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Effect of Infliximab on Renal Injury Due to Methotrexate in Rat

Aynur Kirbas,[1] Medine Cumhur Cure,[1] Yildiray Kalkan,[2] Erkan Cure,[3] Levent Tumkaya,[2] Osman Zikrullah Sahin,[4] Suleyman Yuce,[3] Bayram Kizilkaya,3 Ahmet Pergel[5]

1Department of Biochemistry, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
2Department of Histology and Embryology, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
3Department of Internal Medicine, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
4Department of Nephrology, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
5Department of Surgery, School of Medicine, Recep Tayyip Erdogan University, Rize, Turkey

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Introduction. Methotrexate, an antagonist of folic acid used in the treatment of many cancers and inflammatory diseases, is associated with side effects that limit its usage. Infliximab has been reported to have a protective effect against nephrotoxicity induced by some drugs and ischemic reperfusion. We aimed to investigate whether infliximab has a protective effect against methotrexate-induced nephrotoxicity.

Materials and Methods. We administered methotrexate at a dose of 20 mg/kg as a single intraperitoneal injection in 10 rats (methotrexate group). Another group of 10 rats received a single dose of infliximab, 7 mg/kg, intraperitoneally (infliximab group). The methotrexate and infliximab group received a similar single injection of infliximab 72 hours prior to methotrexate injection. After 72 hours a single dose of methotrexate, 20 mg/kg, was administered intraperitoneally. Five days after methotrexate injection, blood samples were collected and the kidney tissues were removed for biochemical and histological examination.

Results. The methotrexate group had significantly higher tissue levels of tumor necrosis factor-α ($P = .008$), interleukin-1β ($P = .036$), nitric oxide ($P < .001$), and adenosine deaminase ($P < .001$) than the methotrexate and infliximab group after the 5-day study. The methotrexate group also had significantly higher total histological scores ($P < .001$) and carbonic anhydrase-II activity ($P < .001$) when compared to the methotrexate and infliximab group.

Conclusions. Infliximab has a strong protective effect against methotrexate-induced nephrotoxicity by suppressing cytokines release. It may decrease methotrexate-induced nephrotoxicity by regulating carbonic anhydrase-II enzyme activities and slowing down purine metabolism.

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INTRODUCTION

Methotrexate is a folic acid antagonist that blocks the synthesis of purine and pyrimidine. Generally, it is used in the treatment of many malignancies and diverse autoimmune diseases such as rheumatoid arthritis or psoriasis due to its anti-inflammatory and immunosuppressive effects.[1] Side effects such as nephrotoxicity and hepatotoxicity are seen frequently during methotrexate treatment.[2] The formation of reactive oxygen species (ROS) and the release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β are considered to be responsible for methotrexate induced nephrotoxicity.[3-5] Nitric oxide (NO) is extremely an important substance for the organism. Methotrexate toxicity increases NO in the tissues, leading to generation of peroxynitrite radicals that damage the cell.[6,7] Additionally, methotrexate leads

to ROS formation by increasing purine metabolism turnover.[8] Adenosine deaminase (ADA) activity of the enzyme is a good indicator of purine metabolism intermediates and increased ROS.[9]

Infliximab is a chimerical anti-TNF-α monoclonal antibody. It blocks the interaction of this cytokine inducing lyses of the TNF-α producing cell.[10] It is widely used for the treatment of various inflammatory diseases.[11] Infliximab has been shown to decrease the damage of ischemia reperfusion of the kidney, liver, and intestine by inhibiting TNF-α. It decreases cell injury caused by increasing both tissue injury stimulant cytokines and ROS formation by inhibiting TNF-α.[12-14] Carbonic anhydrase (CA), which is found in many tissues especially renal tubules, is an important enzyme involved in acid-base buffering.[15] Carbonic anhydrase-II is one of the 11 CA subtypes, which is a cytosolic enzyme that is found in abundance in the renal tubules.[16] It catalyzes the production of hydrogen and bicarbonate ions in the reversible reaction between carbon dioxide and water. Carbonic anhydrase-II level is in equilibrium in the organism. While elevated CA-II levels increase bicarbonate and carbonate radical formation, its decreased activity increased hydrogen ion leading to metabolic acidosis and cellular injury.[17-19] The deficiency of this enzyme due to an autosomal recessive disorder or its suppression by some drugs leads to renal tubular acidosis. Some solid and hematologic cancers may lead to overexpression of CA-II which increases bicarbonate production that is used by tumor cells.[16]

In this study, we aimed to investigate whether infliximab has a protective effect against methotrexate-induced nephrotoxicity and to investigate TNF-α, IL-1β, ADA, and NO tissue levels in addition to the histopathological examination.

MATERIALS AND METHODS

Animals

We used 40 Wistar-albino male rats aged 12 to 15 weeks old, weighing 250 g to 300 g. They were randomly divided into 4 groups: control group (n = 10), methotrexate group (n = 10), infliximab group (n = 10), and methotrexate and infliximab group (n = 10). This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 1985), and approved by the local ethics committee (Approval No, 2012/24).

Experimental Design

The control group received an intraperitoneal injection of only isotonic saline solution that was equal to methotrexate volume. The animals of the methotrexate group received an intraperitoneal single dose of methotrexate, 20 mg/kg (Emthexate-s, 50-mg ampule), and were sacrificed 5 days after the injection.[20] The animals of the infliximab group received only 1 single dose of infliximab, 7 mg/kg (Remicade), injected intraperitoneally.[21] The animals of the methotrexate and infliximab group received 1 single dose of infliximab, 7 mg/kg, and 3 days later, a single dose of methotrexate, 20 mg/kg, both administered intraperitoneally. All of the animals were sacrificed 5 days after methotrexate injection. All of the groups were sacrificed after being anesthetized with ketamine hydrochloride (Ketalar, 50 mg/kg, intramuscularly; Parke-Davis Eczacibasi, Istanbul, Turkey). The kidney tissues were then removed and stored at -80°C until analysis.

Biochemical Parameters

Blood samples of 10 mL were drawn from all the rats for biochemical tests evaluation. After standing at room temperature for 15 minutes, the samples were centrifuged at 3000 rpm for 10 minutes. The biochemical parameters were checked in the serum by using commercial kits (Architect c16000, Abbott Laboratories, IL, USA).

Tissue Homogenates

The tissue samples were homogenized in phosphate-buffered saline at a pH of 7.4 and centrifuged at 10 000 g for 20 minutes. Aliquots of the supernatant were put into tubes and were frozen at -80°C.

Measurement of Protein

A turbidimetric procedure was used for the tissue homogenate protein assay by applying benzethonium chloride as a protein denaturing agent. Then the proteins in a fine suspension form were quantitated turbidimetrically at 404 ηm (Architect c16000, Abbott Laboratories, IL, USA).

Tumor Necrosis Factor-α, Interleukin-1β, Nitric Oxide, and Adenosine Deaminase

Tumor necrosis factor-α level was measured using a commercially available rat TNF-α enzyme-linked immunosorbent assay kit (eBioscience,

Vienna, Austria). Interleukin-1β level was also measured using a commercially available rat IL-1β enzyme-linked immunosorbent assay kit (eBioscience, Vienna, Austria). The levels of NO were measured using the colorimetric assay method via a commercially available NO kit (Cayman Chemical Company, USA). Adenosine deaminase level was measured using an enzyme-linked immunosorbent assay method via a commercially available kit (Cusabio Biotech Co, China).

Immunohistological Evaluation

Before being immunohistochemically stained, the samples were cut into sections of 3-μm to 4-μm thickness and kept in xylene for 20 minutes before applying alcohol series (50% to 100%). Then they stand for 10 minutes in hydrogen peroxide solution. The sections were washed by phosphate-buffered saline before being heated for 4 to 5 minutes in a citrate buffer solution by 800-Watt power. Then they were allowed to stay in a secondary blocker substance for 20 minutes. The slides were put in different dilutions of primary antibody (CA-II 1/250-/500) for 75 minutes before being stained by anti-CA-II (cod: ab124687, Abcam plc, Cambridge, UK). We used diaminobenzidine solution as an achromogen, and Mayer's hematoxylin as a counterstain for 3 to 5 minutes. Phosphate-buffered saline was used as a negative control. All preparations were photographed after covering them with appropriate covering materials. After the immunohistochemical staining, the preparations were divided into 4 categories according to tissue percentage of immunopositive reaction regions as mild (1+), moderate (2+), severe (3+), and very severe (4+). To prepare the preparations for histopathological investigation, the blocked tissues were cut into sections of 4-μm to 5-μm thickness before applying hematoxylin-eosin stain. The convenient regions for histopathological evaluation were photographed. All the preparations were blindly appraised by 2 histologists.

Statistical Analysis

The results were reported as the means ± standard deviation. We used the SPSS software (Statistical Package for the Social Sciences, version 13.1, SPSS Inc, Chicago, Ill, USA) to perform data analysis. The 1-way analysis of variance, followed by the Bonferroni analyses, was performed to analyze the biochemical parameters. The comparison of histopathologic parameters analyses were performed using the Kruskal-Wallis test. We used the Bonferroni adjusted Mann-Whitney U test to compare the two groups. *P* values less than .05 were considered significant.

RESULTS

The levels of urea and creatinine of the methotrexate group were significantly higher than those of the methotrexate and infliximab group, the infliximab group, and the control group. The tissue levels of TNF-α, IL-1β, NO and ADA of the methotrexate group were significantly higher than those of the other groups. The NO and ADA levels of the methotrexate and infliximab group were higher than those of the infliximab and the control groups. All biochemical results are shown in Table 1.

The control group was observed to have normal

Table 1. Biochemical Measurements in the Study Groups

Parameter	Control	Methotrexate	Methotrexate and Infliximab	Infliximab
Serum				
Urea	38.7 ± 4.4	59.5 ± 10.0†	50.1 ± 11.2¶†	40.1 ± 3.5§#
Creatinine	0.4 ± 0.0	0.9 ± 0.2†	0.6 ± 0.3§‡	0.4 ± 0.04§$
Tissue				
TNF-α	698.2 ± 126.5	892.0 ± 127.9†	743.7 ± 103.4§	679.2 ± 111.6§
IL-1β	330.0 ± 57.2	410.0 ± 57.5†	355.4 ± 53.3¶	313.4 ± 56.5§
Nitric oxide	1.3 ± 0.3	6.8 ± 1.1†	5.5 ± 0.7§†	1.4 ± 0.3§#
ADA	30.4 ± 4.5	46.0 ± 4.3†	39.1 ± 5.2§†	32.2 ± 3.6§#

*TNF-α indicates tumor necrosis factor-α; IL-1β, interleukin-1β; and ADA, adenosine deaminase.
†*P* < .01 compared to the control group
‡*P* < .05 compared to the control group
§*P* < .01 compared to the methotrexate group
¶*P* < .05 compared to the methotrexate group
#*P* < .01 compared to the methotrexate and infliximab group
$*P* < .05 compared to the methotrexate and infliximab group

histological cell morphology (Figures 1 and 2). An intensive deformation of epithelial cell structures of both proximal and distal tubules was observed in the methotrexate group. Particularly in the surroundings of glomeruli, there were intensive degenerative structures related to the swelling of epithelial cells of the proximal tubules and there were cellular shedding due to the dilatations of the lumen and edematous fluid (Figures 1 and 2). While there was an increase in the connective tissue and edema in the cortex-medulla border region, the amount of leukocytes was also increased. Particularly, in the regions close to the glomeruli, the shedding of epithelium of the distal tubules was obvious and the tubules lumens were observed to be dilated due to cellular vacuolization and deformations. Distortion was detected in the structure of the glomeruli and there was an increase in the number of shrunken glomeruli with inwardly curved edges. There was swelling of the Bowman capsule and the visceral cells in addition to hemorrhage and vessels dilatations. Filtration space was more dilated than in the control group and this dilatation was observed in all the glomeruli. The deformation of the cells of the proximal tubules was observed to have eosinophilic stain. The cells had no degeneration but undergoing apoptotic changes were detected to be intensely eosinophilic infiltration (Figures 1 and 2).

Tissue and cellular deformations of the methotrexate and infliximab group were observed to be low in comparison to the methotrexate group (Figures 1 and 2). While there were lower cellular degeneration and shedding of the proximal tubules epithelium, their nuclei had scattered basophilic stain. In the distal tubules, the dilatations were observed to be lower; however, their lumens were dilated and had scattered epithelial shedding with edematous fluid when compared with the control group. While the connective tissue of the

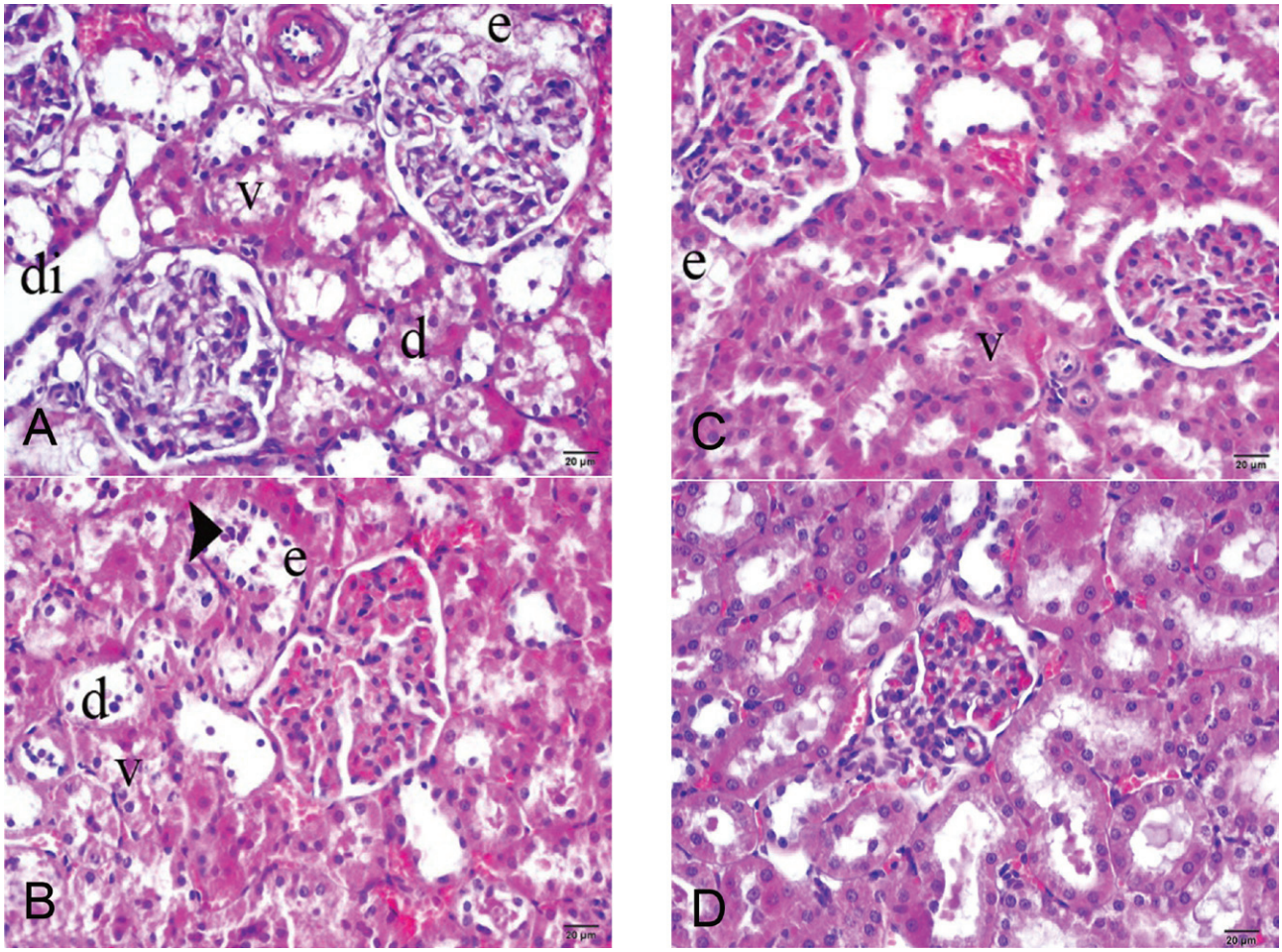

Figure 1. Histopathologic examination of kidney tissue by light microscopy (hematoxylin-eosin). **A,** Methotrexate group. **B,** Methotrexate and infliximab group. **C,** Infliximab group. **D,** Control group. Di indicates dilatation; d, degenerative cell; e, edema; and v, vacuolization.

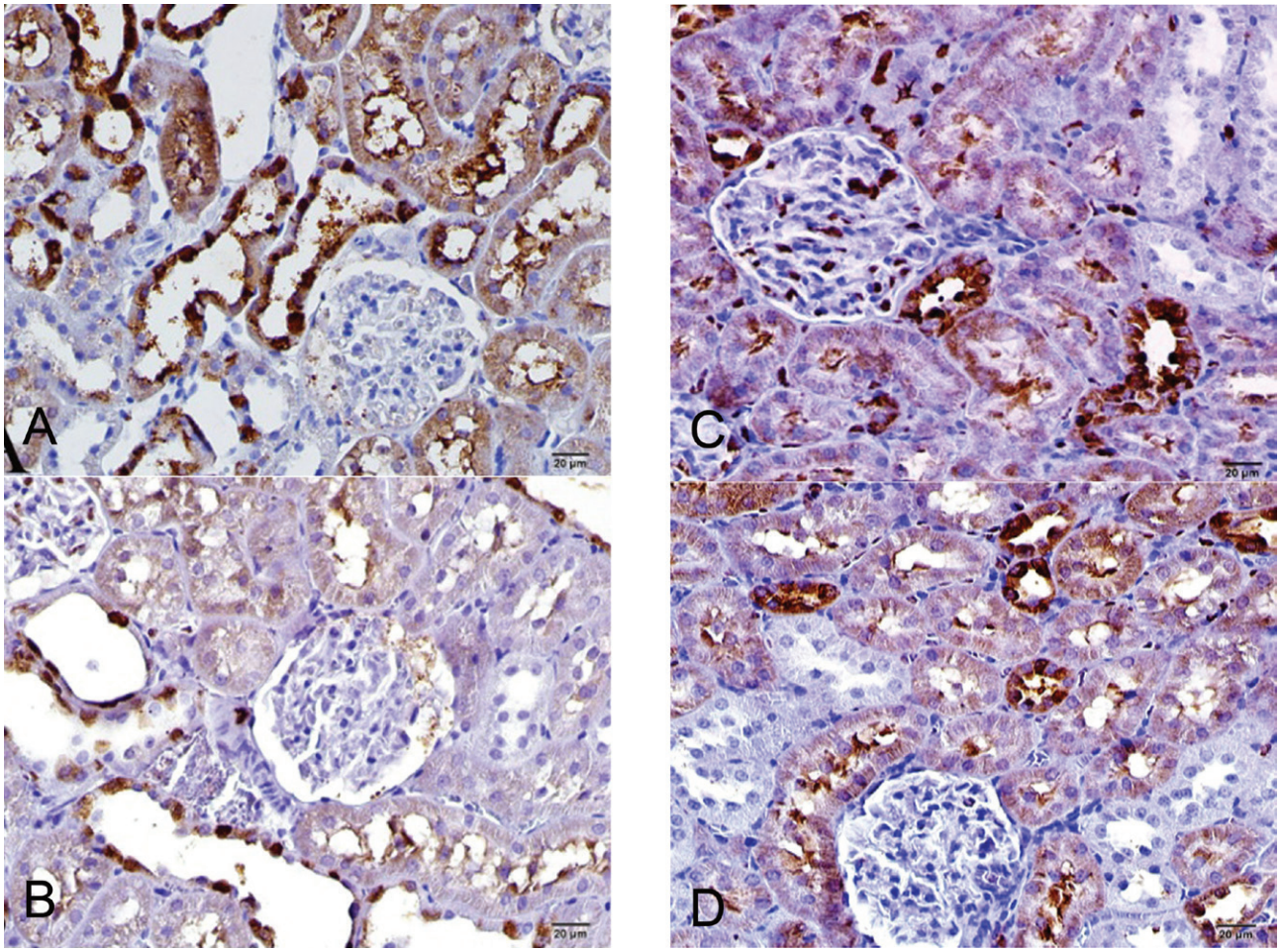

Figure 2. Immunohistochemical staining of kidney tissues with immunoperoxidase method revealed by light microscopy (immunoperoxidase stained anticarbonic anhydrase-II). **A,** Methotrexate group. **B,** Methotrexate and infliximab group. **C,** Infliximab group. **D,** Control group.

cortex-medulla border region was observed to be increased, the vessels had a normal appearance and the amount of lymphocytes was lower when compared to the methotrexate group. While cellular swelling and intensive shedding of tubular epithelium in the outer cortex were decreased in this group, they did not disappear completely. The vacuolization of epithelial cells of both proximal and distal tubules was observed to be decreased by the infliximab effect (Figures 1 and 2).

There was no deterioration found in the epithelial cells and tissue integrity of neither tubules nor glomeruli of the infliximab group. Lymphocytes around the blood vessels in the cortex region were few and the epithelial cells of the proximal tubules were observed to be in normal structure. While there was mild increase in the connective tissue of the cortex-medullar region, the amount of lymphocytes was observed to not be increased. The histological appearance of the cells and capillaries diameters within the connective tissue was observed to be normal (Figures 1 and 2). All histopathological results are shown in Table 2.

DISCUSSION

According to the results of our study, the levels of serum urea and creatinine and tissue levels of TNF-α, NO, and ADA of the methotrexate group were quite higher than the control, the infliximab and the methotrexate and infliximab groups. The histopathological examination of methotrexate revealed massive renal tissue injury. The levels of serum urea and creatinine and tissue levels of TNF-α, NO, and ADA of the methotrexate and infliximab group were lower than those of the methotrexate group and the histopathological injury was found to be less extensive. Only the results of the infliximab group were similar to the

Table 2. Histopathological Examination of Kidney Tissue

Parameter	Control	Methotrexate	Methotrexate and Infliximab	Infliximab
Cell loss	0.2 ± 0.4	3.2 ± 0.4†	1.9 ± 0.3†‡	2.2 ± 0.6†‡
Edema	0.2 ± 0.3	3.1 ± 0.3†	2.1 ± 0.3†‡	1.8 ± 0.4†‡
Capsular dilatation	0.3 ± 0.2	3.0 ± 0.4†	2.0 ± 0.4†‡	2.1 ± 0.5†‡
Lymphocyte infiltration	0.8 ± 0.4	2.9 ± 0.3†	2.2 ± 0.6†‡	1.9 ± 0.5†‡
Tubular deformation	0.1 ± 0.3	2.9 ± 0.7†	1.9 ± 0.3†‡	2.1 ± 0.7†§
Carbonic anhydrase-II	2.0 ± 0.4	3.3 ± 0.5†	2.2 ± 0.4‡	2.2 ± 0.7‡

†$P < .01$ compared to the control group
‡$P < .01$ compared to the methotrexate group
§$P < .05$ compared to the methotrexate group

control group. These findings support the protective effect of infliximab against methotrexate-induced nephrotoxicity. Additionally, we think that it is safe to use infliximab in therapeutic doses.

The effect of infliximab starts after 1 hour of intravenous injection by diffusion into body fluids. It reaches maximum after 1 to 2 days of the injection and its effect lasts for 8 weeks.[19] On the other hand, it reaches serum peak level after 1 to 2 hours of methotrexate injection.[22] In the previous studies conducted on methotrexate nephrotoxicity in rats, methotrexate was administered 5 to 6 days[23]; therefore, we administered methotrexate daily for 5 days after 3 days of single-dose infliximab injection. As infliximab would reach the peak serum level after 3 days of the injection, we investigated its single dosage preventive effect against methotrexate-induced nephrotoxicity. In practice, infliximab is administered once every 8 weeks, so it may be protective against chronic toxicity of methotrexate. Our study is a pilot, so further studies are needed to investigate infliximab protective effect against methotrexate chronic toxicity.

Excessive ROS formation in the kidney leads to injury of the glomeruli and tubules and acute kidney failure.[24,25] Tumor necrosis factor-α and IL-1β are the major pro-inflammatory cytokines. Their excessive release leads to an increase of other pro-inflammatory cytokines and ROS formation. Excessive ROS formation lies on the basis of methotrexate-induced nephrotoxicity.[5] In addition, methotrexate may lead to nephrotoxicity by inhibition of several enzymes related to DNA synthesis.[5,23] It is known that nephrotoxicity is prevented or decreased by suppression of TNF-α.[26] A toxic dose of methotrexate increases the levels of TNF-α and IL-1β.[3] Increased cytokines levels leads to cellular injury and accelerates apoptosis. In a previous study investigating pentoxifylline preventive effects against methotrexate toxicity, a single dose of pentoxifylline was administrated 3 days earlier and was continued 6 days after methotrexate application. Authors reported that pentoxifylline prevents methotrexate-induced nephrotoxicity by blocking TNF-α.[23] Infliximab is a potent TNF-α inhibitor. Experimental studies of ischemia reperfusion have shown that infliximab prevents tissue injuries of some tissues by inhibiting TNF-α and decreasing ROS formation.[12-14] Also, a previous study has reported that infliximab prevents cisplatin-induced intestinal injury by suppressing TNF-α and ROS formation and regulating antioxidant enzyme levels.[27] In our study, while the levels of urea and creatinine were obviously higher in the methotrexate group than the other three groups, their levels in the the methotrexate and infliximab group were obviously lower than the methotrexate group. Acute kidney failure that occurs secondary to excessive ROS formation can be reduced by ROS-formation-lowering agents that prevent or reduce kidney injury and decrease urea and creatinine elevation.[25,28] Our results have shown the increased release of TNF-α and IL-1β to be responsible for nephrotoxicity and infliximab treatment to prevent methotrexate-induced nephrotoxicity by suppressing TNF-α and other pro-inflammatory cytokines.

Nitric oxide produced from arginine is in balance in the organism. While a low NO level leads to vasoconstriction, endothelial dysfunction, and tissue injury, its overproduction leads to formation of peroxynitrite radicals.[29,30] Peroxynitrite radicals lead to cellular injury by formation of lipid peroxidation and ROS formation. Increased peroxynitrite radicals lead to kidney injury by damaging arteries and tubules.[31] Studies conducted on methotrexate have shown its overdose to leads to nephrotoxicity by increasing NO release

and ROS formation.[6] According to the results of the present study, methotrexate increased NO, thus nephrotoxicity might be caused by the stimulation of ROS formation. Previous studies have shown infliximab to decrease NO formation and cellular injury.[19,32] In our study, NO level of the methotrexate and infliximab group was quite lower than the methotrexate group. Tumor necrosis factor-α increased NO production by NO synthetase stimulation. Infliximab may decrease NO production by inhibiting TNF-α; therefore, NO level of the infliximab group was only observed to be similar to the control group. Infliximab may maintain NO balance in the organism.

Adenosine deaminase is an enzyme of purine metabolism that hydrolyzes adenosine to inosine and 2′-deoxyadenosine to 2′-deoxyinosine.[33,34] Adenosine deaminase is required for differentiation and proliferation of T-lymphocytes.[35] Its level is increased in the organism in case of increased need to purine nucleotides as in cancer or tuberculosis.[36] A previous study has reported methotrexate to increase ADA level.[8] Elevated ADA level indirectly indicates ROS formation. Methotrexate increases tissue adenosine level by blocking folic acid-dependent steps in the synthesis of purine and pyrimidine.[8,37] Elevated adenosine level leads to an increase in ADA activity. Cure and colleagues reported that infliximab prevented kidney injury by decreasing the elevation of ADA in cisplatin-induced nephrotoxicity models.[19] In the current study, while ADA level of the methotrexate group was significantly high, it was observed to significantly low in the methotrexate and infliximab group, as elevated ADA level leads to increased production of cytokines from T lymphocytes. Infliximab decreases T lymphocytes stimulation by inhibiting TNF-α, and thus, it may decrease cytokines release. Adenosine deaminase level of the infliximab group was similar to the control group. Infliximab may have a modulator effect on the purine metabolism.

Carbonic anhydrase is an important enzyme in cellular acid-base balance that presents in many tissues, especially in the kidney. Carbonic anhydrase catalyzes the biochemical reaction of carbon dioxide hydration to the bicarbonate ion and proton.[15,38] Overexpression of CA is cause excessive bicarbonate.[39] Increased bicarbonate may lead to the activation of apoptotic pathway as a result of raised free radicals levels and oxidative stress.[40] The reason is that increased bicarbonate level is rapidly converted to carbonate radicals.[41] Infliximab effect on CA-II enzyme is not fully understood due to the limited number of studies in the literature. A study conducted by Cure and coworkers has shown CA-II activity of cisplatin-induced nephrotoxicity model to be excessively lower the enzyme level than the control group.[19] However, this enzyme level of the infliximab group was found to be similar to the control group.[19] The authors reported that infliximab prevented cisplatin-induced nephrotoxicity and metabolic acidosis by preventing renal hydrogen ion excretion. In the current study, CA-II of the methotrexate group was observed to be higher than the control group. However, it was similar in the methotrexate and infliximab group and the control group. Current findings indicate that infliximab regulates tissue CA-II level and prevents its elevation. Infliximab maintains the equilibrium of CA-II enzyme during kidney injury by an unknown mechanism. On the other hand, the enzymes CA and dihydrofolate reductase are released intensively from cancer tissues, so they facilitate the growth and spread of tumor cells.[42] Also, cancer cells use bicarbonate for proliferation.[43] It is known that methotrexate inhibits dihydrofolate reductase enzyme. However, our study has shown that methotrexate overdose increases CA-II enzyme level. Methotrexate may cause nephrotoxicity by increased carbonate radicals. Moreover, excessive dose of methotrexate may facilitate growth of tumor cells. This study is a pilot study, and new studies are needed on this issue.

CONCLUSIONS

Methotrexate leads to nephrotoxicity by excessive cytokine release and increasing ROS formation. Additionally, it leads to nephrotoxicity by increasing the metabolites of purine metabolism. Infliximab is a potent TNF-α blocker that inhibits cytokine release and therefore prevents cellular injury by both cytokine-mediated apoptosis and ROS formation pathways. It may have a protective effect against nephrotoxicity by regulating tissue levels of ADA and CA-II enzymes.

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CONFLICT OF INTEREST

None declared.

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Correspondence to:
Erkan Cure, MD
Department of Internal Medicine, School of Medicine, University of Recep Tayyip Erdogan, Rize 53100, Turkey
E-mail: erkancure@yahoo.com

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