Species differences in methotrexate-induced hepatotoxicity and nephrotoxicity: Rabbits resist methotrexate nephrotoxicity

Jawad H. Ahmed

ABSTRACT

Background: Animal models are important tools for studying drug-induced toxicity. Species differences in drug-induced nephrotoxicity or hepatotoxicity was frequently reported, however, clear evidence of species differences in nephrotoxicity or hepatotoxicity induced by methotrexate (MTX) is not available. Aim: The present study was designed to investigate nephrotoxicity or hepatotoxicity of MTX in mice and rabbits.

Methodology: twelve rabbits and 16 mice were used for the study. Each species of animals was divided into 2 groups; control and MTX treated groups. The control groups were treated with normal saline intraperitoneum (IP), MTX was given IP in a dose of 20 mg/kg/weekly for 38 days for the rabbits and at a dose of 10mg/kg/ weekly for 23 days for mice. Serum levels of liver enzymes, urea, creatinine, Malondialdehyde (MDA) and MDA in liver homogenate were estimated for the rabbits. In mice, glutathione (GSH) and MDA were estimated in liver and kidney homogenates. Histopathological examination of the kidney and liver was done for the 2 species.

Results: In the rabbits, MTX resulted in a significant increase in liver enzymes, and in MDA levels of serum, liver and kidney homogenates, while the levels of urea and creatinine were not affected. Histopathological changes suggestive of liver damage were seen, while the kidneys appeared normal on histopathological examination. In the mice, MTX resulted in a significant increase in MDA in the liver homogenates with a small and insignificant reduction in GSH. In mice kidney homogenates, MTX had not affected MDA, while GSH was significantly reduced. Histopathological examination in mice showed significant damage in both liver and kidney.

Conclusion: MTX produced hepatotoxicity in rabbits and mice but nephrotoxicity occurred in mice only. This suggests that rabbits might be resistant to MTX induced nephrotoxicity.

Keywords: Methotrexate, hepatotoxicity, nephrotoxicity, species difference

الاختلافات بين اجناس الحيوانات في سمية الكبد والكلى الناجمة عن الميثوتريكسيت: الأرانب تقاوم سمية الكلى الناجمة عن الميثوتريكسيت

خلفية الدراسة: النماذج الحيوانية للامراض هي أدوات هامة لدراسة سمية الأدوية على اعضاء الجسم المختلفة. وهناك تباين كبير في سمية الكبد والكلى الناجمة عن الأدوية بين اجناس الحيوانات' ولكن لا يوجد دليل واضح على الاختلاف بين اجناس الحيوانات في سمية الكبد والكلى الناجمة عن الميثوتركسيت.

هدف الدراسة: صممت الدراسة الحالية لتقصى سمية الكبد والكلى التي يسببها الميثوتركسيت في الفئران والأرانب.

منهجية البحث: تم استخدام اثني عشر ارنبا" وستة عشرة فأرا" للدراسة. تم تقسيم كل نوع من الحيوانات إلى مجموعتين، مجموعة السيطرة ومجموعة الميثوتركسيت. وعولجت الأرانب في مجموعة السيطرة بالمحلول الملحي بالزرق البريتوني اسبوعيا" بينما اعطيت المجموعة الثانية ٢٠ ملغ / كغ / اسبوعيا" عن طريق الزرق البريتوني لدواء الميثوتركسيت لمدة ٣٨ يوما بينما اعطيت مجموعة الفئران جرعة الميثوتركسيت ٢٠ ملغ / كغ/ اسبوعيا" عن طريق الزرق البريتوني واعطيت مجموعة السيطرة المحلول الملحي عن طريق البريتوني ولمدة ٣٣ يوما. تم قياس مستوى المالوندايلديهايد (MDA) ومستويات إنزيمات الكبد، واليوريا، والكرياتينين في دم الارانب و (MDA) في متجانس كبد الارانب. اما في الفئران فقد تم قياس الجلوتاثيون(GSH) و (MDA) في متجانس الكبد والكلى. وقد اجري الفحص النسجي في الكلى والكبد لكلا النوعين من الحيوانات.

النتائج: أدى الميثوتركسيت الي زيادة كبيرة في أنزيمات الكبد، وفي مستويات MDA في مصل ومتجانس الكبد والكلى في الارانب[°] بينما لم يؤثر الميثوتركسيت على مستوى اليوريا والكرياتينين في دم الارانب. وشوهدت تغييرات نسجية مرضية تدل على تلف الكبد، في حين ظهرت الكلى طبيعية. اما في الفئران فقد ادى الميثوتركسيت الى زيادة كبيرة في (MDA) و نقص قليل في مستوى GSH في متجانس الكبد ولم يؤثر على مستوى (MDA) في متجانس الكلى بينما ادى الى نقص كبير في مستوى (GSH) . واظهر الفحص النسجي في الفئران ضررا" واضحا" في كل من الكبد والكلى. *الأستنتاج:* ادت المعالجة بالميثوتركسيت الى سمية الكبد في الأرانب والفئران ولكن حدثت سمية الكلى في الفئران فقط. وهذا يشير إلى أن الأرانب

INTRODUCTION

nimal models represent a vital tool for studying drug induced hypersensitivity reactions and rats and mice are the most commonly used models.^[1] Generally, researchers choose laboratory animals for their experiment depending on their personal experience and familiarity with the animal, easy handling, price and availability of animals with little consideration to the suitability of the model.^[1] MTX, an analogue of folate is widely used in the treatment of various types of cancers ^[2] and many other diseases such as arthritis^[3], psoriasis^[4], ectopic pregnancy^[5], inflammatory bowel disease^[6], and bronchial asthma.^[7] It has been reported that MTX causes many side effects including gastrointestinal, neurological, pulmonary, myelosuppression, hair loss. hepatotoxicity and nephrotoxicity.^[8] A study published in 1966, revealed that the rabbit showed strong resistance to hematological and toxicological effects induced by MTX; whether this resistance extended to involve liver or mentioned.^[9] toxicities was not kidnevs Published literature from PubMed, (updated to February 28, 2016), on the other hand, revealed that data on MTX induced nephrotoxicity were obtained from studies on rodents (rats or mice) and very little data were obtained from the rabbits. However, the decision of preferring rodents in study MTX induced nephrotoxicity is not clearly stated and seems not based on firm evidence. Whatever the case, this ultimately stimulates interest in investigating if species differences in the response of rabbits or

rodents to MTX induced nephrotoxicity and hepatotoxicity exist. Therefore, the study was designed to evaluate nephrotoxicity and hepatotoxicity induced by MTX using mice and rabbits as two different species of animals.

قد تكون مقاومة لسمية الكلى الناجمة عن الميثوتركسيت.

MATERIALS AND METHODS Animal handling

The study was conducted between January 2014 and April 2015 on two different species of animals. Two weeks before the study, the animals were kept in separate cages in the animal house of the College of Medicine for acclimatization with a 12:12-hrs light/dark cycle and at $24 \pm 2^{\circ}C$ with free access to water and food. The animals were carefully handled in accordance with the internationally accepted guidelines for handling laboratory animals (National Institutes for Health USA publication, 1985), and measures were undertaken to minimize pain and discomfort during experimentation. The study protocol was submitted to a local institutional ethical committee for approval.

Rabbits:

Twelve sexually mature domestic male rabbits with an age of 8-12 weeks were recruited for the study. The body weight ranged from 1.5-2 kg. Food was carefully selected and restricted to trefoil and lettuce to avoid food with antioxidant properties such as carrot.

Mice:

Sixteen albino male mice ranging in body weight between 20-30 g obtained from the animal house of the College of Medicine, University of Basrah were used for the study. A standard food was prepared in the form of pellets which contains starch, carbohydrate, moisture, and a crude protein not less than 20% and crude fat not less than 6% of the total weight of the pellet. Food and ordinary drinking water were provided ad libitum.

Study design:

The rabbits were randomly selected and divided into two groups, six animals in each group. Group 1 (control group) was left free in the animal house with free access to food and water and injected with 2 ml/kg normal saline IP at days 10, 18, 25, and 32 of the experiment. At the night before sacrificing, the rabbits were transferred to special constraint cages, which prevented the rabbit from eating its own dropping to ensure fasting condition, and were then sacrificed in the morning of day 38. Rabbits in group 2 (MTX group) were treated in a manner similar to the control but injected weekly with 20 mg/kg MTX (Ebewe-Pharma, Austria) IP. The mice were randomly divided into 2 groups, 8 animals in each group. The animals in group 1 (control group) were treated with normal saline (0.3 ml) orally everyday through a special curved smooth tip nontraumatic needle and injected with normal saline (0.25 ml) IP at the days 7,14 and 21 of the experiment. The animals in Group 2 (MTX group) were allocated for the same schedule of the control but injected weekly with MTX (Ebewe-Pharma, Austria) 10 mg/kg IP. The animals were sacrificed at day 23 after an overnight fasting.

Blood sampling

The rabbit was first anesthetized in a close container containing chloroform-soaked cotton for few minutes. A sample of blood was taken from the heart via cardiac puncture immediately before sacrificing. Five to 10 ml of blood were obtained, transferred into a non-heparinized tube, and allowed to clot. Serum was obtained by centrifugation at 3000 rpm for 20 minutes. One milliliter of fresh serum was used for measurement of MDA, the rest of the serum was frozen at -20 °C for estimating liver enzymes, urea, and creatinine. Blood samples for biochemical tests were not taken from mice.

Liver and kidney tissue sampling:

The kidneys and livers were obtained from the rabbits and mice and processed separately. After sacrificing the animal, the abdomen was opened by a scissor, the livers and kidneys were removed. rinsed in a freshly prepared phosphate-buffered saline (pH=7.4). The livers and kidneys were cut into pieces, one piece from each was stored in 10% neutral buffered formalin and send to the Department of Pathology and Forensic Medicine at Basrah College of Medicine for histopathological examination. Two grams from the fresh piece of the liver and 2 gm. from the two kidneys were taken, transferred to two separate small glass flasks containing 20 ml of phosphate-buffered saline, homogenized mechanically at 6000 rpm for 20 minutes in a cold phosphate buffer saline Hiedolph (pH=7.4) using Electrical Homogenizer, Korea to obtain 10% homogenate from each organ. Immediate measurement of liver and kidney MDA (for rabbits and mice) were performed and the other portion of homogenate was frozen at - 20 °C for GSH measurement (mice only).

Measurement of serum MDA:

Thiobarbituric acid assay of Beuge and Aust^[10] was used for measurement of serum MDA using one ml of fresh serum.

Measurement of MDA in kidney and liver homogenates

MDA levels in kidney and liver homogenates were measured by thiobarbituric acid (TBA) test, based on the reaction of MDA with TBA which produces a pink pigment that can be measured by a spectrophotometer at 532 nm. ^[11]

Measurement of GSH in kidney and liver homogenates (mice only)

GSH levels of kidney and liver homogenates were measured by ELISA Kit specific for mice (Cusabio reagents, Cusabio Laboratories, Wuhan, China). This kit employs a technique known as "Double Antibody Sandwich technique. ELISA (Huma Reader HS, Germany) was used for the analysis.

Measurement of serum liver enzymes (rabbits only)

Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were estimated using a commercial kit (Randox Diagnostic Reagents, Randox Laboratories, UK). Serum alkaline phosphatase (ALP) and serum total bilirubin were estimated by commercial kits (Biolabo Reagents, Biolabo SA, France).

Measurement of serum creatinine and serum urea levels

Creatinine level was measured by spectrophotometric method.

Histopathological examination

Histopathological examinations were done for all specimens of the control and after MTX treatment for the rabbits and mice. Slides were prepared in duplicates from each liver or kidney specimens and hematoxylin and eosin (H&E) were used for staining. The slides were coded and blindly examined by a senior histopathologist using light microscopy (Olympus CX-series). Histopathological changes were graded as 0 (normal), 1(mild), 2 (moderate), and 3(severe), for 0, 25, 50, and 75% histopathological changes of fields examined respectively.^[12]

Statistical analysis

SPSS version 19 (IBM, NY, USA) was used for statistical analysis. Variables were compared by a non-parametric Mann–Whitney U-test for independent samples. The data were presented as mean \pm standard deviation, P < 0.05 is considered significant. The data were also presented as 95% confidence interval (95% C.I) . If the 95% interval does not comprise 0; this means the difference achieves statistical significance.

RESULTS

Rabbits

Effect of MTX on liver enzymes, urea and creatinine, MDA in serum of rabbits

Liver enzymes were significantly elevated following treatment with MTX. The rise in liver enzymes was nearly twice the level of the control (Figure-1). While the levels of urea and creatinine were not affected by MTX treatment (Figure-2). MDA was increased from 0.19 \pm 0.04 in the control group to 0.41 \pm 0.1 µmol/l in the MTX treated group (Mean difference of 0.21 \pm 0.11 µmol/l, 95% C.I. 0.3 to 0.09, P = 0.01) (Figure-3a).



Figure 1. Effect of treatment with MTX on liver enzymes in serum of the rabbits. *: P = 0.01



Figure 2. Effect of treatment with MTX on serum urea (a) and creatinine (b) in the rabbits

Effect of MTX on MDA in kidney homogenate in rabbits:

MDA level in kidney homogenate was significantly elevated to 2013 ± 783 in the group treated with MTX compared with 952 ± 226 µmol/l in the control group, with a mean difference of 1062 ± 333, 95% C.I. 321 to 1803, P = 0.01. (Figure-3b).

Effect of MTX on MDA in liver homogenate in rabbits:

MDA level in liver homogenate was significantly elevated to $1722 \pm 760 \mu mol/l$ in the MTX treated group compared with $381 \pm 90 \mu mol/l$ in the control group with a mean difference of $1341 \pm 313 \mu mol/l$, 95% C.I. 645 to 2038, P = 0.02. (Figure-3b).



Figure 3. Effect of MTX treatment on (a) serum MDA, MDA in kidney and liver homogenates (b) in the rabbits, MDA: Malondialdehyde, *: significantly different from the control, P = 0.01.

Histopathological examination (rabbits): Slides for histopathological examination were prepared from all studied animals (6 animals for the liver and 6 animals for the kidneys). Slides were prepared in duplicates for each organ).

Histopathological changes of MTX in the liver of rabbits: Compared with the control

(Figure-4a), MTX caused severe histopathological changes in the liver (score 3); these changes included sinusoidal dilatation, portal and lobular lymphocytic infiltration (Figure-4b) and hepatocytes hydropic degeneration (Figure-4c).



Figure 4. Liver tissue of a rabbit stained with Hematoxylin-Eosin, (a): control group 10X; (b, c) a high power field (40X) of a liver tissue treated with MTX 20 mg/kg IP which revealed sinusoidal dilatation, lobular hydropic degeneration, portal lobular chronic inflammatory cell infiltration.

Histopathological changes of MTX in the kidney of rabbits

In all treated rabbits, MTX for the same dose and duration, did not produce hitopathological changes in renal tissue (Figure-5a,b,c). The scores of histopathological examination for the kidney and liver were shown in (table-1).



Figure 5. (a) A renal tissue of a rabbit stained with Hematoxylin-Eosin (a, b) control group at low (10X) and high power field (40X) respectively, (c) a renal tissue of a rabbit treated with MTX 20 mg/kg IP (40X), which revealed normal glomerulus and tubules in the control and MTX treated groups.

Table 1.	Scores of histopathological changes of kidney and liver damage induced by MTX in
	rabbits

	Number of animals (kidneys)		Number of animals (liver)	
Scores	Control	MTX	Control	MTX
0	6	6	6	0
1	0	0	0	0
2	0	0	0	2
3	0	0	0	4

Number of animals in each group = 6

Scores 0, 1, 2 and 3 are for normal, mild, moderate and severe damage respectively

Mice

Treatment with MTX resulted in a significant increase in tissue MDA from $1199 \pm 717 \mu mol/l$ to 2406 ± 913 with a mean difference of 1207 ± 367 , 95% C.I. 436 to 1978, P = 0.004 (Figure-6a)

Effect of MTX on GSH in liver homogenate in mice:

There was a slight reduction of GSH from 79 ± 9.4 nmol/ml to 73 ± 3.4 in liver homogenate after treatment with MTX. The difference was small and did not achieve statistical significance (a mean difference of 5.95 ± 3.16 , 95% C.I. - 0.68 to 12.6, P = 0.07) (Figure-6b)



Figure 6. Effect MTX on MDA (a), GSH (b) in mice liver homogenate, MDA: Malondialdehyde, GSH: Glutathione, (*) significantly different from the control, P = 0.004

Effect of MTX on MDA in kidney homogenate in mice:

There were small and insignificant differences in the level of MDA in kidney homogenate in the group treated with MTX compared with the control (1770 \pm 1187 versus 1893 \pm 994 µmol/l) with a mean difference of 123 \pm 489, 95% C.I. -905 to 1152, P = 0.804 (Figure-7a). *Effect of MTX on GSH in kidney homogenate in mice:* The level of GSH in kidney homogenate was significantly decreased by MTX from 72.1 \pm 19.3 nmol/ml in the control group to 59.2 \pm 4.3 nmol/ml in the group treated with MTX (a mean difference of 17.4 \pm 17 nmol/ml, 95% C.I. 1.19 to 33.7, P = 0.037) (Figure-7b)



Figure 7. Effect MTX on MDA (a), GSH (b) in mice kidney homogenate, MDA: Malondialdehyde, GSH: Glutathione, (*) significantly different from the control, P = 0.037

Histopathological examination (mice):

Slides for histopathological examination were prepared from all studied animals (8 animals for the liver and 8 animals for the kidneys).

Histopathological changes of MTX in the liver of mice:

MTX caused sinusoidal dilatation, lobular hydropic degeneration, and portal lobular

chronic inflammatory cell infiltration in 7 out of 8 treated animals. These changes were severe (score 3) in 4 animals, moderate (score 2) in 2 and 2 animals with mild or no changes (Table-2, Figure-8 a,b,c).



Figure 8. Histopathological features of a mouse liver treated with MTX, (a) control group which showed normal liver architecture, (b) MTX treated mouse which showed hydropic degeneration, (c) MTX treatment which showed chronic inflammatory cell infiltration (H&E X40)

Histopathological changes of MTX in the kidneys in mice

MTX produced focal degeneration of tubular epithelial cells in 6 out of 8 animals treated with MTX and in 2 animals no changes were seen. Four animals had severe changes (score 3) and 2 animals had moderate changes. There were no changes seen in the glomeruli (Table-2, Figure-9 a,b).



Figure 9. Histopathological features of a mouse kidney treated with MTX, (a) control group which showed normal glomerulus and tubules, (b) MTX treated mouse which showed tubular epithelial cell degeneration with normal glomerulus (H&E X40)

Table 2.	Scores of histopathological	changes of kic	lney and liver	damage induced	by MTX in
	mice				

	Number of animals (kidneys)		Number of animals (liver)	
Scores	Control	МТХ	Control	МТХ
0	8	2	8	1
1	0	0	0	1
2	0	2	0	2
3	0	4	0	4

Number of animals in each group = 8

Scores 0, 1, 2 and 3 are for normal, mild, moderate and severe damage respectively.

DISCUSSION

The study was designed to investigate the hepatotoxicity and nephrotoxicity of MTX in two species of animals, rabbits and mice. MTX was injected to the rabbits in a dose of 20 mg/kg IP and caused hepatoxicity as documented by a marked rise in liver enzymes and parameters of oxidative stress (MDA) in serum and liver homogenate which may suggest that oxidative stress has a role in hepatotoxicity of MTX. This mechanism has been reported by other investigators who proclaimed that hepatotoxicity can occur in rats and rabbits as a result of MTX associated oxidative stress ^[13] or as a result of formation of polyglutamate derivatives of MTX which is toxic to hepatocytes.^[14] Hepatotoxicity of MTX is further confirmed by histopathological changes. These results are in agreement with other workers. ^[15,16] Although MDA in rabbit kidney homogenate was significantly increased by MTX, little effect was observed in serum urea and creatinine and the kidneys appeared normal on histopathological examination. In mice, the administered dose of MTX was half that given to the rabbits (10 mg/kg); the selection of this dose was based on observation of a pilot study which showed that mice treated with higher doses had diarrhea, reduced food and water intake and died. However, there were significant toxicities of the liver and kidneys which are presented as an increase in parameters of oxidative stress in liver and kidney and histopathological homogenates changes suggestive of toxicity in the two organs. We have to admit that biochemical tests in the serum were not done for mice since sufficient amount of blood cannot be obtained. Overall, the study revealed that MTX can lead to hepatotoxicity in rabbits and mice but nephrotoxicity is seen in the mice only despite the relatively lower dose in comparison to rabbits. It is unlikely that absence of nephrotoxicity in the MTX treated rabbits is time-dependent and may appear as a late complication since, in the present study the mice are treated with MTX for a period of time shorter than that in the rabbit, yet severe nephrotoxicity was observed. Nephrotoxicity of MTX may involve two mechanisms; first: direct toxic effect on renal tubules which is expressed as an increased MDA and GSH in kidney homogenates. This finding is in agreement with Devrim et al ^[17], second: precipitation of MTX and its metabolites in the renal tubules.^[18] Secretion of MTX occurs mainly in the kidney and depends mainly on pH of urine. MTX and its main metabolite 7-OH-MTX are poorly soluble in acidic pH and thus precipitate in renal tubules leading to kidney damage. There are published data that rats and other rodents such as mice, as animals feeding on protein, tend to have acidic urine $(pH = 5)^{[19]}$, which enhances precipitation of MTX in the tubules resulting in tubular damage, while the rabbits, as a herbivorous species have alkaline urine (pH 88.2)^[20] which renders MTX and its metabolite more soluble and thus easily excreted in urine without being precipitated in renal tubules. Such differences in urine pH between rabbits and mice may help, in part, explain the observation in the present study that MTX induces nephrotoxicity in mice while the rabbits resist such toxicity. Animal models of nephrotoxicity and hepatotoxicity significantly contribute to our understanding of the pathophysiology of human disease and lead to the founding of an effective strategy for preventing or treating these toxicities. Therefore, more attention should be paid to selecting a proper animal model to avoid repeating the study, saving time and money, and to greatly improving the study outcome and reliability of the results. Although MTX is an old drug and that all pharmacological and toxicological aspects were thought to be thoroughly studied, however, studies on animals are still required particularly emergence of relatively with the new compounds such as Glucarpidase which is found protective against MTX induced nephrotoxicity.^[21,22] In the present study, the rabbit is demonstrated as an inconvenient model for studying MTX induced nephrotoxicity and that models other than rabbits such as rats or mice should be chosen for nephroprotective agents. In fact, there are considerable numbers of trials which demonstrate safety of drugs in one species of experimental animal which turned out later toxic to other species or human. The best example which demonstrated significant species differences is thalidomide which was found non teratogenic in rats while severe teratogenicity was reported in rabbits and human ^[23]

In conclusion, the results of the present study demonstrated that MTX produced hepatotoxicity in rabbits and mice but nephrotoxicity at this dose occurred in mice only and the rabbits appear to resist nephrotoxicity by MTX.

Acknowledgements

Thanks are due to Dr. Isra M Abdul-Majeed, MSc, Pharmacology, for technical assistance, Dr. Sawsan S. Al-Haroon, assistant Professor, Department of Pathology, Basrah College of Medicine, for histopathological examination.

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