RESEARCH ARTICLE

Effects of Co-administration of Pyridoxine in Two Different Doses with Methotrexate-induced Nephrotoxicity in Rats

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ABSTRACT

Nephrotoxicity is defined as rapid deterioration in kidney functions that arises from direct exposure to a drug or its metabolite. Methotrexate is an anti-metabolite used in low doses for autoimmune and inflammatory disorders and high doses for different malignancies. Pyridoxine is the usual form of vitamin B_6 included in pharmaceutical products. This study is designed to examine the effect of pyridoxine in two different doses, each co-administered with methotrexate at 20 mg/kg made renal toxicity in rats through the involvement of Nrf2/HO-1 molecular mechanism in this respect. The animals used in this research were allocated to four distinct groups (each group with 10 rats) of both sexes; as control, MTX, pyridoxine 33 mg/kg and MTX, Pyridoxine 100 mg/kg, and MTX. Serum creatinine, neutrophil gelatinase-associated lipocalin (NGAL), and NrF2/HO-1 signaling pathway were determined. Administration of Pyridoxine (33 mg/kg intraperitoneally) in combination with MTX showed no significant differences ($p > 0.05$) as compared to the MTX group. At the same time, administration of pyridoxine (100 mg/kg intraperitoneally) in combination with methotrexate showed a significant reduction in serum activities of both neutrophil gelatinase associated lipocalin (NGAL) and creatinine ($p < 0.05$) and significant upregulation in Nrf2/HO-1 signaling pathway each compared to corresponding levels in MTX-only group. In conclusion, this study demonstrated that when pyridoxine (100 mg/kg IP) was administered in combination with methotrexate, the renal toxicity was reduced as a result.

Keywords: Methotrexate, Nephrotoxicity, Nrf2/Ho-1 signaling pathway, Pyridoxine, Serum creatinine, Serum NGAL.

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INTRODUCTION

Nephrotoxicity is defined as a fast decline in kidney function caused by direct exposure to environmental toxins or as a side effect of numerous medicines, including nonsteroidal anti-inflammatory drugs (NSAIDs), and aminoglycoside antibiotics, and anticancer treatments.[1]

Drug-induced renal toxicity is becoming well-known as the main cause of renal diseases such as acute and chronic kidney disease.[2]

Methotrexate is a amethopterin, 4-amino-4-deoxy-*N*-10-methylpteroylglutamic acid.[3,4] Its chemical structure shown in Figure 1.[5] Such drug belongs to an anti-folate therapeutic class that can be utilized as a chemotherapeutic agent in high doses for lymphoma, sarcoma, and breast cancer, and since it has an immunosuppressant effect; where it is used in low doses for treatment of psoriasis, rheumatic diseases, and refractory inflammatory bowel syndrome (Figure 1).[6]

Despite its wide range of clinical applications, MTX's efficacy is frequently restricted by extreme adverse effects, most notably renal and liver toxicity as well as duodenal damage,[7] and bone marrow toxicity.[8] Methotrexate-induced renal toxicity has been related to several different damage pathways, comprising oxidative stress and inflammatory reactions, mitochondrial dysfunction, and lastly, apoptosis.[9]

Vitamin B6, a member of the water-soluble vitamin family, is made up of three related compounds: pyridoxine, pyridoxal, and pyridoxamine;[10] as shown in Figure 2 (structure 1).[11]

Pyridoxine (vitamin B_6) is a key cofactor in the metabolic trans-sulfuration process, producing cysteine from dietary methionine. Furthermore, such vitamin is also a moderate

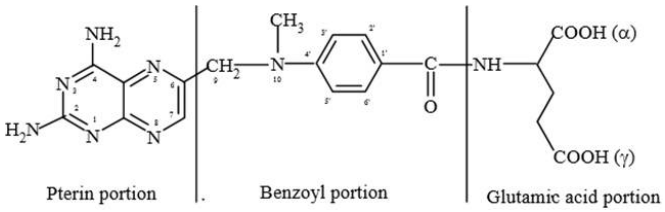

Figure 1: Chemical structure of methotrexate (MTX).[5]

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chelator and works as an antioxidant by promoting the formation of reduced glutathione (GSH).[12] Furthermore, pyridoxal (5) phosphate (PLP) is the active form of pyridoxine, acting as a cofactor in a variety of enzymatic activities as well as an antioxidant.[13] Moreover, it was already reported that pyridoxine supplementation can be utilized as an adjunctive agent for improving oxidative stress (OS) and biological markers for preventing cardiac complications due to the anticancer drug (doxorubicin).[14]

Vitamin B6's specificity in restoring glomerular hemodynamics and the disruption of renal vasodilator metabolism appears to be linked to the accelerated formation of renal PLP, which counteracts nephrotoxicity's oxidative responses, and this is most likely the primary method by which this vitamin exerts its nephron-protective effects.[15]

Aim of Study

This study is designed to investigate the impact of pyridoxine at two different doses, each co-administered with methotrexate at 20 mg/kg induced renal toxicity in rats through the involvement of the Nrf2/HO-1 molecular mechanism in this respect.

MATERIALS AND METHODS

Experimental Animals and Treatments

Forty albino white rats of both sexes, weighing 180 to 200 g were used in this study. Rats got from the Animal House of the College of Pharmacy at the University of Baghdad and were kept under strict temperature, humidity, and light: dark cycle controls. The animals were fed commercial pellets and tap water *ad libitum*.

Drugs and chemicals

Methotrexate vial (50 mg/2 mL) Mylan, France; pyridoxine HCL inj (50 mg/1 mL) HUONS, Korea, Rat NGAL ELISA kit Mybiosource, USA; Rat creatinine ELISA kit Mybiosource, USA. In addition, TRIzol reagent Thermo Fisher Scientific (USA). GoTaq® 1-Step RT-qPCR System,

Promega, USA. PCR primers for Nrf2, HO-1, and β–actin genes were synthesized and purchased from Macrogen, Korea.

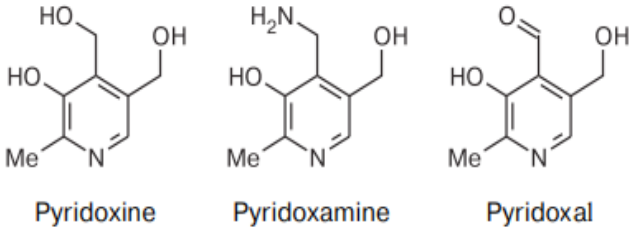

Figure 2: Chemical structures of pyridoxine, pyridoxamine, and pyridoxal.[11]

Table 1: Primers sequences

Primer Name	*Sequence*
Bactin_ F	5`-CCACCATGTACCCAGGCATT-3`
Bactin_ R	5`-ACGCAGCTCAGTAACAGTCC-3`
HO-1-F	5`-GTAAATGCAGTGTTGGCCCC-3`
HO-1-R	5`-ATGTGCCAGGCATCTCCTTC-3`
NRF2-F	5`-TTGTAGATGACCATGAGTCGC-3`
NRF2-R	5`-TGTCCTGCTGTATGCTGCTT-3

Experimental Design

The rats in this study were divided into four groups, each with ten rats, and were treated intraperitoneally (IP) as follows:

Group 1: Control/ Experimental healthy rats intraperitoneally (IP) injected with 0.5ml normal saline (0.9% NaCl) once daily for seven consecutive days.

Group 2: Experimental healthy rats intraperitoneally (IP) injected with 0.5 mL normal saline (0.9% NaCl) once daily for seven consecutive days; and on day 2, a single dose of MTX (20mg/kg)[16] is to be IP injected.

Group 3: Experimental healthy rats IP injected with a 33mg/kg pyridoxine once daily for seven consecutive days,[17] and on day 2, a single IP dose of methotrexate (20 mg/kg) to be injected.

Group 4: Experimental healthy rats IP injected with a 100 mg/kg pyridoxine once daily for seven consecutive days,[17] and on day 2, MTX single IP dose of (20 mg/kg) to be injected.

Rats per each group were euthanized by anesthetic ether 24 hours at the completion of the treatment.

Preparation of Serum Sample

Following euthanization of rats by anesthetic ether, blood was obtained from the neck and placed in a gel tube to allow clotting; the clot was dispersed with a glass rod and then centrifuged at 300 rpm for 15 minutes to get the serum, which was utilized for the estimation of neutrophil gelatinase-associated lipocalin (NGAL) and serum creatinine as biochemical parameters of kidney function tests.

Determination of Renal (Nrf2 and HO-1) mRNA Expression

Total RNA was extracted from kidney tissues depending on the steps of the TRIzol™ Reagent protocol. A Quantus fluorometer was applied for the detection of the extracted RNA concentration. The isolated RNA was ready for use in cDNA synthesis—a one-step RT-qPCR protocol was used to quantitatively investigate the mRNA of the transcription factor Nrf2 and HO-1. Real-time reverse transcriptase-polymerase chain reactions were conducted in a total volume of 10 µL. The thermal profile used was at 37°C for 15 minutes for RT. Enzyme activation at 95°C for 5 minutes for initial activation denaturation, then 40 cycles of 95°C for 20s denaturation, 56°C for 20 s annealing, and 72°C for 20 s extension. As a reference gene, the β-actin is used. Following the PCR amplification, the ΔΔ Ct was utilized for calculating by subtraction of the β-actin Ct from each sample Ct. qRT-PCR primers Nrf2, HO-1, and B-actin sequences[18,19] were shown in Table 1.

Statistical Analysis

The data is presented as a mean ±standard error of the mean (SEM). Graph Pad Prism, version 8, was used to conduct the analysis. An analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was done. Data differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Effect on serum Neutrophil Gelatinase Associated Lipocalin (NGAL)

Rats IP injected with MTX on day 2 at a dose of 20mg/kg (**Group 2**) showed significant elevation ($p < 0.05$) in the NGAL serum level as compared to the control group (**Group 1**) rats. The mean±SEM of serum NGAL levels were respectively 0.05460 ± 0.008388 and 0.02510 ± 0.004406. Figure 3.

Furthermore, in Figure 3, there was a significant reduction ($p < 0.05$) in serum NGAL level in (**Group 4**) rats treated with a pyridoxine dose of 100 mg/kg for 1-week in combination with MTX at day 2 as compared to (**Group 2**); where mean ± SEM of serum NGAL levels were respectively 0.02820 ± 0.004678 and 0.05460 ± 0.008388. Figure 3.

On the contrary, there was no significant difference ($p > 0.05$) in serum NGAL level in (**Group 3**) rats treated with a pyridoxine dose of 33 mg/kg for 1 week in combination with MTX at day 2 as compared to **Group 2**. The mean±SEM of serum NGAL levels were respectively 0.04340 ± 0.006669 and 0.05460 ± 0.0083. Figure 3.

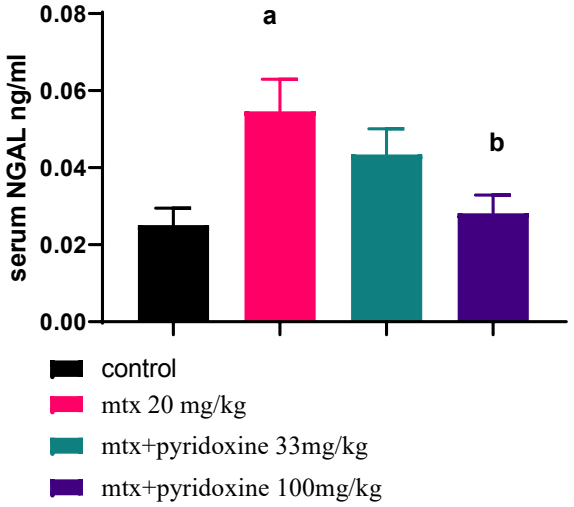

Figure 3: Effect of 20mg/kg MTX and pyridoxine (at two doses) on serum NGAL level
a latter, is significantly different ($P < 0.05$) compared to control group; b latter, is significantly different ($p < 0.05$) compared to MTX group. N = 10.

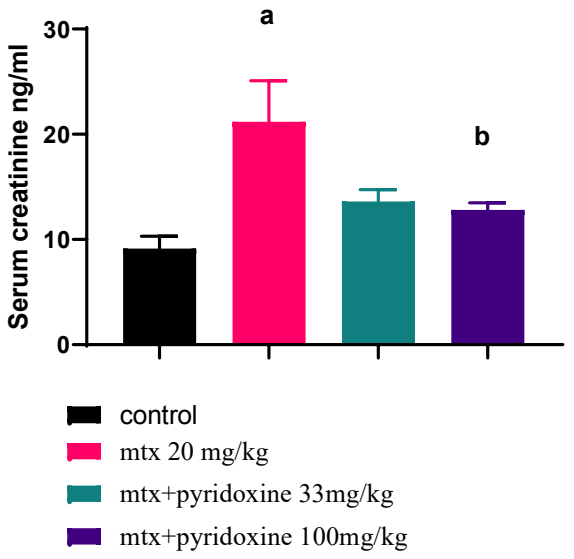

Figure 4. Effects of 20mg/kg MTX and Pyridoxine (in two doses) on serum creatinine level
a letter is significantly different ($p < 0.05$) compared to control (Group 1); b letter is significantly different ($p < 0.05$) compared to MTX (Group 2) treated-rats. N=10

Effects on the Serum Creatinine

Rats IP injected with MTX on day 2 at a dose of 20mg/kg (**Group 2**) produced significant elevation ($p < 0.05$) in the level of serum creatinine compared to the corresponding level in control (**Group 1**) rats. Figure 4. Mean ± SEM of serum creatinine levels were respectively 21.20 ± 3.893 and 9.143 ± 1.176. Furthermore, in figure 4 there was a significant reduction ($p < 0.05$) in serum creatinine level in (**Group 4**) rats treated with a pyridoxine dose of 100 mg/kg for 1-week in combination with MTX at day 2 as compared to group 2. Mean ± SEM of serum creatinine levels were respectively 12.81 ± 0.6767 and 21.20 ± 3.893.

On the contrary, there was no significant difference ($P > 0.05$) in serum creatinine level in (**Group 3**) rats treated with a pyridoxine dose 33mg/kg for 1 week in combination with MTX at day 2 as compared to the corresponding serum level in **Group 2** treated rats. Mean ± SEM of serum creatinine levels were respectively 13.62 ± 1.111 and 21.20 ± 3.893. Figure 4.

Effects on Nrf2/HO-1 Signaling Pathway

Rats IP injected with MTX at day 2 at a dose of 20mg/kg (**Group 2**) downregulated renal Nrf2 and HO-1 mRNA expression significantly ($p < 0.05$) compared to the control group (Group 1). Table 2 and Figure 5 (A, B).

Furthermore, Table 2 Figure 5 (A,B) showed that there were significant up-regulation ($p < 0.05$) in renal Nrf2 and HO-1 mRNA expression in (**Group 4**) rats treated with a pyridoxine dose of 100 mg/kg for 1 week in combination with MTX at day 2 compared to rats treated with MTX (**Group 2**).

While there were no significant upregulation ($p > 0.05$) in renal Nrf2 and HO-1 mRNA expression in (**Group 3**) rat treated with pyridoxine dose 33mg/kg for 1 week in combination with MTX at day 2 compared to rats treated with MTX (**Group 2**). Table 2 and Figure 5 (A, B).

Table 2: Effects of 20mg/kg MTX and Pyridoxine (at two doses) on the mRNA expression of renal nuclear factor erythroid 2-related factor 2 (NRF2) and heme oxygenase-1 (HO-1) genes.

Groups N=10	*Nrf2 folding Mean± SEM*	*HO-1 folding Mean± SEM*
Control	1.982 ± 0.1495	1.501 ± 0.1956
Mtx 20 mg/kg	0.3529 ± 0.05385	0.2253 ± 0.05067
Mtx 20 mg/kg + pyridoxine 33 mg/kg	1.603 ± 0.5078	0.9881 ± 0.2521
Mtx 20 mg/kg + pyridoxine 100 mg/kg	2.169 ± 0.4949	1.679 ± 0.4940

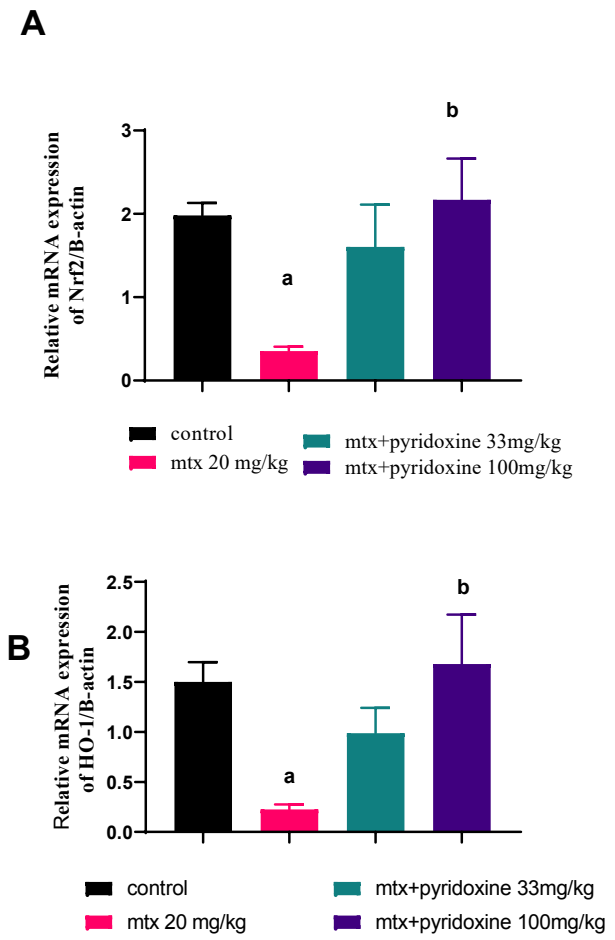

Figure 5: Effects of 20mg/kg MTX and Pyridoxine (at two doses) on each of A – Nrf2 and B-HO-1 genes expression
a significantly different ($P < 0.05$) as compared to control group
b significantly different ($P < 0.05$) as compared to MTX group
n = 10

DISCUSSION

This study revealed that rats treated with 20 mg/kg MTX IP showed significant elevation in serum activity of both NGAL and creatinine as compared to the control group. Figures 3 and 4.

Acute kidney damage (AKI) caused by large dosages of MTX is defined by non-oliguric renal failure, which is marked by a rapid rise in serum creatinine level during or shortly after MTX infusion.[20]

The NGAL is a promising biochemical marker of nephrotoxicity, particularly distal tubular injury, where it rises when tubular epithelial cells are structurally injured. Moreover, serum, plasma, and urine NGAL levels made similarly well as a useful early predictor of AKI. Organs damage could directly increase serum NGAL levels, and diminished glomerular filtration rate (GFR) of NGAL can further increase its level in serum when there was AKI. Moreover, high dose MTX (HDMTX) can induce direct kidney tubular injury, and this can be detected by using serum NGAL levels as a marker.[21]

Results of Figures 3 and 4 showed that in rats co-administered pyridoxine in a dose of 33mg/kg along with MTX (**Group 3**), there were no significant differences ($p > 0.05$) in the serum activities of both NGAL and creatinine compared to MTX group. But, rats co-administered pyridoxine in a dose of 100 mg/kg along with MTX (**Group 4**), and results shown in Figures 3 and 4, there was a significant reduction ($p < 0.05$) in the serum activities of both NGAL and creatinine compared to MTX **(Group 2)** rats.

The mechanism of MTX-induced nephrotoxicity is still unknown. However, researchers found that oxidative damage, which can lead to the formation of free radicals, is the primary cause of MTX-related tissue injury. The function of OS in MTX-induced nephrotoxicity has been established.[22]

Additionally, researchers also showed that MTX increased reactive oxygen species production by overwhelming homocysteine remethylation, NADPH depletion, neutrophils stimulation, NADPH oxidase activation, and mitochondrial dysfunction.[23]

Nephrotoxicity is thought to be caused by crystal nephropathy owing to the existence of MTX and its metabolites in the renal tubules. Therefore plasma MTX concentration monitoring, adequate hydration, urine alkalization and using glucarpidase are recommended during treatment to facilitate renal elimination.[8]

Mechanistically, the link between OS and other signaling pathways is well understood. It has been reported that OS can play a role in the development of a variety of diseases by activating the inflammatory response, inducing apoptosis and cell death; furthermore, OS decreases the expression of NRF-2, with the consequent down-regulation of antioxidant genes.[18]

Nuclear factor erythroid-derived- 2-like- 2 (Nrf2) is a redox-sensitive transcription factor, basic leucine zipper protein that controls the expression of protective and antioxidant genes, including genes that encode antioxidant enzymes such as HO-1, superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase.[9,24] Moreover, researchers showed that activation of the Nrf2/HO-1 signaling pathway in rat kidneys was positively associated with diminished ROS and enhanced antioxidant defenses.[25]

The result of this study revealed that rats IP injected with MTX produced significant dawn-regulation ($p < 0.05$) in Nrf2/HO-1 signaling pathway as compared to the control group. Table 2 and Figure 5 (A, B). Moreover, in rats co-administered pyridoxine in a dose of 33 mg/kg along with MTX, results of Table 2 and Figure 5 (A, and B) showed that there were no significant up-regulation in Nrf2/HO-1 signaling pathway ($p > 0.05$) compared to MTX group. In contrast, in rats co-administered with pyridoxine in a dose of 100 mg/kg along with MTX, there was significant up-regulation in Nrf2/HO-1 signaling pathway as ($p < 0.05$) compared to the MTX group.

CONCLUSION

According to the findings of this research, it could be oncluded the protective effect of pyridoxine at a dose of 100 mg/kg was observed when co-administered with 20 mg/kg MTX but not

observed at a low dose of pyridoxine used at a dose of 33mg/kg. Particularly, co-administration of pyridoxine (100mg/kg IP) with 20mg/kg MTX resulted in a diminution of MTX-induced nephrotoxicity.

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