European Review for Medical and Pharmacological Sciences 2023; 27: 11103-11108

Alpha-linolenic acid protects against methotrexate-induced nephrotoxicity in mouse kidney cells

H.M. KAPLAN[1], M. DEGER[2], K.E. ERDOGAN[3], T. ATES[2], E. DEMIR[2]

[1]Department of Pharmacology, Faculty of Medicine, Çukurova University, Adana, Turkey
[2]Department of Urology, Faculty of Medicine, Çukurova University, Adana, Turkey
[3]Department of Pathology, Faculty of Medicine, Çukurova University, Adana, Turkey

Abstract. – OBJECTIVE: Methotrexate (MTX) is a folic acid antagonist used in chronic inflammatory diseases and various cancer treatments. Although the main mechanism of the toxic effect of MTX is not known, it is stated that it causes oxidative stress and inflammation. Alpha-linolenic acid (ALA) protects against oxidative stress, apoptosis, and inflammation. For this reason, we aimed to find out the useful effect of ALA on MTX-induced nephrotoxicity

MATERIALS AND METHODS: The mice were divided into 4 groups randomly. The control group was treated with physiological saline solution; the ALA group was treated with ALA (200 mg/kg) by gavage; MTX-treated group received 20 mg/kg i.p. (intraperitoneal) MTX; and MTX+ALA treated group received 20 mg/kg i.p. MTX and ALA 200 mg/kg by gavage. All of the drugs were performed once a day for 9 days.

RESULTS: Alpha-linolenic acid significantly decreased oxidative stress parameters and MTX-induced inflammatory and apoptotic mediators. Furthermore, histopathological examination showed that MTX induced significant edematous damage, and ALA treatment attenuated this damage in renal tissue.

CONCLUSIONS: Our results revealed that ALA may be helpful against MTX-induced nephrotoxicity in mice via its antioxidant and anti-inflammatory properties.

Key Words:
Methotrexate, Alpha-linolenic acid, Nephrotoxicity, Renal, Toxicity.

Abbreviations

Alpha-linolenic acid: ALA; Methotrexate: MTX; Gastrointestinal system: GIS; Polyunsaturated fatty acid: PUFA; Myeloperoxidase: MPO; Malondialdehyde: MDA; Superoxide dismutase: SOD; B-cell lymphoma gene-2: Bcl-2; Bcl-2-associated X: Bax; Cyclooxygenase-2: COX-2; Inducible nitric oxide synthase: iNOS; Phospholipase A2: cPAL2; Glutathione peroxidase: GPx; Catalase: CAT; Lipoprotein saccharide: LPS; Mitogen-activated protein kinase: MAPK.

Introduction

Methotrexate (MTX), used in treating various malignancies and chronic inflammatory diseases, is a folic acid antagonist. Since MTX inhibits dihydrofolate reductase, it has an antifolate-type antimetabolite effect; it restricts the synthesis of DNA, RNA, and proteins[1]. Since the kidneys are the primary excretion site (80-90%), it accumulates in patients with impaired renal function, has a toxic effect on the bone marrow, and may cause pancytopenia[2]. MTX can also acutely impair kidney functions by causing direct toxic effects on tubule epithelial cells through its metabolites and MTX crystals, causing intratubular obstruction[3]. It has been reported[1] that MTX may have significant toxic effects on the liver, gastrointestinal system (GIS), lungs, skin, and nerves, as well as in the bone marrow and kidneys. However, the main mechanism of the nephrotoxic effect of MTX is unknown. It is stated that it causes oxidative stress and inflammation. Oxidative stress increases the amount of lipid peroxidation and decreases the antioxidant expression in the liver; it is indicated that histopathologically, it causes changes ranging from fatty changes to fibrosis[4].

Alpha-linolenic acid (ALA) [(9Z, 12Z, 15Z)-9,12,15-octadecatrienoic acid] is an n-3 polyunsaturated fatty acid (PUFA) essential for health. It cannot be produced in the human body[5]. It is found naturally in flaxseed and many plants. Previous studies[1] show that ALA is a protective agent against oxidative stress, inflammation, and apoptosis. The useful ef-

Corresponding Author: Tunahan Ates, MD; e-mail: drtunahanates0101@gmail.com

fect of ALA on oxidative stress was revealed by normalized intracellular glutathione (GSH) concentrations and decreased inducible nitric oxide synthase (iNOS) expression. ALA also shows a useful effect against inflammation by reducing Nuclear Factor kappa B (NF-κB) activation and cyclooxygenase-2 (COX-2) expression[6]. It also has been revealed[7,8] that ALA acid is useful against apoptosis by reducing levels of caspase-3 protein.

In this study, we aimed to find out the useful effects of ALA on MTX-induced nephrotoxicity in mice by analyzing parameters of oxidative stress, and inflammatory and apoptotic mediators.

Materials and Methods

Chemicals

MTX (20 mg/kg) and ALA (all-MTX-9,12,15-octadecatrienoic acid) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Experimental Process

Ethics committee approval was obtained from Çukurova University Experimental Research Animals Ethics Center (TIBDAM) with decision number 9 at meeting number 5 on 03.09.2019. Our study obtained mice (weighing 30 g, 32 males BALB/c albino) from Cukurova University Experimental Research Animal Ethics Center (TIBDAM). Animals were kept in a 12-hour light and 12-hour dark cycle at 20-22°C and 50-55% humidity. Food and water were provided *ad libitum*. Then, the 32 mice were randomly divided into four groups. Control group: 0.09% NaCl (physiological saline) solution; ALA-treated group: 200 mg/kg ALA by gavage; MTX-treated group: 20 mg/kg intraperitoneal (i.p.) MTX; and ALA+MTX treated group: 20 mg/kg i.p. MTX and 200 mg/kg ALA. All of the drugs were administered once a day for 9 days.

Animals were killed by cervical ejection 24 hours after the last injection. The kidneys of mice were frozen at -80°C and stored in Eppendorf tubes for use in quantitative experiments. The levels of B-cell lymphoma gene-2 (Bcl-2), Bcl-2-associated X (Bax), glutathione peroxidase (Gpx), catalase (CAT), activated (cleaved) caspase-3, myeloperoxidase (MPO), malondialdehyde (MDA) and superoxide dismutase (SOD) were examined in kidney tissue samples.

Homogenization of Tissue

3 ml Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma Aldrich, St. Louis, MO, USA), 30 µl phenylmethane sulfonyl fluoride (PMSF) (Sigma Aldrich, St. Louis, MO, USA), 30 µl sodium vanadate, protease inhibitor of 30 µl was added to the tissues in Eppendorf tubes, then, the tissues were lysed on ice by using an ultrasonic disintegrator. The homogenates were then centrifuged at 10,000 rpm for 10 minutes, the supernatants were removed, and the remaining pellets were discarded frozen at -20°C.

Biochemical Analyses

Bradford method was used for the total protein of samples. Determination of MDA, MPO, SOD, GPx, and CAT activity was done as described in a previous work[9].

ELISA (Enzyme Linked Immunosorbent Test) Test

The value of Bcl-2, Bax, COX-2, cleaved caspase-3, iNOS, and phospholipase A2 (cPAL2) enzymes was tested by ELISA according to the manufacturer's test protocol. ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Statistical Analysis

Statistical analysis used Graph Pad Prism 4.0 (Graph Pad Software, San Diego, CA, USA). Data were stated as mean ± standard error. Comparisons of the group were made using a one-way analysis of variance (ANOVA) (Bonferroni post hoc) test. *p*-values were considered significant if lower than 0.05.

Results

MTX-induced MDA and MPO levels compared to the control group. ALA reduced MDA and MPO levels that were induced by MTX compared to only the MTX-treated group ($p<0.05$) (Figure 1).

Bax and cleaved-caspase-3 levels were found to be significantly higher in the MTX-treated group than in the control group ($p<0.05$). However, Bax and activated caspase-3 levels were substantially lower in the ALA + MTX-treated group than in the MTX-treated group (Figure 2).

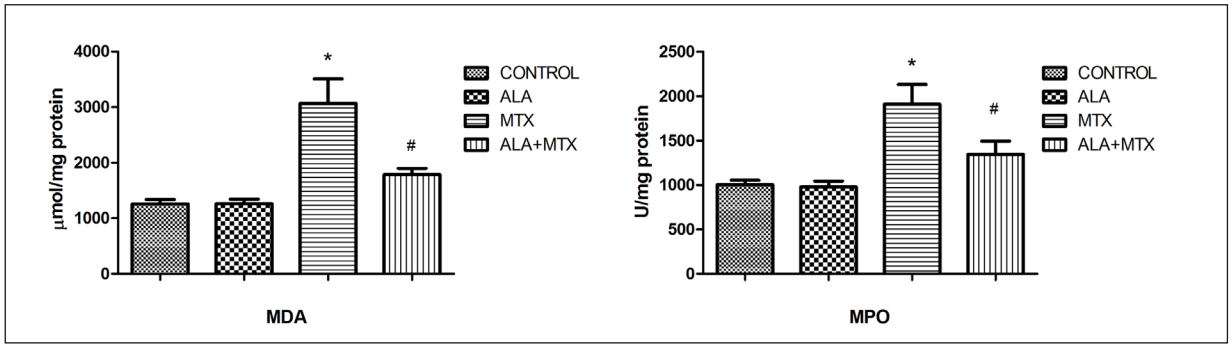

Figure 1. MDA and MPO levels in the groups.

Bcl-2 levels were significantly decreased in the MTX-treated group compared to the control group ($p<0.05$). They were significantly increased in the ALA + MTX-treated group than in the MTX-treated group ($p<0.05$) (Figure 2).

MTX reduced the expression of SOD, CAT, and GPx compared to the control group ($p<0.05$). ALA significantly increased the level of SOD, CAT, and GPx compared to the MTX-treated group ($p<0.05$) (Figure 3).

COX-2, cPLA2 and iNOS levels were found to be significantly higher in the MTX-treated group than in the control group ($p<0.05$). However, COX-2, cPLA2, and iNOS levels were significantly lower in the ALA + MTX-treated group than in the MTX-treated group (Figure 4).

The kidney biopsy specimen consisted of medullary and cortical areas. Damage caused by MTX appeared to affect the interstitial space primarily. Thus, glomerular cells were normal, but there was significant interstitial edema. It was shown that ALA treatment attenuated edema (Figure 5).

Discussion

Nephrotoxicity is a significant side effect of methotrexate[10]. Methotrexate-induced toxicity depends on many factors, such as the duration and dose schedule of treatment with methotrexate[11]. Our findings showed that MTX administration induced the apoptotic pathway. However, MTX application also caused an increase in oxidative stress. In studies[11], it has been demonstrated that the decrease in the level of glutathione with MTX application reduced the activity of the antioxidant defense system that protects the cells by scavenging reactive oxygen radicals such as hydroxyl radicals, superoxide anion, hydrochloric radicals, and hydrogen peroxide. In another study[12], it was shown that oxidative stress, especially neutrophil infiltration, plays a role in the damage to the small intestine

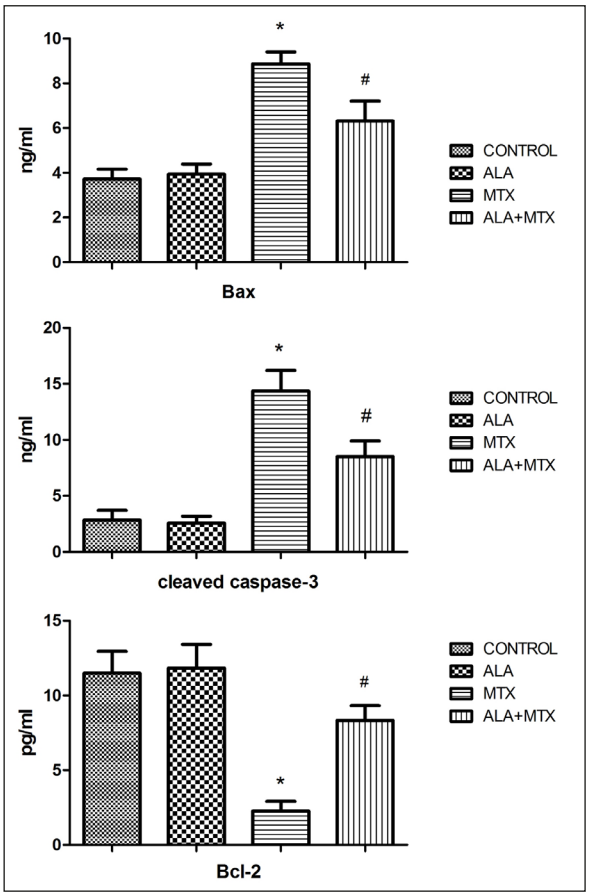

Figure 2. Bax, cleaved caspase-3 and Bcl-2 levels in the groups

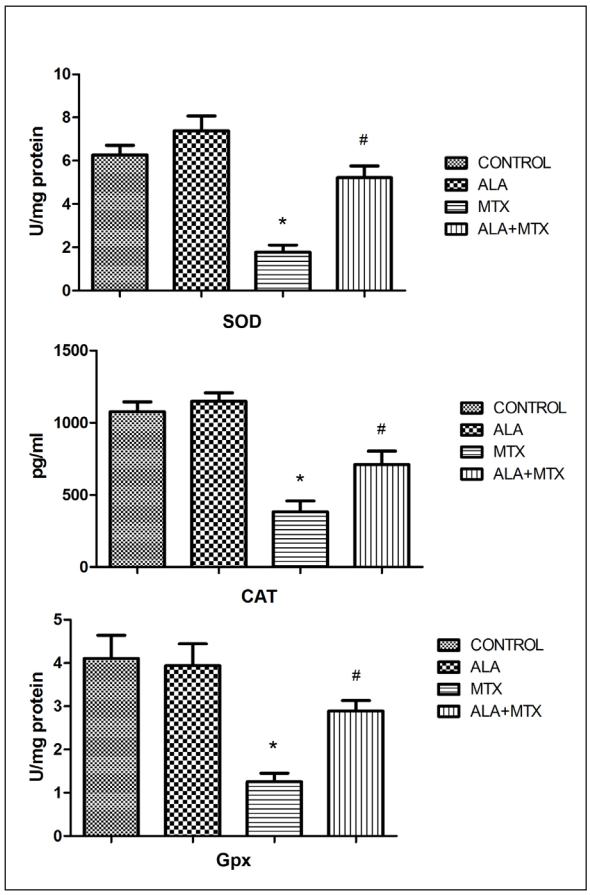

Figure 3. SOD, CAT and Gpx levels in the groups.

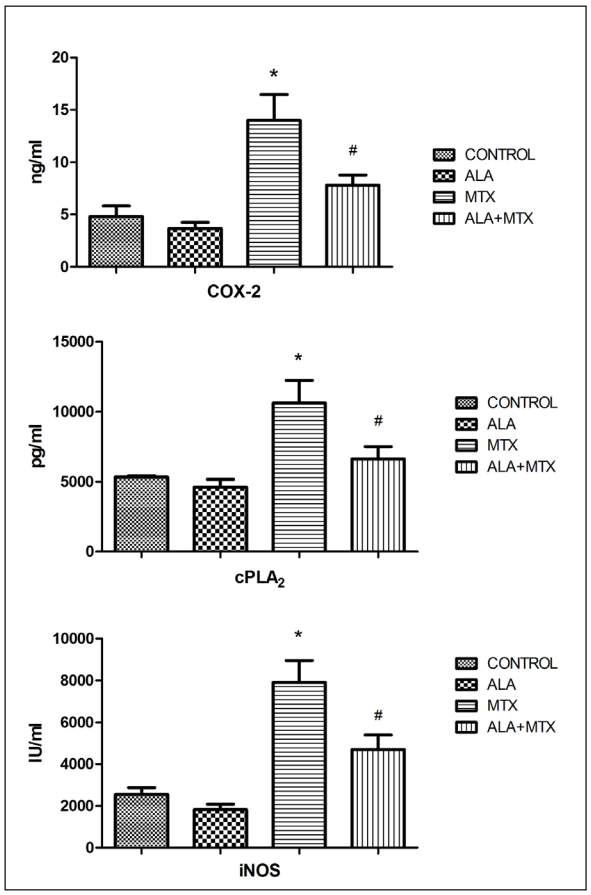

Figure 4. COX-2, cPLA2 and iNOS levels in the groups.

induced by MTX. In another study[13], they revealed that oxidative stress has an initial role in methotrexate nephrotoxicity. When phagocytic leukocytes are overstimulated, they increase their oxygen consumption. This event is called a respiratory/oxidative burst of neutrophils; activated neutrophils secrete some enzymes such as myeloperoxidase, elastase, and protease and release oxygen radicals[14]. Myeloperoxidase is a determinant of neutrophil infiltration and is important for neutrophil functions. In addition to their direct damaging effects on tissues, free radicals also cause tissue damage by collecting leukocytes in the damaged tissue[15]. However, methotrexate has been reported[16] to increase inducible nitric oxide levels in the kidney. ALA, which we used in our study, has both antioxidant and anti-inflammatory properties. Our findings showed that MTX-induced apoptosis induction, inflammation, and reduced oxidative stress.

ALA protects the heart and cardiovascular system[17]. ALA has potent antioxidant and anti-inflammatory activity[18]. Inhibiting oxidative stress also contributes to the prevention of the expression of inflammatory mediators[19]. ALA also attenuates inflammation caused by lipoprotein saccharide (LPS), NF-κB translocation and phosphorylation of mitogen-activated protein kinase (MAPK). This inhibition decreases levels of iNOS, COX-2, and TNF-alpha, which are inflammatory mediators[6]. Studies[8] have reported that ALA is protective against gentamicin-induced nephrotoxicity.

Conclusions

Our study revealed that co-treatment of ALA with MTX reduced the toxic effects of MTX. Considering that MTX has a wide area of use in rheumatology and cancer treatment, it is important to reduce its side effects. In conclusion, combining MTX with alpha-linolenic acid will reduce the nephrotoxic effects of MTX.

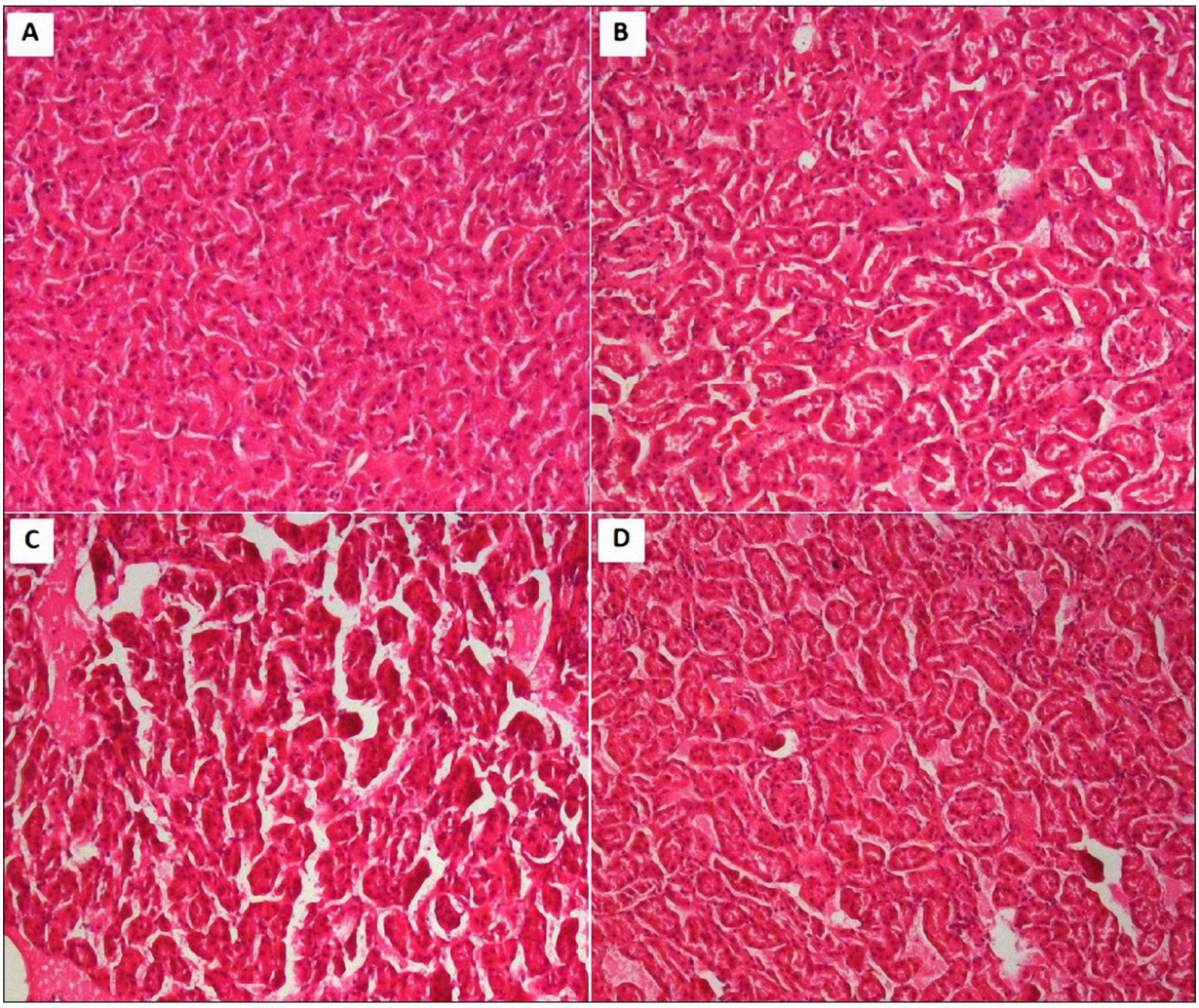

Figure 5. Micrograph of the kidney of control mice (**A**); mice treated with alpha-linolenic acid (**B**); mice treated with MTX (**C**); mice treated with MTX+ALA (**D**). Alpha-linolenic acid indicated no toxic effect on kidneys, while cisplatin administration resulted in edema. ALA administration attenuated edema induced by MTX (Light micrograph sections of the kidney of control mice. Arrow: glomerulus (renal corpuscle), asterisk: tubulus. Scale bar = 100 um)

Conflict of Interest
The authors declare that they have no conflict of interests.

Acknowledgements
Authors want to thank Dr. Halil Mahir Kaplan, who contributed greatly to the conduct of the study.

Funding
Financial support was received from Çukurova University Scientific Research Projects with project number 2018-10148.

Ethics Approval
Ethics committee approval was obtained from Çukurova University Experimental Research Animals Ethics Center (TIBDAM) with decision number 9 at meeting number 5 on 03.09.2019.

Availability of Data and Materials
All data and material is accessible.

Informed Consent
Not applicable.

Authors' Contribution
Conception and Design: Halil Mahir Kaplan. Acquisition of Data: Halil Mahir Kaplan. Analysis and Interpretation of Data: Kıvılcım Eren Erdogan. Statistical Analysis: Tunahan Ates. Obtaining Funding: Erkan Demir. Drafting of The Manuscript: Mutlu Deger.

ORCID ID
H.M. Kaplan: 0000-0002-1911-7327
M. Deger: 0000-0002-8357-5744

K.E. Erdogan: 0000-0002-4951-8703
T. Ates: 0000-0001-9087-290X
E. Demir: 0000-0003-2676-5346

References

1) Pınar N, Kaplan M, Özgür T, Özcan O. Ameliorating effects of tempol on methotrexate-induced liver injury in rats. Biomed Pharmacother 2018; 102: 758-764.
2) Pınar N, Çakırca G, Özgür T, Kaplan M. The protective effects of alpha lipoic acid on methotrexate induced testis injury in rats. Biomed Pharmacother 2018; 97: 1486-1492.
3) Jafaripour L, Naserzadeh R, Alizamani E, Javad Mashhadi SM, Moghadam E, Nouryazdan N, Ahmadvand H. Effects of Rosmarinic Acid on Methotrexate-induced Nephrotoxicity and Hepatotoxicity in Wistar Rats. Indian J Nephrol 2021; 31: 218-224.
4) Ali N, Rashid S, Nafees S, Hasan SK, Shahid A, Majed F, Sultana S. Protective effect of Chlorogenic acid against methotrexate induced oxidative stress, inflammation and apoptosis in rat liver: An experimental approach. Chem Biol Interact 2017; 272: 80-91.
5) Istifli ES, Demir E, Kaplan HM, Ates KE, Doran F. Alpha-linolenic acid confers protection on mice renal cells against cisplatin-induced nephrotoxicity. Cytotechnology 2019; 71: 905-914.
6) Ren J, Chung SH. Anti-inflammatory effect of alpha-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-kappaB and mitogen-activated protein kinase pathways. J Agric Food Chem 2007; 55: 5073-5080.
7) Kaplan HM, Kuyucu Y, Polat S, Pazarci P, Yegani AA, Singirik E, Ertug P. Molecular basis of vascular damage caused by cigarette smoke exposure and a new approach to the treatment: Alpha-linolenic acid. Biomed Pharmacother 2018; 102: 458-463.
8) Kaplan H, Izol V, Aridogan I, Olgan E, Yegani A, Pazarci P, Singirik E. Protective Effect of Alpha-linolenic Acid on Gentamicin Induced Nephrotoxicity in Mice. Int J Pharmacol 2016; 12: 562-566.
9) Pinar N, Cakirca G, Hakverdi S, Kaplan M. Protective effect of alpha lipoic acid on cisplatin induced hepatotoxicity in rats. Biotech Histochem 2020; 95: 219-224.
10) Caglar Y, Ozgur H, Matur I, Yenilmez ED, Tuli A, Gonlusen G, Polat S. Ultrastructural evaluation of the effect of N-acetylcysteine on methotrexate nephrotoxicity in rats. Histol Histopathol 2013; 28: 865-874.
11) Cetinkaya A, Bulbuloglu E, Kurutas EB, Kantarceken B. N-acetylcysteine ameliorates methotrexate-induced oxidative liver damage in rats. Med Sci Monit 2006; 12: 274-278.
12) Miyazono Y, Gao F, Horie T. Oxidative stress contributes to methotrexate-induced small intestinal toxicity in rats. Scand J Gastroenterol 2004; 39: 1119-1127.
13) Devrim E, Cetin R, Kilicoglu B, Erguder BI, Avci A, Durak I. Methotrexate causes oxidative stress in rat kidney tissues. Ren Fail 2005; 27: 771-773.
14) Ascaso FJ, Huerva V, Grzybowski A. The role of inflammation in the pathogenesis of macular edema secondary to retinal vascular diseases. Mediators Inflamm 2014; 2014: 432-685.
15) Martin-Ventura JL, Madrigal-Matute J, Martinez-Pinna R, Romoz-Mozo P, Blanco-Colio LM. Erythrocytes, leukocytes and platelets as a source of oxidative stress in chronic vascular diseases: detoxifying mechanisms and potential therapeutic options. Thromb Haemost 2012; 108: 435-442.
16) Uz E, Oktem F, Yilmaz HR, Uzar E, Ozguner F. The activities of purine-catabolizing enzymes and the level of nitric oxide in rat kidneys subjected to methotrexate: protective effect of caffeic acid phenethyl ester. Mol Cell Biochem 2005; 277: 165-170.
17) Sekikawa A, Doyle MF, Kuller LH. Recent findings of long-chain n-3 polyunsaturated fatty acids (LCn-3 PUFAs) on atherosclerosis and coronary heart disease (CHD) contrasting studies in Western countries to Japan. Trends Cardiovasc Med 2015; 25: 717-723.
18) Kaplan HM, Singirik E, Erdogan KE, Doran F. Protective effect of alpha-linolenic acid on gentamicin-induced ototoxicity in mice. Somatosens Mot Res 2017; 34: 145-150.
19) Alessandri C, Pignatelli P, Loffredo L, Lenti L, Del Ben M. Alpha-linolenic acid-rich wheat germ oil decreases oxidative stress and CD40 ligand in patients with mild hypercholesterolemia. Arterioscler Thromb Vasc Biol 2006; 26: 2577-2578.