamide and Vitamin C separately, reflected by a decrease in ALP, ALT, and LDH, while the combination of (niclosamide and Vitamin C) showed no additive effect (p>0.05) on enhancement of liver function.

Conclusion: Niclosamide alone was found to be superior than in combination with vitamin C for treating methotrexate-induced liver damage.

Key Words: Methotrexate, Chloroform, Niclosamide, Intraperitoneal, Liver, Formaldehyde

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Introduction

Inhibiting the enzyme dihydrofolate reductase (DHFR) by converting it to tetrahydrofolate, methotrexate (MTX) is an antifolate agent[1]. Psoriasis, rheumatoid arthritis, liver cancer, osteosarcoma, lymphomas and acute lymphocytic leukaemia are only a few of the numerous clinical disorders where MTX is employed[2,3]. Among the many toxicities and side effects of MTX, mild hepatitis, cholestasis and severe liver damage are among the most concerning[4]. Although the precise mechanism(s) through which MTX causes liver damage is unknown, it is known that it does occur. MTX by itself induces hepatic injury, inflammation, apoptosis and necrosis. MTX produces a defect in the mitochondria, leading to free radical generation. Increased free radical generation leads to derangement of antioxidant defense mechanism and elevated liver function tests that indicate the cellular damage of hepatocytes[5-9]. Therefore, ascorbic acid, sitagliptin, and naringin, which have a hepatoprotective effect against MTX-induced liver damage, are the subject of clinical and experimental investigations looking for antioxidant or anti-inflammatory drugs that reverse the liver impairment produced by MTX[8].

For quite some time, niclosamide has been the drug of choice for treating tapeworms due to its high oral lethal dose (LD50) value (>1,000mg/kg) and low toxicity to humans when given for just a few days at a time[10]. Niclosamide's capacity to uncouple oxidative phosphorylation and stimulate the activity of adenosine triphosphate (ATP) in mitochondria constitutes its mode of action[11-13].

Ascorbic acid, better known as vitamin C, is a powerful antioxidant that dissolves easily in water. As a result of its antioxidant and reactive oxygen species (ROS) scavenging properties, vitamin C finds widespread use in pharmaceutical and cosmetics industries[14-16] . In order to prevent lipid peroxidation produced by peroxide radicals, vitamin C helps replenish antioxidants like alpha-tocopherol, or vitamin E, in the body. Vitamin C's anti-apoptotic effect makes it useful for treating mitochondrial disorders associated with elevated ROS levels[15-17].

To our knowledge, no study has evaluated the impacts of

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combination treatment on MTX-induced liver injury despite the fact that several medicines and medicinal plants have proved their antioxidant efficiency experimentally by lowering the enhanced oxidative stress in liver damage generated by MTX. The current study was planned to fill the gap by examining the effect of using vitamin C and niclosamide together on liver damage caused by MTX.

Materials and Methods

The study was conducted at the Pharmacology Department and the Iraqi Center for Cancer and Medical Genetics Research, College of Medicine, Mustansiriya University, Baghdad, Iraq, from November 2020 to July 2021, and comprised albino mice. The mice were housed under standard laboratory settings with free access to food and water, temperatures maintained between 23 ± 2◦C with a light-dark cycle of 12:12 hours as per the guidelines of the National Research Council related to experimental animals[18]. The procedure was performed after getting approval from the Animal Care and Use Committee at Al Mustansiriya Medical College, Baghdad, Iraq. The mice were randomly assigned to 5 equal groups. Group 1 comprised healthy controls who received saline alone. Group 2 was given MTX. Group 3 received niclosamide 70mg/kg/day[13,19]. Group 4 was given vitamin C 100mg/kg/day[20]. Group 5 received a comobination of niclosamide 70mg/kg/day and vitamin C 100mg/kg/day. Each day, a new batch of dissolved niclosamide tablet 500mg (Bayer, Germany) for oral administration was prepared by crushing the tablets using a pestle and mortar. Normal saline 35ml was added, and stirring was done with a magnetic stirrer until the powder was completely dissolved. It was then given by oral gavage to mice in groups 3 and 5, with the dose determined by the animals' body weight. Each day, a sachet of vitamin C 1,000mg fine powder (Uniphar, EC(European Comision)) was dissolved in normal saline, and was given through oral gavage to mice in groups 4 and 5, with the dosage determined by the animals' body weight.

After 10 days, hepatotoxicity was generated by a single dose of MTX 20mg/kg (Kocak pharma, Turkey) injection given intraperitoneally to all groups except the control group[8]. After 48 hours of the MTX injection, the mice were put under chloroform anaesthesia and sacrificed. Cardiac blood samples were drawn for biochemical examination. The liver was taken out and washed with distilled water to remove the blood. Then it was divided into two parts. One part was placed in a tube and washed in 0.01 monophosphate buffer solution (MPBS) to eliminate the excess blood. Then it was weighed 300mg and small slices were cut. Cold water and a tissue protein extraction reagent were added at a ratio of 1g:5-10ml. The blended substance was centrifuged at 5000 rpm for 5 minutes. The supernatant was extracted and placed at -20^{0}C until the histological examination was performed. The other part was preserved in formalin 10% for histopathological analysis.

Lactate dehydrogenase (LDH) was measured in serum while Superoxide dismutase (SOD), malondialdehyde (MDA), and the glutathione (GSH) reductase were measured in a liver tissue sample, using enzyme-linked immunosorbent assay (ELISA) depending on the instructions given in the kit by the manufacturer (MyBioSource,USA).

Liver histopathological examination was done using the paraffin-embedded technique, as described in literature[21]. The tissue was stained with haematoxylin and eosin (H&E). MTX-induced liver damage was scored semi-quantitatively using a histological scoring method, which ranked tissue lesion severity to determine the level of histopathological alterations brought on by MTX, with 0 = no change, 1 = changes in <20% of fields, 2 = changes in 20-60% of fields, and 3 = changes in >60% of fields(22).

Data was analysed using SPSS 16. Data was expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare mean values$P \leq 0.05$ was considerd statistically significant.

Results

Of the 35 mice, there were 7(20%) in each of the 5 groups. The overall age ranged 9-12 weeks and weight 18-38gm.

Tissue levels of oxidative stress indicators SOD and GSH) were significantly lower in the MTX group compared to the control group. There was a significant rise in MDA ($p<0.05$) level. Serum levels of hepatocellular and hepatobiliary markers alanine aminotransferase (ALT),

Table-1: Changes in tissue and serum level of biochemical parameters among mice in methotrexate (MTX) and control groups (N=7 each).

[A]: Parameters	Groups (x±S.D)	
	Control	MTX
SOD(u/ml)	476.92±33.52	65.77±34.57*
GSH(µg/ml)	67.61±7.22	43.09±9.19*
MDA(nmol/ml)	1.07±0.22	4.58±0.217*
ALT(IU/L)	33.42±4.92	50.71±7.67*
AST(IU/L)	27.42±4.15	34.71±8.78
ALP(IU/L)	267.28±65.63	458±74.91*
LDH(ng/ml)	21.83±5.20	38.48±3.62*

* significant difference ($p<0.05$). SD: Standard deviation, SOD: Superoxide dismutase, GSH: Glutathione, MDA: Malondialdehyde, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

Table-2: Changes in tissue and serum level of biochemical parameters between mice groups treated with niclosamide 70mg, vitamin C 100mg and their combination for 10 days (N=7 each).

[B]:Parameters	Groups (x±S.D)			
	MTX	Vitamin C100mg& niclosamide70mg + MTX	Niclosamide 70mg+ MTX	Vitamin C 100mg +MTX
SOD(u/ml)	65.77±34.57	78.93±51.80	62.79±23.91	294.97±205.97•
GSH(µg/ml)	43.09±9.19	54.45±7.27	50.82±6.83	34.31±13.61
MDA(nmol/ml)	4.58±0.217	2.78±0.84•	2.47±0.91•	4.00±0.65
ALT(IU/L)	50.71±7.67*	37.14±2.67•	34.00±3.60•	40.28±10.16•
AST(IU/L)	34.71±8.78	30.14±7.12	30.71±7.52	32.85±6.51
ALP(IU/L)	458±74.91*	264.85±95.70•	265.28±47.51•	200.91±141.01•
LDH(ng/ml)	38.48±3.62*	22.63±4.82•	25.42±3.47•	22.89±4.53•

• significant difference (p<0.05). SOD: Superoxide dismutase, GSH: Glutathione, MDA: Malondialdehyde, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

Table-3: Liver injury assessment of study groups.

[C]: Score components		GR.1	GR.2	GR.3	GR.4	GR.5
Depletion of glycoprotein	Score	+/-	++	++	+/-	_
	Extent	<5%	>40%	>30%	<5%	no
Inflammatory cellular infiltrate	Score	–	++	++	–	++
	Extent	No	>40%	>30%	no	>30%
Cellular necrosis	Score	–	++	++	–	++
	Extent	No	>40%	>30%	no	>30%
sinusoid dilation with an accumulation of fat droplets	Score	–	–	–	+/-	–
	Extent	No	no	no	<5%	no

(–) No pathological lesion; (+/–) Very mild changes in <5% fields; (+) Histopathology changes in <20% fields; (++) Histopathology changes in 20-60% of fields. GR: Group.

elevated significantly (p<0.05), but AST elevation was not significant (Table 1, Figure 1A-B).

In group 2, there was increase in tissue levels of SOD and GSH (p<0.05), but no significant change in MDA (p>0.05). ALT, ALP and LDH were significantly low (p<0.05), while AST was not significantly low (P>0.05).

In group 4, a significant increase was noted in the tissue level of SOD (p<0.05), but the change was non-significant for GSH and MDA (p>0.05). Ther was a significant decrease in ALT, ALP and LDH (p<0.05), but the change was non-significant for AST (p>0.05).

In group 5, SOD and GSH levels were not significant (p>0.05), but MDA was significantly lower (p<0.05). ALT, ALP and LDH levels were low (p<0.05), but AST level was not significantly different (p>0.05) (Table 2, Figure 1A-B).

Histological changes showed no significant liver abnormality (p>0.05) and very mild depletion of glycoprotein in the diffuse area of liver tissue in group

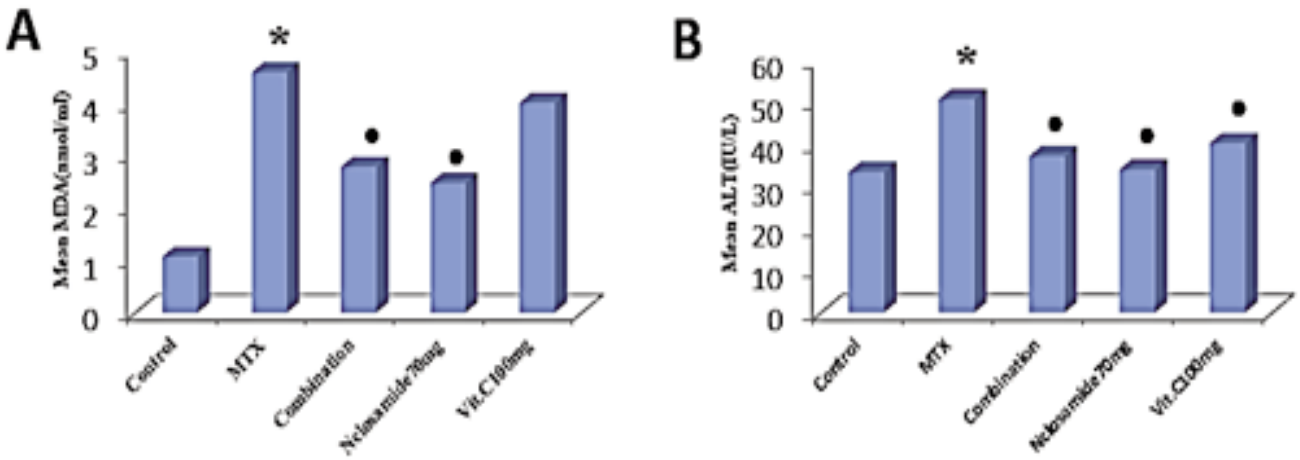

Figure-1: (A) Effect of treatment regimens on malondialdehyde (MDA) tissue level. (B) Effect of niclosamide 70mg/kg/day, vitamin C 100mg/kg/day, and their combination on serum alanine aminotransferase (ALT) level.
* significant difference from the control group (p<0.05)", • significant difference from the methotrexate (MTX) group (p<0.05).

aspartate aminotransferase (AST), and alkaline phosphatase (ALP), as well as LDH were elevated compared to the control group. ALT, ALP and LDH were

1 (Figure 2A)), whereas group 2 showed the highest score (Figure 2B), and group 5 showed lower score (Figure 2 C, Table 3).

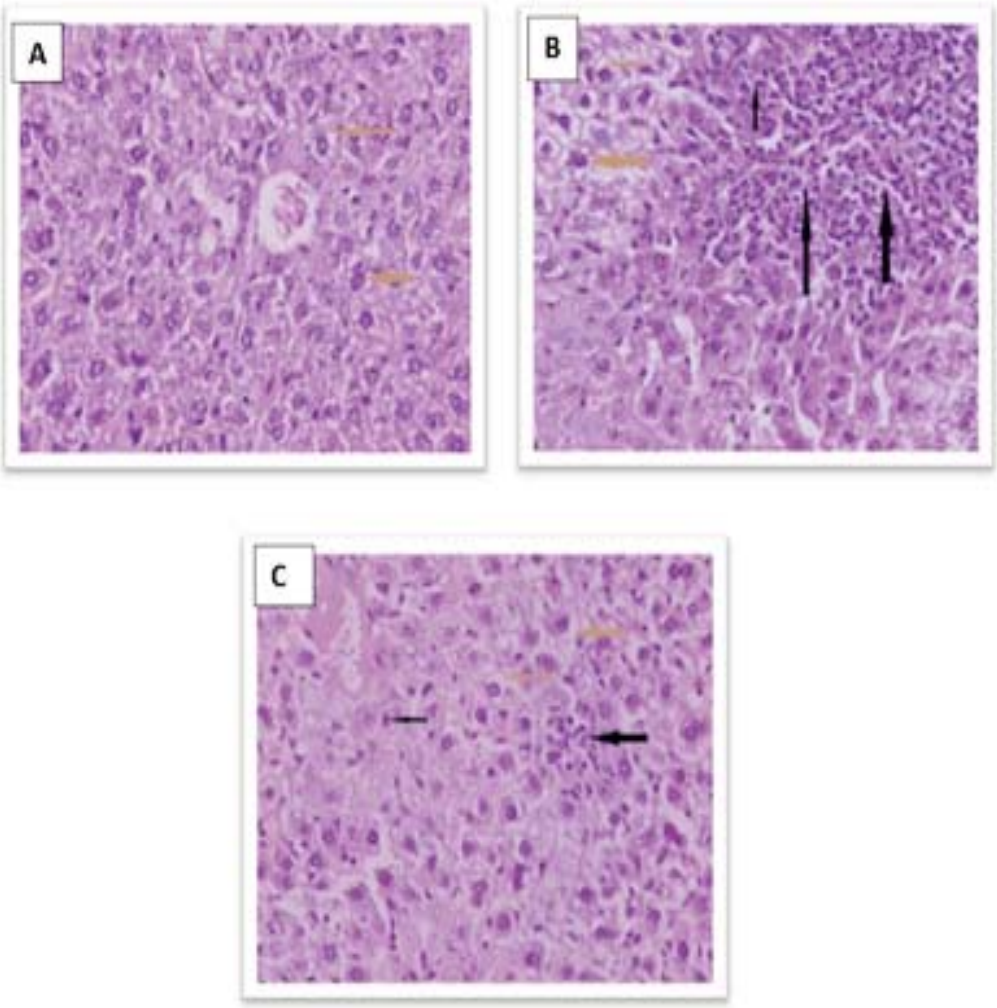

Figure-2: (A) Liver section of normal control mice showing very mild depletion of glycoprotein in the diffuse area of liver tissue after being stained with haemotoxylin and eosin (H&E). (B) Liver section of mice treated with methotrexate (MTX) showing the area of necrosis with inflammatory cell infiltration with depletion of glycoprotein after being stained with H&E. (C) Liver section of mice treated with a combination of niclosamide and vitamin C showing the focal area of inflammatory cell infiltration with dispersed necrotic hepatocyte cells after being stained with H&E.

Discussion

The high doses of MTX used in certain clinical conditions are associated with organ toxicity, including acute liver toxicity, progressive liver fibrosis and cirrhosis. The current study, the first of its kind, aimed at proving the effective hepatoprotective activity of niclosamide, vitamin C, and their combination on MTX-induced liver injury.

The results indicated that MTX-treated mice exhibited significant liver damage, as shown by significant increase in liver transaminase, ALP and LDH. These cytoplasmic enzymes are the best indicators of liver necrosis. Increased serum activity indicates cell membrane leakage, which, in turn is related to liver cell death[5,6]. The results of the histopathological examination supported the biochemical changes, and showed obvious liver damage in the MTX group. It is well-known that accumulation of polyglutamate-MTX inside the hepatic cells results in the depletion of hepatic folate stores and induction of mitochondrial dysfunction. Mitochondria are the powerhouses of the cell, constantly churning out ATPs. Liver damage caused by MTX is in large part due to ROS, which are produced in large quantities in dysfunctional mitochondria. The vulnerability of cells to free radicals is also increased by MTX, which raises the homocysteine level. Additionally, MTX lowers cellular GSH via decreasing nicotinamide adenine dinucleotide phosphate (NADPH) levels. This makes hepatocytes more liable to the damaging effects of ROS[5-9]. The elevated level of MDA in the liver of mice by MTX suggested

increased lipid peroxidation causing liver injury and indicating that the antioxidant defense mechanisms failed to deal with the formation of excessive free radicals. This causes damage to the cellular membrane, the release of intracellular contents, and elevation of serum liver function tests ALT, AST, ALP and LDH levels. They are located in the cytoplasm and serve as markers for plasma membrane injury in hepatocytes. Significant alterations in biochemical markers were also reported to be confirmed by histological findings in liver tissue[4,21-24].

Pretreatment of mice with niclosamide significantly reduced biochemical markers and histopathological assessment of MTX-induced liver damage in the present investigation. It is possible that niclosamide's capacity to restore mitochondrial dysfunction and its antioxidant properties were responsible for the drug's dramatic impact on liver enzymes. By blocking oxidative phosphorylation, niclosamide keeps mitochondria healthy. Niclosamide increases fatty acid oxidation by inducing mitochondrial uncoupling, which lowers the proton gradient across the inner mitochondrial membrane. Increased mitochondrial oxidation of lipids lowers cellular lipid buildup. As a result, oxidative stress and the forms of ROS that cause tissue damage get diminished. This suggests that niclosamide may control MTX-induced mitochondrial dysfunction, leading to reduced free radical production and cellular damage[11-13].

The current study strongly supported the remarkable antioxidant activity of vitamin C, which is one of the well-known antioxidant defense systems that act as cofactors for many enzymes, protecting cells against damage by free radicals by acting as free radical scavenger. The protective results of vitamin C against hepatotoxicity was owing to its ability to neutralise free radicals and antioxidants. By scavenging free radicals, vitamin C aids in protecting mitochondria from damage caused by the electrical donation/transfer process and its accompanying rise in ROS. Supplementing with vitamin C may aid the body's antioxidant enzymes in neutralising the free radicals produced at an excessive rate by MTX. This will lead to reducing the effect of ROS on mitochondria. Vitamin C leads to decreased oxidation byproducts, like MDA. Vitamin C acts as a complement to SOD in fighting the free radicals[16,25,26].

The current study showed that the hepatoprotective effect of mice administered simultaneously with niclosamide and vitamin C was smaller than the hepatoprotective effect obtained when mice were treated with only the drug.

Conclusion

Niclosamide and vitamin C together could reduce the liver toxicity caused by methotrexate, but niclosamide alone was more effective than the combination.

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Conflict of Interest: None.

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