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The effect of alpha lipoic acid on rat kidneys in methotrexate induced oxidative injury

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Abstract. – OBJECTIVE: The purpose of this study is to determine the antioxidant and anti-inflammatory effects of alpha lipoic acid (ALA) on methotrexate (MTX) induced kidney injury in rats.

MATERIALS AND METHODS: Thirty-two rats were equally divided into four groups; control, ALA, MTX and MTX with ALA groups. A single dose of MTX (20 mg/kg) was administered to make kidney injury to groups 3 and 4, intraperitoneally. The ALA was administered intraperitonealy in groups 2 and 4 and the other groups received saline injection for five days. On the sixth day the blood samples and kidney tissues were obtained for the measurement of TNF-α, IL-1β, malondialdehyde, glutathione, myeloperoxidase and sodium potassium-adenosine triphosphatase levels and histological examination.

RESULTS: Administration of MTX caused a decrease in tissue GSH, and Na^+, K^+-ATPase activity significantly. A significant increase in tissue MDA and MPO activities were also seen. The pro-inflammatory cytokines (TNF-α, IL-β) were increased in the MTX group significantly. ALA treatment reversed all biochemical indices as well as histopathological alterations induced by MTX administration.

CONCLUSIONS: MTX made oxidative damage on kidneys of rat and it was partially prevented by anti-inflammatory and antioxidant effects of ALA treatment.

Key Words:
Alpha lipoic acid, Methotrexate, Kidney, Oxidative injury.

Introduction

Folic acid antagonist Methotrexate (MTX) is the commonly used antimetabolite drug in the treatment of malignant diseases, autoimmune and inflammatory disorders. High dose MTX in higher dosages used with leucovorin rescue treat a variety of cancer types such as; acute lymphoblastic leukemia, lymphoma, osteogenic sarcoma, cancer of the head and neck region[1,2]. Because of its side effects and toxic effects such as hepatotoxicity, renal toxicity and bone marrow suppression MTX has a limited use. MTX depletes folic acid species and the lack of folic acid affects the purine metabolism. This is responsible for therapeutic or toxic effects of MTX[3-5]. Acute renal failure and nephrotoxicity have been reported according to use of MTX in high doses[6-8].

The mechanism of renal toxicity has been due to direct toxic effect of MTX[9], inhibition of some enzymes with synthesis of DNA[10] and reactive oxygen species (ROS) production[11].

Nephrotoxicity is one of the important side-effect of MTX. MTX induced nephrotoxicity can be reversed by anti-inflammatory and anti-oxidant agents, such as pentoxyfilline[12]. Thus, ALA, an organosulfur compound derived from octanoic acid, can ameliorate the MTX induced renal toxicity.

ALA synthesized in mitochondria[13] works as co-factor of the pyruvate dehydrogenase complex[14]. ALA protects kidney from ischemia-reperfusion injury[15] and cisplatin- induced nephropathy[16]. It is, however, still unclear whether ALA prevents kidney from MTX-induced injury.

In this study we aimed to investigate the protective effect of ALA against the MTX-induced renal oxidative injury.

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Material and Methods

Animals and Experimental Design

All experimental protocols were approved by the Animal Care and Use Committee of nönü University Faculty of Medicine. Thirty-two Wistar albino rats of both sexes of 200-250 g aged 3 months were provided from animal laboratory and used in this experiment. All animals maintained at a constant temperature (22°C) with a 12/12-hours light-dark cycle and randomly divided into four equal groups of eight rats each. Group 1 (control group): rats in this group received no additional treatment except physiological saline. Group 2 (α-lipoic acid group): rats in this group received intraperitoneal α-lipoic acid (Sigma, St. Louis, MO, USA) for five days (60 mmol/kg). Group 3 (Methotrexate group): rats received MTX (Onco-Tain; Faulding Pharmaceutics Plc, Leamington Spa, UK) treatment at a single dose intraperitoneally (20 mg/kg). Group 4 (Methotrexate group-α-lipoic acid group): rats received both a single dose of MTX and intraperitoneal injection of α-lipoic acid for five days. ALA was dissolved in 0.1% dimethyl sulfoxide (DMSO). At the end of the sixth day, the experimental rats were euthanized by decapitation and blood samples were obtained for the tumour necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) measurement. The malondialdehyde (MDA) levels, reduced glutathione (GSH), myeloperoxidase (MPO) and sodium potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity were also analysed in the kidney tissue samples. Furthermore, the degree of inflammation and histopathologic damage (glomerular, tubular, and interstitial changes) were evaluated via histological examination under a light microscope.

Measurement of Malondialdehyde Levels

To determine the MDA levels, kidney tissue samples were homogenized in ice cold 150 mm KCl. The MDA levels (nmol MDA/g tissue) were assayed for the products of lipid peroxidation[17].

Measurement of Reduced Glutathione Levels

To determine the GSH levels, kidney tissue samples were homogenized in ice cold 150mm KCl. The GSH levels (mg GSH/g tissue) were measured by spectrophotometric method using Ellman's reagent[18].

Measurement of Myeloperoxidase Activity

MPO (U/g tissue) activity was measured according to the procedure reported by Hillegas et al[19] Kidney tissue samples were homogenized in buffer of 50 mm potassium phosphate (PB, pH 6.0) and than homogenates were centrifuged at 41 400 g for 10 min; pellets were suspended in 50 mm phosphate buffered (PB) containing 0.5% hexadecyltrimethylammonium bromide. Three cycles of freezing and thawing were done with sonication between the cycles, the samples were centrifuged at 41 400 g for 10 min. Volumes of 0.3 ml were added to 2.3 ml of reaction mixture containing 50 mm PB, o-dianisidine, and 20 mm H_2O_2 solution. 1 unit of enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 minutes.

Measurement of Na^+/K^+-ATPase Activity

The measurement of Na^+/K^+-ATPase activity was based on the measurement of inorganic phosphate produced from 3 mm disodium adenosine triphosphate added to the incubation medium[20]. The medium [containing in mm: 100 NaCl, 5 KCL, 6 MgCl2, 0.1 EDTA and 30 Tris HCL (pH 7.4)] was incubated at 37°C in water bath for 5 min. Following this preincubation period, Na2ATP, at a final concentration of 3 mm, was added into each tube and incubated at 37°C for 30 min. After the incubation, the tubes were placed in an ice bath to stop the reaction. The mixture was then centrifuged at 3500 g, and Pi in the supernatant fraction was determined by the method of Fiske and Subarrow[21]. The specific activity of the enzyme was expressed as nmol Pi mg-1 protein h-1. The protein concentration of the supernatant was measured by the Lowry et al method[22].

Biochemical Analysis

TNF-α and IL-1β were analyzed using the enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium). They were selected particularly because of their high degree of sensitivity, specificity and inter-assay and intra-assay precision, and due to requiring a small amount of plasma sample.

Renal Histpathological Evaluation

Each kidney tissue samples were processed for light microscopic examination. The samples were placed in 10% neutral formalin for 48 h and prepared for routine parafin embedding. Kidney tis-

Table I. Effect of ALA on some biochemical parameters in the serum of control, MTX, MTX-ALA groups consisting of 8 animals each. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

Parameters	Control group	MTX	MTX-ALA
TNF-α (pg/ml)	9.4 ± 1.1	35.6 ± 4.1***	11.8 ± 2.3^{+++}
IL-1β (pg/ml)	10.1 ± 1.8	29.8 ± 2.7***	13.3 ± 2.4^{++}

Data are means ± SD, ***$p < 0.001$ compared to control group. $^{++}p < 0.01$, $^{+++}p < 0.001$, compare to MTX group. ALA: Alpha lipoic acid, MTX: Methotrexate.

sue samples were cut into 5 μm thick sections, mounted on slides and stained with hematoxylin-eosin (H&E).

Kidney tissue sample sections were examined by blind histologist in a light microscope (Leica DFC280, UK) and analyzed by the Leica Q Win Plus V3 Image Analysis system (Leica Micros Imaging Solutions, Cambridge, UK).

Kidney damage was scored by grading glomerular, tubular, and interstitial changes with a maximum score of 15. Glomerular damage (sclerotic changes such as matrix expansion, narrowing or disappearance of the Bowman's space, adhesion of capillary tuft to the Bowman's capsule, capillary collapse) was evaluated as 0, absent; 1, < 25% of glomeruli affected; 2, 25-50% of glomeruli affected; 3, > 50% of glomeruli affected.

Tubular injury was defined as, tubular epithelial degeneration and tubular necrosis. Grading for each of these tubular changes was scaled as 0, absent; 1, < 25% of tubules affected; 2, 25-50% of tubules affected; 3, > 50% of tubules affected. The presence of interstitial inflammation and congestion were each judged as 0, absent; 1, mild; 2, moderate; 3, severe[23].

Statistical Analysis

Statistical analysis of this study was performed by GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). The data were expressed as the mean±standard error of the mean (SEM). Comparisons of the groups were performed with the variance analysis followed by Tukey's tests. $p < 0.05$ was considered as statistically significant.

Results

The TNF-α levels were found significantly increased in the MTX group ($p < 0.001$) when compared to the control group, with the α-lipoic acid treatment this MTX-induced rise of serum TNF-α levels were abolished ($p < 0.001$). Similarly in the MTX group IL-1 β proinflammatory cytokine was also found increased ($p < 0.001$), however treatment with α-lipoic acid following MTX administration, TNF-α and IL-1 β levels were back to levels at the control (Table I).

In accordance with these findings, in MTX group levels of the major cellular antioxidant GSH of kidney samples were found lower than those of the group significantly ($p < 0.001$). On the other hand, treatment with α-lipoic acid to MTX group restored the GSH levels ($p < 0.01$, Figure 1).

When compared with the control group the mean of MDA level, a major degradation product of lipid peroxidation was found increased in all tissues after MTX administration ($p < 0.001$), while treatment with α-lipoic acid of the MTX group caused a decrease in MDA levels ($p < 0.01$, Figure 2).

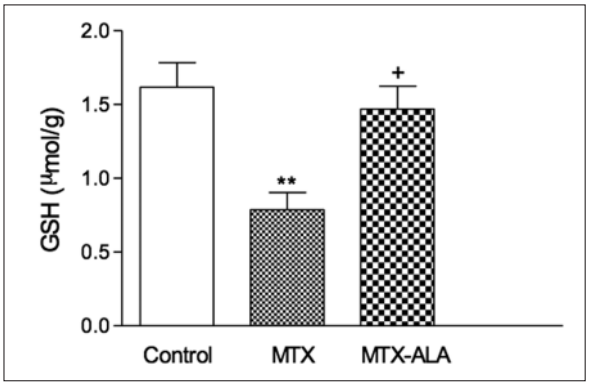

Figure 1. Glutathione (GSH) levels in the kidney tissues of control, methotrexate (MTX), MTX-ALA (α-lipoic acid) groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. ***$p < 0.001$; compare to control group $^{++}p < 0.01$; compare to MTX group.

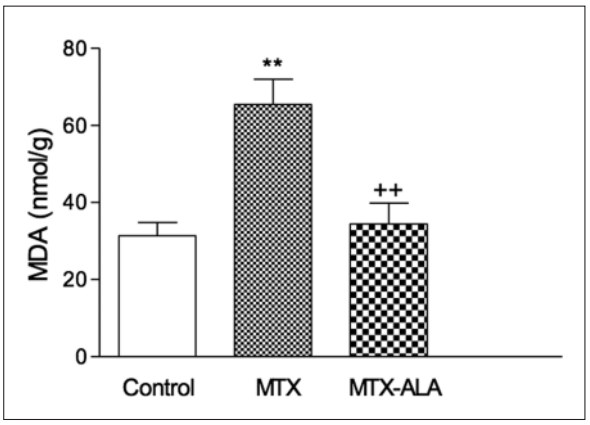

Figure 2. Malondialdehyde (MDA) levels in the kidney tissues of control, methotrexate (MTX), MTX-ALA (α-lipoic acid) groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. ***$p < 0.001$; compare to control group $^{++}p < 0.01$; compare to MTX group.

When compared to control group myeloperoxidase activity, an indicator of neutrophil infiltration was found higher in the kidney tissues of the MTX group significantly ($p < 0.001$). The α-lipoic acid treatment in MTX group was found decreased all tissues MPO level significantly ($p < 0.001$, Figure 3) than that of the control group.

As compared with the control group the Na^+-K^+-ATPase activity was shown to be decreased in the kidney tissues of saline treated MTX group significantly. The α-lipoic acid treatment in MTX group was found increased in all tissues Na^+-K^+-ATPase activity significantly ($p < 0.001$, Figure 4).

The kidney sections of control (Figure 5A, B) and ALA (Figure 6A, B) groups rats were having normal histological appearance.

Kidney specimens of MTX administered groups showed histopathological alterations such as glomerular sclerosis and necrosis, capillary collapse, narrowing or disappearance of the Bowman's space, and tubular necrosis, tubular atrophy, tubular epithelial degeneration, accumulation of fibrinoid material in the lumen of renal tubules additionally interstitial inflammation and vascular congestion (Figure 7A, B, C).

In the kidney section of MTX+ALA administered rats, the histopathological evidence of MTX related kidney damage was markedly reduced. However, mild glomerular sclerotic changes and mild tubular epithelial degeneration and vascular congestion were occasionally observed (Figure 8A, B, C).

Discussion

In this study we aim to investigate the oxidative damage in MTX-induced kidney and the protective effect of ALA on kidney tissues. With its free radical scavenging effect the ALA prevented lipid peroxidation and neutrophil infiltra-

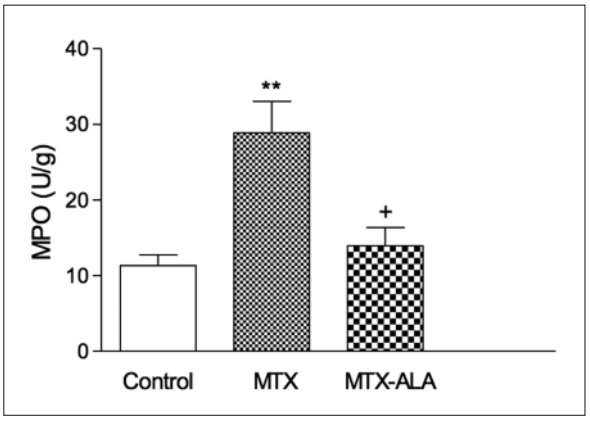

Figure 3. Myeloperoxidase (MPO) activity in the kidney tissues of control, methotrexate (MTX), MTX-ALA (α-lipoic acid) groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. ***$p < 0.001$; compare to control group $^{++}p < 0.01$, $^{+++}p < 0.001$; compare to MTX group.

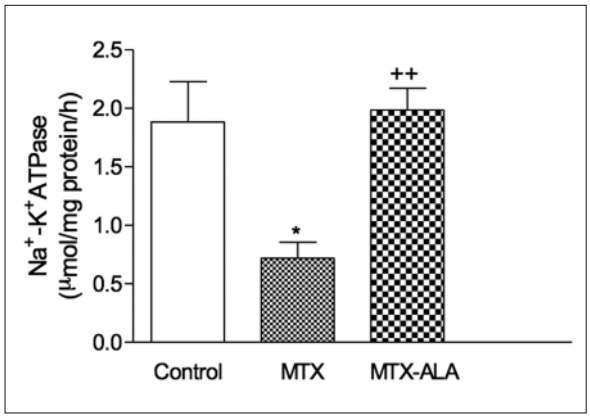

Figure 4. Na^+-K^+-ATPase activity in the kidney tissues of control, methotrexate (MTX), MTX-ALA (α-lipoic acid) groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. **$p < 0.01$, ***$p < 0.001$; compare to control group $^{++}p < 0.01$, $^{+++}p < 0.001$; compare to MTX group.

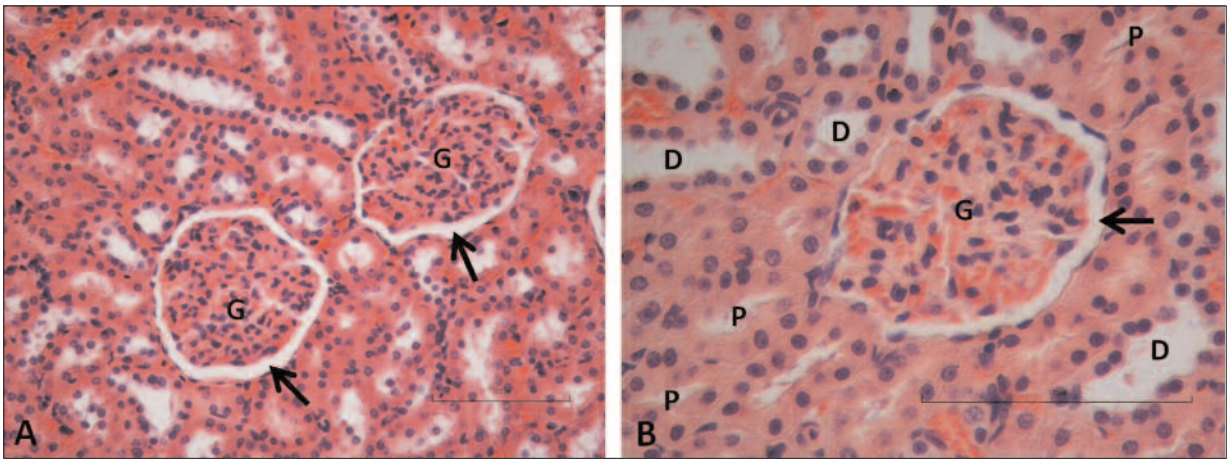

Figure 5. Control group; Normal histological appearance of kidney tissues. ***A,*** Glomerulus (G), Bowman's space (*arrow*). ***B,*** Distal tubules (D), proximal tubules (P). H&E, Scale bar = 100 µm.

tion of the tissues of MTX-induced rat kidney. Furthermore, ALA treatment reduced the plasma cytokines and improved the kidney tissue morphological changes caused by MTX.

Methotrexate is an antimetabolite that competitively inhibits the folic acid metabolism resulted with the impairment of synthesis of purine end pyrimidine thus DNA[24]. Kidney toxicity due to MTX treatment can occur both at low or high doses. High doses of MTX make kidney damage with two ways; tubular injury with the precipitation of MTX in kidney tubules and decrease on the glomerular filtration rate[24]. It can mostly be ameliorated with hydration and make the urine alkaline. In patients receiving MTX treatment, the risk of kidney toxicity is 2%[25].

ALA is an effective free radical scavenger[26] found in mitochondria as cofactor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase[11,27]. Tissue cell membranes contain unsaturated fatty acids, nucleic acids and proteins. Lipid peroxidation by free oxygen radicals is an important cause of destruction and oxidative damage[28]. It has contribute to develope methotrexate associated tissue damage[17,29]. Some studies showed mitochondrial functional impairment with oxygen free radicals following treatment with MTX[27]. By the free oxygen radicals attack, lipid peroxidation increase and fail the Na^+/K^+-ATPase activity[30]. Na^+/K^+-ATPase is the other target of oxidative damage[28]. In this work, MTX treatment caused to a significant kidney

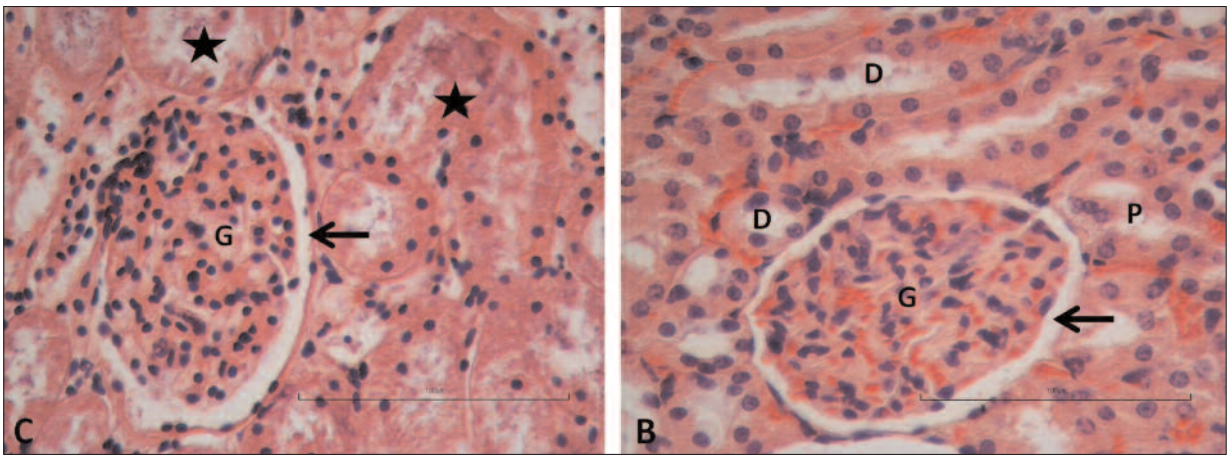

Figure 6. ALA group; Normal histological appearances of kidney tissues. ***A,*** Glomerulus (G), Bowman's space (arrow). ***B,*** Distal tubules (D), proximal tubules (P). H&E, Scale bar = 100 µm.

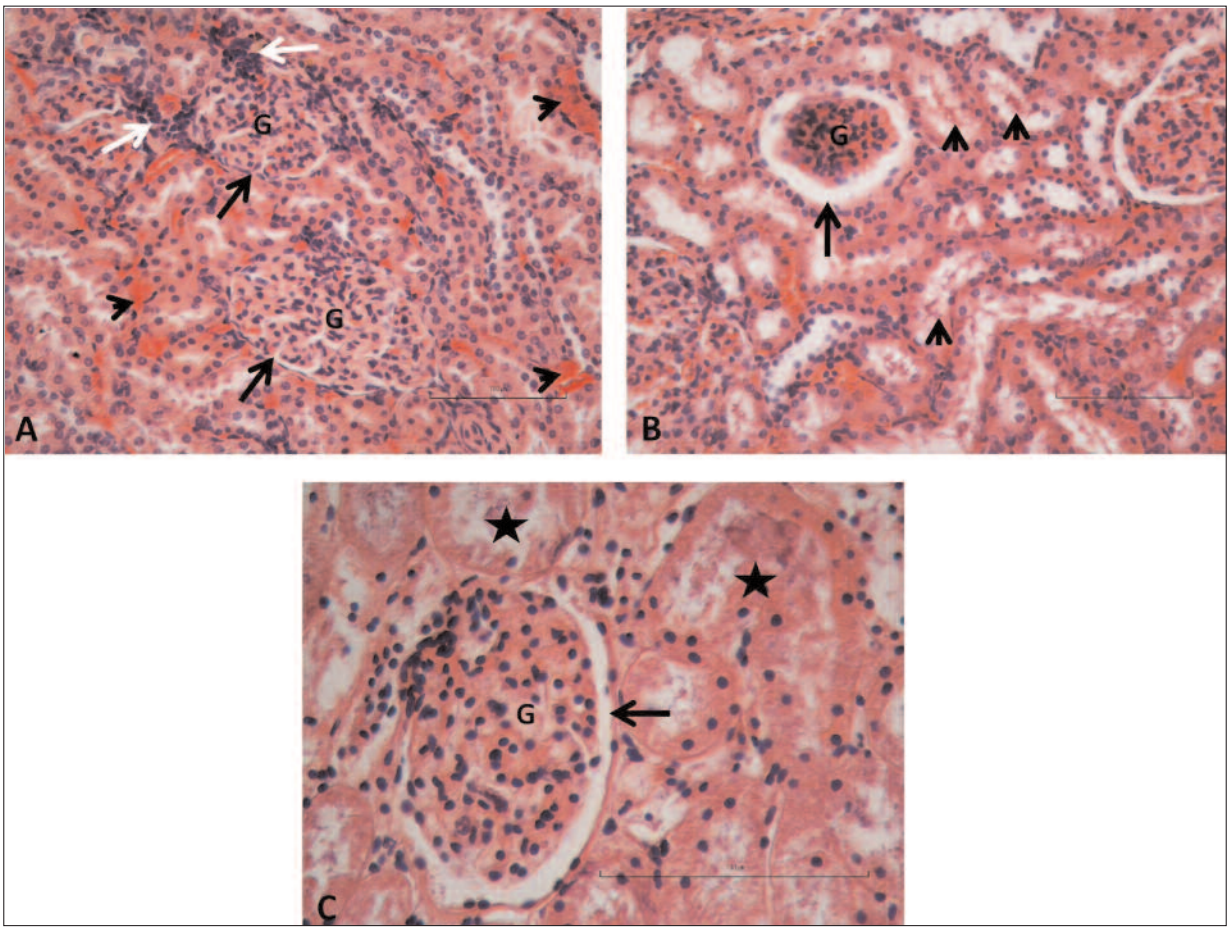

Figure 7. ***B,*** MTX group; ***A,***, Glomerular sclerosis and capillary collapse (G), disappearance of the Bowman's space (*black arrows*), interstitial inflammation (white arrow), vascular congestion (arror heads). ***B,*** Glomerular sclerosis and capillary collapse (G), narrowing of the Bowman's space (black arrows), accumulation of fibrinoid material in the lumen of renal tubules (arrow heads). ***C,***, Glomerular necrosis (G), tubular necrosis (asters), Bowman's space (arrow). H&E, Scale bar = 100 µm.

tissue damage since MDA; a major oxidative end product of cell membrane which was the indicator of the lipid peroxidation is increased while Na^+/K^+-ATPase activity is decreased due to the cell membrane damage.

Free oxygen radicals trigger the leukocytes accumulation in tissue and activate the enzyme (myeloperoxidase, protease and elastase) secretion of neutrophils, thus, leads to further tissue damage. Therefore, MPO plays role in production of oxidants by neutrophils[31,32]. The polimorphonuclear neutrophil infiltration and oxidative stress may play a critical role in MTX-induced kidney damage[33]. In our study, MPO level which is an index of polimorphonuclear leukocyte infiltration was seen to be increased. This indicates that neutrophil accumulation contributes to MTX induced oxidative injury in kidney tissues. Treatment with ALA decreased the MPO activity

MTX-induced kidney toxicity is associated with the activation of the systemic inflammatory response and proinflammatory cytokines[34]. The levels of systemic inflammatory response indicators TNF-α and IL-1β were also be found increased. Treatment with ALA decreased the levels of TNF-α and IL-1β at the plasma.

Reduced glutathione (GSH) has a role in the maintenance and regulation of the thiol-redox status of the cell[35]. In our study tissue GSH depletion is one of the primary factors permitting kidney tissue damage associated with oxidative stress caused by MTX. In the other studies it has been demonstrated that MTX administration results an increase on the MDA levels of the kidneys[36,37].

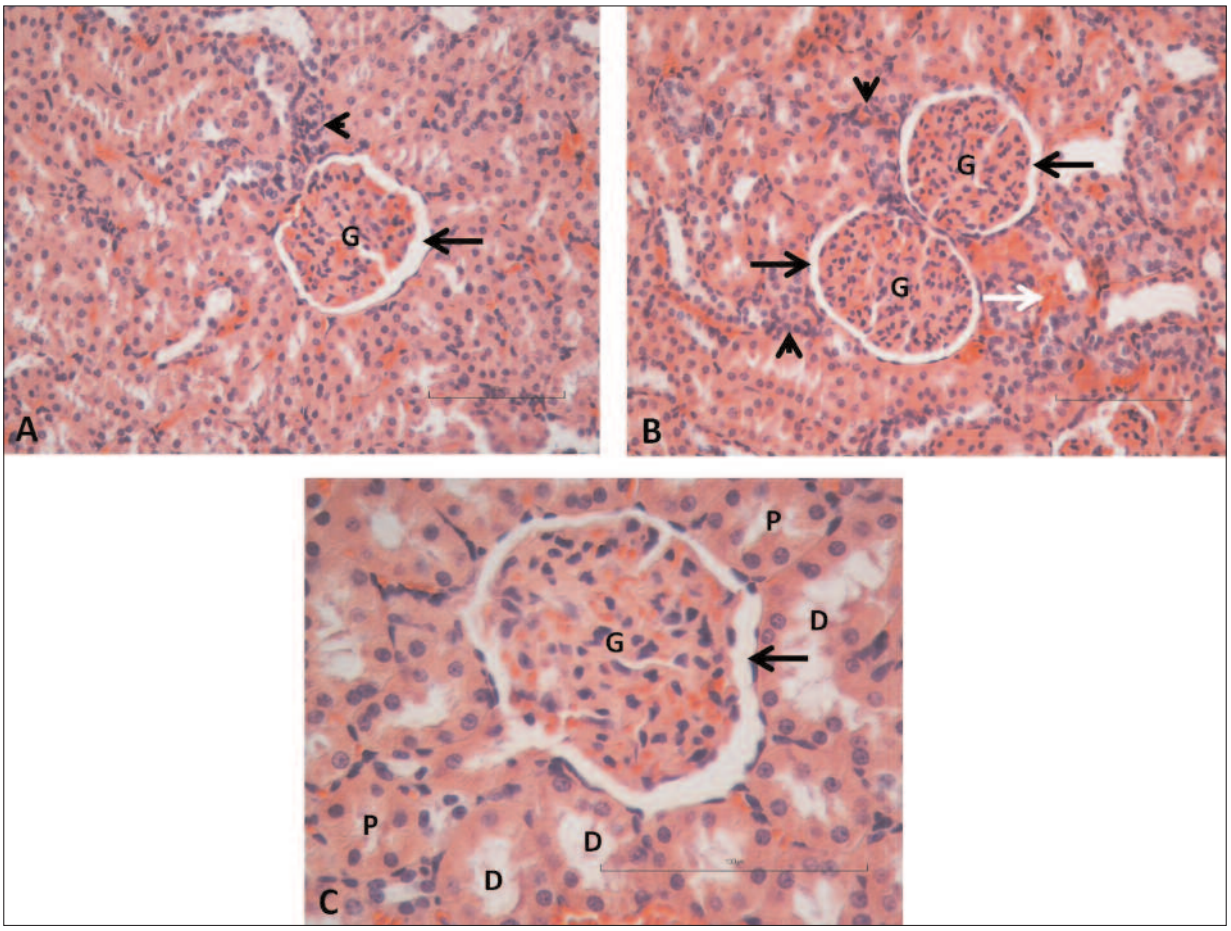

Figure 8. MTX+ALA group; ***A,*** Glomerulus G, Bowman's space (*arrows*), interstitial inflammation (*arrow head*). ***B,*** Glomerulus G, Bowman's space (*black arrows*), interstial inflamation (*arrow head*), vascular congestion (*white arrow*). ***C,*** Glomerulus G, Bowman's space (*arrows*), distal tubules (D), proximal tubules (P). H&E, Scale bar = 100 μm.

In this report, following the MTX administration, ALA treatment was significantly reduced the MDA levels and increased the Na^+/K^+-ATPase enzyme activity, while histological appearance was observed normally in kidney tissue samples.

Conclusions

We have found a reduction of oxidative injury in the MTX-induced kidney by ALA. It can be due to anti inflammatory and antioxidative effects of ALA.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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