Expression of Orai1/STIM1 and Caspase-3 in Methotrexate-induced Kidney Injury in Rats; and Protective Effect of N-acetylcysteine

Tuba Demirci[1] and Semin Gedikli[2]

***Abstract*—** In the present study, we investigated the probably curable effects of N-acetylcysteine (NAC) on Methotrexate (MTX)-induced renal damage as histopathologically and immunohistopathologically. For this purpose 18 male rats were divided into three groups as; control, MTX, and NAC+MTX groups. Control and MTX groups were given 0.09% NaCl solution for 7 days; and MTX group was administrated single dose MTX at fourth day, additionally. NAC+MTX group was given NAC during 7 days via gavage; and NAC+MTX group was applied single dose MTX at fourth day. On the eighth day, rats were sacrificed, and kidneys were taken. Histopathologically, tubular dilatation and epithelial desquamation, vascular congestion and contracted glomerular tufts seen in the MTX group were decreased with NAC administration. MTX increased caspase-3, STIM1 and Orai1 immunopositivity, but NAC decreased caspase-3, STIM1 and Orai1 immunopositivity. Findings obtained in the current study suggest that NAC may be effective on the MTX-induced nephrotoxicity..

***Index Terms*—**Kidney, Methotrexate, N-acetylsystein, Orai1/STIM1.

I. Introduction

Methotrexate (MTX) is a therapeutic drug widely used for the treatment of many types of cancer such as lymphoma, breast cancer, osteosarcoma, head and neck cancers, and some autoimmune diseases in humans [1]. MTX blocks dihydrofolate reductase, an enzyme needed for the survival of the cells, and so, it prevents the growth of cancer cells [2]. The presence of its many side effects limits the use of MTX [3]. Because of excretion of more than 90% of MTX through the kidneys, it causes severe nephrotoxicity [4], [5], increasing of serum creatinine levels, uremia, and hematuria, and this situation limits its use as an anticancer agent [6].

By resulting in toxic effects in the renal tubules due to increased reactive oxygen species (ROS) in the kidneys [7], MTX causes oxidative stress, inflammation, apoptosis [8], and fibrosis [9]. Reactive oxygen species also play a role in the pathogenesis of MTX-induced renal injury [10]. Whereas caspases are found as inactive proenzymes in living cells, they become activated when the apoptosis signal begins in the cell [11]. In recent studies, it has been shown that MTX causes to increase of caspase-3 expression in kidney and liver [12], [13].

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Calcium ions take charge as a secondary messenger in the regulation of various cellular functions. The increase in the intracellular Ca^{2+} ion concentration plays an important role in the development of renal diseases such as renal fibrosis [14]. Store-operated Ca^{2+} channels (SOC) are the most major pathway for entry of Ca^{2+} ions into the cell [15], [16]. STIM1 and Orai1proteins have been described as components of SOC channels found on the plasma membrane and it's been seen that the increase in expression of these proteins in renal injuries [14].

N-acetylcysteine (NAC) is a strong antioxidant [17], and a cytoprotective drug protecting the cells from the hazardous effects of free radicals [18]. In studies, it has been reported that NAC has the protective effect in some diseases as fibrosis [17], coronary diseases and MTX-induced renal injury [19]. It has also been detected that NAC reduces oxidative stress, apoptosis, cytokine levels, and the entry of Ca^{2+} ions into the cell through the channels [20].

In this study, the presence of apoptosis in the MTX-induced nephrotoxicity in rat kidney tissue, and the effects of NAC on apoptosis and entry of Ca^{2+} ions through SOC channel were investigated by using histochemical and immunohistochemical techniques.

II. Material and Method

A. Experimental Procedure

In this study, eighteen adult male Sprague Dawley rats were used. The rats were obtained from Atatürk University Medical Experimental Application and Research Center. During the experiment, all subjects were kept in rooms under a 12/12 hour light / dark cycle. They were fed with standard rat chow and tap water ad libitum. Rats were divided randomly into three groups as control group (n=6), MTX group (n=6), NAC+MTX group (n=6).

The rats of the control group were not applied any drug administration, only 0.09% NaCl solution was given with 0.5 mL/day dosage intraperitoneally (i.p.) during seven days. In MTX group, 0.09% NaCl solution was administered 0.5 mL/day i.p. for seven days, and on the fourth day, MTX (Methotrexate® 10 mg/ml, Kocak Pharma) was applied as i.p in a single dose of 20 mg/kg. The rats of NAC+MTX group were given 50 mg/kg/day oral NAC via gavage every day for seven days, and MTX was applied as i.p in a single dose of 20 mg/kg only on the fourth day. At the eighth day of the experiment, the rats were anesthetized with intramuscular 10

mg/kg xylazine hydrochloride and 70 mg/kg ketamine hydrochloride as intramuscular. The abdomen was cut with midline section the kidneys were removed and the rats were sacrificed. Kidney tissues were used for histopathological and immunohistochemical examinations.

B. Histopathology

Kidneys were fixed with 10% neutral buffered formaldehyde solution for histopathological evaluation. After routine histological procedure, paraffin-embedded kidney tissues were cut in 4-5 μm thicknesses with Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). Sections of all groups were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS) stains. Kidney tissue sections of the all groups were examined under the light microscope (Nikon Eclipse E600, Tokyo, Japan).

C. Immunohistochemistry

Sections were treated with 3% hydrogen peroxide after they were deparaffinised and rehydrated. Then, they were washed in phosphate buffered saline (PBS) and stained using caspase-3 primary antibody (dilution: 1/50, Abcam, Cambridge, UK), STIM1 primary antibody (dilution: 1/50, Santa Cruz Biotechnology, Oregon, USA) and Orai1 primary antibody (dilution: 1/50, Santa Cruz Biotechnology, Oregon, USA) with streptavidin-biotin-peroxidase method. The primary antibodies were not added to the negative control slides and incubation was made with only PBS. The sections were evaluated under the Nikon Eclipse E600 light microscope (Tokyo, Japan) and photographs of sections were taken.

III. RESULTS

A. Histopathological Findings

Light microscopic examinations of H-E stained sections showed normal renal corpuscles and tubules in the control group. The tubular arrangement of epithelial cells exhibited the normal histological appearance (Fig. 1a). In the MTX group, architectural impairment was remarkable with tubular dilatation and epithelial desquamation, and also dilatation and congestion of the peritubular vessels was seen. The glomerular structure disrupted in some areas and contracted glomerular tufts were observed (Fig. 1b). NAC+MTX group exhibited moderate degeneration (Fig. 1c).

The PAS-positive stained brush borders at the luminal surface of the proximal tubules were observed in the control group (Fig. 1d) in the PAS stained sections of renal tissues. Brush border loss was seen in the epithelium of rat kidney proximal tubules of the MTX group (Fig. 1e). The view of the NAC+MTX group was similar to those of the MTX group (Fig. 1f).

B. Immunohistochemical Findings

Immunohistochemical staining was performed using the avidin–biotin method to determine the kidney tissue expression of Orai1, STIM1 and caspase-3. In the slides prepared as the negative control, positive immunoreaction was not observed because of primary antibody was not used (Fig. 2a, 2e, and 2i).

In Orai1- and STIM1-stained sections, we observed that the cells of tubules had a weak brown reaction in few in rats of Control group (Fig. 2band 2f). Expression of these two proteins increased in the MTX group (Fig. 2c and 2g), however the immunoreactivity for Orai1 became stronger than STIM1. When compared with the MTX group, expression of Orai1 and STIM1 prominently decreased in the rats of NAC+MTX group (Fig. 2d and 2h).

When the sections examined according to the caspase-3 immunostaining, there was no markedly immunopositive staining in the Control group (Fig. 2j). In comparison between all groups, we observed that the comparatively weak caspase-3 immunoreactivity was observed in the NAC+MTX group (Fig. 2l), whereas at the tubules of the rats of MTX group strong labeling occurred (Fig. 2k).

IV. DISCUSSION

Exogenous toxic agents and drugs can cause nephrotoxicity because of they excreted via kidneys. Nephrotoxic drugs lead to inflammation at the glomerulus, proximal tubules, and around of cellular matrix. Inflammation causes increasing of oxidative stress and ROS production in the renal tubules and disruption at tubular transport system [21].

It has been shown that MTX, used for the treatment of some autoimmune diseases and cancer types, causes the occurring of toxic situations associated with kidneys, liver, skin, bone marrow, lungs, and gastrointestinal system [1], [22]. In a recent study, it has been shown that MTX-induced nephrotoxicity leads dilatation and congestion in renal vessels, necrosis of epithelial cells, leucocyte infiltration, glomerular atrophy and decreased Bowman's space in some glomerulus [21]. Also, in another study, especially resultant deteriorations of brush border of the proximal tubules via PAS stain have been reported [23]. We observed that tubular dilatation and epithelial desquamation, dilatation, and congestion of the peritubular vessels, and contracted glomerular tuft in MTX group. And also, we detected the disruption of proximal tubular brush border in MTX-treated rats. In the present study, MTX administration to rats resulted in marked pathological changes and NAC supplemented for 7 days either before or after MTX, remarkably healed the MTX-induced histological alterations in the kidney.

Free radical-mediated lipid peroxidation in membranes and apoptotic cell death are considered among the reasons led MTX-induced nephrotoxicity. Caspases are effector molecules played role in apoptosis, and caspase-3 is an important protease activating both extrinsic and intrinsic apoptosis pathways. It has been reported that apoptosis increases due to caspase-3 expression increasing in MTX-induced nephrotoxicity [23]. In another study, it has been shown that NAC inhibits irradiation-induced apoptosis in hippocampus via the inhibition of caspase-3 in rats [24]. Our findings were similar to the literature. In our study, we observed that concomitant applied NAC with MTX leads the reduction of caspase-3 expression.

In nonexcitable cells, the Ca2+ entry into the cell occurs mainly via SOC channels. STIM1 and Orai1 proteins, the main components of SOC channels, are responsible for calcium oscillation in the kidney, and also they have important tasks in the regulation of cell migration and proliferation, and gene

expression [14]. Orai1 reduces extracellular matrix protein expression in glomerular mesengial cells, and so protects the kidney in diabetes [25]. Additionally, it has been reported the increase of Orai1 expression in the occurred renal fibrosis event in the rats fed high-fat diet. Also, Orai1/STIM1 expressions increase in renal cancers [26]. In present study, we detected that Orai1 and STIM1 expression increased in MTX-induced renal injury, and this expression decreased with NAC usage accompanying MTX. Hu et al. reported that in the kidneys of rats with diabetic renal ischemia/reperfusion injury, increased intracellular Ca2+ concentration augments renal tubular epithelial cell damage and apoptosis [27]. We detected the intense immunostaining with Orai1, STIM1 and caspase-3 in the sections of MTX group, and in MTX-induced renal injury, we thought that Ca2+ concentration increases in the cells a result of enhanced expression of Orai1 and STIM1 proteins, and so apoptosis can induce. According the results of the present study we considered that the inhibition of expression of Orai1, STIM1 and caspase-3 with NAC usage may reduce MTX-induced renal injury, and NAC may be a potential therapeutic agent.

V. Conclusion

In conclusion, the present study demonstrates the potent protective effects of NAC against MTX-induced renal injury. This protective effect of NAC on the kidneys depends on the ability to develop the antioxidant defense system, the ability to reduce the pro-inflammatory and apoptotic signal pathway. However, further studies are needed to exactly understand the molecular mechanism.

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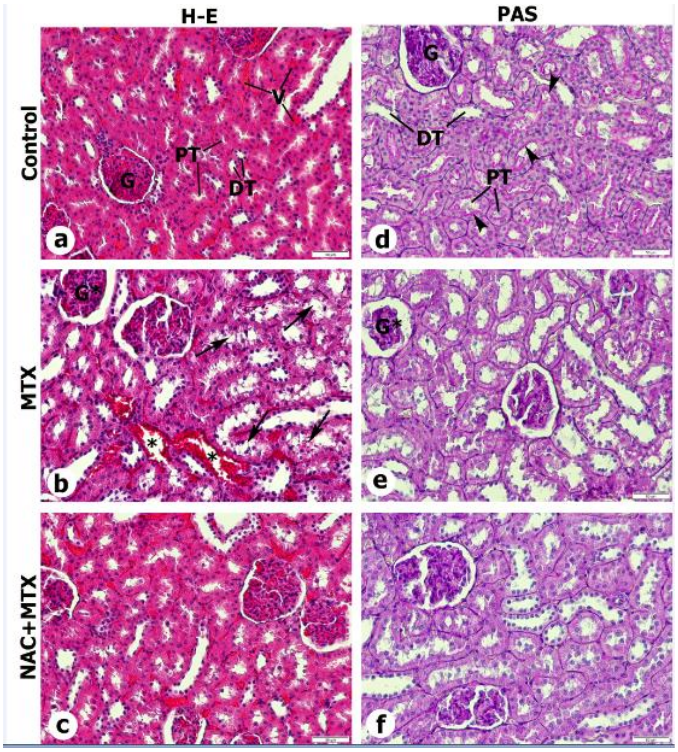

Fig. 1. Light micrographs of kidney tissues are shown for the control (a and d), MTX (b and e) and NAC+MTX groups (c and f). DT, normal distal tubule; G, glomerulus; G*, contracted glomerular tuft; PT, normal proximal tubule; arrow, tubular dilatation and epithelial desquamation into the lumen of the tubules; arrow head, PAS-positive stained brush borders at the luminal surface of the proximal tubules (they are not seen in MTX and NAC+MTX groups); v, vessel; *, dilatation and congestion of the peritubular vessels. Stains: H&E (a-c) and PAS (d-f). Bars: 50 μm.

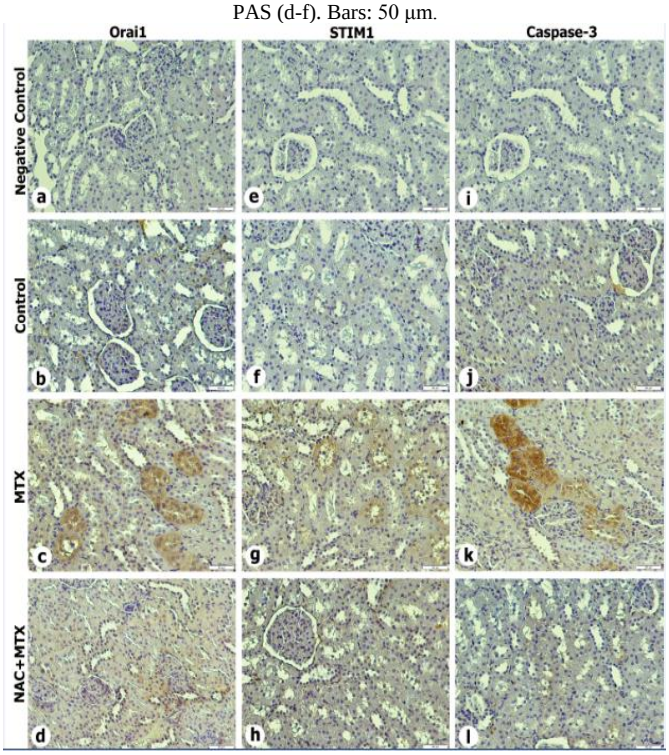

Fig. 2. Light micrographs of immunohistochemistry staining of Orai1 (**b, c** and **d**), STIM1 (**f, g** and **h**), and caspase-3 (**j, k and l**) of rat kidney tissues. Negative control (**a, e,** and **i)** slides were stained without primary antibody. **Stains:** avidin–biotin method. **Bars:** 50 μm.