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Original Article

Amlodipine protects against methotrexate nephrotoxicity by modulating inflammation, oxidative stress, and apoptosis through NF-κB/PPAR-β/caspase 3 pathways

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ABSTRACT

Methotrexate (MTX), a folic acid antagonist used for treating malignancies and autoimmune disorders, has limited use due to its toxicity, including bone marrow suppression and hepatic and renal damage. High doses of MTX (>500 mg/m²) are particularly harmful. Calcium channel blockers, such as amlodipine, may alleviate MTX-induced toxicity. This study investigated amlodipine's potential to protect against MTX-induced acute kidney injury in rats. Thirty-two male Wistar rats were divided into four groups: a control group receiving saline, a group treated with MTX alone, and two groups receiving MTX followed by oral amlodipine at doses of 5 mg/kg or 10 mg/kg. Serum levels of kidney function biomarkers (urea, creatinine, uric acid, albumin, KIM-1, NGAL), inflammatory markers (ICAM-1, VCAM-1, IL-6, TNF-α), and oxidative stress markers (SOD, GSH, TBARS) were measured. Histopathological and immunohistochemical analyses were also performed. MTX increased serum levels of kidney function biomarkers and decreased glomerular filtration rate (GFR), which were significantly improved by amlodipine treatment. Amlodipine also reversed increases in inflammatory markers and the expression of NFκB and Caspase-3, while normalizing PPARβ levels. Histopathological findings supported these results. Thus, amlodipine demonstrates nephroprotective effects and may be a viable intervention for MTX-induced acute kidney injury.

Keywords: Methotrexate, Amlodipine, NF κB, Acute kidney injury, Caspase 3

Introduction

Acute kidney injury (AKI) is a growing health challenge in terms of morbidity, mortality, and financial impact worldwide [1]. The patient outcomes of Acute Kidney Disease (AKD) were subjected to meta-analysis by Su *et al*. in 2023. The study revealed that 4.60% of AKD cases were acquired within the community, 5.67% occurred in hospitalized patients, and 26.11% and 2.11% were acquired in the hospital with and without a previous AKI occurrence, respectively [2]. Moreover, AKI is becoming well-recognized as a potential cause of chronic kidney disease and end-stage renal disease [3].

AKI may be caused by a variety of clinical conditions, including sepsis, acute illness, circulatory shock, radiocontrast agents, and nephrotoxic medications [4]. Drug-induced acute kidney (D-AKI) injury is kidney damage caused by medicines or their metabolites within 7 days after exposure to one or more drugs. Because the kidneys have high blood flow and acidify urine, they

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are a prime target for drug-induced toxicity [5]. Preventable variables for D-AKI include exposure to nephrotoxic medicines or radiocontrast agents [6]. Further studies are needed to identify interventions that reduce the risk of D-AKI in diabetes, heart failure, and the use of non-nonsteroidal anti-inflammatory drugs (NSAIDS) [7].

Methotrexate (MTX) is a medication that blocks the action of folate and is used to treat different types of cancerous and autoimmune conditions [8]. The most severe consequences of exposure to MTX are bone marrow suppression, oral mucositis, elevated liver enzymes, and acute kidney injury (AKI). High-dose methotrexate, ranging from 500-1000 mg/m2, is a significant contributor to renal damage. Individuals with obesity, diabetes, heart failure, or who are over the age of 60 are particularly susceptible to developing acute kidney injury (AKI) as a result of methotrexate (MTX) treatment [9]. When administered in large amounts in acidic conditions, MTX forms crystals (7-hydroxy-MTX) that cannot dissolve in urine and instead settle in the distal renal tubules. Furthermore, the presence of significant accumulations of uric acid and calcium might lead to the development of acute renal failure as a consequence of tumor rupture occurring during chemotherapy [10].

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate several physiological activities, including lipid metabolism, glucose regulation, cell differentiation, and inflammation [10].

Second messenger Ca2+ controls the activity of several important cellular processes, including transcription, motility, cell shape, proliferation, apoptosis, mitochondrial function, and immunological responses [11]. It has been hypothesized that cellular Ca2+ plays a role in a variety of diseases and conditions, including cardiovascular disease, cancer, diabetes, and the immunological response (including inflammation) [12]. L-, T-, N-, and P/Q-type voltage-gated calcium channels are present in renal vascular and tubular tissues. Renal microcirculation is also affected differently by blocking these various calcium channels [13].

Several antihypertensive drugs, such as calcium channel blockers (CCB), may prevent chronic renal failure by inhibiting some of the mechanisms associated with kidney damage [14]. Because of their potent blood pressure (BP) reducing characteristics and the lack of undesirable side effects, CCB targeting voltage-dependent calcium channels are widely prescribed in combination with renin-angiotensin-aldosterone system inhibitors for CKD patients [15]. AMO is a calcium channel blocker that inhibits the entry of calcium into specific tissues and arteries [16]. This causes the arteries to relax and facilitates blood flow more freely to the heart. As a result, BP is reduced, thus decreasing the likelihood of a heart attack or stroke. During non-proteinuric CKD, dihydropyridine CCBs (such as AMO) may be used as a first-line treatment either alone or in combination with other drugs [17]. Based on these findings, we hypothesized that AMO can prevent AKI caused by MTX treatment; thus, we examined the underlying mechanism of the renoprotective potential of these compounds in MTX-induced AKI.

Materials and Methods

Drugs and chemicals

Methotrexate (Lot # G024412AB, Hospira Pharmaceutical Co., Illinois, United States) and amlodipine besylate (Lot # XM0221, Jamjoom Pharmaceuticals Co., Jeddah, Saudi Arabia) were obtained. All ELISA kits to measure biochemical factors were purchased from MyBioSource, Inc. (San Diego, CA, USA). All chemicals used were of analytical grade.

Preparation of solutions for dosing

Ten amlodipine besylate (5 mg) capsules were dissolved in 20 ml of distilled water to yield a final concentration of 2.5 mg/ml. The solutions were prepared for oral delivery daily.

Experimental design

All work involving animals, such as euthanasia and the collection of blood and tissues was done following international norms with special emphasis on human nature. This experiment was conducted following ethical approval from the Institutional Ethics Committee of the Faculty of Pharmacy, King Abdulaziz University (reference #PH-1442-75). We complied with the protocol for animal care and experimentation according to the regulations of Saudi Arabia.

From the animal facility at the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia, thirty-two male Wistar rats in all, weighing between 170 and 190 g, were acquired. The animals spent one week adjusting to relative humidity (55%–65%), room temperature (24°C–26°C), and a 12-hour light/dark cycle. Every drug has a five-day dosage schedule. AMO was orally (PO) given gavage on the first day; one hour following a single MTX dosage was intraperitoneally (IP). **Table 1** shows the four groups the rats were randomly assigned to n=8 in each group. They had free access to standard nourishment and tap water and were housed in 8 propylene cages with 4 animals/cage.

Table 1. Animal groupings and treatment

Group	Treatment
Control	Normal Saline (1 ml/kg, i.p.)
MTX	MTX (20 mg/kg, single dose, IP) [18]
AMO 5	MTX (20 mg/kg, single dose, IP) + AMO (5 mg/kg, PO) [19]
AMO 10	MTX (20 mg/kg, single dose, IP) + AMO (10 mg/kg, PO) [19]

On day 6, the animals were weighed and anesthetized using ether. Blood samples were collected in a blood collection tube (Clot Activator with Gel) through the retro-orbital plexus. The animals were euthanized and the kidneys were subsequently harvested and washed with ice-cold PBS. The kidneys were cryopreserved at a temperature of -80°C and utilized for the quantification of biochemical markers.

Preparation of kidney tissue homogenate

To prepare a 10% kidney homogenate, 100 mg of frozen kidney tissue was weighed and placed in an Eppendorf tube. The tissue was then homogenized using a rotary homogenizer Bio-Gen PRO200 (PRO Scientific Inc, Connecticut, USA) with 1 ml of 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was subjected to centrifugation using an Eppendorf high-speed benchtop centrifuge 5804/5804R (Eppendorf, Hamburg, Germany) at a speed of 10,000 revolutions per minute for 15 minutes at a temperature of 4 degrees Celsius. Next, the liquid portion was moved to new Eppendorf tubes and kept at a temperature of -80 °C until it could be tested.

Biochemical estimations

Kidney function tests

Using MyBioSource ELISA Kits, urea (Cat No. MBS2600001), creatinine (Cat No. MBS2749827), uric acid (Cat No. MBS7606443), albumin (Cat No. MBS564099), KIM-1(MBS264966), and NGAL (MBS260195) were measured following the manufacturer's instructions.

Estimation of glomerular filtration rate

The glomerular filtration rate of the animal kidneys was estimated according to the formulas below [20].

When serum creatinine <52µmol/ml (0.588 mg/d)

1. GFR (ml/min) = 880 × body weight(g)$^{0.695}$ x creatinine concentration (µmol/ml) $^{-0.660}$ x urea concentration (mmol/ml) $^{-0.391}$

When serum creatinine ≥52µmol/ml (0.588 mg/dl)

2. GFR (ml/min) = 5862 × body weight(g)$^{0.695}$ x creatinine concentration (µmol/ml) $^{-1.15}$ x urea (mmol/ml) $^{-0.391}$

Renal oxidative stress marker analysis

The concentration of TBARS (Cat. No. MBS1600368), GSH (Cat. No. MBS2609227), and SOD (Cat. No. MBS037536) were measured in the supernatants of the kidney tissue homogenates using commercially available ELISA kits following the manufacturer's instructions.

Serum inflammatory marker analysis

ICAM-1, VCAM-1, IL-6, and TNF-α concentrations were measured in the rat serum samples using the ELISA kits MBS700189, MBS2502676, MBS726707, and MBS2507393, based on the manufacturer's instructions.

Histopathology and immunohistochemical analysis

The process of tissue staining for immunohistochemistry (IHC) involves the application of specific antibodies to target antigens inside tissue samples, followed by the visualization of these antigens using various staining techniques.

The kidney tissues were first immersed in a solution of 10% neutral formalin for fixation, followed by the process of paraffinization. The tissues were sectioned into slices with a thickness of 5 µm. The slices were stained with hematoxylin and eosin (H&E) and subsequently imaged using light microscopy (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan). The tissue slices underwent deparaffinization, followed by tissue rehydration using ethanol serial dilutions. Subsequently, the tissue samples were subjected to boiling in a 0.1 M citrate buffer (pH 6.0) for 10 minutes. Subsequently, a 2-hour incubation phase was conducted in a solution containing 5% bovine serum albumin (BSA) in tris-buffered saline (TBS). The tissue incubation involved the use of primary antibodies for 12 hours at a temperature of 4 °C. The primary antibodies utilized were NFκB (Cat. No. sc-8414, Santa Cruz, TX, USA), Caspase-3 (Cleaved) kit (Concentrated and Prediluted Polyclonal Antibody (Cat. No. CP 229 A, B, C and PP 229 AA respectively, Biocare Medical, CA, USA) and PPARβ (Cat. No. sc-74440, Santa Cruz Biotechnology, Inc., MA, USA). Following tissue flushing with TBS, a further incubation was performed using an antirabbit biotinylated secondary antibody, selected based on the reactivity of the primary antibody (Cat. No. MA100_RUO, biocyc Biotechnologie GmbH & Co. KG, Potsdam, Germany).

Statistical analysis

The data are represented as the mean ± SEM. Graphs and statistical analysis were done using a one-way ANOVA followed by Sidak's multiple comparisons test using GraphPad Prism V6.0 software (GraphPad Software, San Diego, CA). $p < 0.05$ was considered statistically significant.

Results and Discussion

Amlodipine attenuates MTX-induced nephrotoxicity

Compared with the control group, MTX administration resulted in a significant increase in serum creatinine (164%) **(Figure 1a)**, uric acid (75%) **(Figure 1b)**, KIM-1 (319%) **(Figure 1d)**, and NGAL (200%) **(Figure 1e)** levels as well as a significant decrease in GFR (80%) **(Figure 1e)**. Rats that were administered AMO at doses of 5 and 10 mg/kg were protected from the nephrotoxic effects of MTX as evidenced by a respective decrease in serum creatinine (44% and 65%) **(Figure 1a)**, uric acid (26% and 42%) **(Figure 1b)**, KIM-1 (17% and 77%) **(Figure 1d)**, NGAL (36 and 61%) **(Figure 1e)** concentrations and a significant increase in GFR with at an AMO dose of 10 mg/kg (450%) **(Figure 1c)**.

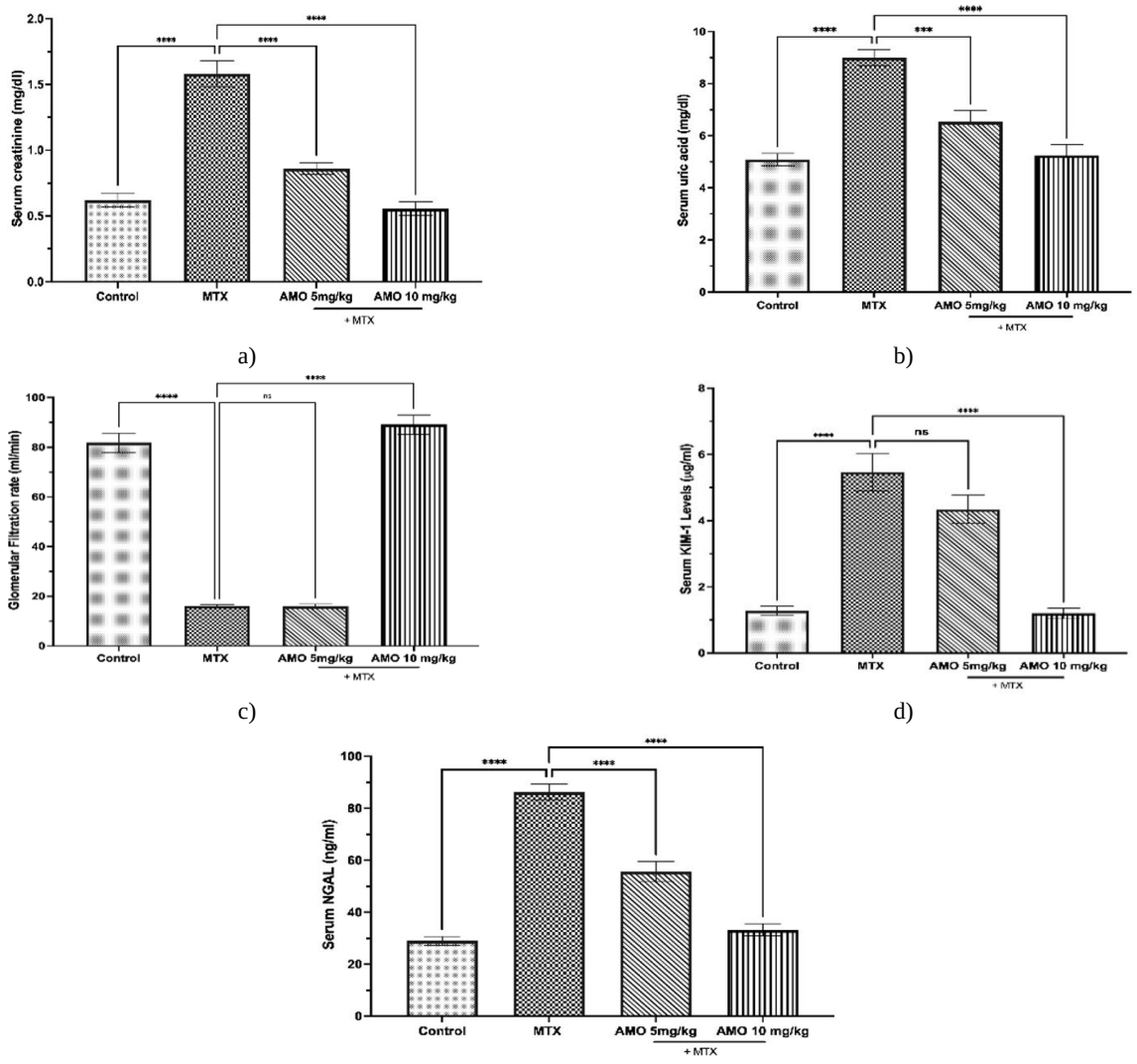
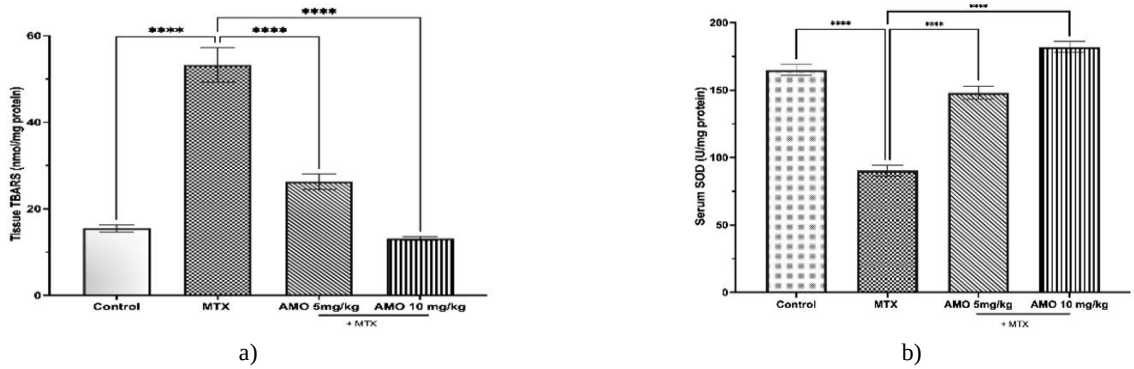
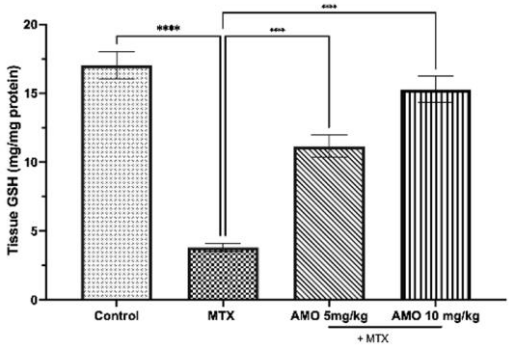


Figure 1. Amlodipine attenuates MTX-induced nephrotoxicity, Effect of amlodipine administration (5 and 10 mg/kg/rat/day: PO) against MTX-induced renal injury in rats on a) creatinine, b) uric acid, c) GFR, d) KIM-1, and e) NGAL (n = 8) levels in a dose-dependent manner. Data are expressed as the mean±SEM. One-way ANOVA followed by Sidak's multiple comparisons test. ns: non-significant, *: $p < 0.05$; **: $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Amlodipine attenuates MTX-induced renal oxidative stress

MTX-induced oxidative stress in renal tissues was evident as it significantly increased the renal TBARS concentration by 232% **(Figure 2a)** and significantly decreased the level of SOD by 48% **(Figure 2b)** and GSH by 79% **(Figure 2c)** compared with the normal group. AMO at both 5 mg/kg and 10 mg/kg doses was protective against MTX-induced oxidative stress as it significantly decreased renal TBARS levels (49% and 75%) **(Figure 2a)** and significantly increased SOD by 66% and 107% and GSH by 224% and 364% **(Figures 2b and 2c)** levels, respectively, compared with the MTX group.



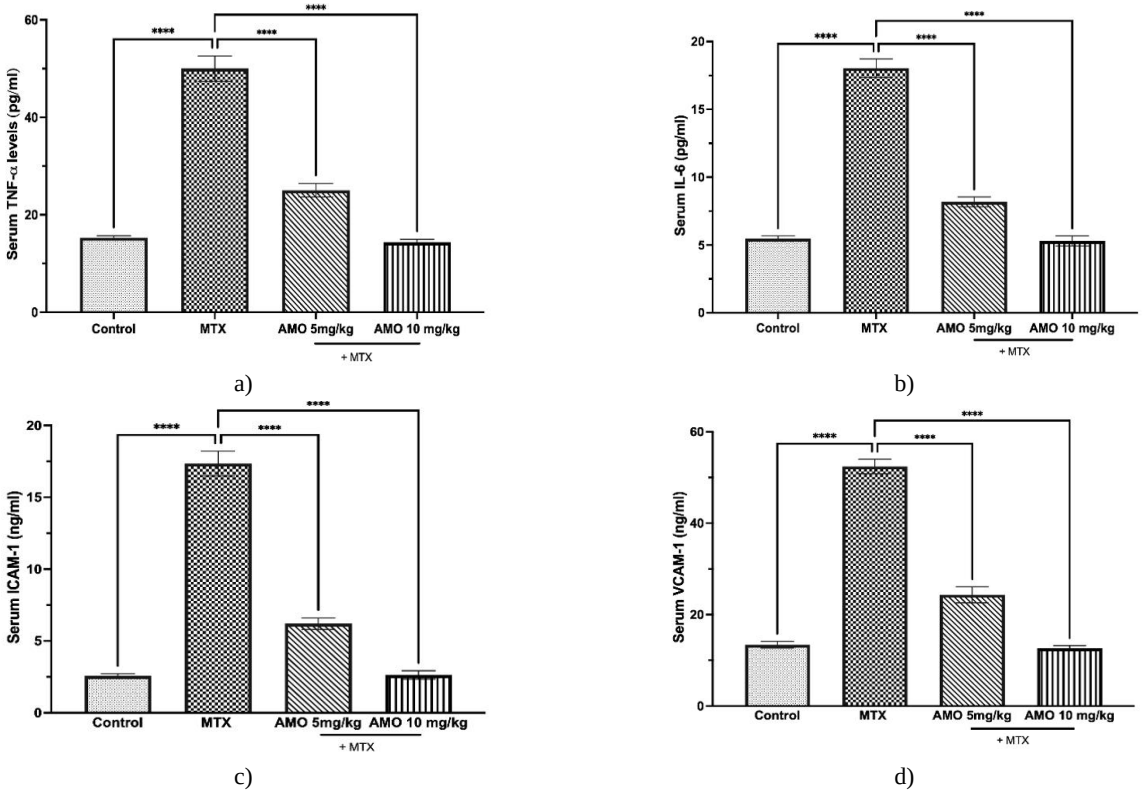


c)

Figure 2. Amlodipine modulates MTX-induced renal oxidative stress. Effect of amlodipine administration (5 and 10 mg/kg/rat/ day: PO) against MTX-induced oxidative stress in rats on a) TBARS, b) SOD, and c) GSH concentrations ($n = 8$) in a dose-dependent manner. Data are expressed as the mean±SEM. Data were analyzed by a one-way ANOVA followed by Sidak's multiple comparisons test. $p < 0.05$ was considered significant; **** $p < 0.0001$.

Amlodipine modulates the MTX-induced increase in inflammatory markers

MTX significantly increased the serum levels of TNF-α (246%) **(Figure 3a)**, IL-6 (229%) **(Figure 3b)**, ICAM-1 (539%) **(Figure 3c)**, and VCAM1 (287%) **(Figure 3d)** compared with the control group. Interestingly, AMO exhibited a dose-dependent decrease in the serum levels of these markers **(Figures 3a-3d)** compared with the MTX group.



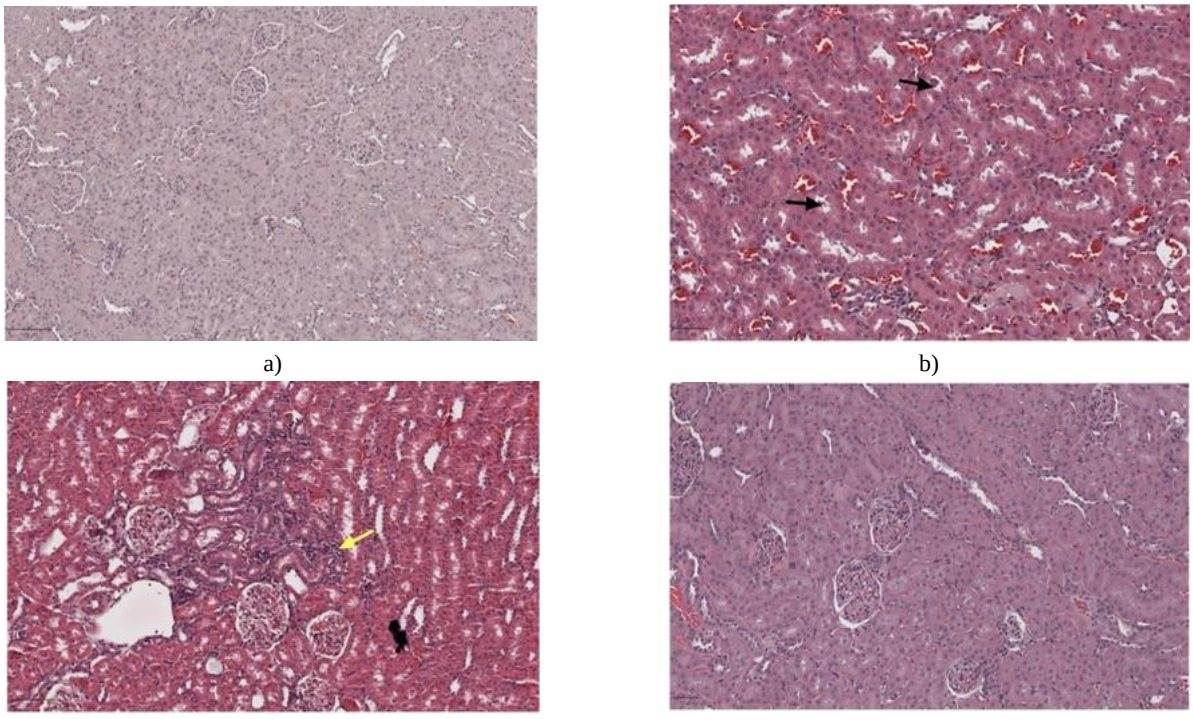
a) b) c) d)

Figure 3. Amlodipine modulates MTX-induced dysregulation of TNF-α, IL-6, VCAM-1, and ICAM-1. The effect of amlodipine administration (5 and 10 mg/kg/rat/day: PO) to MTX-induced nephrotoxicity on a) tumor necrosis factor-α (TNF-α), b) interleukin-6 (IL-6), c) vascular adhesion molecule-1 (VCAM-1), and d) intercellular adhesion molecule-1 (ICAM-1) concentration (n = 8) in a dose-dependent manner. Data are expressed as the mean±SEM. Data were analyzed by a one-way ANOVA followed by Sidak's multicomparisons test. **** $p < 0.0001$.

Amlodipine prevents MTX-induced histological alterations in the kidney

Histopathological analysis has always been considered a reliable and validated investigational component in the preclinical and clinical setting. Sections from the H & E-stained kidney of Control animals showed normal histology, including renal corpuscle, glomerular capillaries, and renal tubules **(Figure 4a)**.

However, Sections revealed Peri-tubular capillary congestion, congested and distended glomeruli, focal tubular vacuolization, focal luminal eosinophilic secretions (black arrows), and minimal interstitial chronic inflammatory cell infiltrate **(Figure 4b)**. Additionally, Sections show **(Figure 4c)** chronic lymphocytic interstitial inflammation (yellow arrows) in a background of non-significant renal cortical structure changes and mild congestion confirmed in animals treated with AMO 5mg/kg with MTX. Furthermore, the administration of AMO 10 mg/kg dose-treated section reveals unremarkable renal cortical structures, demonstrating the effect of AMO at 10 mg/kg dose in renal protection against MTX-induced renal injury **(Figure 4d)**.



a) b) c) d)

Figure 4. H & E-stained kidney of a) Control animals showed normal histology, including renal corpuscle, glomerular capillaries, and renal tubules, b) MTX-treated rats sections revealed Peri-tubular capillary congestion, congested and distended glomeruli, focal tubular vacuolization, focal luminal eosinophilic secretions (black arrows), and minimal interstitial chronic inflammatory cell infiltrate, c) showing chronic lymphocytic interstitial inflammation (yellow arrows) in a background of non-significant renal cortical structures changes and mild congestion confirmed in animals treated with AMO 5mg/kg with MTX, d) Scale bars = 50 µm; Zoom 400X.

Amlodipine protects against MTX induced NF-κB, caspase-3, and PPARβ alterations in kidney

The immunohistochemical results demonstrated elevated levels of NF-κB and caspase 3 expressions in the kidneys of rats treated with MTX. Tubular epithelial cells in the rat kidneys showed frequent expression of these markers in the MTX group. The Amlo therapy resulted in a reduction of expression levels in the kidneys **(Figures 5a and 5b)**.

Figure 5c showed that PPARβ expression in the glomerular capillaries of renal corpuscles and the peritubular capillaries of the renal medulla, indicating an immunoreactive reaction in the normal control group. However, the MTX group exhibited a lack of PPARβ immunostaining in the glomerular capillaries of renal corpuscles and the peritubular capillaries of the renal medulla (shown by red arrows). The rats treated with AMO 5 $mgkg^{-1}$ exhibited a restoration and considerable increase in the intensity of PPARβ immunostaining in the glomerular capillaries of renal corpuscles and the peritubular capillaries of the renal medulla (shown by black dotted arrows). The administration of AMO at a dosage of 10 mg kg^{-1} resulted in a significant and pronounced enhancement of the immunostained intensity of PPARβ in the glomerular capillaries of renal corpuscles and peritubular capillaries of the renal medulla, surpassing the effects observed with a dosage of AMO at 5 mg kg-1.

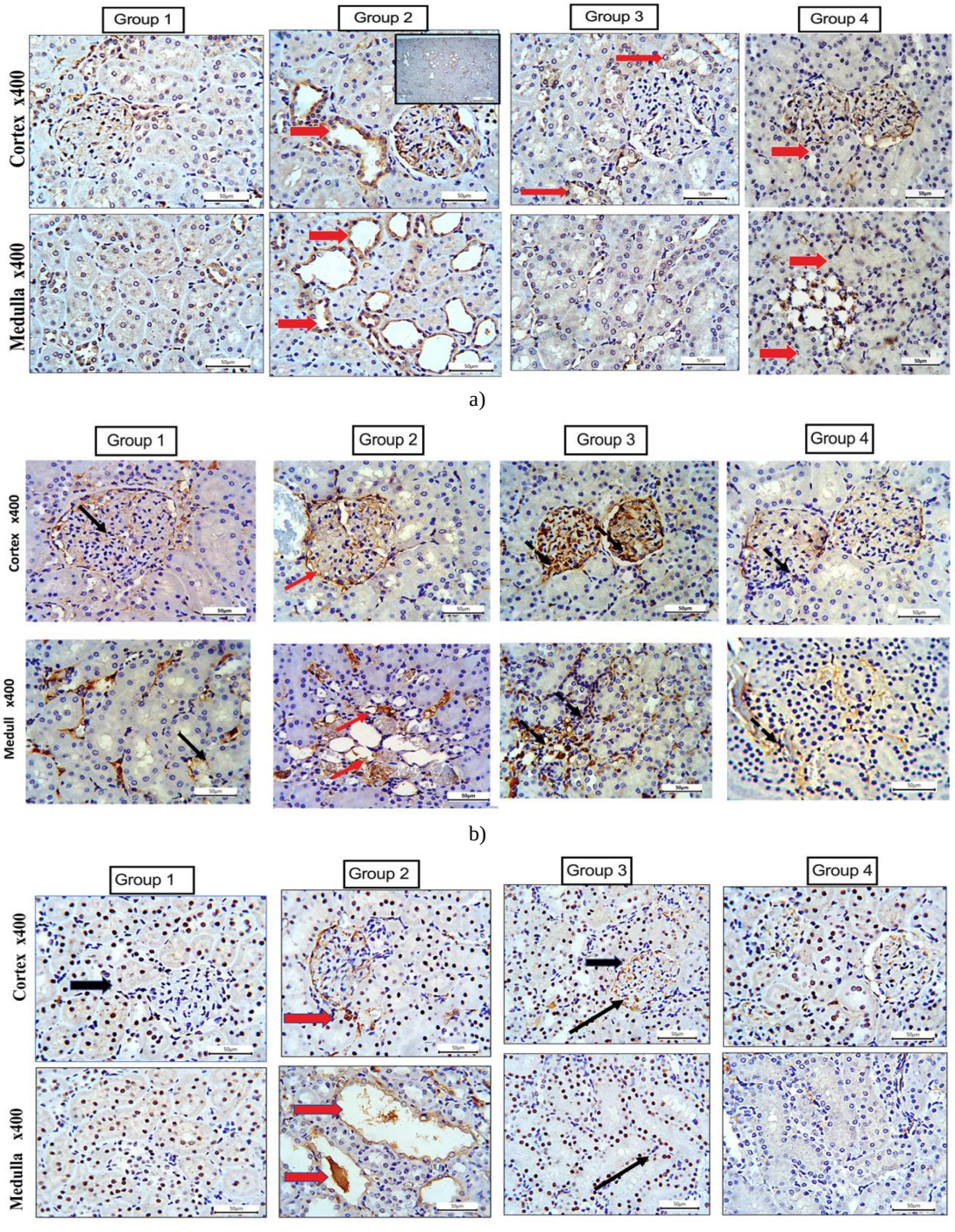


Figure 5. a) An immunohistochemical staining of the kidney after the administration of methotrexate (MTX) and amlodipine, showing the presence of Nuclear factor-κB (NF-κB). Group 1 consists of normal control animals, Group 2 consists of animals treated with MTX, Group 3 consists of animals treated with Amlodipine at a dose of 5 mg per kg of body weight, and Group 4 consists of animals treated with Amlodipine at a dose of 10 mg per kg of body weight. Positive immunostaining is indicated by black arrows, whereas negative immunostaining is indicated by red arrows. The scale bars indicate a length of 50 µm, and the image is magnified at a zoom level of 400 X.Scale bars = 50 µm; Zoom 400X, b) Representative caspase 3 immunohistochemical staining of the kidney after high-dose methotrexate (MTX) and amlodipine administration. Group 1 consists of normal control animals, Group 2 consists of animals treated with MTX, Group 3 consists of animals treated with Amlodipine at a dose of 5 mg per kg of body weight, and Group 4 consists of animals treated with Amlodipine at a dose of 10 mg per kg of body weight. Black arrows indicate decreased immunostaining, red arrows indicate increased immunostaining of epithelial lining and dilated tubule. Scale bars = 50 µm; Zoom 400X, c) Representative PPARβ immunohistochemical staining of the kidney after high-dose methotrexate (MTX) and amlodipine administration. Group 1 consists of normal control animals, Group 2 consists of animals treated with MTX, Group 3 consists of animals treated with Amlodipine at a dose of 5 mg per kg of body weight, and Group 4 consists of animals treated with Amlodipine at a dose of 10 mg per kg of body weight. Black arrows are Positive immunostaining, red arrows are negative immunostaining. Scale bars = 50 µm; Zoom 400X.

Although methotrexate is a good immunosuppressive and anticancer medication, its use is limited by strong adverse effects. The mechanism of MTX-induced nephrotoxicity includes inflammation and oxidative damage [21]. Despite a growing understanding of MTX nephrotoxicity, effective therapies for preventing or treating these serious side effects are lacking. As a result, there is a need to develop new strategies for the treatment and/or prevention of MTX-induced nephrotoxicity. In this study, we evaluated the protective effect of AMO against MTX-induced AKI in rats. AMO is a calcium channel blocker with potential pharmacological activity [22].

In MTX-treated rats, serum creatinine, urea, and renal Kim-1 levels all rose, suggesting AKI. Among markers of glomerular function include creatinine, albumin, uric acid, urea, and GFR [23, 24]. Kim-1 and NGAL are markedly elevated following renal injuries, particularly caused by renal proximal tubular injury [25, 26]. As a result, increased levels of these kidney damage indicators suggest renal failure. Previous studies linking MTX treatment to altered renal function [27, 28] have corroborated these findings. Kidney damage can result from the precipitation of MTX and its 7-hydroxy metabolite in renal tubules, which leads to tubular blockage and impaired renal clearance [29]. AMO exhibited a marked renoprotective effect as evidenced by its ability to protect and improve kidney function in MTX-treated rats.

MTX-induced cytotoxicity is linked to an increase in reactive oxygen species [30, 31]. The pathophysiology of nephrotoxicity involves several mechanisms, which indicates that direct nephrotoxic damage is associated with oxygen radicals, hydrogen peroxides, and functional changes resulting from excess apoptosis [32, 33]. Rising ROS levels in reaction to MTX may happen in a number of ways, such as by stopping homocysteine remethylation, lowering NADPH levels, activating NADPH oxidase, and causing mitochondrial dysfunction [28, 34]. ROS may oxidize lipids and proteins, block antioxidant enzymes, and damage DNA, which may result in a faulty cellular defense response [35]. The loss of membrane integrity can be caused by lipid peroxidation, which alters membrane fluidity and permeability and inactivates associated receptors and enzymes. Protein-protein crosslinks and the oxidation of amino acid side chains are two other ways in which ROS contributes to cellular chaos [28, 34]. Many studies have found that MTX enhances renal ROS, lipid peroxidation, and decreases GSH and antioxidant enzymes, such as SOD [31, 33, 34]. There is also evidence that direct and indirect mitochondrial damage is an important effector of nephrotoxicity. Damage to mitochondria from ROS leads to mitochondrial permeability transition pore formation, which in turn, disrupts oxidative phosphorylation and leads to the gradual depletion of ATP and, in extreme cases, cell death [36, 37]. Our data demonstrate that MTX treatment increased TBARS and decreased SOD and GSH levels in the kidney. Thus, protecting the cellular redox balance may be an effective strategy for preventing MTX-induced AKI.

Various medical conditions are linked with oxidative stress and AMO exhibits antioxidant effects [14, 38]. Thus, we inferred that the nephroprotective effect of AMO is associated with its antioxidant properties. Our findings indicate that mitochondrial activity is restored in MTX-treated rats through reduced ROS production and increased levels of cellular antioxidants. Thus, AMO protected cells against MTX-induced AKI by lowering oxidative stress and restoring antioxidant defense and mitochondrial function.

Not only does MTX-induced ROS generation lead to oxidative stress, but it can also activate other types of stress signalling like NF-kB. This, in turn, raises the expression of cytokines like TNF-α, IL-6, COX-2, and iNOS [27, 39]. Serum levels of TNF-α and IL-6 increase following MTX treatment, which indicates an inflammatory response. NF-kB controls the transcriptional expression of proinflammatory cytokines in response to glomerular damage and tubulointerstitial disorders [30, 31, 40]. An experimental study by Mahmoud and colleagues showed that the kidneys of MTX-treated animals exhibited activation of the reactive oxygen species/nuclear factor kappa B/NLRP3 inflammasome axis [27]. The anti-inflammatory effect of AMO against MTX-induced nephrotoxicity was evident by a reduction in circulating levels of proinflammatory cytokines/chemokines in MTX-treated rats; thus, inhibition of this ROS/NF-kB signaling pathway may be involved. Consistent with earlier reports [31, 41, 42], our results indicated that MTX induced a marked proinflammatory response as evidenced by the significant elevation in serum levels of inflammatory cytokines IL-6, ICAM-1, VCAM-1, and TNF-α. Importantly, AMO significantly reduced MTX-induced levels of IL-6, ICAM-1, VCAM-1 and TNF-α. These results are consistent with multiple studies that reported an inhibitory effect of AMO on various inflammatory cytokines/chemokines included in the present study [38]. The histological findings in MTX-treated rats mirrored the changes observed in biochemical parameters and they are in line with the earlier reports.

The present study found a correlation between elevated levels of reactive oxygen species (ROS) and inflammation and the occurrence of oxidative DNA damage. Additionally, the expression of caspase-3 was found to be upregulated. Bax promotes programmed cell death by causing the release of cytochrome c from the mitochondria, leading to the activation of caspases. Moreover, the excessive generation of reactive oxygen species (ROS) in mitochondria during the metabolism of MTX can harm the mitochondrial membrane. This damage leads to the disruption of membrane potential and the subsequent release of cytochrome c. Ultimately, this process triggers renal apoptosis by activating caspase-3 [35]. In this study, the kidneys of rats treated with MTX exhibited elevated levels of caspases-3, indicating the activation of apoptotic signaling. Thus, the activation of MAPK and the subsequent stimulation of NF-κB led to the production of inflammatory cytokines, which caused apoptosis in the kidneys of rats treated with MTX. The primary cause of MAPK/NF-κB signaling activation in the kidneys of MTX-induced rats can be attributed to the elevated generation of reactive oxygen species (ROS) [43]. Protecting against apoptosis can be achieved by inhibiting the development of

reactive oxygen species (ROS) and the production of proinflammatory cytokines induced by MTX. By administering AMO before MTX, the harmful effects of MTX-induced oxidative injury and inflammation were reduced. This was demonstrated by the decrease in caspases and the increase in the protective Bcl2 protein, which prevented apoptotic signaling. Similarly, prior research has demonstrated the induction of caspase-dependent apoptosis by various dihydropyridine CCBs [44-46].

PPARβ is a member of the nuclear receptor superfamily of ligand-activated transcription factors. It plays a crucial role in the regulation of various physiological processes, including lipid metabolism, glucose homeostasis, inflammation, and cell differentiation. PPARβ is expressed in a variety of tissues, and its role in kidney function has been a subject of interest in recent research [47, 48].

In the context of kidney function, PPARβ has been implicated in renal lipid metabolism, inflammation, and fibrosis. Dysfunction in these processes can contribute to the development and progression of chronic kidney disease (CKD) and other renal disorders. Understanding the role of PPARβ in kidney function has potential implications for the treatment of such conditions [48, 49].

Platelet PPARβ expression can be increased by using calcium channel blockers (CCBs) such nifedipine. Letavernier *et al*. found that PPARβ/δ can protect the kidney from I/R injury by boosting the proliferation of tubular epithelial cells and activating the antiapoptotic Akt signalling pathway [50]. Thus, we conducted experiments to evaluate MTX's effects, and our results corroborated those of Letavernier *et al*. research has shown that diabetic kidneys treated with PPARβ/δ agonists have less renal failure, leukocyte infiltration, and IL-6 and TNF-α production after I/R injury. In models of ischemia/reperfusion (I/R) injury, the expression of suppressor of cytokine signaling-3 (SOCS-3), an important participant in the signalling pathway activated by cytokines, was increased after treatment with a PPARβ/δ agonist [51, 52]. Our research shows that AMO therapy increases PPARβ/δ expression, which in turn decreases kidney injury by reducing inflammatory and immunological responses. The findings corroborate the study by Yang *et al*. which demonstrated that administering the PPARβ agonist GW501516 to mice in a nephropathy model led to less severe tubulointerstitial lesions, decreased macrophage infiltration, and decreased synthesis of monocyte chemotactic protein (MCP-1) and TNFα mRNA. To do this, the TGF-β activated kinase (TAK1)-NF-κB pathway was directly inhibited [53, 54].

Conclusion

Collectively, MTX reduces the expression of PPARβ, potentially caused by the stimulation of oxidative stress. Enhanced expression of NF-κB leads to an upsurge in proinflammatory markers, including ICAM-1, IL-6, VCAM-1, and TNF-α. The findings of our study suggest that AMO protects rats treated with MTX by reducing renal oxidative damage, inflammation, and apoptosis. This protection is likely achieved by improving the activity of PPARβ, while also reducing caspase 3 and NF-κB induced renal changes.

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