



## HEPATOPROTECTIVE EFFECTS OF VITAMIN C AGAINST METHOTREXATE INDUCED ACUTE LIVER INJURY: AN EXPERIMENTAL STUDY

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*Methotrexate (MTX), a synthetic antimetabolite with a wide range of therapeutic indications, although its liver toxicity induced mainly through oxidative stress limits its clinical use. Vitamin C (ascorbic acid) possesses potent antioxidant and anti-inflammatory properties, Therefore, has a possible hepatoprotective effect. This study seeks to address the hepatoprotective effects of vitamin C against MTX-induced liver injury in albino mice. MTX showed significant elevation in both serum Alanine aminotransferase (ALT) and liver tissue Malondialdehyde (MDA), indicating hepatic injury, while vitamin C pre-treatment will hold down this elevation significantly and dose-dependently, causing amelioration of the toxic effect of MTX; the histopathological findings also support this finding. Also, MTX causes consumption of liver tissue content of superoxide dismutase (SOD) and Glutathione (GSH), while vitamin C pre-treatment boosts the SOD hepatic tissue level while GSH is diminished even more. In conclusion, vitamin C has a dose-dependent amelioration effect on the toxic effect of MTX on the liver.*

**Keywords:** methotrexate, vitamin C, hepatotoxicity, liver, oxidative stress

### INTRODUCTION

Drug-induced liver injury (DILI) is the most critical cause of hepatotoxicity and can lead to all forms of liver injuries ranging from mild asymptomatic transaminitis to liver failure<sup>1&2</sup>; one of the most common drugs known to cause hepatotoxicity is Methotrexate, which is a cytotoxic antifolate drug, highly effective with many clinical applications ranging from anti-inflammatory and immunosuppressant at relatively low doses and antineoplastic at higher doses; however, liver toxicity of MTX has attracted considerable attention. Methotrexate exhibits many mechanisms of action; the most important one is inhibiting the dihydrofolate reductase enzyme, so decreasing tetrahydrofolate level, the building blocks for many essential cellular nucleic acids and proteins, therefore MTX will inhibit the synthesis of purines, pyrimidines, DNA, RNA, thymidylate, and other proteins<sup>3-5</sup>.

Several mechanisms have been investigated for liver injury induced by MTX; the most accepted one is the loss of oxidative equilibrium inside liver tissue due to overproduction of reactive oxygen species (ROS) induced by mitochondrial dysfunction, depletion of endogenous anti-oxidant due to NADPH inhibition, and influenced by long periods of hepatic cellular deposition of both MTX and its polyglutamate metabolite<sup>4,6&7</sup>. This disturbance in the oxidative balance will shift it toward oxidative stress and stimulate lipid peroxidation process, and ultimately, induction of the autophagic cell death and apoptosis<sup>8&9</sup>, leading to an oxidative stress-induced liver injury<sup>10-13</sup>.

Recently, many studies demonstrated using different herbal and chemical agents with antioxidant properties to prevent DILI, and the result was quite promising. For example, vitamin C (ascorbic acid), which is a six-carbon compound with many vital functions in

the living systems, most important one is through its antioxidant effect<sup>14-16</sup>.

Vitamin C is one of the potent antioxidants capable of scavenging ROS, protecting tissues from damage caused by free radicals; Vitamin C's antioxidant effect comes from donating a hydrogen atom, converting reactive, unstable radicals to stable unreactive ones. Moreover, Vitamin C effectively regenerates another antioxidant called vitamin E<sup>15</sup>.

Vitamin C exhibited potent antioxidants activity in the biological system by acting as a scavenger free radicals like hydroxyl radical ( $\cdot\text{OH}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and singlet oxygen ( $^1\text{O}_2$ ). So, Vitamin C will reduce ROS overload and ameliorate oxidative stress, the leading cause of many chronic and degenerative diseases like cancer, cardiovascular disease, and neurodegenerative diseases. Furthermore, several reports have shown that vitamin C can enhance conventional anticancer drug-induced cytotoxicity on cancer cells<sup>17-19</sup>.

Because of this antioxidant activity, many studies demonstrate the hepatoprotective effect of vitamin C against many drugs that induce hepatic injury, since most of these drugs induce hepatic damage through induction of oxidative stress pathway<sup>14-16&20</sup>.

## METHOD AND MATERIALS

### Experimental design

This study was performed in December 2020 in the animal's house of the Iraqi centre for cancer research and medical genetics/ Mustansiriyah university/ Iraq-Baghdad.

Because female gender is one of the risk factors for DILI and to eliminate the gender difference effects<sup>21</sup>, twenty-eight healthy female albino mice weighted  $30 \text{ g} \pm 2$  was randomly collected after one week adaptation period without any intervention, they are separated into four groups and placed into four sterilizer cages, each with seven mice and placed in a room of (21-25) °C temperature and 12-hour light/dark cycles, also a free excess to food and water was provided. The human care for animals was according to the guide and care of laboratory animals. these mice were separated into four groups (fig. 1) as follow:

- 1- Group 1: Control group (n= 7):** mice have received 0.5 ml of distilled water orally for ten consecutive days, and on the tenth day, sodium chloride was injected intraperitoneally.
- 2- Group 2: MTX group (n= 7):** mice have received 0.5 ml of distilled water orally for ten consecutive days, and on the tenth day, Methotrexate was injected intraperitoneally in a dose of 20 mg/kg.
- 3- Group 3: group C100 (n= 7):** mice have received 100 mg/kg of Vitamin C orally for ten consecutive days, and on the tenth day, Methotrexate was injected intraperitoneally in a dose of 20 mg/kg.
- 4- Group 4: group C200 (n= 7):** mice have received 200 mg/kg of Vitamin C orally for ten consecutive days, and on the tenth day, Methotrexate was injected intraperitoneally in a dose of 20 mg/kg.

Routs and doses of the drugs were determined according to previous studies<sup>22&23</sup>.



**Fig. 1:** Scheme of the experimental design. MTX, Methotrexate; NaCl, sodium chloride; DW, distilled water.

### Serum sample preparation

On the 12<sup>th</sup> day, the mice were anesthetized by using chloroform, and a 5ml syringe was used to collect the blood from the heart; the collected blood was put in a gel & clot activator tube and left in the stand for 30minute, then centrifuged for 15 min. at 5000rpm at room temperature. The yielded isolated sample was stored in a freezer at -20°C to be used later.

### Tissue sample preparation

After collecting the blood sample from the heart, the mice are sacrificed by cervical dislocation, and sharp scissors were used to isolate the liver. After this, the liver was weighed and divided into two pieces; one of them is 3-gram weighted piece prepared for homogenization by moving it to a Teflon homogenizer contain 1 ml of ice-cold PBS (phosphate-buffered saline); after homogenization, homogenate will be centrifuged for 30 min. at 3000 rpm, and the yielded supernatant isolated and stored at -20°C to be used later for biochemical analysis. At the same time, the remaining part of the liver was soaked in a 10% buffer formalin solution and stored at room temperature for later histopathological examination.

### Measurement of liver function test

Serum levels of alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) were measured using an automatic analyser (flexor-EL80, Vitalab, South Africa).

### Measurement of liver tissue level of malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD)

After preparing the tissue homogenate, the double-sandwich ELISA technique is used to measure MDA, GSH, and SOD concentration in the supernatant using ELISA kit for each marker from MyBioSource, USA.

The wells of the kit were pre-coated with mouse monoclonal antibodies of either MDA, GSH and SOD. Samples were added to the wells and after one-hour of incubation, the wells were washed out with PBS (Phosphate Buffered Saline), after this avidin-peroxidase conjugate are added to the wells and washed out with PMS. Then, TMB

(Tetramethylbenzidine) as the substrate is added, which will turn into blue colour, and the acid (stop solution) will be added to stop the reaction and turns the colour into yellow; the intensity of the colour is positively proportional to the concentration of the targeted compounds in the sample. The concentration was obtained by plotting a standard curve relating the colour (O.D.) intensity to the concentration of standards by using an automated ELISA reader (ELISA-humareder, Germany).

### Histopathological analysis

Liver samples intended for histopathological studies were soaked in 10% buffer formalin, then the dehydration process was implied using graded (50-100%) ethyl alcohol and embedded in paraffin wax. The liver tissue was then cut into small sections (4-5 micrometre thick), and these sections were stained with haematoxylin and eosin (H & E) stain for observation and photomicroscope assessment using a light microscope (M83MPTR-C140U; AmScope, California, USA). Histopathological assessment and scoring were done using the Ishak Modified HAI system which depends on the extent of the histopathological changes<sup>24&25</sup>, including degeneration of the hepatocyte and extent of the inflammatory reaction, and as follows:

- 1- **Score 1:** No abnormality.
- 2- **Score 2:** Mild degenerative changes (10%).
- 3- **score 3:** Moderate degenerative changes (25%).
- 4- **score 4:** Severe degenerative changes (50%).
- 5- **score 5:** Extensive and marked changes (>75%).

### Statistical analysis

All statistical analysis were performed using SPSS (version 16) for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with the post-hoc test was used for data analysis. Expression of the data was as means and stander deviation (SD) values, and p-value of less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Results

#### Effect on oxidative stress biomarker (GSH, SOD, and MDA) (table 1):

Significant consumption of the antioxidant capacity represented by glutathione (GSH) and superoxide dismutase (SOD) was shown in the liver tissue after injection of methotrexate, ( $p < 0.001$ ) compared to the control group).

Pre-treatment with Vitamin C causes a non-significant reduction of tissue glutathione level compared to the MTX group with a p-value of (0.129 & 0.246) for both 100&200 mg/kg

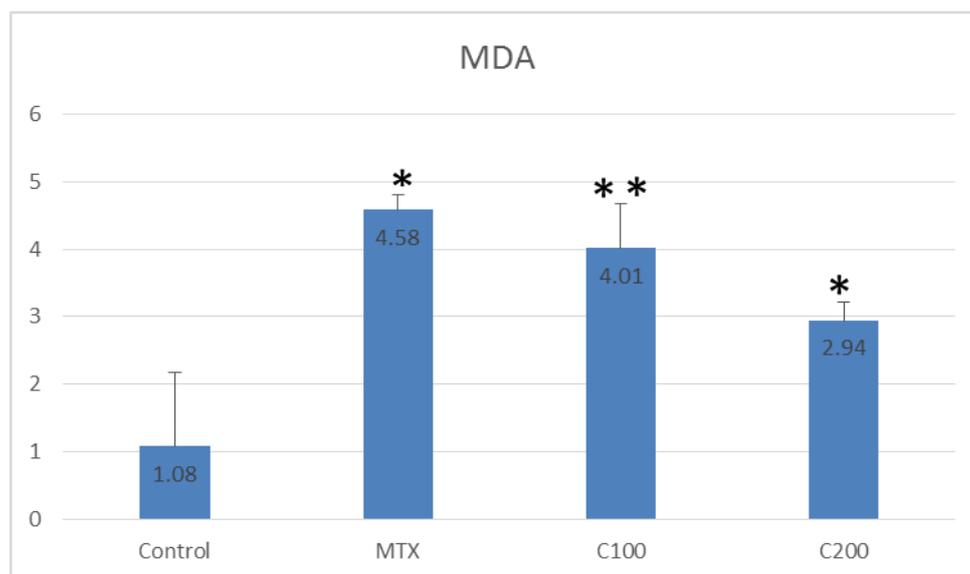
doses, respectively. On the other hand, significant enhancement of tissue SOD level was observed in group C100 ( $p \leq 0.001$ ) while a non-significant increase was observed at the higher dose of vitamin C ( $p = 0.364$  for Group C200).

Malondialdehyde (MDA) as a by-product of oxidative stress was raised significantly ( $p < 0.001$ ) after MTX injection in a dose of 20 mg/kg IP compared to the control group. Although, Vitamin C pre-treatment significantly and dose-dependently held down the MDA level (fig. 2) (100 mg/kg ( $p = 0.012$ ), 200 mg/kg ( $p < 0.001$ )).

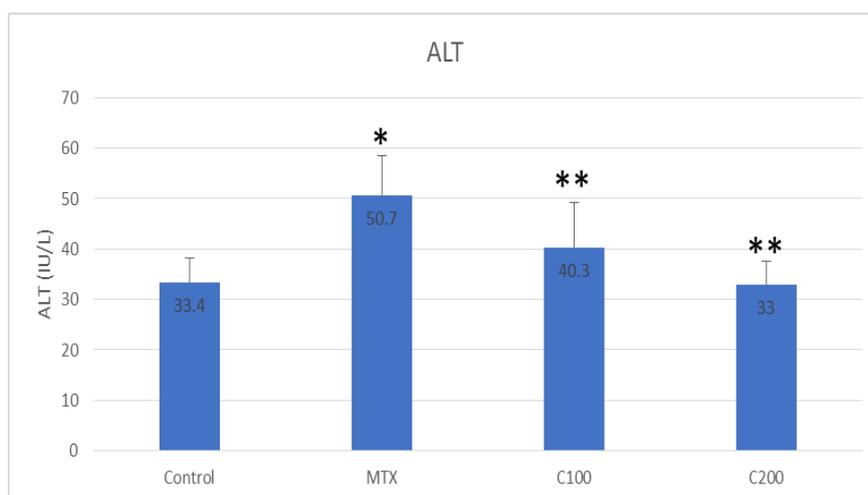
**Table 1** : Effect of Methotrexate and Vitamin C on liver tissue level of oxidative stress biomarker (GSH, SOD, and MDA)

Parameter	Groups			
	control	MTX	C100	C200
GSH (mcg/ml)	66.5±7.2	42.1±9.1*	36.4±17.6^^	37.2±8.1^^
SOD(U/ml)	477.9±36.5	65.3±36.6*	293±205.7**	124.5±146^^
MDA (nmol/ml)	1.07±1.09	4.6±0.23*	4.11±0.67**	2.95±0.27**

Data are presented as means ± SD; n = 7 for each treatment group. \* P < 0.05, significantly different from the control group. \*\*P<0.05, significantly different from the MTX group. ^^p ≥0.05, non-significant different with the MTX group. MTX, Methotrexate; C, ascorbic acid; GSH, glutathione; SOD, superoxide dismutase; MDA, malonaldehyde



**Fig. 2:** MDA level comparison. Data are presented as means ± SD; n = 7 for each treatment group. \* P < 0.05, significantly different from the control group. \*\*P < 0.05, significantly different from the MTX group. MTX, Methotrexate; C, ascorbic acid; MDA, malondialdehyde.



**Fig. 3:** ALT level comparison. ALT level comparison. Data are presented as means  $\pm$  SD; n = 7 for each treatment group. \* P < 0.05, significantly different from the control group. \*\*P<0.05, significantly different from the MTX group. MTX, Methotrexate; C, ascorbic acid; ALT, alanine aminotransferase

**Table 2:** Effect of Methotrexate and Vitamin C on serum level of enzyme biomarker (ALT, ALP, and LDH)

Parameter	Groups			
	control	MTX	C100	C200
ALT (IU/L)	31.3 $\pm$ 4.8	52.2 $\pm$ 7.8*	41.1 $\pm$ 9**	32.1 $\pm$ 4.6**
ALP (mcg/L)	266.7 $\pm$ 66.6	460 $\pm$ 74.6*	206 $\pm$ 122**	123 $\pm$ 30.3**
LDH (ng/ml)	21.8 $\pm$ 5.23	39.5 $\pm$ 3.71*	23.2 $\pm$ 4.6**	23.6 $\pm$ 4.3**

Data are presented as means  $\pm$  SD; n = 7 for each treatment group. \* P < 0.05, significantly different from the control group.  $\wedge$ p $\geq$ 0.05, non-significant different with the control group \*\* P < 0.05, significantly different from the MTX group.  $\wedge$ p  $\geq$ 0.05, non-significant different with the MTX group. MTX, Methotrexate; C, ascorbic acid; ALP, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase

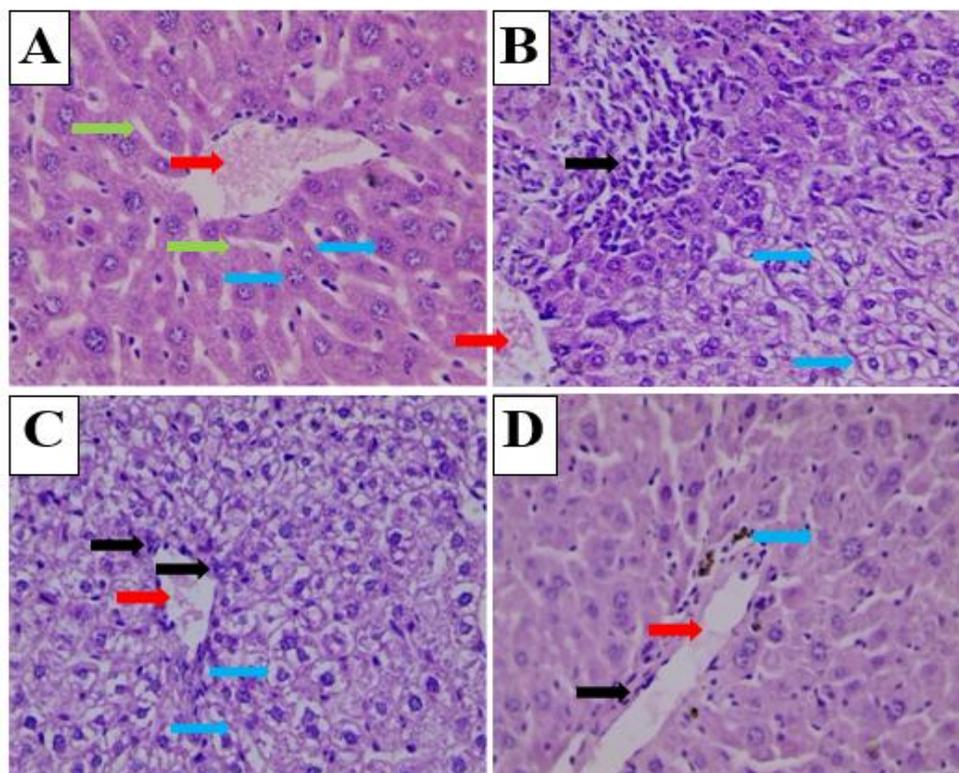
#### Effect on the activity of serum marker enzymes (ALT, ALP, and LDH) (table 2):

When Methotrexate is injected into the MTX-only group, a significant elevation was observed in the serum ALT (Fig.3), LDH and ALP level ( $p < 0.001$ ) in respect to the control group. Pre-treatment with vitamin C in both doses (100&200 mg/kg) will hold down this elevation significantly and dose-dependently when compared to the MTX group ( $p < 0.001$  for both Vitamin C groups).

#### Effect of MTX and different treatment regimens on liver tissue histopathological changes (table 3)

Figure 4 briefly describes the histopathological changes in all groups involves in this study.

As expected, the control group (fig. 4A) generally shows typical liver tissue architecture (score 1). On the other hand, the Methotrexate group (fig. 4B) showed severe degeneration and inflammation of the liver tissue (score 5). Pre-treatment with Vitamin C has a positive effect on the degeneration of the hepatocyte, and this effect seems to be dose dependent. Group C100 (fig. 4C) shows slight improvement on hepatocyte degeneration (score 4), while more improvement was noticed (score 3) in group C200(fig. 4D).



**Fig. 4:** Photomicrograph of the liver mice histopathological section of the control group (A), MTX group (B), group C100 (C) and group C200 the (D). (A) the control group showed normal liver architecture with mild congested central vein (red arrow), normal hepatocyte (blue arrow), and normal sinusoids (green arrow). (B) MTX group showed extensive inflammatory cells reaction (black arrow) with dilated and congested central vein (red arrow) and multifocal marked hepatocyte degenerative changes and cytoplasmic vacuolation (blue arrow). (C) group C100 showed severe hepatocyte degenerative changes (blue arrow) with mild inflammatory cells reaction (black arrow) and mildly dilated and congested central vein (red arrow). (D) group C200 showed moderate degenerative changes (blue arrow) with mild perivascular inflammatory cells (black arrow) reaction and mild congestion in the central vein (red arrow). Magnification= 40X and Haematoxylin-eosin (H&E) staining of liver sections was used for all (A, B, C, and D).

**Table 3:** Effect of Methotrexate and Vitamin C on liver tissue histopathological changes

Groups	Histopathological score	Degeneration changes
CONTROL	1	No abnormality
MTX	5	Extensive and marked changes (>75%)
C100	4	Severe degenerative changes (50%).
C200	3	Moderate degenerative changes (25%).

**Discussion**

This study evaluates the hepatoprotective effect of pre-treatment with different doses of vitamin C against methotrexate induce liver injury. The result revealed the potential of vitamin C to significantly and dose-dependently preserves the hepatic tissue from acute injury caused by methotrexate.

Injection of Methotrexate in this study reduces both GSH level and SOD activity in

the hepatic tissue. Direct inhibition of MTX on NADPH, a co-factor for enzyme glutathione reductase to make reduced glutathione, together with overproduction of ROS, will cause a reduction in GSH cellular availability<sup>6,9,26&27</sup>. Furthermore, the influence of Methotrexate on Nrf2 expression might also reduce glutathione tissue level<sup>3</sup>.

Superoxide dismutase is an essential endogenous antioxidant enzyme, induces the

conversion of mitochondrial generated ROS, mainly superoxide (O<sub>2</sub><sup>-</sup>), into less toxic hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or molecular oxygen (O<sub>2</sub>)<sup>28</sup>. On the other hand, Methotrexate causes mitochondrial membrane damage, and more superoxide (O<sub>2</sub><sup>-</sup>) free radical production will lower SOD activity<sup>24</sup>.

Overproduction of ROS and loss of endogenous antioxidants will shift the oxidative balance toward oxidative stress, ROS will cause mitochondrial dysfunction, and more ROS are produced, causing lipid peroxidation of the cell's lipid contents with MDA liberation<sup>29</sup>. Therefore, in this study, MTX increases MDA hepatic tissue level more than four-fold compared to the control group.

In addition, Both lipid peroxidation and mitochondrial dysfunction will eventually cause cell injury and damage to its membrane, this will make the cell loses its integrity, and the liver cell contents, significantly ALT, LDH, and ALP, will be shifted outside the injured cell to the blood stream<sup>30</sup>. Furthermore, neutrophil activation caused by ROS will cause augmentation of the cell injury<sup>31</sup>.

Vitamin C is well known for its antioxidant properties with free radical scavenger activity, well documented in many previous studies. Therefore, pre-treatment with vitamin C will effectively scavenge ROS, mainly superoxide radical ions, decreasing ROS availability<sup>15</sup>. Thus, this study shows that Vitamin C preserves SOD activity and decreases MDA liberation. Although, the higher dose of vitamin C increases SOD level non-significantly, and this might be justified by the small sample size used in this study.

In this study, vitamin C significantly and dose-dependently ameliorate the hepatotoxic effect of Methotrexate; this was evident by the significant reduction in ALT, LDH, and ALP. Moreover, the histopathological finding supports the biochemical findings.

However, vitamin C causes a significant reduction in glutathione concerning the control group. Suggested explanation to this odd result may be due to the interruption of the glutathione-ascorbate cycle by Methotrexate.

In the process of hydrogen peroxide scavenging by vitamin C, the last will goes throughout many processes ended with the formation of dehydroascorbate which is reduced back to ascorbate using

dehydroascorbate reductase and glutathione (GSH). During this process, glutathione will be converted to oxidizing glutathione (GSSG), which in turn need NADPH to return back to glutathione (GSH), this process is known as the glutathione-ascorbate cycle<sup>15</sup>. Therefore, the inhibition of NADPH caused by methotrexate may deplete the conversion of oxidized glutathione to the reduced one, resulting in GSH level reduction<sup>27</sup>. In addition, this result also suggests that Vitamin C amelioration of MTX induces oxidative stress does not mainly depend on GSH level.

### Acknowledgements

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### Conclusion

From this study, the following can be concluded:

- 1- Disturbance of the oxidative balance in the liver tissue and induction of lipid peroxidation represented by the increased level of MDA caused by MTX is related to the progress of hepatotoxicity.
- 2- Vitamin C show the ability to bring back the oxidative balance and decrease the level of MDA, therefore, ameliorating the hepatic injury caused by MTX.
- 3- The protective effect of vitamin C is proportionally dose dependent.

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## نشرة العلوم الصيدلانية جامعة أسيوط



### التأثيرات الوقائية الكبدية لفيتامين سي ضد إصابة الكبد الحادة التي يسببها الميثوتريكسات: دراسة تجريبية

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قسم علم الأدوية ، كلية الطب ، جامعة المستنصرية ، بغداد ، العراق

الميثوتريكسات (MTX) ، مضاد للايض اصطناعي لديه طيف واسع مع الاستخدامات العلاجية ، مع ذلك سمية الكبد الناتجة بشكل رئيسي من خلال الإجهاد التأكسدي تحد من استخدامه السريري. يحتوي فيتامين C (حمض الأسكوربيك) على خصائص قوية مضادة للأكسدة ومضادة للالتهابات ، لذلك ، له تأثير محتمل في حماية الكبد. تسعى هذه الدراسة إلى بيان التأثيرات الوقائية الكبدية لفيتامين C ضد إصابة الكبد التي يسببها MTX في الفئران البيضاء. أظهر MTX ارتفاعاً كبيراً في كل من Alanine aminotransferase (ALT) ومحتوى أنسجة الكبد من Malondialdehyde (MDA) ، مما يشير إلى إصابة الكبد ، في حين أن العلاج المسبق بفيتامين C سوف يخفض هذا الارتفاع بشكل كبير ويعتمد على الجرعة ، مما يؤدي إلى تحسين التأثير السام لـ MTX ؛ كما تدعم النتائج النسيجية المرضية هذه النتيجة. في الختام ، لفيتامين C تأثير وقائي على التأثير السام لـ MTX على الكبد وهذا التأثير يعتمد على الجرعة بطريقة تناسبية .