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Ultrastructural evaluation of the effect of N-acetylcysteine on methotrexate nephrotoxicity in rats

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Summary. The aim of this study is to investigate the possible protective effect of N-Acetylcysteine (NAC) against the likely methotrexate (MTX) toxicity on the kidney using ultrastructural together with biochemical data. Moreover, the immunohistochemical detection of Ki67 nuclear antigen is to be evaluated. Fifteen male Wistar albino rats, weighing 240-290 g, were divided into three equal groups: Rats receiving MTX alone, rats receiving MTX plus NAC treatment, and rats comprising the control group. MTX (18 mg/kg/day, body weight) in dissolved physiologic saline was administered intraperitoneally to rats during 3 days. For the MTX plus NAC group, N-Acetylcysteine (300 mg/kg/day, body weight) was administered together with MTX. At the end of the third day, all the rats were killed with cervical dislocation to obtain blood and tissue samples. Application of MTX principally induced prominent large vacuolization in the proximal convoluted tubule cells, and focal thickening in the glomerular basal lamina of some glomeruli. A decrease in tissue SOD (superoxide dismutase) and GSH-Px (glutathione peroxidase), and an increase in serum urea nitrogen and creatinine and in tissue MDA (malondialdehyde) levels were also seen in the MTX group. These changes were significantly reversed in the MTX-plus-NAC-treated group. Most of the vacuoles in the proximal convoluted tubule cells disappeared. Furthermore, an increase in antioxidant enzyme activities, a decrease in serum urea nitrogen and creatinine, and tissue MDA levels were all significant. Additionally, an increase in the number of Ki67 positive-stained cells in proximal tubules was also noted. In conclusion, NAC may be a promising substance against MTX-induced renal damage. It might be useful to use NAC supplementally to minimize MTX-induced nephrotoxicity.

Key words: Kidney, Methotrexate, N-Acetylcysteine, Ultrastructure, Toxicity

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Introduction

Methotrexate (MTX) belongs to the group of medicines known as antimetabolites. MTX, a folic acid antagonist, is widely used as a chemotherapeutic agent (Jolivet et al., 1983). MTX is frequently used to treat dangerous types of malignant tumours, including blood, lymph, breast and uterus. It is also used in treatment of nononcological diseases including rheumatoid arthritis or psoriasis (Hornung et al., 2008; Kuhn et al., 2010). MTX primarly intervenes in the growth of cancer cells by blocking dihydrofolate reductase which is an enzyme needed by the cell for life (Purcell and Ettinger, 2003).

Although MTX is necessary, common side effects such as gastrointestinal ulceration, hair desquamation, cough, nausea, vomiting and diarrhea have been shown to follow methotrexate administration (Weisman et al. 2006). In various experimental studies, it has been shown that MTX may lead to suppression of intestinal absorption, DNA fragmentation in epithelial cells (Naruhashi et al., 2000; Gao et al., 2001; Li et al., 2009), damage in neuronal tissue (Vezmar et al., 2003), A decrease in osteoblastic function (Fan et al.,2009), distortion of morphology of Leydig cells, a reduction in diameter of seminiferous tubules (Shrestha et al., 2007), and structural changes and fibrosis in the hepatocytes (Hytiroglou et al., 2004; Al-Ali et al., 2005). The presence of tubular morphological changes has been noted in the kidney after MTX administration (El-Badawi et al., 1996; Skretkowiez et al.,1996). MTX

administration in high doses has been reported to cause acute renal failure (Miguel et al., 2008).

Renal toxicity is one of the most serious toxic effects in MTX treatment. It has been observed that various antioxidants such as melatonin and caffeic acid ester have a protective effect on MTX-induced renal damage (Jahovic et al., 2003; Uz et al., 2005). N-acetylcysteine (NAC) is a strong antioxidant. In recent years, studies regarding NAC are rarely encountered. In various clinical studies, it has been demostrated that N-acetylcysteine may have a protective effect in some diseases, such as respiratory fibrosis (Behr et al., 1997; Tirouvanziam et al., 2006), coronary disorder, and renal distortion during angiography (Ramesh et al., 2006).

The aim of this study is to investigate the possible protective effect of NAC against likely MTX toxicity on the kidney by studying ultrastructural evaluation, together with biochemical data, and to contribute to clinical applications.

Materials and methods

Animals and chemicals

In our study, fifteen adult male Wistar albino rats weighing between 240-290 g were used. All rats were obtained from the Medical Sciences Experimental Research Center of the University of Çukurova. The rats were fed a standard laboratory diet at normal room temperature (23°C) and humidity (65%). The rats were housed in steel cages with a 12-h light-dark cycle (lights on at 6:00 a.m.). MTX (ebewe, 500 mg/5ml, F.H. Faulding, Co. Ltd., Australia) at a dosage of 18 mg/kg/day, and NAC (Hüsnü Arsan Drug Company, Turkey) at a dosage of 300 mg/kg/day were administered intraperitoneally (i.p.).

Experimental design

The rats were divided equally into three groups. Group 1: rats receiving MTX alone (n=5) - the rats received MTX (18 mg/kg/day body weight) dissolved in 0.5 ml physiological saline via i.p. injection during 3 days. Group 2: rats receiving MTX plus NAC (n=5) - MTX was injected together with NAC (300 mg/kg/day). Group 3: control group (n=5) - the control group received only physiological saline. At the end of the third day, all the rats were killed with cervical dislocation to obtain blood and kidney tissue samples. Blood samples were taken from the heart (about 2 cc). The kidneys were also quickly removed for microscopic examinations and biochemical analyses. Body weight was determined at the end of the experiment (data not shown).

Electron microscopic analysis

Kidney tissue samples were immediately placed in 5% glutaraldehyde buffered at pH 7.4 with Milloning phosphate for 4 hours and subsequently fixed in 1% osmium tetraoxide for two hours. The samples were dehydrated in graded ethanol, and embedded in araldite. Thin sections were stained with lead citrate and uranyl acetate, and examined with a JEOL JEM 1400 transmission electron microscope.

Light microscopic analysis

For light microscopic observation, kidney tissue samples were fixed in 10% formaldehyde and were embedded in paraffin. Kidney tissues were cut into 4 Ìm-thick sections, mounted on slides, and deparafinized. Tissue sections were treated using an antigen retrieval method in a microwave oven (600W), heating for 15 minutes in citrate buffer solution, then incubated with Rabit Anti-human Ki67 monoclonal antibody (1:200; Clone SPA). Then, sections were conjugated with biotin-avidin, and coloured with AEC. Cellular proliferation was assessed with immunohistochemical detection of Ki67 nuclear antigen. Ki67 positive-stained cells were counted in each sample and divided by 10. Nuclei that had been stained red were scored as positive for Ki67 nuclear antigen.

Biochemical analysis

Blood samples taken from the heart were centrifuged for 10 minutes at 3000 rpm for serum urea nitrogen and creatinine. After that, the serum was separated. The kidney tissues and serum samples were stored at -20°C until the biochemical studies. The tissues were homogenised with three volumes of ice-cold 1.15% KCI. Then, the activities of antioxidant enzymes (SOD, GSH-Px) and MDA were measured in the supernatant obtained from centrifugation at 4000 rpm. SOD (superoxide dismutase) activity was measured in the tissue samples according to the method described by Fridovich (Fridovich, 1974). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitrotetrazlium violet (INT) to form a red formazan dye, which was measured at 505 nm. SOD activity was expressed as U/g tissue. GSH-Px (glutathione peroxidase) activity was determined according to the method of Beutler (Beutler, 1975). Oxidation of GSH to oxided glutathione (GSSG) is possible in the presence of GSH-Px by the addition of t-butil hydroperoxide. For the transformation reaction of GSH, oxidation of NADPH to NADP was measured at 340 nm. GSH-Px activity was expressed as U/g tissue. The MDA (malondialdehyde) level in the tissue samples was measured with the TBA (thiobarbituric acid) test (Ohkawa et al., 1979). After centrifugation, the absorbance of the organic layer was measured at 532 nm. The MDA level was expressed as nmol/g tissue. Serum urea nitrogen (BUN) and creatinine (Cr) were determined by glutamate lactate dehydrogenase and

calorimetric methods.

Statistical analysis

The SPSS programme was used for the Mann-Whitney U test. All data were expressed as the mean (X) ± standard deviation (SD). The difference was considered significant when the probability was less than 0.05, ($p<0.05$).

Results

Ultrastructural findings

Control group

Electron microscopic observation of the kidney tissue in the control group showed normal ultrastructure. In micrographs, glomerular basal lamina in renal

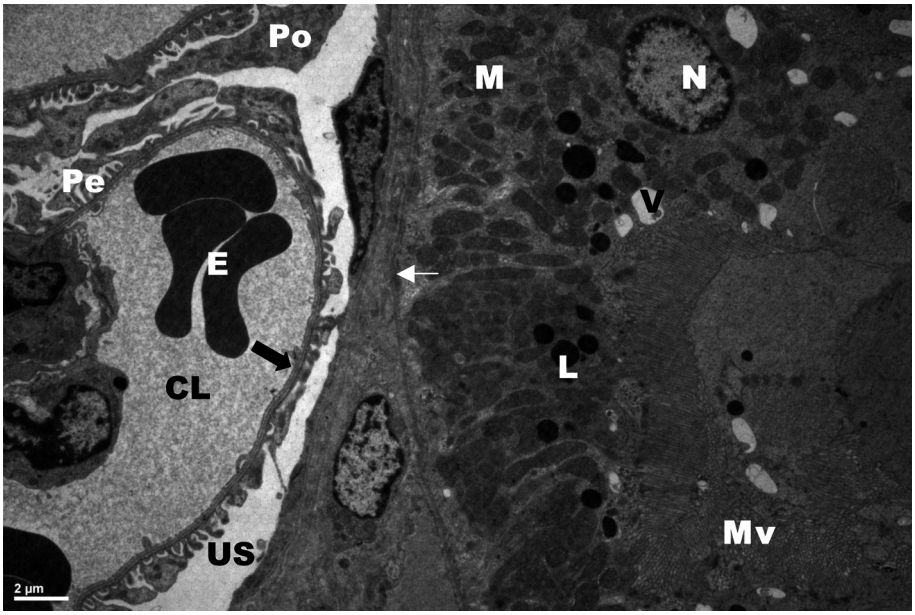

Fig. 1. Control group. Renal glomerulus and proximal convoluted tubule cell show normal ultrastructure. The nucleus (N), mitochondria (M), vacuoles (V), microvilli (Mv), lysosomes (L), tubular basal lamina (thin arrow), glomerular basal lamina (thick arrow), capillary lumen (CL), erythrocytes (E), podocytes (Po), pedicels (Pe), urinary space (US) are indicated.

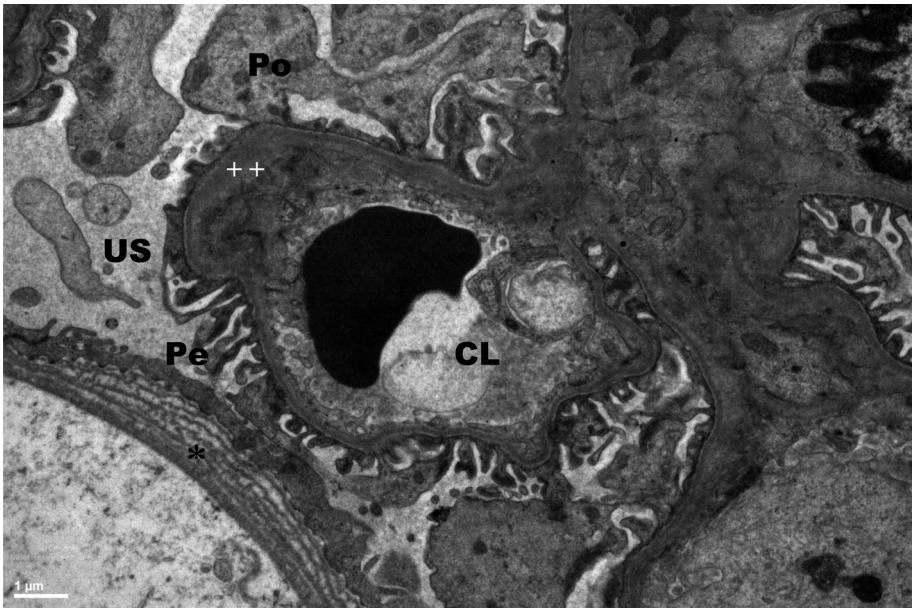

Fig. 2. MTX-treated group. Renal glomerulus. As well as a slight increase in some areas of glomerular basal lamina (++), thickened glomerular basal lamina showing lamellation (asterisk). Urinary space (US), capillary lumen (CL), podocytes (Po), pedicels (Pe) are indicated.

corpuscles appeared intact. The cytoplasmic organelles, nuclei, and brush border in the proximal convoluted tubular cells had a normal structure. Small apical vacuoles beneath the brush border were rarely observed (Fig. 1).

Experimental group

In samples obtained from the animals after MTX administration, there were no significant histological changes in the glomerules. However, a slight increase in the thickening of glomerular basal lamina was focally observed in some glomeruli. In addition, in some areas, thickened glomerular basal lamina were seen to be lamellated (Fig. 2). Marked ultrastructural alterations were observed in proximal convoluted tubule cells. Increased endositotic activity beneath the brush border was also seen. There were vacuoles of various sizes in the cytoplasm of proximal convuluted tubule cells. The presence of large vacuoles in the cytoplasm of most cells

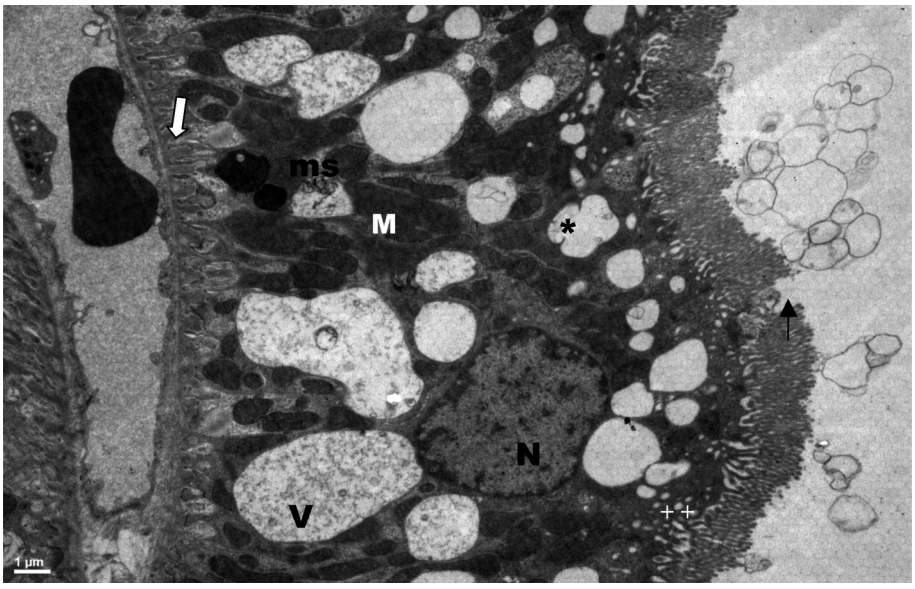

Fig. 3. MTX-treated group.Proximal convoluted tubule cell. The large vacuoles (V) containing dense granular content and membranous structure (ms). Fusion of some neighbouring vacuoles (asterisk), partial destruction on the brush border (thin arrow). Increased endositotic activity beneath the brush border (++). Nucleus (N), slight pleomorphic and dense mitochondria (M), basal infoldings of plasma membrane (thick arrow) are indicated.

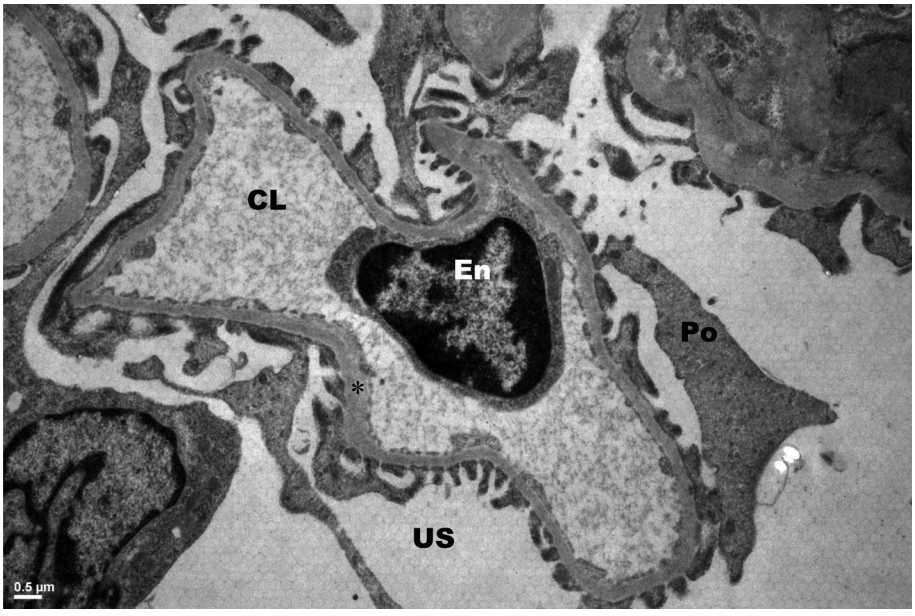

Fig. 4. MTX plus NAC-treated group.Renal glomerulus. Slight thickening in some areas of glomerular basal lamina (asterisk) was seen. Urinary space (IS), podocytes (Po), capillary lumen (CL) and endothelial cell (En) are indicated.

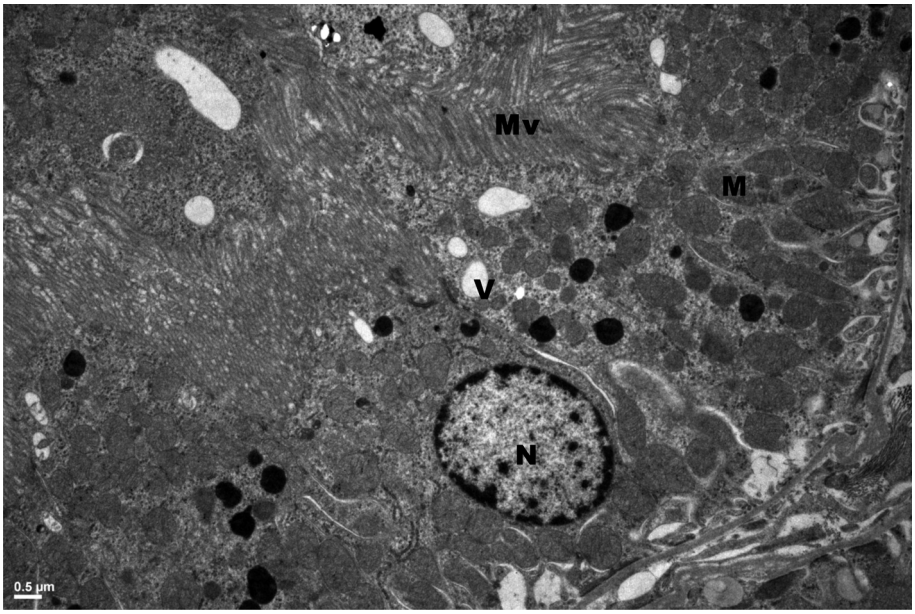

Fig. 5. MTX plux NAC-treated group. Proximal convoluted tubule cell. Loss of large vacuolar structures with dense granular content. Small apical vacuoles (V) beneath the brush border. Nucleus (N), mitochondria (M), and inteact micorvilli (Mv) were indicated.

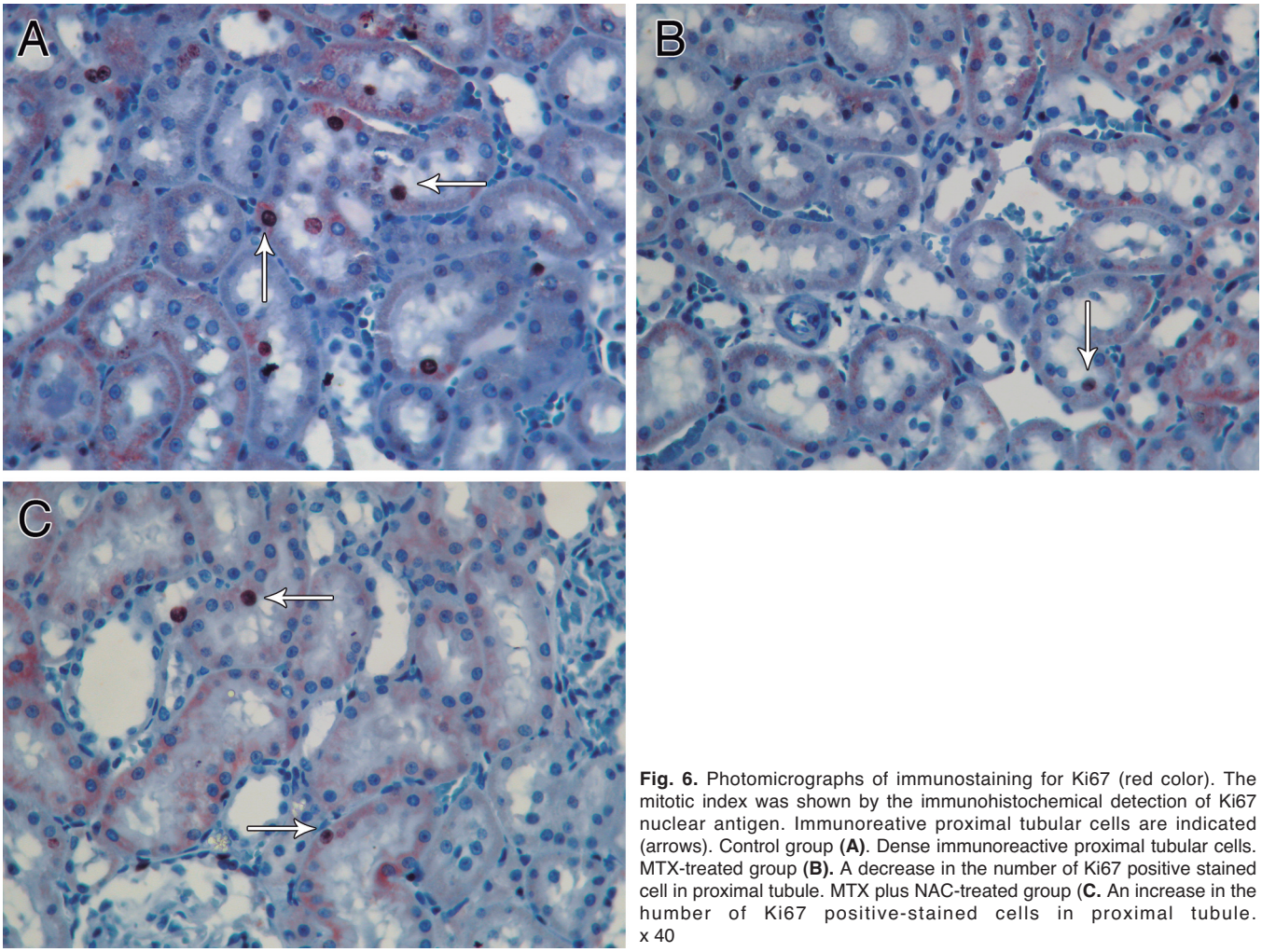

Fig. 6. Photomicrographs of immunostaining for Ki67 (red color). The mitotic index was shown by the immunohistochemical detection of Ki67 nuclear antigen. Immunoreative proximal tubular cells are indicated (arrows). Control group **(A)**. Dense immunoreactive proximal tubular cells. MTX-treated group **(B).** A decrease in the number of Ki67 positive stained cell in proximal tubule. MTX plus NAC-treated group (**C.** An increase in the humber of Ki67 positive-stained cells in proximal tubule. x 40

in the proximal tubule was striking. Some of those vacuoles revealed granular and membranous structures. Furthermore, most of the large vacuoles had more dense granular content. Fusion was frequently observed in some of the neighbouring vacuoles. While the size of vacuoles towards the middle part of the proximal convoluted tubule cells increased, their numbers decreased. There was slight pleomorphism and an increase in density of some mitochondria in the tubular cell cytoplasm. Destruction was also seen in some areas of the brush border in some proximal convuluted tubule cells (Fig. 3).

In the methotrexate-plus-NAC group, although there was slight thickening in some areas of the glomerular basal lamina, lamellation of the glomerular basal lamina was not observed (Fig. 4). Also, a decrease was apparent in this group in the proximal convoluted tubule cells' vacuoles. Variously sized small apical vacuoles were observed beneath the brush border (Fig. 5).

Light microscopic findings

The values of expression of Ki67 positive cells are shown in figure 7. The mitotic index was evaluated by the immunohistochemical detection of Ki67 nuclear antigen in proximal tubular cells. The MTX-administered group was significantly different in terms of the number of Ki67 positive-stained cells compared with that of the control group. In the MTX-administered group, the number of Ki67-positive cells was significantly decreased in proximal tubular cells compared to that of the control group. The methotrexate-plus-NAC group exhibited differences compared to the MTX-administered group, and a partial increase in the number of Ki67 positive-stained cells was noted (Fig. 6).

Biochemical findings

The values of SOD, GSH-Px and MDA activities are shown in figure 8.

The differences in biochemical analysis values between the experimental group and the control group were statistically significant. In rats given only MTX, the levels of SOD and GSH-Px were significantly reduced compared with those of the control group. Furthermore, a significant increase in the concentration of MDA in kidney tissue was seen. In the group given both MTX and NAC, the levels of SOD and GSH-Px increased compared with those of the group treated with MTX only. In addition, a decrease was seen in the level of MDA in the NAC-supplemented group compared to that of the MTX group.

The values of serum urea nitrogen (BUN) and creatinine (Cr) are shown in figure 9. BUN and Cr levels increased in the MTX-only-treated group compared with those of the control group. However, in the MTX-plus-NAC-treated group, levels of BUN and Cr decreased compared to the MTX-only-treated group.

Discussion

The kidney is sensitive to many foreign compounds. Because of its physiological and anatomical features, the kidney is the target of many toxins and drugs. The metabolites of foreign chemicals are one of the most common causes of toxicity. The metabolites of MTX are known to be nephrotoxic. Specifically, formation of the main metabolites such as 7hydroxymethotrexate and 2.4-diamino-N^{10}-methylpteroic acid are observed in plasma (Lankelma et al., 1980; Smeland et al., 1996; Fuskevag et al., 2000). MTX is primarily cleared by renal excretion (Widemann and Adamson, 2006). MTX-induced renal dysfunction may be reported in various patients with such diseases as rheumatoid arthritis and osteosarcoma (Siedeman et al., 1993; Widemann et al., 2004). The toxicity of MTX can be prevented by vigorous hydration and alkalinisation of the patient. In spite of these preventive measures, MTX-induced nephrotoxicity still occurs (Kintzel, 2001).

It is believed that precipitates and metabolites of MTX may play a part in the pathogenesis of MTX-induced renal dysfunction (Winick et al., 1987; Schwartz et al., 2007). In our study, significant vacuolization in proximal convoluted tubule cells has been described following the intraperitoneal administration of MTX. Dense granular and membranous structure was also observed in large vacuoles in various sizes. The metabolites of MTX may affect membrane permeability and endostotic activity. Dense granular content observed in large vacuoles could be related to the metabolites of MTX. These vacuoles in proximal tubular cells may be

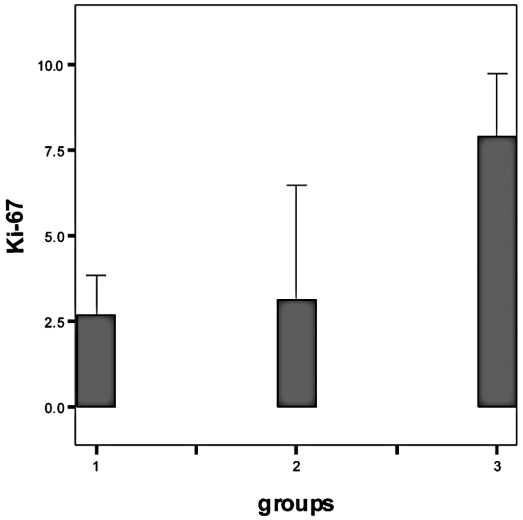

Fig. 7. Number of Ki 67 positive-stained cells in the proximal tubular cells. Groups: 1(MTX), 2 (MTX+NAC), 3 (Control).Values are expressed as mean (X) ± Standard deviation (SD) from five rats in each group. The mitotic index in both groups was significant compared to control group ($p<0.05$).

lysosomal changes due to active internalisation of MTX. Therefore, it may be speculated that the amount of lysosomal enzyme activities decreased in the tubular cells of the MTX-treated rats' kidneys. This situation may cause lysosomal accumulation in the cell cytoplasm. So it may be speculated that the effect of MTX on the kidney may be via the directly toxic effect of its metabolites. Abraham et al. (2007) reported that lysosomal enzymes may be a factor leading to cyclophosphamide (CYP)-induced renal damage. They said that the decrease in the activity of lysosomal protein digestive enzymes may play a role in CYP-induced renal failure. The accumulaton of abnormal amounts of the protein may be due to a disorder of lysosomal enzyme activity. Thus, lysosomal dysfunction may contribute to CYP-induced renal damage. Benesic et al. (2006) showed that chloroacetaldehyde, which is a product of ifosfamide metabolism, led to inhibition of lysosomal cathepsins and a significant increase in lysosomal size in proximal tubule cells. So they said that chloroacetaldehyde-induced renal damage may be associated with a decrease in the activity of lysosomal enzymes and with lysosomal overload in the kidney.

According to the results of some clinical studies, a transient decline in glomerular filtration rate (GFH) is a relatively common observation during MTX treatment (Hempel et al., 2003). In our study, increased endostotic activity could be related to the possible precipitation of MTX in lumen of proximal tubules due to elimination of delayed MTX metabolites. This damage in proximal tubule cells might also develop as a result of the possible connection between the MTX metabolites and the glomerular filtration rate. Grönroos et al. (2006) suggested that MTX has a direct toxic effect on renal tubuler cells, and MTX may lead to permanent tubular damage if its elimination is delayed. Also, in our study, the presence of thickened glomerular basal lamina in some areas is attributable to the toxic effect of metabolites during the passage through the glomerular filtration barrier. So the presence of increased serum

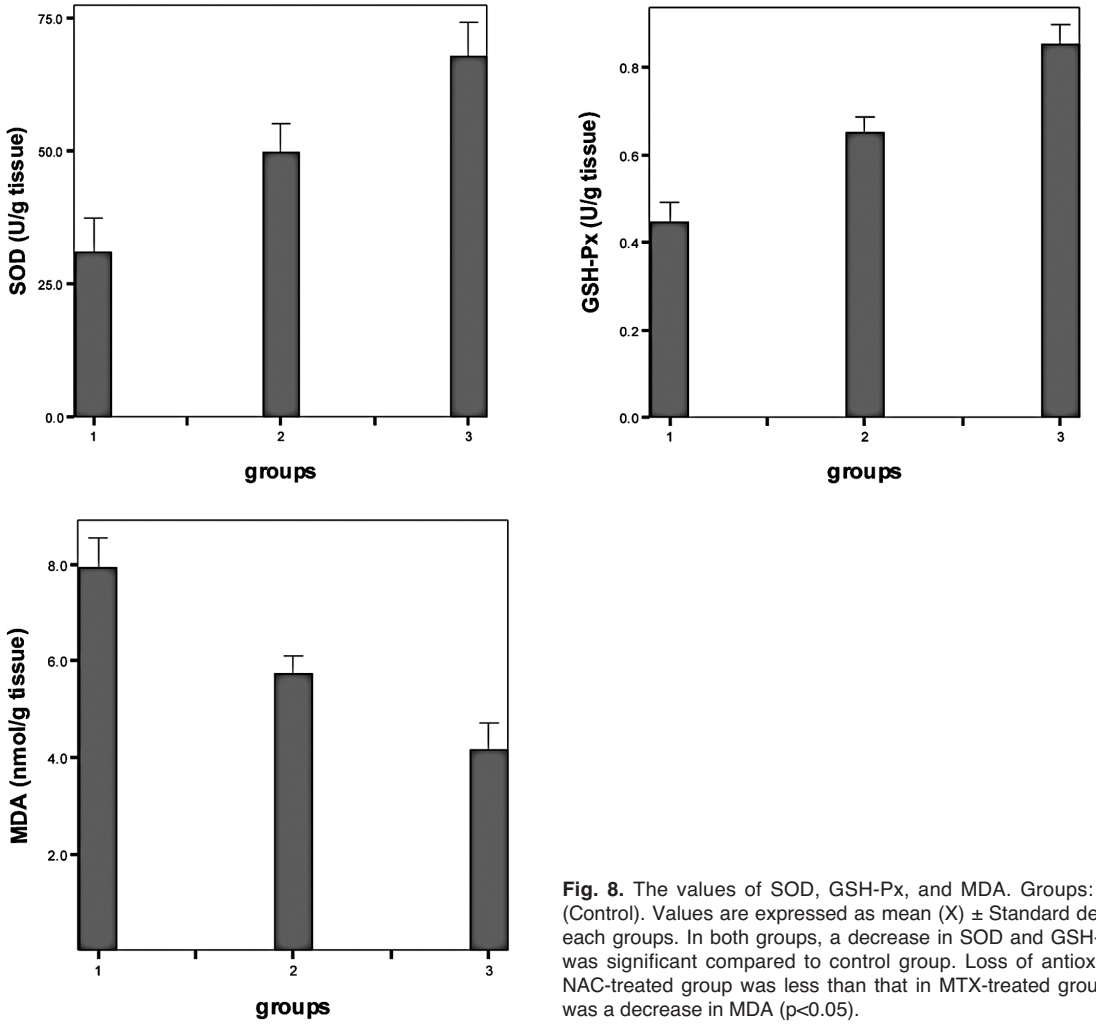

Fig. 8. The values of SOD, GSH-Px, and MDA. Groups: 1(MTX), 2 (MTX+NAC), 3 (Control). Values are expressed as mean (X) ± Standard deviation (SD) from five rats in each groups. In both groups, a decrease in SOD and GSH-Px and an increase in MDA was significant compared to control group. Loss of antioxidant enzymes in MTX plus NAC-treated group was less than that in MTX-treated group. On the other hand, there was a decrease in MDA ($p<0.05$).

urea and creatinin may be related with the toxic effect of methotrexate.

Furthermore, in our study the reactive oxygen radicals were investigated biochemically. The formation of reactive oxygen radicals (ROS) is one of the main causal factors related to MTX-induced renal injury. The endogenous antioxidative defence system is known protect against the production of free radicals in the cell. The antioxidative enzymes SOD and GSH-Px form the major defence against ROS-induced oxidative damage. The depletion of SOD and GSH-Px activities in a cell is one of the primary factors resulting in lipid peroxidation, which may cause an increase in the MDA level (Babiak et al., 1998; Kolli et al., 2009). According to our biochemical findings, in the MTX-treated group, the level of SOD and GSH-Px significantly decreased compared to the control group. In addition, the MDA level was higher in the MTX-treated group than in the control group. It might be hypothesised that the effect of MTX metabolites may cause oxidant damage to the cell. Thus, these changes may reflect the presence of increasing free radicals in the cell. A probable link between oxidative stress and the nephrotoxic effect of various drugs has also been shown by other studies (Mohamadin et al., 2005; Behling et al., 2006).

N-acetylcysteine (NAC) is the N-acetyl derivative of the amino acid L-cysteine, and is a precursor in the formation of the antioxidant glutathione in the body (Akturi et al., 2007; Sansone and Sansone, 2011). In our study, an increase in antioxidant enzyme activity was seen in the MTX-plus-NAC group comparead to the MTX group. In this group, increased SOD and GSH-Px enzyme activities and decreased MDA levels were noted. It may be speculated that NAC supplies defence in the cell against the cytotoxic effect formed by MTX. Thus, decreased vacuolization in proximal convoluted tubule cells may be related to the protecting effect of NAC via membrane stabilisation. Tariq et al., (1999) said that CsA-induced nephrotoxicity was significantly attenuated by NAC, and intracellular glutathione increased in the renal tissue.

MTX also influences several metabolic pathways, including purin metabolism. It is said that MTX inhibits the dihydrofolate reductase related with DNA synthesis, and prevents the conversion of folic acid to tetrahydrofolate. Thus, MTX leads to inhibition of the proliferation of cells by transient disruption of DNA synthesis (Jansen et al., 1997; Frouin et al., 2001). In our study, a decrease was seen in the number of cells stained as positive with Ki67, which is a cellular proliferation marker in the proximal tubule cells following MTX administration. In addition, with MTX treatment antioxidant enzyme levels decreased in the renal tissue. This situation is indicative of possible nucleic acid destruction due to MTX toxicity via oxidative stress in renal tissue. Vardi et al. (2008) established a decrease in the number of mitotic figures in the epithelium of the small intestine in MTX-induced damage, an increase in malondialdehyde and myeloperoxidase, and a decrease in antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione. A slight increase in the number of Ki67 positive-stained cells was seen in the MTX-plus-NAC-treated group. It may be that NAC regulates DNA synthesis in the cell.

According to our findings, ROS may play a role in the pathogenesis of MTX damage. NAC may play a partially protective role against MTX-induced toxicity in the kidney. It may be deduced that the free radical scavenging activity of NAC reduces the toxic effect of MTX. In conclusion, it has been suggested that NAC may be a promising protective substance against MTX-induced renal damage. It might be useful to use NAC supplementally to minimize MTX-induced nephrotoxicity in human.

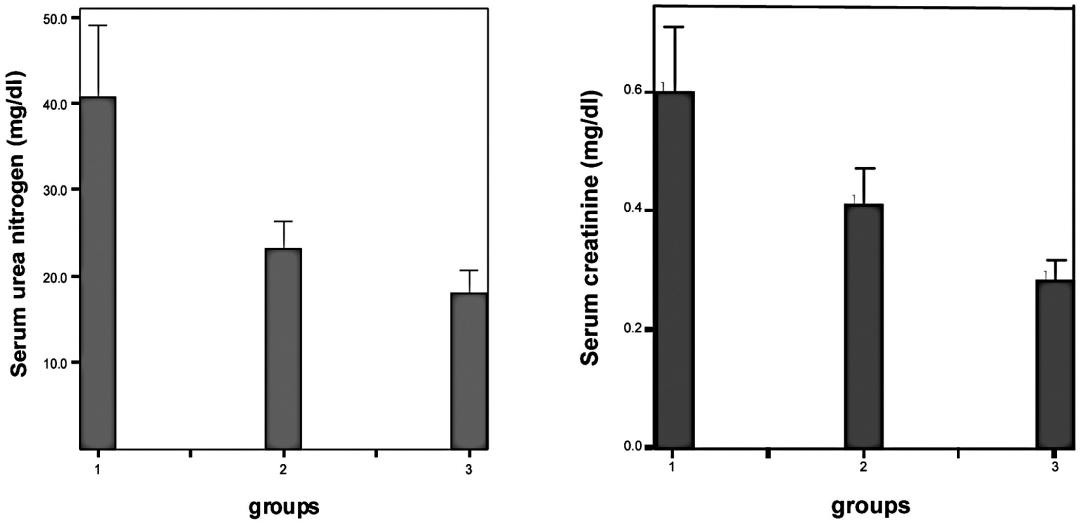

Fig. 9. The values of serum urea nitrogen and creatinine. Groups: 1(MTX), 2 (MTX+NAC), 3 (Control). Values are expressed as mean (X) ± Standard deviation (SD) from five rats in each group. Increment in both groups was significant according to control . In MTX plus NAC-treated group, a decrease in levels of serum urea nitrogen and creatinine was significant compared to MTX-treated group ($p<0.05$).

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