Original Article

Comparative Study between Effect of Niclosamide and Vitamin C on Methotrexate-Induced Liver Injury in Mice

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Abstract

Background: Niclosamide is an old drug used before in the treatment of tapeworms, its main mechanism of action is through the amelioration of mitochondrial-free radical generation. It has been thought that free radical generation has an important role in methotrexate-induced liver injury. Vitamin C, an antioxidant agent, has an essential role in the methotrexate-induced oxidative stress (OS) pathways in mice hepatocytes. **Aim:** The aim of the study was to evaluate the effect of pretreatment with Vitamin C in different doses and niclosamide in different doses on liver injury, when we induced liver injury with methotrexate. **Materials and Methods:** Forty-two albino mice were divided equally into six groups, the first group was considered as "the control group," which received a normal saline solution, the second group was considered as "Methotrexate group," third and fourth groups were orally pretreated with "niclosamide at a dose (70 mg/kg/day) and (140 mg/kg/day)" respectively, fifth and sixth groups were orally pretreated with "Vitamin C at a dose (100 mg/kg/day) and (200 mg/kg/day)," respectively, all groups, except the control group, were injected with "Methotrexate (20 mg/kg)" intraperitoneally on the 10th day, to induced hepatotoxicity and assessed the effect of the pretreatment with these medications on OS biomarker and histopathological alteration that induced by methotrexate-induced hepatotoxicity. **Results:** It was found that pretreatment with niclosamide 70 mg/kg/day and 140 mg/kg/day and Vitamin C 100 mg/kg/day and 200 mg/kg/day, in mice injected with methotrexate, led to a decrease in "liver function tests, OS parameters, as well as improvement in liver tissue." There was an improvement in "serum alanine aminotransferase, alkaline phosphatase, malondialdehyde" in addition to an improvement in "histological appearance," but it was noted that pretreatment with niclosamide gives a better improvement in "liver function, OS, and liver tissue." **Conclusion:** Niclosamide is better than Vitamin C in protecting the hepatocytes against methotrexate-induced liver injury, also niclosamide and Vitamin C have a dose-dependent protecting effect against methotrexate-induced liver injury.

Keywords: Hepatotoxicity, methotrexate, niclosamide, oxidative stress, Vitamin C

Introduction

Methotrexate is an antifolate agent that works by inhibiting the action of the enzyme dihydrofolate reductase by converting it to tetrahydrofolate.[1] MTX is used in many clinical conditions such as autoimmune diseases like psoriasis and rheumatoid arthritis and different types of malignancies counting liver cancer, osteosarcoma, lymphomas, and acute lymphocytic leukemia[2-4] Methotrexate has several adverse effects and toxicities; one of the most dangerous adverse reactions of methotrexate treatment is liver injury from mild hepatitis, cholestasis, and acute liver injury.[5] The main cause of MTX-induced hepatic injury is unclear, but it is believed that there are a group of mechanisms that cause liver damage, from these mechanisms; MTX produces a defect in the mitochondria by depleting hepatic folate stores due to the accumulation of polyglutamate in the liver cells, leading to mitochondrial dysfunction and generation of reactive oxygen species (ROS). In addition, MTX increases homocysteine and thus increases cellular sensitivity to ROS and reactive nitrogen species, as well as lipid peroxidation (LPO) of biological membranes and reduces the level of nicotinamide adenine dinucleotide phosphate (NADPH) within the

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cells, thus depleting the cellular glutathione, exposing the cells to damage to ROS. Furthermore, the increase in homocysteine with oxidative stress (OS) leads to a tension in the endoplasmic reticulum, which affects the metabolism of cholesterol and triglycerides and thus causes the leakage of fats in the liver[6-10] Therefore, clinical and experimental studies look for antioxidant or anti-inflammatory agents that reverse the liver injury caused by MTX such as ascorbic acid, sitagliptin, and naringin that has a hepatoprotective effect against MTX-induced liver injury.[9]

Vitamin C, famous as ascorbic acid, is an oxidizing agent that is fine soluble in water. Vitamin C is an antioxidant and ROS scavenger and has a fundamental role in the pathways of OS and therefore is used in the field of pharmaceutical and cosmetics[11-14] The mechanism of action of Vitamin C is its ability to neutralize OS during the process of donation/electronic transfer. It can decrease unsteady types of oxygen, nitrogen, and sulfur radicals and also works to replenish antioxidants in the body such as alpha-tocopherol (Vitamin E) and also works to avoid LPO caused by peroxide radicals. Increased ROS concentration is connected with mitochondrial diseases and can be treated with Vitamin C because it has antiapoptotic activity.[13-15]

Niclosamide has been used for several years to treat tapeworms and it is fine tolerated in humans with severely oral LD50 values of >1000 mg/kg and used for a short period.[16,17] The mechanism of action of niclosamide is its ability to uncoupling oxidative phosphorylation and induce the action of adenosine triphosphate in the mitochondria. The main function of mitochondria is to produce ATP, it is a center for the metabolism of fatty acids and carbohydrates, proteins associated with the production of ATP, and a rise in lipid oxidation through them reduces the accumulation of lipids accumulation in all cells. Niclosamide protects the mitochondria through its ability to uncoupling the oxidative phosphorylation that reduces the ROS responsible for tissue injury and thus reduces OS[18-20] Many drugs and medicinal plants have demonstrated their antioxidant efficacy experimentally by decreasing the elevated OS in liver damage induced by methotrexate; however, no research has been done to assess the influences of niclosamide on liver damage by methotrexate in mice so far.

Aim of the study

This study was conducted to study the comparison of the effect of niclosamide and Vitamin C on MTX-induced liver injury in mice.

Materials and Methods

This study was conducted in the Pharmacology Department, and the Iraqi Center for Cancer and Medical Genetics Research, College of Medicine, Al-Mustansiriya University From November 2020 and lasted for 9 months. Forty-two albino mice were used. All appropriate international, national, and/or institutional guidelines for the care and handling of animals were followed. All animal study protocols approved by the Animal Care and Use Committee in Al-Mustansiriya Medical College. The experimental protocol of the present study was also approved by Scientific Committee and of the Pharmacology Department, College of Medicine, Al-Mustansiriya University. Forty-two albino mice weigh between (18 and 38) grams and their ages range between 9 and 12 weeks. Mice were kept under the control of room temperature between 23 ± 2°C and humidity with a light-dark cycle (12:12 h), and they were housed under normal laboratory conditions with food and water provided *ad libitum*. The mice were divided into six groups as follows: Control group: mice receive normal saline until the termination of the experiment; MTX group: mice were left untreated for 10 days followed by giving a single injection of MTX (20 mg/kg, i.p.) on the 10th day;[9] niclosamide pretreated group: mice treated with niclosamide in a dose 70 mg/kg body weight/day orally through mice oral gavage followed by intraperitoneally injected with 20 mg/kg MTX on the 10th day;[20,21] niclosamide pretreated group: mice treated with niclosamide in a dose of 140 mg/kg body weight/day orally through mice oral gavage followed by intraperitoneally injected with 20 mg/kg MTX on the 10th day;[20,21] Vitamin C pretreated group: mice treated with Vitamin C in a dose of 100 mg/kg body weight/day orally through mice oral gavage followed by intraperitoneally injected with 20 mg/kg MTX on the 10th day;[22] Vitamin C pretreated group: mice treated with Vitamin C in a dose 200 mg/kg body weight/day orally through mice oral gavage followed by intraperitoneally injected with 20 mg/kg MTX injectable solution (KOCAK pharma/Turkey) on the 10th day.[22] Niclosamide tablet (500 mg) (Bayer/Germany) was prepared freshly every day to be administered orally, was converted into a fine powder by crushing it with a pestle and mortar, then transferring the powder to a beaker and adding 35 ml of normal saline to it, after that, it was stirred well using a magnetic stirrer to dissolve it until the substance was well dissolved. Then, it was administered orally through mice's oral gavage according to the weight of the mouse. Vitamin C sachet (1000 mg) fine powder (Uniphar/EC) was prepared freshly every day by dissolving it in distilled water and then it was administered orally through mice's oral gavage according to the weight of the mouse. At the end of the experiment, 48 h after MTX administration, the mice were anesthetized with chloroform and sacrificed. The blood sample was taken from the heart and collected for biochemical analysis and the liver was divided into two parts, one part was "taken and placed in a plane tube and washed in 0.01 monophosphate buffer solution to eliminate the excess blood. Then, it was weighed 300 mg and chopped into small slices; then, the tissue protein extraction reagent was added according to the ratio of 1 g: 5–10 ml and mixed with ice water. After being mingled, the mixture was centrifuged for 10 min at 5000 rpm. It was taken supernatant of tissue extract and placed at −20°C until the histological examination was performed," and the other parts were preserved in formalin for histopathological study.

Biochemical analysis

Serum assessment

Assessment of serum level of ALT, AST, and ALP using Flexor-EL80 automated device and assess the level of

LDH in liver serum using a competitive (enzyme-linked immunosorbent assay [ELISA]) kit.

Liver tissue assessment

The level of SOD in liver tissue is estimated using the quantitative sandwich ELISA technique. The level of GSH in liver tissue is estimated using the double sandwich ELISA technique, and LPO was estimated by assessing the level of MDA in liver tissue using the double sandwich ELISA technique.

Liver histopathological examination

Followed the traditional processing procedure (paraffin-embedded method) according to Bancroft,[23] then the tissue was dyed with hematoxylin and eosin (H and E). Liver architecture assessment of MTX-induced liver injury using a histological scoring system for semi-quantitative assessment of methotrexate-induced liver injury by ranking tissue lesion severity to assess the grade of histopathological changes induced by MTX therapy. Ranking from 0 to 3 according to the grade and extent of the change as follows: (0) no histopathology changes, (1+) histopathology changes in <20% of fields, (2+) histopathology changes in 20%–60% of fields, and (3+) histopathology changes in >60% of fields. This ranking was employed by Benli *et al*.[24] to establish an evaluation rate of the histopathological lesion for studied animal tissues.

Statistical analysis

The resulting data were analyzed using International Business Machines Corp. SPSS version 16, package for windows 8, (IBM, New York, USA). Data in this study were displayed as mean ± standard deviation. The significance of differences of different means was tested using one-way ANOVA analysis for differences between more than two independent means. Statistical significance was evaluated whenever the probability value ($P < 0.05$).

Results

Methotrexate effect on hepatic function and oxidative stress markers

Treating mice with a single dose of 20 mg/kg MTX only intraperitoneally injected on the 10th day resulted in a significant decrease in tissue level of OS markers (SOD and GSH) compared with the control group. MDA was increased significantly ($P < 0.05$). Furthermore, there was a significant increase in serum level of hepatocellular and hepatobiliary markers (ALT, AST, and ALP) and LDH compared with the control group. ALT, ALP, and LDH were increased significantly ($P < 0.05$), while nonsignificantly ($P > 0.05$) increased in serum level of AST [Table 1 and Figures 1, 2].

Effect of pretreatment with niclosamide and vitamin C on hepatic function and oxidative stress markers

The present study shows that the four pretreated mice groups with niclosamide 70 mg/kg, niclosamide 140 mg/kg, Vitamin C100 mg/kg, and Vitamin C200 mg/kg in comparison with the methotrexate-treated group resulting from the following changes in parameters:

Table 1: Changes in tissue and serum level of biochemical parameters among mice treated with methotrexate and control group, ($n=7$) for each

Parameters	Groups (x±SD)	
	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 from the control group ($P<0.05$); Unpaired *t*-test, data expressed as "Mean±SD", *n*: Number of the animal for each group. SOD: Superoxide dismutase, GSH: Glutathione reductase, MDA: Malondialdehyde, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, SD: Standard deviation, MTX: Methotrexate

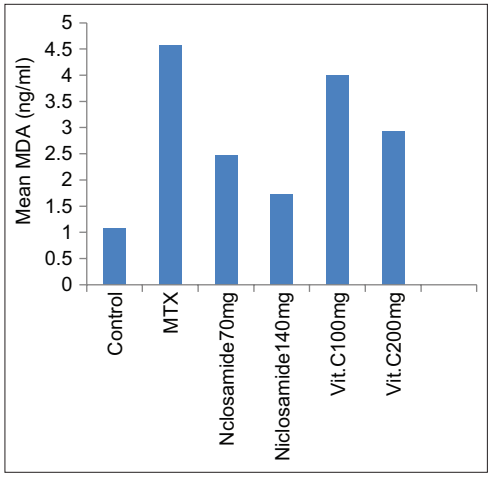

Figure 1: Effect of treatment regimens on "MDA tissue level." * = "significant difference from the control group ($P < 0.05$)," = "significant difference from the methotrexate group ($P < 0.05$)"

Pretreatment with niclosamide 70 mg/kg causes a nonsignificant increase ($P > 0.05$) in tissue level of SOD and GSH while causes a significant decrease ($P < 0.05$) in tissue level of MDA. It also causes a significant decrease ($P < 0.05$) in serum level of ALT, ALP, and LDH, while a nonsignificant ($P > 0.05$) decrease in serum level of AST.

Pretreatment with niclosamide 140 mg/kg causes a nonsignificant increase ($P > 0.05$) in tissue level of SOD, while significant increase ($P < 0.05$) in tissue level of GSH, and MDA decreased significantly ($P < 0.05$). It also causes a significant decrease ($P < 0.05$) in serum level of ALT, ALP, and LDH while a nonsignificant ($P > 0.05$) decrease in serum level of AST.

Pretreatment with Vitamin C100 mg/kg causes a significant increase ($P < 0.05$) in tissue level of SOD while a nonsignificant increase ($P > 0.05$) in tissue level of GSH, and MDA decreased nonsignificantly ($P > 0.05$). It also causes a significant

decrease ($P < 0.05$) in serum level of ALT, ALP, and LDH while a nonsignificant ($P > 0.05$) decrease in serum level of AST.

Pretreatment with Vitamin C 200 mg/kg causes a nonsignificant increase ($P > 0.05$) in the tissue level of SOD and GSH while causes a significant decrease ($P < 0.05$) in the tissue level of MDA. It also causes a significant decrease ($P < 0.05$) in serum level of ALT, ALP, and LDH, while a nonsignificant ($P > 0.05$) decrease in serum level of AST [Table 2 and Figures 1, 2].

Treatment effect on the histopathological findings of the liver

The histopathological findings from drug-induced liver injury were evaluated by ranking tissue lesion severity, ranking from 0 to 3 depending on the degree and extent of the alteration. They were studied in six groups each containing seven mice. According to the semi-quantitative scoring of histological changes, Table 3 and Figure 3 shows no significant liver abnormality and very mild depletion of glycoprotein in the diffuse area of liver tissue in the control group (score +/–<5%), while in the MTX group, observed the highest score (Score >20% (++) =40%) as shown in Figure 4, and the groups [Figures 5-8] pretreated with niclosamide 70 mg/kg, niclosamide 140 mg/kg, Vitamin C 100 mg/kg, and Vitamin C 200 mg/kg observed lower scores with moderate histopathological alterations. The lowest score is shown in niclosamide 140 mg and Vitamin C 200 mg treated mice. The extent and severity of histopathological lesions were attenuated in comparison with those of the MTX group.

Discussion

In this study, the effect of Vitamin C and Niclosamide in different doses on MTX-induced liver injury in mice was examined. The "high doses of MTX" used in certain clinical conditions are associated with "organ toxicity including acute

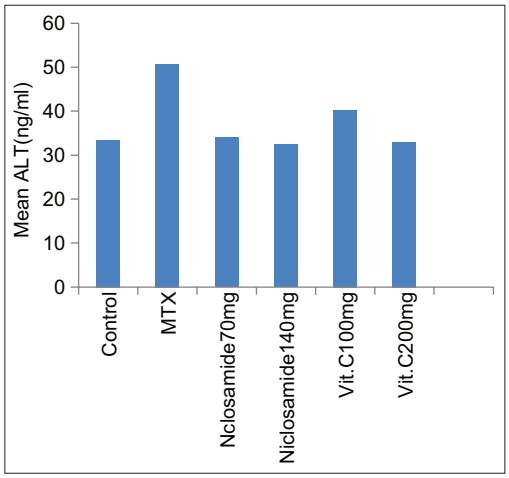

Figure 2: Effect of treatment regimens on "serum ALT level." * = "significant difference from the control group ($P < 0.05$)," = "significant difference from the MTX group ($P < 0.05$)"

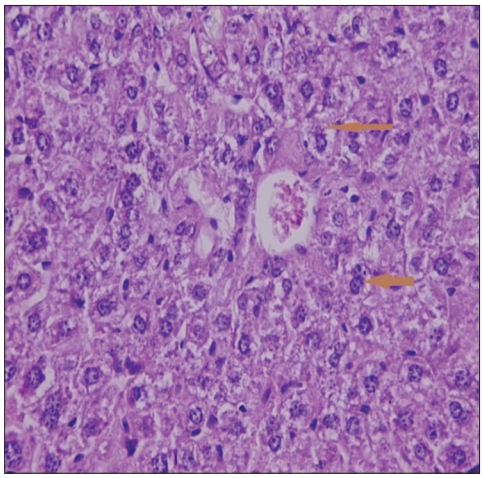

Figure 3: Liver section of normal control mice showing very mild depletion of glycoprotein in diffuse area of liver tissue. Stained with hematoxylin and eosin

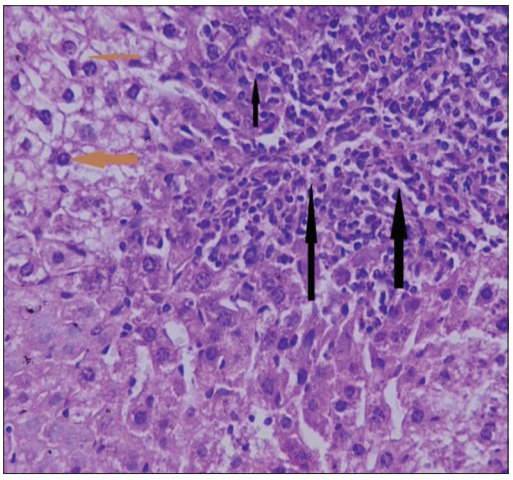

Figure 4: Liver section of MTX treated mice showing the area of necrosis with inflammatory cells infiltration with depletion of glycoprotein. Stained with hematoxylin and eosin

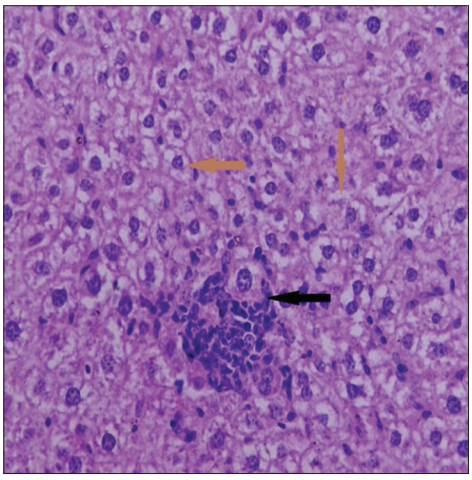

Figure 5: Liver section of niclosamide 70 mg treated mice show a focal area of necrosis and inflammatory cells infiltration with depletion of glycoprotein. Stained with hematoxylin and eosin

Table 2: Changes in tissue and serum level of biochemical parameters between mice treated with niclosamide and Vitamin C in different doses (pretreatment groups) for 10 days, (n=7) for each

Parameters	Groups (x±SD)				
	MTX	Niclosamide 70 mg + MTX	Niclosamide 140 mg + MTX	Vitamin C 100 mg + MTX	Vitamin C 200 mg + MTX
SOD (IU/ml)	65.77±34.57	62.79±23.91	76.83±48.23	294.97±205.97$^{\$}$	122.61±150.10
GSH (µg/ml)	43.09±9.19	50.82±6.83	58.99±13.63$^{\$}$	34.31±13.61	36.42±15.02
MDA (nmol/ml)	4.58±0.217	2.47±0.91$^{\$}$	1.73±0.87$^{\$}$	4.00±0.65	2.93±0.24$^{\$}$
ALT (IU/L)	50.71±7.67	34.00±3.60$^{\$}$	32.42±6.72$^{\$}$	40.28±10.16$^{\$}$	33.00±4.54$^{\$}$
AST (IU/L)	34.71±8.78	30.71±7.52	32.28±7.84	32.85±6.51	33.14±8.27
ALP (IU/L)	458±74.91	265.28±47.51$^{\$}$	311.85±127.60$^{\$}$	200.91±141.01$^{\$}$	126.00±31.85$^{\$}$
LDH (ng/ml)	38.48±3.62	25.42±3.47$^{\$}$	23.25±3.87$^{\$}$	22.89±4.53$^{\$}$	24.40±4.52$^{\$}$

*Significant difference from the control group (P<0.05), $^{\$}$Significant difference from the methotrexate group (P<0.05) One-way ANOVA test, data expressed as "mean±SD", n: Number of the animal for each group". SOD: Superoxide dismutase, GSH: Glutathione reductase, MDA: Malondialdehyde, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase, SD: Standard deviation, MTX: Methotrexate

Table 3: Liver injury assessment of study groups according to ranking tissue lesion severity

Score components	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Depletion of glycoprotein						
Score	±	++	++	−	±	±
Extent (%)	<5	>40	>30	No	<5	<5
Inflammatory cellular infiltrate						
Score	−	++	++	±	−	±
Extent (%)	No	>40	>30	<5	No	<5
Cellular necrosis						
Score	−	++	++	±	−	±
Extent (%)	No	>40	>30	<5	No	<5
Sinusoid dilation with an accumulation of fat droplets						
Score	−	−	−	−	±	−
Extent (%)	No	No	No	No	<5	No

Ranking from 0-3 depending on the degree and extent of the alteration between the treatment groups MTX, Vitamin C 100 mg and 200 mg and niclosamide 70 mg and 140 mg, (pretreatment groups), (n=7) for each. (−) No pathological lesion, (±) Very mild changes in <5% of fields, (+) Histopathology changes in <20% of fields, (++) Histopathology changes in 20%-60% of fields. N: Number of the animal for each group, Group 1: Control group, Group 2: MTX-treated animals, Group 3: 70 mg-treated animals, Group 4: 140 mg treated animals, Group 5: Vitamin C 100 mg-treared animals, Group 6: Vitamin C 200 mg-treared animals, MTX: Methotrexate

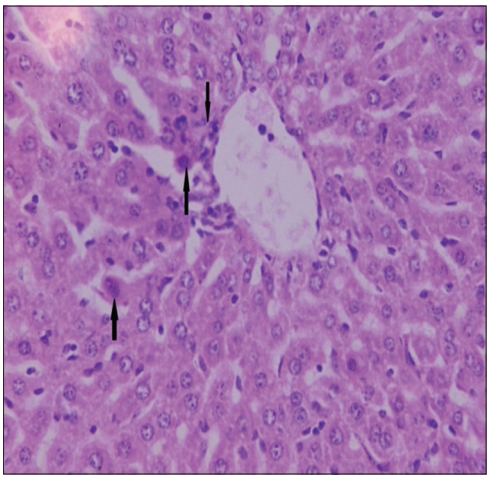

Figure 6: Liver section of niclosamide 140 mg treated mice show very mild changes in which there was dispersed necrotic cell with very mild inflammatory cells infiltration. Stained with H and E ×40

liver toxicity, progressive liver fibrosis, and cirrhosis." Here, we intend to prove the effective hepatoprotective activity of niclosamide and Vitamin C in different doses on MTX-induced liver injury. "It is worth mentioning that there are no previously published studies describing the effects of niclosamide on methotrexate-induced hepatotoxicity."

The results of this study indicate that MTX-treated mice exhibited "significant liver damage," as shown by "significant increases 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.[6,7] The results of histopathological examination supported the biochemical changes and showed obvious liver damage in the MTX group. It's well known that the accumulation of "polyglutamate-MTX" inside the hepatic cells resulted in "depletion of hepatic folate stores" and induction of "mitochondrial dysfunction." Mitochondria represent the generator of energy production inside the cells. "Mitochondrial dysfunction" is associated with over "a generation of ROS" and these free radicals have an essential part in methotrexate-induced liver injury. Moreover, MTX "increases homocysteine" and thus "increases cellular

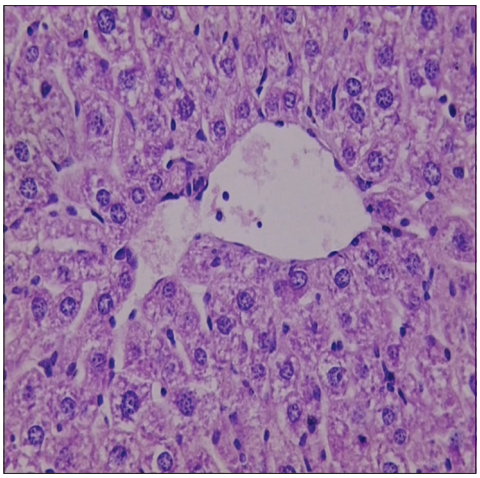

Figure 7: Liver section of Vitamin C 100 mg treated mice showing very mild depletion of glycoprotein inside the hepatocyte cells. Stained with hematoxylin and eosin

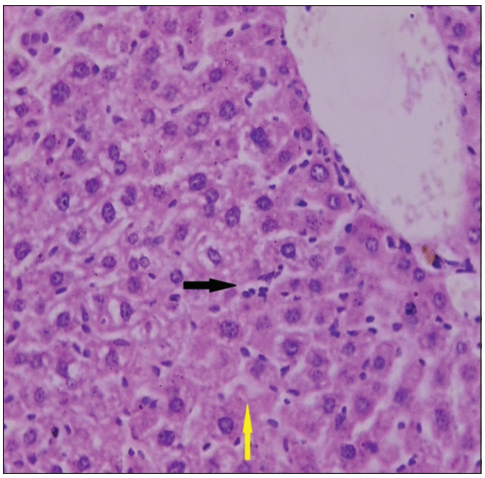

Figure 8: Liver section of Vitamin C 200 mg treated mice show very mild changes in which there was dispersed necrotic cell with very mild inflammatory cells infiltration, and mild depletion of glycoprotein. Stained with hematoxylin and eosin

sensitivity" to free radicals. Furthermore, MTX "reduces" the level of "NADPH" within the cells, thus "depleting the cellular glutathione," and this making hepatocytes more liable to damaging effects of ROS[6-10] Hence, the "elevated level of MDA," in the liver of mice by methotrexate suggested increased "LPO" causing liver injury and indicating that "the antioxidant defense mechanisms were failed" to deal with excessive free radical formations. This will leads to damage to "the cellular membrane," "the release of intracellular contents," and "elevation of serum liver function tests ALT, AST, ALP," and "LDH levels," which are "cytosolic enzymes" that indicate "the damage to the plasma membrane" of the hepatocytes. It was also noted that these significant changes in biochemical parameters are supported by histopathological results of liver tissue.[5,23-26]

In the current study, pretreatment of mice with "niclosamide" markedly ameliorated MTX-induced liver injury in terms of biochemical parameters and histopathologic examination. The ability of niclosamide to significantly improve "liver enzymes" may be due to its antioxidant effects and its capability to "normalize mitochondrial dysfunction." Niclosamide protects the mitochondria by its ability to "uncoupling the oxidative phosphorylation process." Mitochondrial uncoupling that induced by niclosamide causes "a decrease in the proton gradient across the inner membrane of the mitochondria" and this leads to more fatty acid oxidation. A rise in "lipid oxidation" through the mitochondria reduces "the accumulation of lipids in all cells." This will reduce the types of ROS responsible for tissue injury and eventually reduces OS. As a result, Niclosamide might "regulate mitochondrial dysfunction" induced by MTX and therefore less free radical will generate with less cellular damage[18-20] As the result of the above, it is evident that the pretreatment with niclosamide 140 mg improved cellular status hepatocytes more than pretreatment niclosamide 70 mg.

The results of this study strongly support the remarkable antioxidant activity of Vitamin C in previous studies. Vitamin C 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 scavengers." The protective results of Vitamin C against hepatotoxicity in this study are consistent with other studies. In the present study, pretreatment of mice with Vitamin C was shown to attenuate MTX-induced liver injury in comparison with those of the MTX group. This can be attributed to its "antioxidant and ROS scavenger properties." Vitamin C can "neutralize OS during the electronic donation/transfer process," which participates in the protection of the mitochondria from the increased levels of ROS. Vitamin C acts to help endogenous antioxidant enzymes reduce the excess amount of free radicals generated during treatment with MTX. This will lead to reducing the effect of ROS on mitochondria. Vitamin C leads to decreased "oxidation byproducts (MDA)." Vitamin C act as "a complement to SOD" in fighting the free radical generating[12,14,27,28] As the result of the above, it is evident that the pretreatment with Vitamin C has a cytoprotective effect on hepatocytes and this is manifested by improving the level of ALT in was dose-dependent manner. Regarding the total antioxidant capacity of hepatocytes, pretreatment with Vitamin C increasing total antioxidant capacity and improving oxidative damage and is manifested by a decreased level of MDA in way dose-dependent manner.

The results of this study showed that the hepatoprotective effect of mice administered with Vitamin C was smaller than the hepatoprotective effect obtained when mice were pretreated with niclosamide.

Conclusion

The present study suggests that niclosamide is better than Vitamin C in protecting the hepatocytes against MTX-induced liver injury, also niclosamide and Vitamin C have a dose-dependent protecting effect against MTX-induced liver injury.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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