

## Histopathological effects of Methotrexate in mice livers

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### Abstract

**Background:** Methotrexate is a folic acid antagonists that inhibits dihydrofolate reductase, resulting in a block in the synthesis of thymidine and inhibition of DNA synthesis. Methotrexate has been used for the treatment of malignancy, rheumatic disorders, and psoriasis and termination of intrauterine pregnancy. Recently, methotrexate has become a standard treatment for ectopic pregnancy. However, the use of methotrexate is limited due to high incidence of serious dose-dependent toxicity of methotrexate, including hepatotoxicity, renal damage, bone marrow suppression and gastrointestinal mucosal inflammation .

**Objective:** Evaluate the effect of the treatment with multiple doses of methotrexate in the livers of mice.

**Materials & Methods:** A total of (70) white mice at the ages of (4-8 weeks) and body weight of about (25+2 gm) were used in the study. Mice were divided into seven groups where each group received multiple doses of (0.05mg/Kg) of methotrexate during an interval of six weeks where the animals of each group had injected with a single dose of (0.05mg/Kg) of methotrexate at the beginning of each week and then, they were killed and autopsied after five days. On the other hand, the control group had received (0.1ml) of normal saline. Liver slide sections were examined by light microscope and photographed. Then, images were assessed histopathologically and comparisons were made between mice groups received different doses of methotrexate.

**Results:** Results showed that, a single dose of (0.05mg/kg) of methotrexate produced no liver tissue histopathological effects on the liver of the treated mice. While, six weeks of methotrexate treatment produced significant changes in the liver of the treated mice including lymphocyte infiltration, necrosis, liver tissue fatty degeneration and congestion and dilatation of the hepatic vein.

**Conclusions:** It was concluded that, a single dose of (0.05mg/Kg) of methotrexate had no effect on mice liver histology. While, multiple doses of methotrexate were associated with significant side effects included a hallmark of cellular infiltration and lesions of necrotic hepatocytes with congestion and dilatation of hepatic vein. Thus, the administration of multiple doses of methotrexate for the treatment of various medical conditions should be considered carefully to avoid liver damage of the patient especially in the case of ectopic pregnancy.

**Key words:** Histopathological effects, Methotrexate, mice, liver.

### التأثيرات المرضية النسيجية للميثوتريكسيت في أكباد الفئران

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### الخلاصة

**المقدمة:** الميثوتريكسيت هو من مقابلات حمض الفوليك والذي يمنع اختزال الديهايدروفولات مما يؤدي إلى إيقاف تركيب الثيميدين وتشبيط تخليق الحامض الرايبوزي منقوص الاوكسجين. وقد استخدم الميثوتريكسيت لعلاج الأورام الخبيثة، والاضطرابات الروماتيزمية، والصدفية وإنهاء الحمل داخل الرحم. ومؤخراً، أصبح الميثوتريكسيت من المعالجات المعيارية للحمل خارج الرحم. ومع ذلك، فإن استخدام الميثوتريكسيت محدود بسبب ارتفاع معدل التلف في الاعضاء نتيجة سمية الميثوتريكسيت المرتبطة بالجرعة، بما في ذلك تلف الكبد، تلف الكلى، نخاع العظم والتهاب الغشاء المخاطي المعدي المعوي.

**الهدف:** تقييم تأثير العلاج بجرعات متعددة من الميثوتريكسيت في أكباد الفئران.

المواد وطرق العمل: استخدام في الدراسة (٧٠) من الفئران البيضاء وبأعمار تتراوح ما بين (٤-٨ أسابيع) وبمعدل وزن الجسم ما بين (٢٥ ± ٢ غم). تم تقسيم الفئران إلى سبع مجموعات حيث حقنت كل مجموعة بجرعات متعددة من (٠.٥، ٠.٥، ٠.٥ ملغم / كغم) من الميثوتريكسيت خلال فترة ستة أسابيع وتمحقن الحيوانات من كل مجموعة بجرعة واحدة من (٠.٥، ٠.٥، ٠.٥ ملغم / كغم) من الميثوتريكسيت في بداية كل أسبوع ومن ثم تم قتل الحيوانات وتشريحها بعد خمسة أيام من كل جرعة، في حين حقنت مجموعة السيطرة بمقدار (١، ٠ مل) من المحلول الملحي. تم فحص شرائح الاكباد بواسطة المجهر الضوئي وتصويرها ومن ثم تقييم التغيرات المرضية النسجية في صور الاكباد والمقارنة بين التغيرات المرضية النسجية والجرع المختلفة من الميثوتريكسيت.

النتائج: أظهرت النتائج أن جرعة واحدة من (٠.٥، ٠.٥، ٠.٥ ملغم / كغم) من الميثوتريكسيت لم تنتج أي تغييرات نسيجية مرضية في أكباد الفئران المعاملة، في حين أظهرت نتائج الحقن بالميثوتريكسيت لمدة ستة أسابيع حصول تغييرات نسيجية مرضية كثيرة تضمنت ارتشاح الخلايا للمفاوية، تنخر، تنكس الدهونم احتقان وتوسع الوريد المركزي.

الاستنتاجات: تم استنتاج أن حقن الفئران بجرعة واحدة من (٠.٥، ٠.٥، ٠.٥ ملغم / كغم) من الميثوتريكسيت لم يكن لها تأثير على انسجة اكباد الفئران المعاملة في حين أظهرت نتائج حقن الفئران بجرع متعددة من الميثوتريكسيت اثارا جانبية واسعة السمية شملت ارتشاح الخلايا للمفاوية، تنخر، تنكس الدهونم احتقان وتوسع الوريد المركزي وبالتالي ينبغي النظر في استخدام جرع متعددة من الميثوتريكسيت لعلاج مختلف الحالات الطبية وبالأخص حالة الحمل خارج الرحم.

الكلمات الدالة: التأثيرات المرضية النسجية، الميثوتريكسيت ، الفئران ، الكبد..

## Introduction

Methotrexate (MTX) is a structural analogue of folic acid that acts as a dihydrofolic acid analogue that binds to the dihydrofolic acid reductase enzyme by inhibiting the synthesis of tetrahydrofolate that is required for DNA synthesis and thus inhibiting cell proliferation [1&2]. It is a well-known chemotherapeutic and immunosuppressive agent that is widely used in many rheumatologic, dermatologic and hematologic diseases successfully [3-6]. Recently, methotrexate has become the drug of choice for the treatment ectopic pregnancy showing benefits in being used as an alternative to laparoscopy in selected cases for the resolution of the pregnancy [7-9]. However, the typical doses of methotrexate administration for the treatment of various medical cases depended on the protocol used and on the patient response [9-11]. However, its use is limited due to high incidence of serious dose-dependent toxicity, including hepatotoxicity, renal damage, bone marrow suppression and gastrointestinal mucosal inflammation making the use of methotrexate a significant clinical problem that affect the compliance with MTX-containing treatment regimens [5,6&12].

## Materials and methods

### 1. Animals management:

Healthy, adult mice animals of *Mus musculus* strain were used in this study (35 females and 35 males), ranging in age between (4-8) weeks old and their weight were about (25 ± 2g). Animals were divided into seven equal groups (each group consisted of 5 males and 5 females). These animals were kept in an air condition room at a temperature of (22-24 °C), with about (12-14 )

hours of day light exposure. Animals were housed in cages measuring (29\*15\*12 cm) and each seven animals were kept in one cage contained wooden shave. Water and Feed composed of (wheat, barely mixed with 250mg of milk powder) were freely excess able and animals were kept for at least two weeks for adaptation. Animal cages were cleaned and sterilized with 70% ethanol once a week regularly according to the procedure mentioned by [13].

### **2.Treatment:**

Methotrexate (Trixilem®) is a clear yellowish solution, vial of (5mg/2ml) for injection, (Lemery-Uppsala Sweden).Each (5mg/2ml) was diluted with (100 ml) physiological normal saline and the mixture was injected intramuscularly to animals. Animals were injected once weekly and for six weeks, untreated controls received equivalent amount of(0.1ml)physiological normal saline according to standard procedure [10].

### **3.Experimental Design:**

Methotrexate administration Protocol ( the concentration, route of administration and interval of administration) was followed according to standard procedure [10&14]used for the treatment of ectopic pregnancy. Mice were divided into seven equal groups; where six groups of them (therapeutic groups n=10) were injected intramuscularly with methotrexate at the beginning of the week and were autopsied for liver examination after (120 hours-five days) according to table (1).While, the control group (n=10) was injected intramuscularly with (0.1ml) normal saline only.

**Table (1): Methotrexate administration Protocol.**

<b>Group number</b>	<b>Methotrexate dose mg/Kg</b>	<b>Exposure Time (Week)</b>	<b>Total concentration of MTX / dose</b>	<b>Time of Autopsy/ Week</b>
M1	0.05	1	0.05 mg/Kg	1
M2	0.05	2	0.10 mg/Kg	2
M3	0.05	3	0.15 mg/Kg	3
M4	0.05	4	0.20 mg/Kg	4
M5	0.05	5	0.25 mg/Kg	5
M6	0.05	6	0.30 mg/Kg	6
Control	0	6	0mg/Kg	6

### **4. Histological assessment:**

Livertissues were fixed in 10% formalin and was embedded in paraffin. Tissue sections were cut at 5µm, mounted on slides, stained with hematoxylin- eosin (H-E) for general liver structure examination. The sections were examined by light microscope (Optika- Italy) and photographed by (Optica- Italy 4083-B5- camera) according to standard procedures[14&15].

### 5. Statistical analysis:

Data from the study were analyzed using T- test by using SPSS program Ver.10 for Windows. A *P* value of <0.05 was considered indicative of a statistically significant difference .

### Results&Discussion

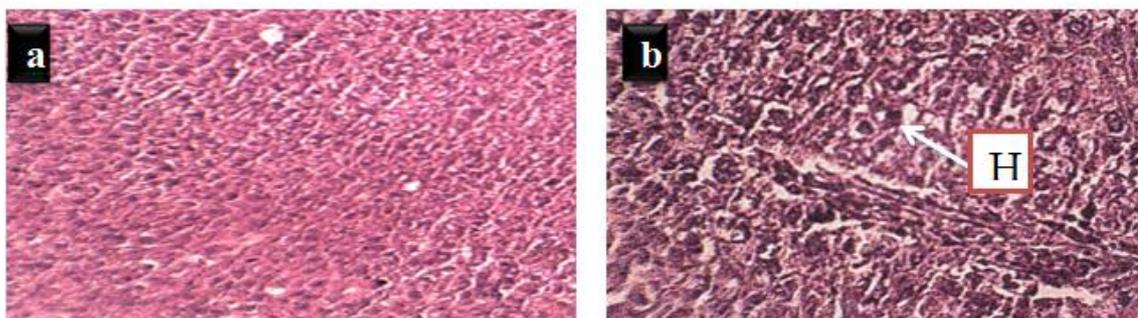
Although, methotrexate is a well-known chemotherapeutic and immunosuppressive agent that is widely used for the treatment in many rheumatologic, dermatologic and hematology diseases successfully [3-5]. This drug could be replaced in the absence of the patient's suitability except in the case of ectopic pregnancy where methotrexate administration substitute the laparoscopy. Accordingly, groups of mice injected with multiple doses of methotrexate were autopsied at different times and results of macroscopic examination of the livers were shown in table(2).

**Table (2):Macroscopic changes observed in the livers of mice treated with different concentrations of methotrexate.**

Group	Methotrexate dose mg/Kg	Histopathological changes in livers
Control	0 mg/kg	Normal
M1	0.05 mg/Kg	Normal
M2	0.1 mg/Kg	Normal
M3	0.15 mg/Kg	Congested
M4	0.2 mg/Kg	Congested
M5	0.25 mg/Kg	Congested
M6	0.3 mg/Kg	Enlarged

Results of table (2) had shown that, there were no significant macroscopic changes noted in the control untreated mice and their livers appeared normal in shape and size. The same results were observed in the livers of the mice groups treated with low doses (0.05mg/Kg-M1 group) and (0.1mg/Kg-M2 group) of methotrexate. While, the macroscopic examination of the groups (M3, M4 and M5) which were injected with the therapeutic doses of methotrexate(0.15,0.20 and 0.25mg/Kg) respectively showed congested livers. Finally, the livers of mice from group (M6) that received a high dose(0.30mg/Kg) of methotrexate were enlarged.

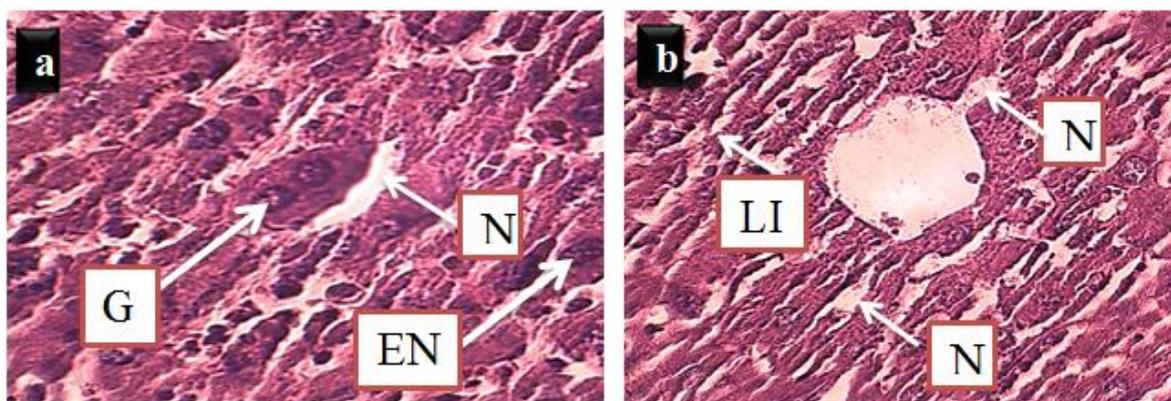
The same results were obtained by[16&17] where they declared that, methotrexate produced no changes in mice livers at low doses though, methotrexate therapeutic doses produce mild effects that could be reversible. Whereas, high doses of methotrexate had severe effects on mice livers. On the other hand, the histopathological findings of this study showed that, a single dose of (0.05mg/Kg) methotrexate produced no liver lesions and livers of group M1 mice appeared as normal as the control livers (Figure-1).



**(Figure-1):** Histological sections of mouse liver showing;  
**a- Liver section of normal control mouse at (10X), b- Hepatocytes (H) of liver section of M1 mouse group at (40X). (H &E staining).**

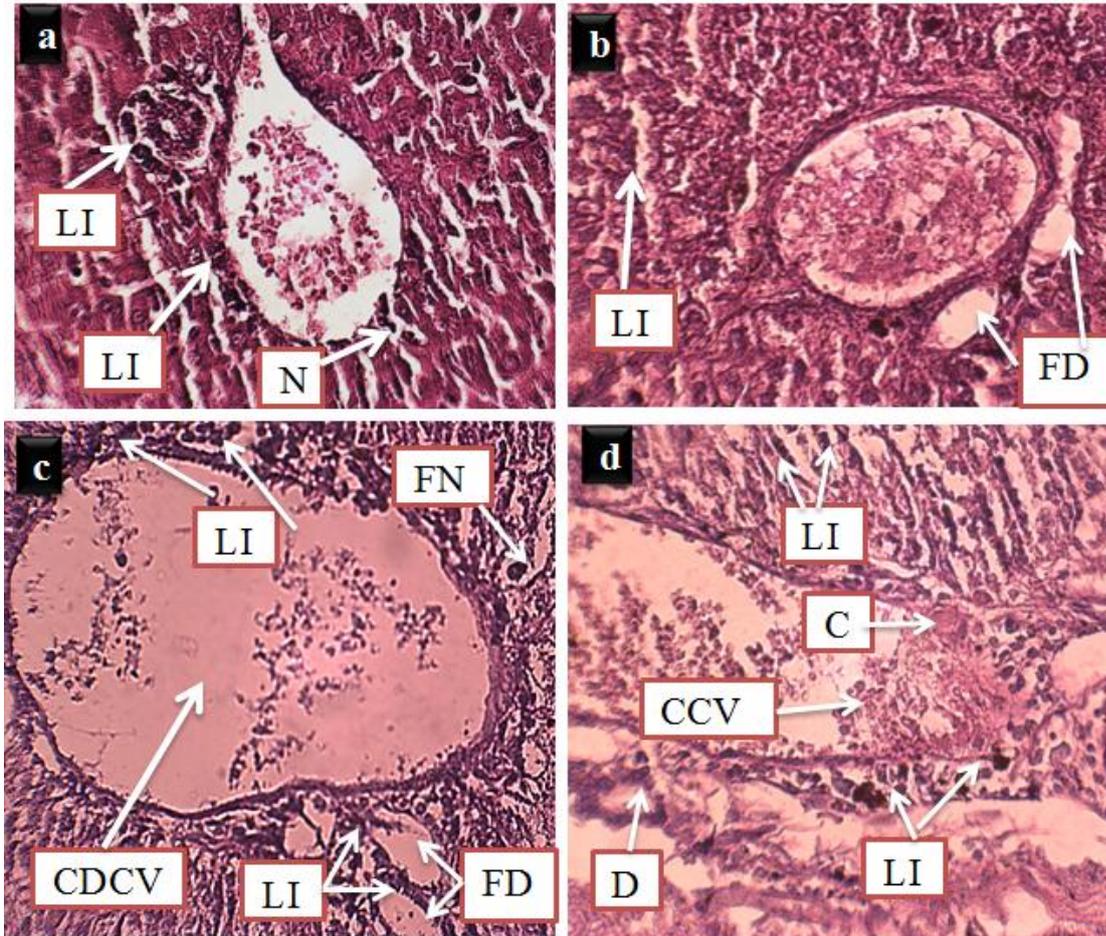
Histological examination of mice liver tissues of group M1 (low dose-.05mg/Kg) showed normal liver histology (figure-1b) with radial hepatic cord and clear hepatic sinusoids. This result was recorded in all of the mice autopsied from group M1 indicating that, the administration of a single low dose of (0.05mg/Kg) methotrexate brought no effect on normal liver tissue. This result came in agreement with the results obtained in [18&19] where administration of single, low dose of methotrexate was associated with fewer side effects.

Yet, the histological examination of liver tissues from mice groups (M2, M3 and M4) which were treated with two to four doses of (0.05mg/Kg) methotrexate showed a significant increase ( $P < 0.05$ ) in giant cells, necrosis and cellular infiltration of lobules with no fibrosis (Figure- 2).



**(Figure-2):** Liver section of methotrexate treated mouse (M2 group) showing;  
**a- Cloudy swelling giant hepatocyte and the appearance of enlarged nuclei with parties necrosis, b- Chronic inflammatory cells and necrosis. G=Giant cell, LI= Lymphocyte infiltration, N=Necrosis and EN=Enlarged nuclei.(H &E staining, 400X).**

The figure above showed that, treatment of mice with two doses of (0.05mg/Kg) methotrexate produce mild histological changes in the liver tissue such as the enlargement of some nuclei in addition to chronic inflammatory cells mainly lymphocytes and neutrophils indicative of necrosis. In addition, some hepatocytes showed dense nuclei suggestive of necrosis. Moreover, treatment of mice with further doses of methotrexate increased the liver injuries where the histopathological findings in groups (M5&M6) showed a complete loss of architecture as shown in (Figure-3).



**(Figure-3):** Liver sections of methotrexate treated mice (M3, M4, M5 &M6 groups) showing; a- Liver section of methotrexate treated (M3 group) appearing (LI= Lymphocyte infiltration & N=Necrosis), b- Liver section of methotrexate treated (M4 group) appearing (FD= Fatty degeneration),c- Liver section of methotrexate treated (M5 group) appearing (FD= Fatty degeneration, FN= Focal necrosis & CDCV= Congestion and dilatation of central vein),d- Liver section of methotrexate treated (M6 group) appearing (CCV= Congestion of central vein, D=Degeneration and C= Congestion).( H.&E. staining, 400X).

Results of (Figure-3) showed that, in approximately 90% of the mice treated with three to four doses of (0.05mg/Kg) methotrexate, there were a significant increase ( $P<0.05$ ) in lymphocyte infiltration and necrosis. Fat was present in the liver tissue of mice killed after three doses only. While, fatty degeneration was randomly distributed in more than 85% of the mice killed after four to six doses. Moreover, the degenerative lesions were more severe and more cells were necrotic in mice given five to six doses were significantly increased ( $P<0.05$ ), in addition to the congestion of the central vein and congestion and dilatation of the hepatic vein. However, the histopathological changes score for each group was determined and results were given in table (3).

**Table (3): Percentage of the histopathological liver changes scored among mice groups treated with different concentrations of methotrexate.**

Groups of Mice	Methotrexate Doses/ Week	The percentage of histopathological changes in livers					
		LI%	N%	FD%	FN%	CDCV%	C&D%
Control	0mg/Kg	0%	0%	0%	0%	0%	0%
M1	0.05mg/Kg	0%	0%	0%	0%	0%	0%
M2	0.1mg/kg	30%	2%	1%	3%	0%	0%
M3	0.15mg/Kg	52%	14%	13%	16%	0%	2%
M4	0.2mg/Kg	75%	26%	60%	23%	24%	21%
M5	0.25mg/Kg	96%	48%	78%	42%	73%	71%
M6	0.3mg/Kg	100%	89%	88%	84%	82%	91%

The effect of Methotrexate multiple doses was very obvious in the table above where the lymphatic infiltration, necrosis and fatty degeneration along with the congestion and dilatation of the central vein were all significantly increased ( $P<0.05$ ) with the improvement in methotrexate doses. Consistent with these findings were the observations of [20,21&22] were they declared that, methotrexate hepatotoxicity is the most important potential major side effect. Also, it has been reported that chronic application of methotrexate may lead to hepatotoxicity, including steatosis, cholestasis, fibrosis, and cirrhosis [22&23]. The mechanisms of methotrexate-hepatotoxicity can be related to its accumulation inside the cells in a polyglutamated form, where this form causes decreasing folat levels and hepatotoxicity [17]. The other way; it is well known that oxidative stress plays a role in tissue damage caused by methotrexate [16& 23].

## Conclusions

The results of this study matched features of similar clinical conditions where;

1. In particular, a single dose of (0.05mg/Kg) of methotrexate had no detectable effect on mice liver histology.
2. Multiple doses of methotrexate were associated with significant side effects included cellular infiltration and lesions of necrotic hepatocytes with congestion and dilatation of hepatic vein.
3. Livers appeared to be affected in all mice treated with multiple doses of methotrexate especially in the sixth dose group. Thus, the administration of multiple doses of methotrexate for the treatment of various medical conditions should be considered carefully to avoid liver damage of the patient.
4. Treatment of patients of ectopic pregnancy with multiple doses of methotrexate results in the administration of this medication to a woman known to be pregnant. The misdiagnosis of ectopic pregnancy in a woman with an intrauterine pregnancy or treatment of a woman with a coexisting ectopic and intrauterine pregnancy can result in methotrexate exposure of a continuing pregnancy. Thus care should be taken due to avoid the high toxicity of this drug.

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