

The role of antioxidant (vit-A and glutamine) in ameliorating methotrexate induced hepatic toxicity in rats

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Abstract:

The aim of this study was to investigate the status of antioxidant glutathione peroxidase (GSH-Px) during oxidative stress in blood serum of rats subjected to oral methotrexate administration at a dose 10 mg/kg B.W and administered antioxidant therapy (vit A and glutamine) to reduced or ameliorate such stress. A total of thirty six, male Sprague-Dawley rats weighing (250 – 300) g divided equally into six groups. The first group, control, were fed only standard rat chow as their diet and water ad libitum. The second group administered orally a single dose of methotrexate at a dose of 10 mg/kg B.W. The third group, given a single dose of methotrexate 10 mg/kg B.W + Vit-A (5000 IU/kg B.W) orally by stomach tube, twice daily. The fourth group, given a single dose of methotrexate 10 mg/kg B.W + Glutamine (500 mg/kg B.W), twice daily dosing. fifth group, given vit-A at dose 5000 IU/kg B.W orally, twice daily. Sixth group, given glutamate at dose 500 mg/BW orally, twice daily, all this dosing continue for 8 days. At the end from all animal, draw blood from heart by syring to obtained serum to determine its level then sacrificed and take section from liver for histopathology resulted in.

It was found that methotrexate caused a significant increase in GSH levels (an important marker of lipid peroxidation) when compared with the control group while the level GSH were significantly decreased in the methotrexate + vitamin A group, methotrexate+ glutamine group, vitamin A group and glutamine group. The results indicate that methotrexate cause oxidative stress by reducing the activities and consequently the effectiveness of the antioxidant enzyme defense system. while the antioxidant therapy reducing of oxidative stress. Histopathological examination observed was normal in methotrexate treated rats and MTX with glutamine and vitmine A . but showed hydropyknosis in treated groups of MTX+vit A while the glutamine treated group showed a necrosis and amyloid change.

Key ward: methotrexate; oxidative stress; Antioxidant enzymes.

**دور المضادات الأكسدة (فيتامين A والكلوتامين) في معاكسة الميثوتريكسيت
المحدث سمية الكبد بالجرذان**

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الخلاصة:

كان الهدف من هذه الدراسة للتحقيق حالة مضادات الأكسدة (GSH) خلال أجهاد الأكسدة في مصل دم الجرذان المتعرضة للعلاج بالميتوتريكسات عن طريق الفم والمتعرضة للعلاج بالمضادات للأكسدة (فيتامين A و الكلوتامين) لتقليل أو تحسين أجهاد الأكسدة. استخدمت مجموعة 36 من الذكور سبراغ داوولي الجرذان وزنها (250-300) مقسمة إلى ست مجموعات بالتساوي. غذيت المجموعة الأولى (السيطرة)، بنظام غذائي وبالطعام الجرذان والمياه. جرعت المجموعة الثانية عن طريق الفم مرة واحدة من ميتوتريكسيت بجرعة 10 ملغ /كغم وزن الجسم. جرعت المجموعة الثالثة، جرعة واحدة من الميتوتريكسيت 10 ملغ /كغم وزن الجسم + A فيتامين (5000 وحدة دولية) فمويًا عن طريق أنبوب المعدة ، مرتين يوميًا. أعطيت المجموعة الرابعة، مرة واحدة من الميتوتريكسيت 10 ملغ/كغم وزن الجسم + الكلوتامين 500 ملغ /كغم وزن الجسم مرتين يوميًا. أعطيت المجموعة الخامسة فيتامين A بجرعة IU5000/ وزن الجسم ، مرتين يوميًا عن طريق الفم. أعطيت المجموعة السادسة الكلوتامين بجرعة 500 ملغ /كغم وزن الجسم، مرتين يوميًا ، وجميع هذه المجموعات استمر التجريب لفترة 8 أيام. وفي النهاية سحبت من كل الحيوانات دم من القلب بواسطة سرنج للحصول على مصل لتحديد مستوى الكلوتوتايون ثم شربت واخذ مقطع من الكبد للفحص النسيجي وجد أنه تسبب الميتوتريكسات زيادة معنوية في مستويات GSH (علامة هامة من أكسدة الدهون) بالمقارنة مع مجموعة السيطرة بينما انخفض معنويًا في مستوى GSH في مجموعة الميتوتريكسيت+ فيتامين A، مجموعة ميتوتريكسات + الكلوتامين ، مجموعة فيتامين A والمجموعة الكلوتامين. وتشير النتائج إلى أن ذلك الميتوتريكسيت يؤدي إلى زيادة انزيمات أجهاد الأكسدة في الكبد وبالتالي عن طريق نظام الدفاع المضادات للأكسدة الحد من فعالية الأنشطة الانزيم. في حين أن المعالجة المضادة للأكسدة حدثت من الأكسدة .

لاحظ في الفحص النسيجي للكبد كان طبيعي في المجموعة التي عولجت بالميتوتريكسيت فقط وكذلك في المجاميع التي عولجت مع فيتامين A والكلوتامين ولكن ظهر تحلل الدهني في مجموعة MTX+vit A وكذلك ظهر التنخر وارتشاح البروتينات بين خلايا الكبد في المجموعة التي عولجت بالكلوتامين فقط.

Introduction:

Methotrexate (MTX), as a competitive folic acid antagonist. It is an antimetabolite. Its affinity for dihydrofolate reductase is about 10^5 times higher than that for its physiological substrate dihydrofolic acid. By inhibiting formation of the product tetrahydrofolic acid it disrupts synthesis of thymidine and nucleotides purines. It is currently the most common anti-rheumatic drug prescribed for the treatment of rheumatoid arthritis and other rheumatic disorders(1). Recently, MTX earned a new indication with its efficacy in the treatment of refractory inflammatory bowel

disease (2). However, the efficacy of this drug is often limited by severe side effects and toxic sequelae. Since the cytotoxic effect of MTX is not selective for cancer cells, it also affects the normal tissues that have a high rate of proliferation, including the hematopoietic cells of the bone marrow and the actively dividing cells of the gut mucosa. (3) Methotrexate has also been used in the treatment of psoriasis, a non-neoplastic disease of the skin characterized by rapid proliferation of epidermal cells, as well as in allogenic bone marrow and organ transplantation.(4)

Oxidative stress is more and more recognized by the scientific community to be an important factor in the genesis of chronic diseases, cancers, cardiovascular diseases and aging (5). represents an imbalance between the production and manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Some reactive oxidative species can even act as messengers through a phenomenon called redox signaling.(6) Oxidative stress has emerged as a key player in the pathogenesis of MTX-induced hepatotoxicity. The increased generation of reactive oxygen and nitrogen species, together with the decreased antioxidant defense, promotes the development and progression of hepatotoxicity. Furthermore, it has been found that MTX increases the number of Ito cells(fat-storing, vitamin A-containing stellate cells). Ito cells can be transformed in to myofibroblasts, capable of secreting collagen. This collagenisation consequently leads to liver fibrosis. (7)

Biological oxidative stress of free radicals is controlled by endogenous antioxidants including the scavenger

enzymes superoxide dismutase (SOD) glutathione peroxidase (GSH-Px) and catalase (CAT) and by exogenous dietary antioxidants vitamin E, C carotenoids and flavonoids . Briefly, antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes.(8) The organism exerts several defense mechanisms against free radicals. The anti-radical defense system comprises endogenous anti-oxidants (synthesised by the organism) and exogenous anti-oxidants (external supply). There are two classes of antioxidants, “scavenger” antioxidants trap-ping ROS (reactive oxygen species) and “preventive” antioxidants. Preventive antioxidants inhibit the synthesis of ROS (9). Taking these supplements antioxidant may support or otherwise help these medication work better and may help reduce the likelihood and/or severity of a potential side effect caused by the medication.(10).

The aim of this study was to investigate the role of vit-A and glutamine, antioxidant, on methotrexate-induced liver oxidative stress in rats.

Material and Method:

Animal

Male Sprague-Dawley rats weighing (250 – 300) g were used throughout the experiments. The animals were placed in Kufa Medical College's animal house. The rats were maintained in cages in the

animal care facility, subjected to alternate 12-hour periods of dark and light and were allowed ad libitum intake of chow and water.

Chemicals and drugs

Methotrexate 2.5 mg/BW the manufacturer by Australia, Vitamine A 50000 IU manufacture by Egypt and glutamine 500mg manufacture by Holland were purchased from a local pharmacies , corn oil and ether.

Animal group

Thirty six adult male rats were equally divided into six experimental groups as follows:

Group 1: (control group 6 animals) : were given diet and tap water, add libitum and served as control .

Group 2: (methotrexate-treated group 6 animals) were given a single dose of methotrexate at dose 10 mg/BW orally by stomach tube, daily for period 8 days

Group 3: (methotrexate - vit A treated group, 6 animals) , were given a single dose of methotrexate 10 mg/BW + Vit-A (5000 IU) orally by stomach tube, twice daily for period 8 days .

Group 4: (methotrexate- glutamine treated group, 6 animals) were given a single dose of methotrexate 10 mg/BW + Glutamine (500 mg/BW) orally by stomach tube, twice daily dosing for period 8 days.

Group 5:(vitamin A group, 6 animals) were given vit-A at dose 5000IU/BW orally, twice daily for period 8 days.

Group 6: (glutamine group, 6 animals) were given glutamate at dose 500mg/BW orally, twice daily for period 8 days.

At the end of the experiment, all rats were anesthetized using ether and drew blood 4ml from heart using a 23-gauge needle syringe. The blood placed in centrifuge 3000/minute for 10 minutes to obtain serum for analyzing Oxidation parameters including serum GSH.

Measurement of oxidative stress.

Determination of Total Glutathione in serum(Mmol/L).

A) Principle:

5, 5-Dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration⁽¹⁰⁸⁾:

B) Reagents:

1. 4 % 5-sulfosalicylic acid.
2. 0.1 mM Ellman's reagent was prepared from DTNB (5-5-dithiobis (2-nitrobenzoic acid) ; M.Wt = 396.3 gm/mole) in phosphate buffer pH 8.
3. Phosphate buffer pH 8 (this solution was prepared by a mixture of 0.6 M KH_2PO_4 , and 0.8M Na_2HPO_4).

C) Procedure:

Two sets of tubes were

Reagent	Sample	Blank
Sample	150 μ l	-
4% 5-sulfosalicylic acid	150 μ l	150 μ l
sample test tube was mixed, and centrifuged at 450 X g at 4°C for (5) minutes, then:		
Supernatant	150 μ l	-
Ellman's Reagent	4.5 ml	4.5 ml

prepared as follow:

All tubes were mixed, and the absorbance of sample was read at 412 nm by using spectrophotometer instrument.

D) Calculation:

The concentration of glutathione is calculated by using the following equation:

$$\text{The concentration of GSH} = \frac{A_{\text{of sample}}}{\epsilon \times L}$$

Where:

ϵ = Extinction coefficient ($13600 \text{ M}^{-1} \text{ cm}^{-1}$), L = Light path (cm).

Histopathological preparation :

Section from different groups were prepared by routine techniques . the section of liver were fixed in neutral buffered formalin (10%). 5-6 μ m sections were stained by H&E (hemotoxylin and eosin stains), change in liver tissue were diagnosed through examination of section by light microscope.

Statistic analysis

Results were expressed as mean \pm SD (standard deviation). Using ANOVA- one way, the level of statistical significance was set at $P < 0.05$.

Result:

Table showed the result concentration of reduced glutathione in the serum of different experimental groups.

Table –reduce GSH (mimole) in serum of different experimental groups

Group n-6	GSH
Control	252.5 ± 4.08 A
Methotrexate	336.5 ± 5.9 B
Methotrexate + Vit A	240 ± 1.7 A
Methotrexate + glutamine	224.5 ± 2.02 A
Vit A	150 ± 3.4 C
Glutamine	157.5 ± 10.7 C

Result represent mean ± S.E

Different capital letter due to significant difference at level P-value <0.05

Administration methotrexate alone caused increased in GSH levels (B) compared with control group (A) ($P < 0.05$), while GSH level in glutamine-methotrexate and vit-A- methotrexate groups caused a significant decrease in GSH levels in their serum when compared with methotrexate alone group. While the administration glutamine and vitamin A treatment alone (C) cause significant decrease $P < 0.05$ in GSH level compared with control group.

Light Microscopic Evaluations In the livers of rats from the control and methotrexate treated rats and MTX with glutamine and vitmine A and vit A hepatocytes showed a normal histological appearance (Figure 1). but showed hydrodegenerate in treated groups of MTX+vit A (Figure 2). while the glutamine treated group showed a necrosis and amyliod appearance (figure 3&4).

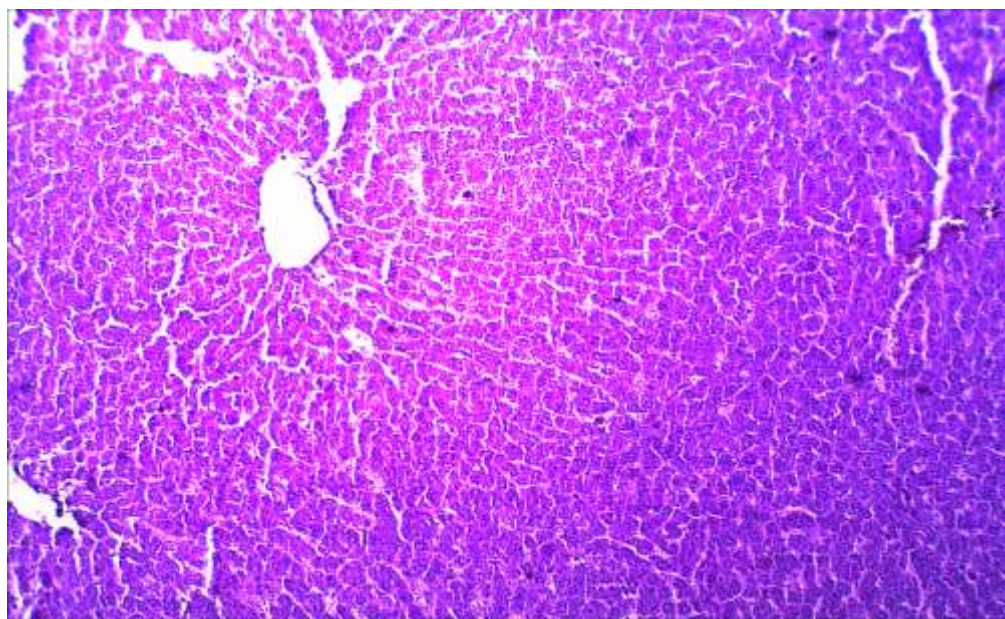


Figure 1.—Normal histological appearance of liver in control group and no change in liver of treated groups with methotrexate , MTX+glutamine and vit A(H&E stain, 40x).

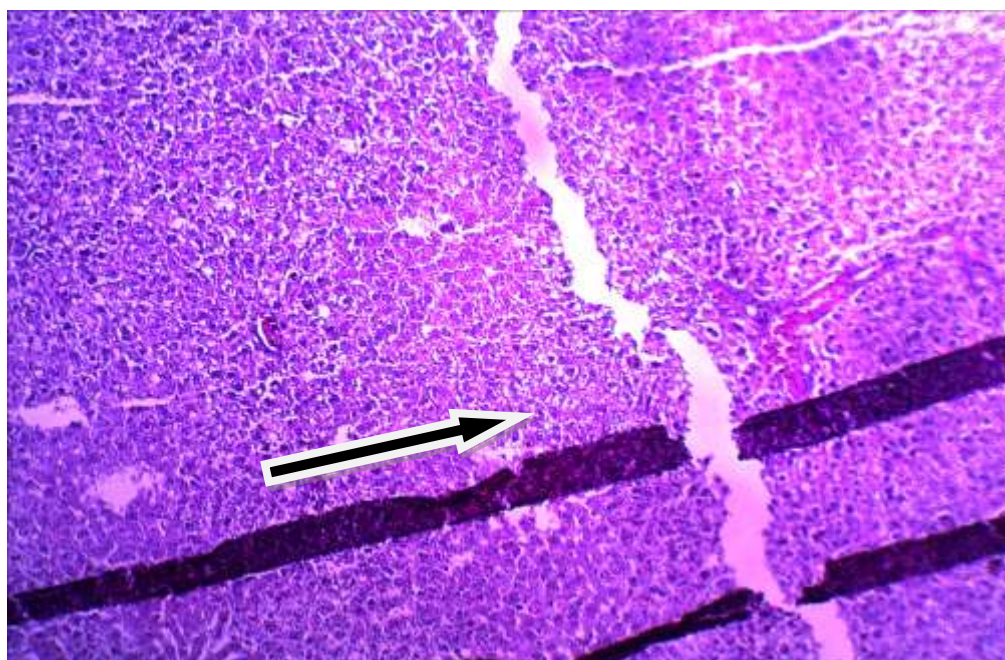


Figure 2-The appearance of hydropic degeneration in hepatocyte in MTX+vit A group (H&E stain, 40x)

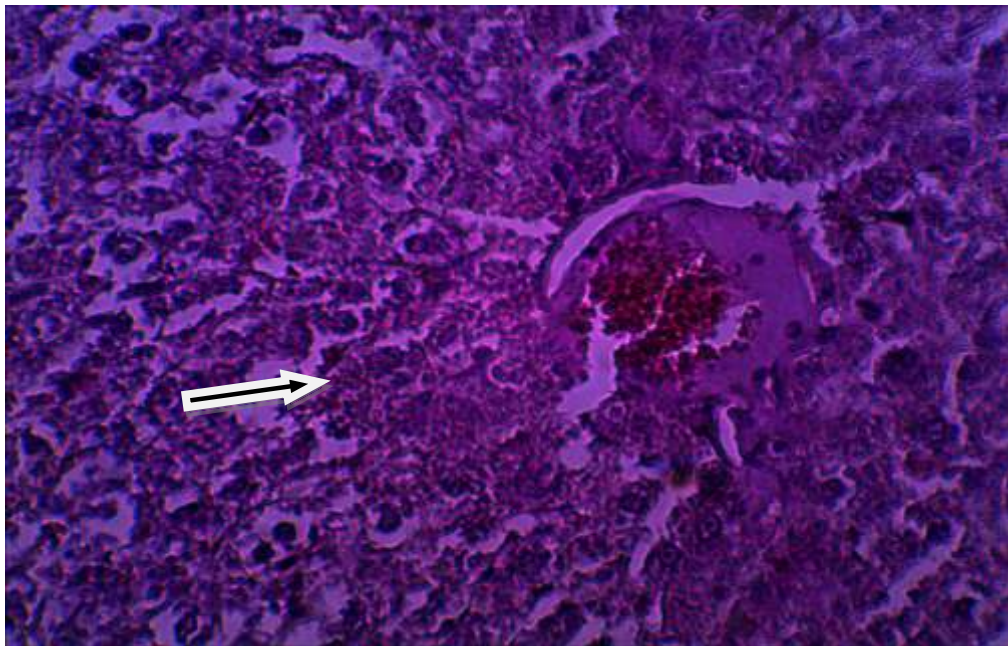


Figure 3- The appearance of necrosis of hepatocyte in Glutamine group (H&E stain, 40x)

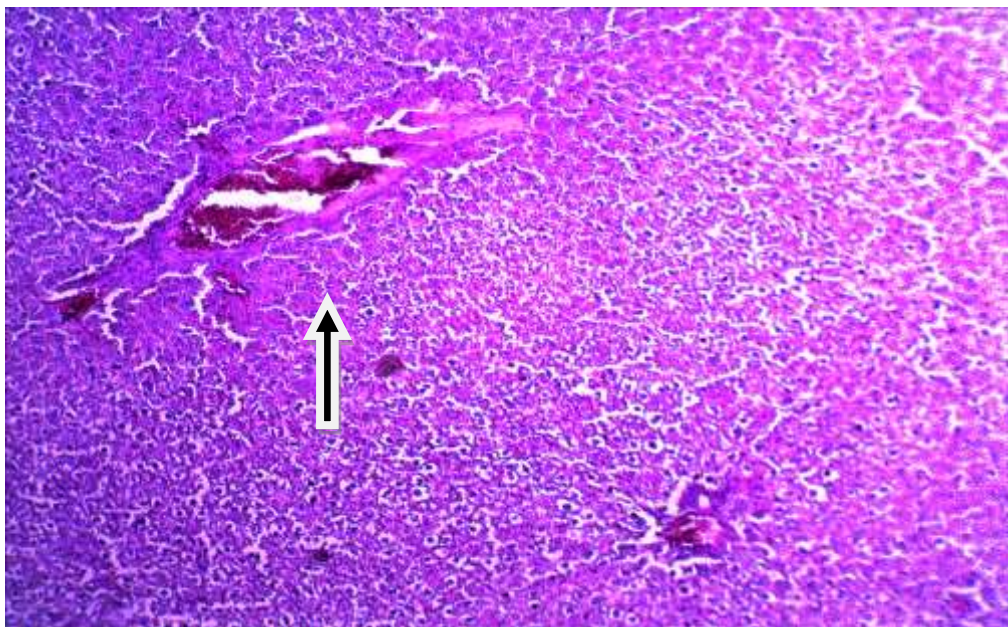


Figure 4- The appearance of amyloid in glutamine group (H&E stain, 40x)

It is now known the cause for the destruction of the myelin in the lesions is overactivation of the microglia in the region of the myelin. An enzyme that converts glutamine to glutamate called glutaminase increases tremendously, thereby greatly increasing excitotoxicity. Mercury also activates microglia, even in subtoxic doses. (note to self: microglia destroys the myelin) Any dietary excitotoxin can activate the microglia, thereby greatly aggravating the injury.

Discussion:

In the present study, methotrexate-treated rats had a significantly increased level of GSH when compared to a control group of rats. Similar results were reported by (11) who found that MTX administration caused oxidative stress and significantly reduced antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver, intestinal mucosa and spinal cord tissues of rats.

Rats treated with vitamin A and methotrexate had a significantly reduced level of GSH when compared to rats treated with methotrexate alone, demonstrating the protective effect of vitamin A. Similar results were reported by (12) who observed that intake of vitamin A reduced oxidant in rat hepatocytes. Antioxidants inhibit the synthesis of reactive oxygen species (ROS) and the same result reported

by (13) who showed that vitamin A, interact with singlet oxygen and can thus prevent the oxidation of polyunsaturated fatty acids.

Rats treated with glutamine and methotrexate had a significantly reduced level of GSH when compared to rats treated with methotrexate alone. Similar results were reported by (14) who described the benefits of use glutamine enriched diet in rats receiving methotrexate (MTX) with i.v in causing enhancement of the ability of methotrexate to kill tumor cells, while decreasing methotrexate toxicity.

Rats treated with glutamine alone and the rats treated with vit A alone had a significantly reduced level of GSH compared with control group. Similar results were reported by (15) who observed that conjugated double-bonds of β -carotene (precursor of vit A) are able to open and scavenge singlet molecular oxygen and peroxy radicals. Glutathione is an intracellular antioxidant and a co-enzyme of GSH-Px. Glutamine and cysteine are precursors of the antioxidant GSH.

It seems methotrexate leads to a reduction in antioxidant enzymatic defense capacity and causes lipid peroxidation in liver tissue while, Vit A and glutamine exhibits protective effects on methotrexate-induced liver oxidative impairment in rats, since vit A and glutamine administered alone lead to reduction in GSH level compared with control group similar result in

reported by (16) who showed recently, glutamine has been demonstrated to protect against ischemia-reperfusion (I/R) injury of gut, heart and skeletal muscle and its possible mechanism of action is partially related to the preservation of the content of glutathione (GSH).

The sections of liver of treated groups with methotrexate , MTX +vit A , MTX + glutamine and vit A normal histological appearance compared with control group (figure 1), but showed hydrodegenerate in treated groups of MTX+vit A (figure 2) similar results were reported by (17). It is known that oxidative stress plays a role in the tissue damage caused by MTX. It is demonstrated that the cytosolic nicotinamide adenosine diphosphate (NADP) dependent dehydrogenases and NADP malic enzyme are inhibited by MTX, suggesting that the drug could decrease the availability of NADPH (nicotinamide adenosine diphosphate hydrogen) in cells. While the glutamine treated group showed a necrosis and amyliod appearance (figure 3&4) similar results were reported by (18) he showed, oxidative damage of proteins results in chemical modification of a variety of amino acid residues. Protein carbonyls formed by oxidation of arginine, lysine, threonine or proline residues are often employed as a marker of protein oxidation.

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