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Original Article

Evaluating the effects of different doses of fimasartan on methotrexate-induced renal inflammation in rats

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ABSTRACT

The nephrotoxicity induced by methotrexate is a severe condition that greatly affects its therapeutic potential and has a significant inflammatory component. Fimasartan is an angiotensin receptor blocker that offers organ-protective effects and may be useful in mitigating renal injury. The present study explored the anti-inflammatory potential of two doses of fimasartan against methotrexate-mediated nephrotoxicity. Albino rats were intraperitoneally administered a single methotrexate (20 mg/kg). Intraperitoneal treatment with fimasartan (5 or 10 mg/kg/day) was initiated on day two after methotrexate injection and continued for seven consecutive days. Methotrexate significantly increased serum urea, creatinine, and NGAL concentrations. It also substantially elevates the proinflammatory cytokines (namely, tumour necrosis factor-alpha, interleukin-1 beta, and interleukin-6) levels while reducing renal tissue's immunomodulatory (interleukin-10) levels. Treatment with both doses of fimasartan significantly restored renal function parameters, lowered the renal concentration of proinflammatory cytokines, and upregulated the renal concentration of the anti-inflammatory mediator interleukin-10. The high fimasartan dose resulted in more pronounced effects on the inflammatory parameters. The obtained data suggested that fimasartan effectively mitigates methotrexate-induced nephrotoxicity by inhibiting inflammation in renal tissue in a dose-dependent manner.

Keywords: Methotrexate, Nephrotoxicity, Inflammation, Fimasartan

Introduction

Acute kidney injury (AKI) is a problematic worldwide condition accompanied by the establishment and progression of chronic kidney disease (CKD) and is linked to clinically significant morbidity and mortality [1]. Notably, it was estimated that (19–26) % of all AKI-reported cases among adult hospitalized patients were induced by prescription drugs [2]. Among the medications that were found to be inherently nephrotoxic is methotrexate (MTX) [3]. Methotrexate is a chemotherapeutic agent and immunosuppressant widely used over a wide dosage range to treat various malignancies and autoimmune disorders [4, 5]. Despite high efficacy, nephrotoxicity remains a serious problem, impacting about 12 % of MTX-administered patients, especially in patients with malignant tumours receiving high doses of the drug. In addition to the possibility of progression to CKD or the development of other severe systemic toxicities, the interruption of therapy due to the development of AKI may worsen the prognosis of the baseline disease [2, 6-8]. Thus, MTX-induced nephrotoxicity is clinically important, and appropriate interventions are required to alleviate its harmful effects.

Methotrexate-induced AKI has been linked to inflammation and is assumed to arise mainly through crystal nephropathy, mediated by intratubular precipitation of MTX and its metabolite, leading to obstruction of renal tubules. Subsequently, interstitial

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inflammation may develop, which can progress to tubular necrosis, initiating an innate immune reaction and complex series of events that create a vicious cycle of cell death-inflammation. Moreover, direct tubular cytotoxic injury has also been reported and mediated through the uptake of MTX and its metabolite into renal tubular cells with subsequent mitochondrial dysfunction, oxidative stress, and inflammation in the renal microenvironment [4, 9, 10]. Furthermore, disturbance in the kidney's local renin-angiotensin-aldosterone system (RAAS) can provoke many cellular events, contributing to renal tissue inflammation and fibrosis [11]. Angiotensin II (Ang-II), a chief element of RAAS, can facilitate such effects by activating the angiotensin II type 1 receptor (AT1R) [12], and studies have reported that tissue expression of AT1R becomes upregulated in stressful conditions [11, 13, 14]. When bound to the AT1R, Ang-II can provoke an inflammatory cascade by inducing nicotinamide adenine dinucleotide phosphate (NADPH) oxidases with a resultant reactive oxygen species (ROS) overproduction [13, 15]. It has been reported that all elements of RAAS can be locally generated in the kidney, and the renally produced Ang-II is about 1,000 times higher than the circulating levels [16]. Since inflammation was known to be a key player in the nephrotoxicity induced by MTX, Ang-II-mediated signaling may be an important player in disease progression due to its prooxidative, proinflammatory, and proapoptotic potential [4, 16]. Accordingly, attempts to curb the inflammatory reactions via RAAS blockade may be of value in the mitigation of renal injury and, thus, the progression to CKD.

There is a growing interest in RAAS blocking agents, including angiotensin II receptor blockers (ARBs), regarding their favorable effects on renal inflammation in the context of AKI, partly because they are widely used antihypertensives [17]. In addition, several human as well as animal studies have shown that ARBs effectively alleviate the progression of renal disease [14, 18-20]. Fimasartan (FMS), the ninth-developed ARB, represents a good candidate to inhibit RAAS activity since it selectively and potently blocks the coupling of Ang-II to AT1R [21]. It has been developed in South Korea and approved for hypertension and heart failure. Along with its excellent efficacy and strong affinity for AT1R, FMS has good tolerability, is available at a convenient cost, and is primarily excreted via the bile [22]. Experimental data confirmed that FMS exerts organ-protecting effects independent of its effects on blood pressure, and previous studies elucidated its nephroprotective efficiency as an antioxidant against renal injury [14, 23]. Thus, it represents an attractive candidate to be examined for the possible amelioration of the deleterious inflammatory effects of MTX on renal tissue. In light of the above, the current study was intended to explore the anti-inflammatory potentials of two FMS dosages on MTX-induced AKI in a rat model.

Materials and Methods

Chemicals and reagents

Fimasartan was purchased from Novachemistry (Loughborough, UK). An injectable form of MTX (50mg/2ml) was obtained from Mylan (Saint-Priest, France). Dimethyl sulfoxide (DMSO) from Thomas Baker Chemicals (Mumbai, India), phosphate-buffered saline (PBS) from EuroClone (Milan, Italy), and diethyl ether from Romil (Cambridge, UK) were utilized as well. Solutions of 0.2% fimasartan potassium trihydrate were prepared by dissolving the drug in a 4% solution of DMSO [24]. Appropriate mixing of the prepared solutions by a vortex mixer (Labinco BV, Netherlands) was done to ensure adequate drug dissolution. Rat creatinine and urea kits were purchased from BIOLABO SAS (Maizy, France). All kits of the enzyme-linked immunosorbent assay (ELISA) that have been used were acquired from MyBioSource (California, USA) and included ELISA kits for the following biomarkers: rat tumour necrosis factor-alpha (TNF-α), rat interleukin-1 beta (IL-1β), rat interleukin-6 (IL-6), and rat interleukin-10 (IL-10); the only exception was the neutrophil gelatinase-associated lipocalin (NGAL) ELISA kit, which was obtained from Boster Biological Technology (Pleasanton CA, USA).

Experimental animals

Adult Wistar rats, males and females, within a weight range of 180-240 g, were utilized in the study. The rats had been supplied by and kept within the Laboratory Animal House Unit in the College of Pharmacy-University of Baghdad under controlled conditions of temperature, humidity, and 12-hour light/dark cycles and given access to standard pelleted diets and water *ad libitum*. All tested rats were acclimatized for a week in the above-stated conditions before the experiment. The study procedures comply with the international guidelines on preclinical experimentation.

Experimental design

The present study was done after approval by the Scientific and Research Ethics Committees of the University of Baghdad-College of Pharmacy. Thirty-six adult Wistar rats (males and females) were allocated randomly into five groups (n=6). Rats in the control group received vehicle only (4% DMSO) by intraperitoneal (IP) injection for seven consecutive days, starting on day 1 [24]. Nephrotoxicity was induced in the model (MTX) group by treating the animals with a single injection of MTX (20 mg/kg, IP) on the first day [3], followed by vehicle administration (4% DMSO, IP) once daily for seven successive days, started on day 2. Rats in the (FMS10) group received once-daily injections of FMS (10 mg/kg, IP) for seven consecutive days [15]. The treatment groups, (FMS5+MTX) and (FMS10+MTX), were administered on day 1 with a single injection of MTX (20 mg/kg, IP); they were then injected intraperitoneally with FMS (5 mg/kg and 10 mg/kg), respectively, daily for seven consecutive days starting on day 2 [15, 25].

Sample collection

Twenty-four hours after the last administered dose, blood was collected from the jugular vein under diethyl ether anaesthesia. Then, cervical dislocation using diethyl ether anaesthesia was done for all tested animals, and the kidney samples were excised and processed for subsequent analysis [26, 27].

The obtained blood samples were allowed to stand for thirty minutes after collection. The clotted samples were then centrifuged for 10 minutes at 3200 rpm in an EBA20® centrifuge (Andreas Hettich GmbH & Co. KG, Germany), and aliquots of supernatant were obtained and stored at −20°C to be analyzed for urea, creatinine, and NGAL [27, 28].

Also, after sacrifice, the right kidneys were rapidly excised from each rat to prepare renal tissue homogenate. First, they were cleaned, washed with PBS, blotted onto filter paper, chopped into fine pieces, and then mixed with PBS (pH=7.4, precooled) to prepare a 10% renal-tissue homogenate as described previously and following the manufacturer's instructions [23]. After 10 minutes of centrifugation of the homogenized mixture at 10,000 rpm in the Hermle Labortechnik GmbH's refrigerated centrifuge (Wehingen, Germany), the resultant clear supernatants were obtained instantly, and aliquots were stored at −20°C for determination of TNF-α, IL-1β, IL-6, and IL-10 levels.

Serum biochemical analysis

Amounts of creatinine and urea in serum samples were determined spectrophotometrically (SEMCO S/E-UV spectrophotometer) using reagent kits as stated by the manufacturer's instructions [29]. Moreover, the concentration of serum NGAL was quantified by the sandwich ELISA method following the manufacturer's instructions [27].

Assay of inflammatory markers in renal tissue

Concentrations of the proinflammatory TNF-α, IL-1β, IL-6, and the immunomodulatory IL-10 cytokines in kidney homogenate samples were quantified via ELISA methods according to the kit manufacturer's instructions. Absorbance at 450 nm was determined by a HumaReader HS® plate reader (Human Diagnostics, Wiesbaden, Germany), which is proportional to the cytokine concentration in the sample [30]. The obtained values were expressed as picograms per millilitre (pg/ml).

Statistical analysis

The expressed values represent the mean ± standard deviation (SD), and data were analyzed by the Statistical Package for Social Sciences (SPSS) Statistics. Comparisons were made utilizing the unpaired Student's *t*-test; the *p*-values of less than 0.05 ($p<0.05$) were considered significantly different.

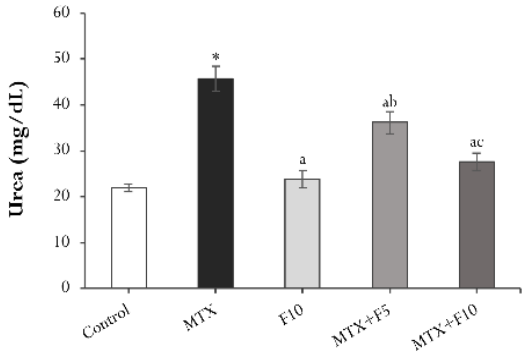
Results and Discussion

Effect of fimasartan on kidney function and injury

To assess kidney function and injury, serum creatinine, urea, and NGAL levels were determined. Methotrexate-only treated rats showed a significant ($p<0.05$) elevation in serum urea and creatinine when compared to rats in the control group **(Figures 1a and 1b)**. Similarly, MTX administration induced significantly augmented ($p<0.05$) serum NGAL levels compared to control **(Figure 1c)**. Rats treated with FMS only showed a nonsignificant difference ($p>0.05$) from the control rats **(Figures 1a-1c)**. However, treatment with both doses of FMS (5 mg/kg or 10 mg/kg) exhibited significantly ($p<0.05$) decreased levels of creatinine, urea, and NGAL as compared to the MTX group **(Figure 1)**. Interestingly, a significant ($P<0.05$) difference in urea and NGAL levels can be observed in rats that received the low FMS dose (5 mg /kg) as compared to the high FMS dose (10 mg/kg). On the other hand, both FMS doses similarly reduced serum creatinine, with a nonsignificant difference ($p>0.05$) between both FMS-treated groups **(Figures 1a-1c)**.

Effect of fimasartan on renal tissue inflammation

To evaluate the inflammatory response after renal injury, concentrations of the cytokines TNF-α, IL-1β, IL-6, and IL-10 in renal tissue homogenates were assessed, and the obtained results were respectively displayed in **(Figures 2a-2d)**. Fimasartan treatment alone did not significantly alter any of the inflammatory markers being tested ($p>0.05$). In contrast, MTX triggered a dramatic elevation ($p<0.05$) in the proinflammatory cytokines (namely TNF-α, IL-1β, and IL-6) in comparison to control. Treatment of rats with both doses of FMS (5 mg/kg/day or 10 mg/kg/day) revealed significantly ($p<0.05$) lowered concentrations of renal TNF-α, IL-1β, and IL-6 in contrast to the MTX-only rats. Notably, FMS at a dosage of 10 mg/kg following MTX treatment displayed a more significant ($p<0.05$) decrease in the levels of these cytokines compared with the FMS 5 mg/kg dose. On the other hand, MTX injections significantly ($p<0.05$) decreased IL-10 concentration when compared to animals in the control. Treating rats with FMS significantly ($p<0.05$) upsurges the amount of this anti-inflammatory cytokine in renal tissue. Furthermore, when the FMS dose was doubled to 10 mg/kg, significantly ($p<0.05$) increased levels of renal IL-10 were achieved compared to the low (5 mg/kg) FMS dose **(Figure 2d)**.



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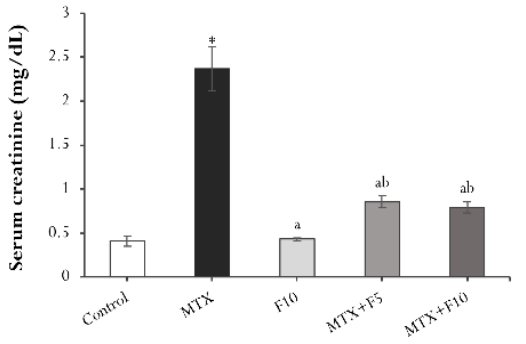

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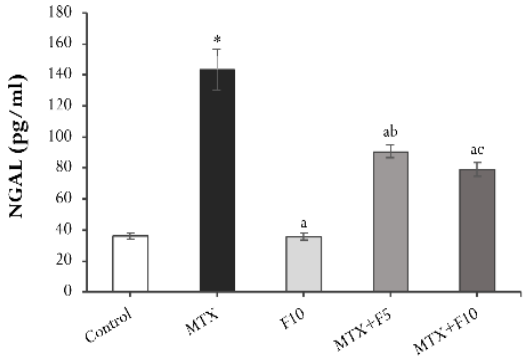
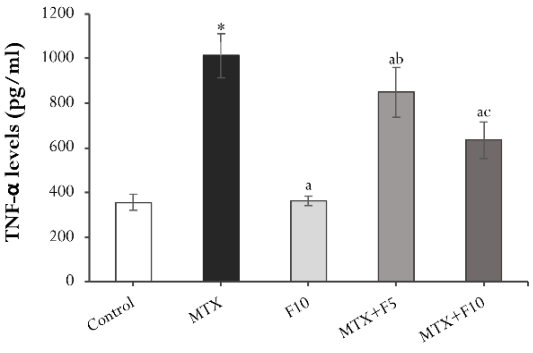


c)

Figure 1. Effects of 5 mg/kg and 10 mg/kg doses of FMS on (a) serum urea; (b) serum creatinine; and (c) serum NGAL in MTX-induced AKI of rats. All displayed data represent (mean ± SD), n=6. The superscripts designates the following:
[*] P<0.05 compared to normal control rats; [a] P<0.05 compared to the MTX-only group; nonidentical small letters [b and c] referred to a P <0.05 between the treatment groups.



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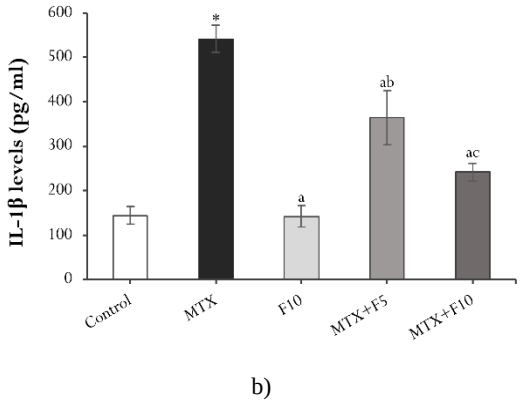

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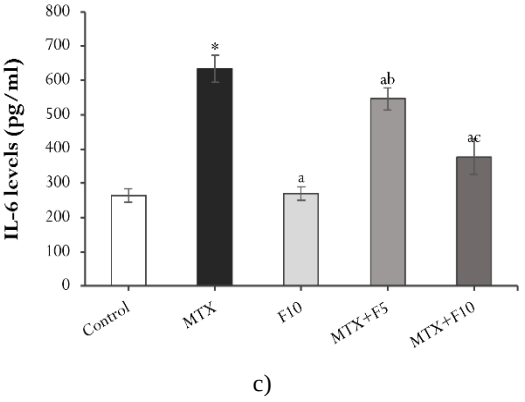

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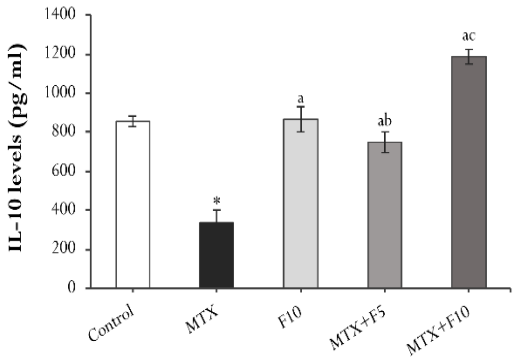

d)

Figure 2. Effects of FMS (5 and 10 mg/kg) on renal-tissue concentration of (a) TNF-α; (b) IL-1β; (c) IL-6; and (d) IL-10, respectively, in MTX-mediated nephrotoxicity. All values were presented as (mean ± SD), n=6. The superscripts designates the following:
[*] P<0.05 compared to normal control rats; [a] P<0.05 compared to the MTX-only group; nonidentical small letters [b and c] referred to a P <0.05 between the treatment groups.

Methotrexate-induced nephrotoxicity is a debilitating pathology with various causal factors, among which is inflammation [9, 31]. In addition to conventional supportive care, appropriate interventions are required to mitigate renal injury and improve survival [3]. Growing evidence has indicated that treatment with anti-inflammatory agents exhibited beneficial effects in such conditions [4, 32, 33]. Since locally produced Ang-II in renal tissue was linked to the activation of multiple signaling pathways that promote inflammation, blocking RAAS signaling with the ARB, fimasartan, maybe a rational mitigation strategy to be tested. The current study highlights the role of two doses of FMS on renal inflammation, contributing to MTX-induced AKI in rats.

The study revealed that renal injury was induced upon injection of rats with MTX, as evidenced by the increase in serum urea and creatinine, the traditional biomarkers of renal dysfunction [34, 35]. Besides, the data confirmed a dramatic elevation in NGAL, a 25 kDa neutrophil-related protein whose upregulated expression in renal tubular epithelia was found to be involved in mediating inflammation during acute renal injury. Since NGAL elevation reflects renal damage and infiltration by neutrophils, it can predict kidney dysfunction and survival [11, 27]. The direct renal damaging effect of MTX and reduced glomerular filtration may explain the dramatic increase in serum NGAL [27]. These outcomes are consistent with previous studies, where MTX in 20 mg/kg dosage was recognized to cause a significant kidney injury in rats [4, 9, 27]. Likewise, MTX therapy was previously reported to give rise to uremia and a characteristic abrupt elevation in serum creatinine levels when administered in high doses to patients with malignancies [7].

Importantly, the study revealed that kidney damage mediated by MTX was accompanied by upregulation of the proinflammatory biomarkers TNF-α, IL-1β, and IL-6, along with downregulation of IL-10, which acts as an anti-inflammatory. This correlates well with previous preclinical research of MTX-induced AKI, which revealed that the activated nuclear factor kappa B (NF-κB) signaling due to ROS overproduction contributes to intensified renal inflammatory reactions [3, 9]. In response to stress, tissue-resident and circulating immune cells drive an inflammatory response by interacting with vascular endothelial and parenchymal cells in renal tissue. Increased levels of proinflammatory cytokines have been linked to the activation of a multiprotein oligomer known as the NLR pyrin domain-containing protein 3 (NLRP3) inflammasome, promoting the expression and maturation of numerous proinflammatory cytokines, including IL-1β. The activation of NLRP3 inflammasome within the activated immune and tissue-resident cells was suggested to arise through the engulfment of MTX crystals deposited in the kidney by these cells—especially macrophages and dendritic cells—as well as through the ROS-mediated activation of the transcription factor NF-κB [3, 36]. IL-1β, IL-6, and other cytokines can then lead to cellular dysfunction and promote pyroptosis, a catastrophic inflammatory mode of programmed cell death, and thus may contribute to the progression of disease [3].

Furthermore, elevated TNF-α levels enhance various vasoactive mediators, which may diminish renal blood flow and glomerular filtration, and are known to activate caspase, resulting in apoptosis [15]. On the other hand, reduction in secreted levels of IL-10 after MTX-induced toxic insult can be linked to the upregulated expression of NF-κβ and may reflect reduced production by T-regulatory (Treg) lymphocytes, which generate IL-10 upon stimulation by kidney-resident dendritic cells in an attempt to limit inflammation and offer renoprotection [11, 37]. IL-10 potent immunomodulatory effects may be explained by its inhibitory effect on the genes involved in leukocyte activation and adhesion coupled with its inhibitory action on the secretion of proinflammatory cytokines, among which are TNF-α and IL-1β [37]. The resultant inflammatory response principally aimed to protect from the damaging effects of ROS and uremic toxins may lead to irreversible injury and organ failure unless appropriately managed [31]. The resolution or the progression of the resultant AKI is determined by the extent of cell death, which largely relies on an intricate balance between proinflammatory and anti-inflammatory reactions. Acutely elevated levels of cytokines post-injury can modulate the adaptive immune response, which could augment and sustain the inflammatory responses and culminate in renal fibrosis, a common outcome of kidney disease. Conversely, it has been established that surviving tubular epithelial cells have regeneration potential, i.e., they can proliferate and replace damaged cells following acute renal injury. Importantly, unresolved inflammation was linked to maladaptive renal repair after injury, with abnormal tissue remodeling and dysfunction. Therefore, minimizing injury is an important approach to prevent severe forms of AKI [10].

In the study, treating rats with FMS attenuated renal MTX injury, as revealed by the dramatic drop in uremia and creatinine levels accompanied by a decline in the sensitive renal tubular injury marker, NGAL, indicating the beneficial effects of FMS on kidney function and structure after MTX injury. Of note, the reduction of serum urea and NGAL levels in the 10 mg/kg FMS-treated rats was more pronounced than in the 5 mg/kg FMS group. High-dose FMS also effectively reduced the MTX-induced upregulation in all proinflammatory cytokines measured in renal tissue (i.e., IL-1β, IL-6, and TNF-α). The results agreed with earlier reports, which demonstrated the ability of FMS to ameliorate renal dysfunction and inflammatory injury in both obstructive [14] and ischemia-reperfusion injury [15] models of AKI. In parallel, fimasartan's anti-inflammatory effects were also found to be responsible for stabilizing atherosclerotic plaques [21], mitigating neuronal injury induced by intracerebral haemorrhage in rats [38] and the hyperplastic changes in carotid arteries' neointima of mice. These effects were attributed to the blockade of AT1R signaling by FMS, which confers antioxidant, anti-inflammatory, and antiapoptotic activity [22]. Based on available data, reduced production of proinflammatory cytokines in the study could be linked to the ability of FMS to reduce renal oxidative stress via suppression of NADPH oxidases and upregulation of Nrf2 signaling, thereby inhibiting ROS-mediated activation of NFκB/ NLRP3 inflammasome signaling, with resultant ameliorative effect on inflammation and apoptosis in renal tissue of MTX-treated rats [14, 15, 38].

On the other hand, elevated levels of IL-10 can be attributed to FMS's ability to upregulate renal Treg lymphocyte expression, promoting anti-inflammatory signaling in affected tissue [21]. It should be mentioned that in previous work, we have verified that a 3 mg/kg dose of FMS exerted ameliorative effects against MTX-induced renal injury via restoration of renal antioxidant defences, which was in line with the present study and coincided with previous reports regarding ARB's nephroprotective potentials [23]. Both FMS dosages utilized in the study effectively ameliorated proinflammatory cytokines and kidney injury markers. However, the high FMS dose was more effective in reducing proinflammatory cytokines, NGAL, and uremia and

upregulating the anti-inflammatory mediator IL-10. Such results could be attributed to the dose-dependent response to acute inflammation in the kidney or to sufficient amount of FMS available near its intrarenal target that is required to counteract the negative effects of MTX.

Undeniably, previous investigations indicated that ARBs can modulate the immune response directly and provide renoprotection via multiple mechanisms independent of its hemodynamic function [11, 15, 18], including reduction of intrarenal Ang-II and angiotensinogen (AGT) as well as lowering renin generation in collecting ducts [12]. Moreover, it has been reported that angiotensin (1–7), abbreviated Ang-(1–7), can neutralize many effects of the Ang-II/AT1R Signaling, and AT1R blockade by ARBs may shift the synthesis pathway toward Ang-(1–7) production from Ang-II by angiotensin-converting enzyme 2 (ACE2), contributing to their renal protective potential [39].

Conclusion

In conclusion, treatment with FMS significantly ameliorated renal injury in MTX-mediated nephrotoxicity in rats. Fimasartan's beneficial effects were found to be dose-dependent and mediated by its anti-inflammatory effects. However, further studies are required to provide more detailed mechanistic insights and validate the safety and efficacy of this promising drug.

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Conflict of interest: None

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Ethics statement: All experiments were conducted after being approved by the Research Ethics Committee at the University of Baghdad - College of Pharmacy.

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