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ORIGINAL RESEARCH

Protective Effects of Alpha-Lipoic Acid on Methotrexate-Induced Oxidative Lung Injury in Rats

Huseyin Arpag, MD,[1] Mehmet Gül, MD,[2] Yusuf Aydemir, MD,[3] Nurhan Atilla, MD,[1] Birgül Yiğitcan, MD,[2] Tugrul Cakir, MD,[4] Cemal Polat, MD,[5] Özer Þehirli, MD,[6] Muhammet Sayan, MD[7]

[1]*Department of Chest Disease, Kahramanmaras Sutcu Imam University Medical Faculty, Kahramanmaras, Turkey,* [2]*Department of Histology, Malatya Inonu University Medical Faculty, Malatya, Turkey,* [3]*Department of Chest Diseases, Sakarya University Medical Faculty, Sakarya, Turkey,* [4]*Department of General Surgery, Antalya Education and Research Hospital, Antalya, Turkey,* [5]*Department of Biochemistry, Public Health Laboratuary, Kütahya, Turkey,* [6]*Department of Pharmacology, Marmara University Medicine Faculty, Istanbul, Turkey and Near East University Faculty of Denstry, Nicosia, North Cyprus,* [7]*Department of Thoracic Surgery Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkey*

ABSTRACT

Objective: Oxidative stress is one of the major causes of methotrexate induced lung injury (MILI). Alpha-lipoic acid (ALA), which occurs naturally in human food, has antioxidative and anti-inflammatory activities. The aim of this study was to research the potential protective role of ALA on MILI in rats. *Methods*: Twenty one rats were randomly subdivided into three groups: control (group I), methotrexate (MTX) treated (group II), and MTX+ALA treated (group III). Lung injury was performed with a single dose of MTX (20 mg/kg) to groups 2 and 3. On the sixth day, animals in all groups were sacrificed by decapitation and lung tissue and blood samples were removed for histological examination and also measurement the levels of interleukin-1-beta (IL-1β), malondialdehyde (MDA), glutathione (GSH), tumour necrosis factor-alpha (TNF-α), myeloperoxidase (MPO), and sodium potassium-adenosine triphosphatase (Na+/K+ATPase). *Results*: In MTX group tissue GSH, Na+/K+ATPase activities were lower, tissue MDA, MPO and plasma IL-1?, TNF-? were significantly higher than the other groups. Histopathological examination showed that lung injury was less severe in group 2 according to group 3. *Conclusions*: Oxidative damage of MTX in rat lung is partially reduced when combined with ALA.

Keywords: lung; oxidative stress; alpha-lipoic acid; methotrexate; rat; injury

INTRODUCTION

Methotrexate (MTX) is an antiproliferative folic acid antagonist used in the treatment of various cancers and chronic inflammatory diseases [1]. In 60 to 93 percent of patients treated with MTX, there have been a number of adverse reactions, including nonproductive cough, dyspnea, fever, pneumonitis, interstitial lung disease, and lung fibrosis [2]. Most of these reactions are dose-dependent in nature and are not life-threatening, but up to 30 percent of patients treated with MTX for more than 5 years discontinue the therapy because of unacceptable toxicity [3].

Although the precise mechanisms by which MTX cause lung injury are unknown, some explanations have been proposed. One of the possible reasons associated with the pathogenesis of methotrexate-induced lung injury (MILI) is increased oxidative stress.

Malondialdehyde (MDA) is known to be an oxidant and glutathione (GSH) an antioxidant parameter [4]. Therefore, increased MDA and decreased GSH amounts indicate the development of oxidative stress. Myeloperoxidase (MPO) as an endogenous oxidant lysosomal enzyme available in polymorphonuclear neutrophils and monocytes catalyzes the reaction between the chloride ion and hydrogen peroxide

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Address correspondence to Nurhan Atilla, Kahramanmaras Sutcu Imam Universitesi, Kahramanmaras, 46100 Turkey. E-mail: nurhanatilla@yahoo.com.tr

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and generates hypochlorous acid and other reactive oxygen species [5]. Interleukin-1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are proinflammatory cytokines. They are produced predominantly by activated macrophages and are involved in the upregulation of inflammatory reactions [6].

MTX has been reported to decrease the GSH level and to significantly increase levels of MPO, MDA, IL-1β, and TNF-α, which are indicators of inflammatory response [7]. In addition, overdose of MTX can lead to proinflammatory cytokine release due to the increase in oxidative stress and reactive oxygen species (ROS) formation [8]. The function of the Na+/K+-ATPase is to transfer sodium ions out of the cell and potassium ions into the cell by using ATP. The cell will remain alive as long as this task is performed and the intensity difference is continuous. Without nutrients and oxygen, there is no ATP, and as a result the enzyme becomes inoperative and the cell dies.

Recently, the popularity of antioxidant herbal medicine is increasing worldwide. Alpha-lipoic acid (ALA) also named as thioctic acid is a synthetic version of lipoic acid and synthesized by both animals and plants. Basically, ALA is found in every cell, where it helps turn glucose into energy [9]. Other antioxidants work only in water (such as vitamin C) or fatty tissues (such as vitamin E) [10]. However, ALA is both fat and water soluble, meaning that it can work throughout the body [10]. Antioxidants in the body are used up as they attack free radicals [11]. But evidence suggests ALA may help regenerate these other antioxidants and make them active again [12].

MILI can be reversed with early recognition, drug withdrawal and high dose anti-inflammatory agents, such as methylprednisolone. It has been shown in experimental studies that, ALA protects lung from oleic acid and lipopolysaccharide induced injury [13, 14].

In an effort to develop treatment modalities that reduce lung toxicity following MTX use, we investigated whether ALA as an antioxidant, inhibit MILI in a rat model.

MATERIALS AND METHODS

Animals and Experimental Design

We used 21 male Wistar rats weighing between 190–240 g. The rats were maintained under optimal laboratory conditions (40–60% humidity, 24±3°C, 12/12 h light /dark cycle), drinking water amounts were not limited and fed on a standard nutrition.

Three groups each consisting of seven rats was formed. The organizations of experimental groups were as follows:

Group 1 (control group): received 100 mg/kg physiological saline intraperitoneally.
Group 2 (MTX group): received 20 mg/kg single dose of MTX (Emthexat-s, 50 mg ampoule) intraperitoneally.
Group 3 (MTX-ALA group): received a single dose of MTX and also received 60 mg/kg ALA (Thioctacid, 50 ml ampoule) which was dissolved in 0.1% dimethyl sulfoxide, intraperitoneally for 5 days.

Blood samples were obtained by decapitation at the sixth day. IL-1β and TNF-α levels were measured in plasma. Also, the lungs of all animals were removed. The levels of sodiumpotassium adenosine triphosphatase (Na+/K+-ATPase), MDA, MPO, and GSH were analyzed in lung tissues. Additionally, degree of inflammation and histopathologic damage were evaluated histologically.

Evaluation of Glutathione and Malondialdehyde Levels in Tissue

Lung tissue specimens were homogenized in ice cold 150 mm KCl to determine the GSH and MDA levels. Lipid peroxidation of lung tissue specimens was expressed with MDA (nmol MDA/g tissue) [15]. Ellman's spectrophotometric reagent was used for measuring the GSH levels (μmol GSH/g tissue) [16].

Evaluation of Myeloperoxidase Levels in Tissue

We used the method described from Hillegass et al for measuring the MPO levels in lung tissue [17]. Lung tissue specimens were intermixed in 50 mM potassium phosphate buffer (PB, pH 6.0), then centrifuged at 41400 g for 10 minutes. The pellet was obtained after separating the supernatant of sample. Finally; a suspension was formed containing pellet, 50 mM PB, 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 41400 g for 10 minutes after three freeze and thaw cycles. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM hydrogen peroxide (H2O2) solution. The amount of MPO that caused a change in the absorbance measured at 460 nm for 3 minutes was considered as one unit of enzyme activity (U/g).

Evaluation of Na+/K+-ATPase Levels in Tissue

The value of inorganic phosphate (Pi) produced from 3 mm disodium adenosine triphosphate (Na2ATP) added to the incubation medium was considered as Na+/K+-ATPase activity [18]. The contents of

medium in mm were 6 MgCl2, 5 KCL, 0.1 EDTA, 100 NaCl, and 30 Tris HCL (pH 7.4). The medium was incubated in water bath at 37°C during 5 minutes. Subsequently 3 mm of Na2ATP was added into each tube then incubated at 37°C for 30 minutes. Finally; the tubes were placed in an ice bath to stop the reaction and then centrifuged at 3500 g. The method of Fiske and Subarrow was used for determination of Pi in the supernatant fraction [19]. Lowry method was used for measuring the protein concentration of the supernatant [20].

Biochemical Analysis of Plasma

Enzyme-linked immunosorbent assay kits (Biosource International, Nivelles, Belgium) were used for measuring the plasma IL-1β and TNF-α.

Histopathologic Evaluations

Lung tissue samples were separated for histopathologic and biochemical examinations. Each lung sample was processed for the examination of light microscope then placed in 10% neutral formalin for 48 h and prepared for parafin embedding. Tissue samples were cut into 6 µm thick sections, placed on slides and stained with hematoxylin–eosin (H&E). Sections were examined by blind histologist in a light microscope (Leica DFC280) and analyzed by the Leica Q Win Plus V3 Image Analysis System (Leica Micros Imaging Solutions, Cambridge, UK).

Statistical Analysis

GraphPad Prism 3.0 (GraphPad Software, San Diego, USA) was used for statistical analysis. Post-hoc Tukey's honestly significant difference test and one-way analysis of variance (ANOVA) was used for multiple comparisons between groups. The data was expressed as mean±standard deviation (SD). Differences with values of $p < 0.05$ were accepted statistically significant.

RESULTS

Biochemical Data of Tissue

The MDA (48.2 ± 7.3 nmol/g) and MPO (42.1 ± 9.6 U/g tissue) values of the MTX group were significantly higher than those of the control (28.8 ± 4.8 nmol/g, 18.1 ± 4.6 U/g tissue) and MTX+ALA (28.9 ± 6.3 nmol/g, 21.9 ± 8.2 U/g tissue) groups. (Figure 1b, 1c). The GSH and Na+/K+-ATPase values of the MTX group (1.75 ± 0.1 µmol/g, 1.32 ± 0.1 µmol/mg) were statistically lower than those of the control (2.86 ± 0.5 µmol/g, 4.12 ± 1.1 µmol/mg) and MTX+ALA group (2.78 ± 0.9 µmol/g, 3.87 ± 0.7 µmol/mg). (Figure 1a, 1d).

TABLE 1 Effect of ALA on some biochemical parameters in the plasma of control, MTX, MTX-ALA groups consisting of 7 animals each

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.
Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

Biochemical Data of Plasma

In the MTX group plasma TNF-α levels were higher than control group ($p < 0.05$), whereas this MTX-induced rise was abolished ($p < 0.05$) with ALA treatment. Similarly IL-1β was also increased in serum of MTX group ($p < 0.05$), however, when rats were treated with ALA following MTX administration, these cytokines were revert to control levels (Table 1).

Histopathologic Data

The lumen of bronchi and bronchioles was open, respiratory epithelial and mucosal structures, lung parenchyma, interalveolar septas, and alveoli were evaluated histologically in normal structure in control group (Figure 2a, 2b, 2c).

The lung sections from the MTX-treated group showed some histopathological changes such as the loss of cilia and bronchial squamous metaplasia in the respiratory epithelium in the form of common and large areas. Degenerative changes in the epithelium of bronchioles and cellular debris in the lumen of bronchioles were detected. Furthermore intense lymphocyte infiltration into epithelium was detected in the mucosa of bronchi and bronchioles. Diffuse and severe mononuclear cell infiltration in the interstitial space of lung parenchyma, congestion and thickening of interalveolar septa and loss of alveoli revealed (Figure 3a, 3b, 3c, 3d).

In MTX+ALA group degenerative changes and damage in lung sections was significantly less than MTX group. Interalveolar septa were thicker than control group, while that in the MTX group was found to be markedly thinner (Figure 4a, 4b, 4c, 4d).

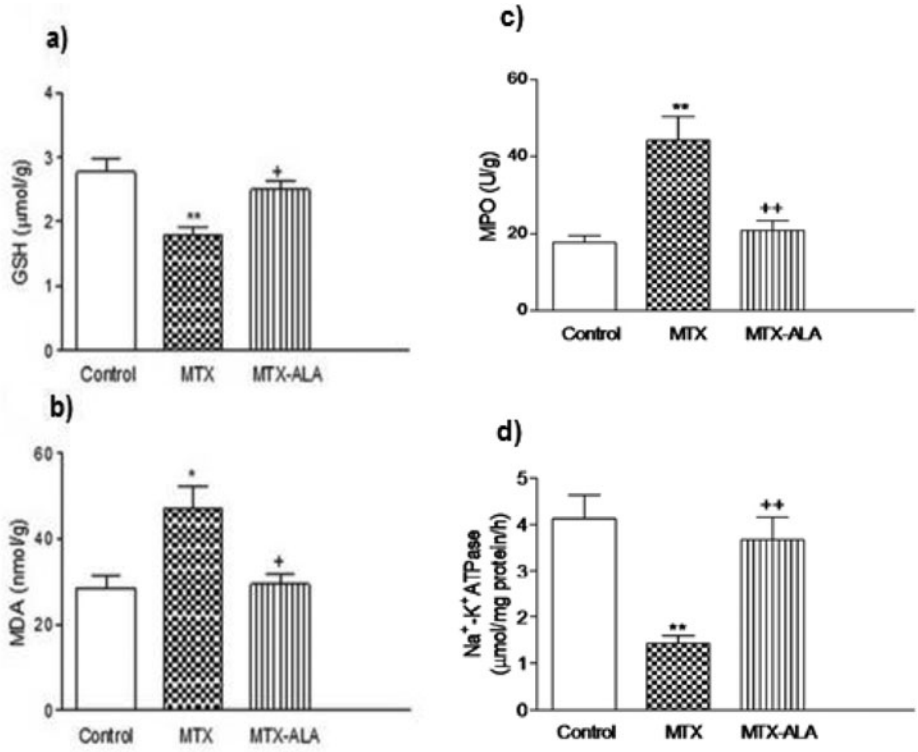

FIGURE 1 Biochemical findings of lung tissue of control, MTX, MTX-ALA groups consisting of 7 animals each. (a) Glutathione (GSH) (b) Malondialdehyde (MDA) levels in the lung tissues of control, methotrexate (MTX), and MTX-ALA (α -lipoic acid) groups. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $*$ $p<0.05$, $**$ $p<0.01$; compared to control group. $+$ $p < 0.05$; compared to MTX group. (c) Myeloperoxidase (MPO) (d) Na+-K+ ATPase activity in the lung tissues of control, MTX and MTX-ALA (α -lipoic acid) groups. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $**$ $p<0.01$; compared to control group. $++$ $p < 0.01$; compared to MTX group.

We evaluated the presence of tissue inflammation, congestion, edema and epithelial degeneration histopathologically. Inflammation scores (IS), congestion scores (CS), edema scores (ES), and epithelial degeneration (EDS) of all groups were shown in Table 2.

In MTX group, IS, CS, ES, and EDS were significantly higher than those in control and MTX+ALA groups ($p < 0.05$).

DISCUSSION

In this present study, it is histopathologically and biochemically demonstrated that; MILI is significantly inhibited by posttreatment with ALA at rat lung. As far as we know, this is the first study that has investigated ALA's protective effects against MILI.

Our result is compatible with other experimental rat studies showing that oxidative stress has a critical role

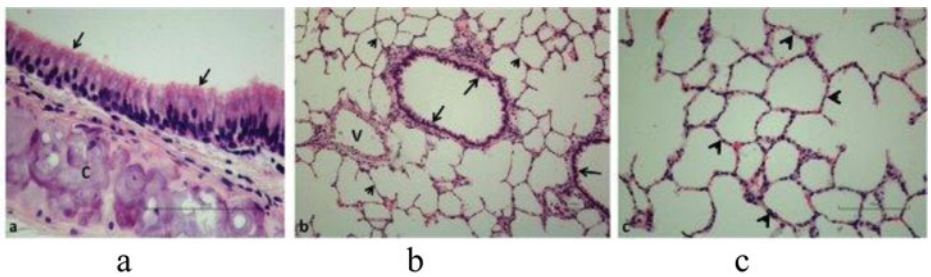

FIGURE 2 Histopathological findings of control group (n:7). (a) Normal ciliated respiratory epithelium of bronchus (arrows), cartilage (C). H&E, Scale bar = 100 µm. (b) Normal bronchiole epithelium (arrows), vessel V), interalveolar septum (arrow head). H&E, Scale bar = 100 µm. (c) Interalveolar septum (arrow head). H&E, Scale bar = 100 µm.

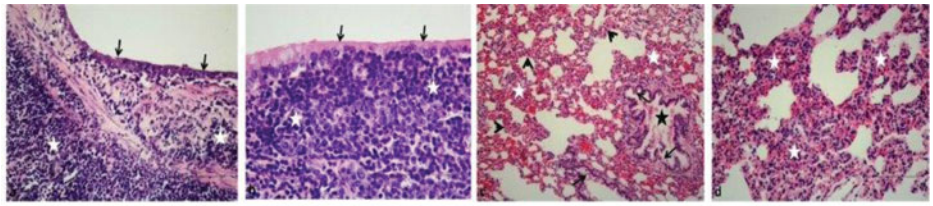

FIGURE 3 Histopathological findings of MTX group (n:7); (a) Squamous metaplasia and loss of cilia on the bronchus epithelium (arrows), mononuclear cell infiltration on in the bronchial mucosa (asters). H&E, Scale bar = 100 μm. (b) Loss of cilia on the bronchus epithelium (arrows), Inflammatory cell infiltration in the bronchial mucosa (asters). H&E, Scale bar = 100 μm. (c) Thickening of interstitial space (arrow head), mononuclear cell infiltration on and congestion (asters), degeneration of bronchial epithelium, cellular debris in the bronchiolar lumen. H&E, Scale bar = 100 μm. (d) Thickening of interstitial space, mononuclear cell infiltration on and congestion (asters). H&E, Scale bar = 100 μm.

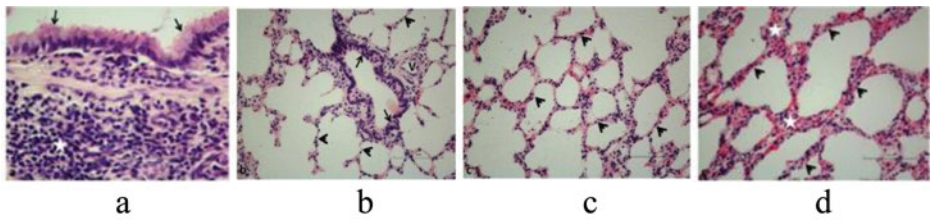

FIGURE 4 Histopathological findings of MTX+ALA group (n:7). (a) Partial loss of cilia in the bronchus epithelium (arrows), inflammatory cell infiltration in the bronchial mucosa (aster). H&E, Scale bar = 100 μm. (b) Normal bronchiole epithelium (arrows), vessel (V), interalveolar septum (arrow head). H&E, Scale bar = 100 μm. (c) Interalveolar septum (arrow head). H&E, Scale bar = 100 μm. (d) Thickening of interstitial space (arrow head), mononuclear cell infiltration on and congestion (asters). H&E, Scale bar = 100 μm.

in MTX induced tissue damage and antioxidants such as erythropoietin (EPO) and N-acetyl-cysteine (NAC) decreases the side effects of MTX [21, 22].

MTX is prescribed for the treatment of a wide range of conditions, including cancer, rheumatoid arthritis, psoriasis, uveitis, asthma, granulomatosis with polyangiitis, sarcoidosis, primary biliary cirrhosis, and inflammatory bowel disease. Over the past 3 decades there have been numerous reports of MILI, mainly acute interstitial pneumonitis. It is estimated that acute lung toxicity develops in 1–8% of patients that receive MTX treatment for rheumatologic conditions, but several reports suggest that the incidence is as high as 33% [23, 24]. Although the pathophysiology of MILI is still outstanding, most researchers think that MTX generates a type IV delayed hypersensitivity reaction presenting lymphocytic proliferation and alveolitis [23, 24]. In our study, we determined congestion and lymphocytic infiltration in histopathological examination of MTX group. By administration of ALA to MTX, IS, CS, ES, EDS, was significantly decreased.

ALA which is known as antioxidant, found in mitochondria as cofactor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase and is an effective free radical scavenger [25]. A diet rich in phytochemicals and antioxidants helps prevent certain diseases. Natural compounds and derivatives that are rich in antioxidants reduce the adverse effects resulting from the toxicity of many chemicals [26]. The antioxidant effect of ALA on tissues like sciatic nerve, kidney, and liver has shown in several rat studies [27–29]. When we searched the literature, we found two stud-

TABLE 2 Histopathologic findings of lung tissue

	Control	MTX	p	MTX+ALA	p
Inflammation	0.00 ± 0.32	3.05 ± 0.69	0.002	1.06 ± 0.54	<0.001
Congestion	0.00 ± 0.44	3.48 ± 0.51	<0.001	1.98 ± 0.01	<0.001
Edema	0.00 ± 0.32	2.04 ± 0.48	0.001	1.00 ± 0.21	<0.001
Epithelial degeneration	0.00 ± 0.41	3.09 ± 0.42	0.008	1.38 ± 0.59	0.002

MTX, methotrexate; ALA, alpha-lipoic acid

ies related ALA effects on oxidative lung injury. But in these studies none of the researchers used MTX, they used lipopolysaccharide and oleic acid to create MILI [13, 14].

MTX has anti-inflammatory and immunosuppressive properties because it facilitates the production of reactive oxygen species (ROS) [30]. ROS that result from MTX treatment enhance its effectiveness as a medication, as well as its toxicity. Experimental studies have demonstrated that there is an increased production of oxygen-free radicals following MTX treatment, and these free radicals might lead to mitochondrial impairment [31, 32].

MTX-induced toxicity activates an inflammatory response and significantly increases the production of pro-inflammatory cytokines [33]. Lipid peroxidation by free oxygen radicals is an important cause of oxidative damage to cell membranes [34, 35]. MDA is a highly biologically active oxidative degradation product from membrane unsaturated fatty acids. As such, MDA serves as a reliable biomarker of lipid peroxidation [33]. MTX administration results in increased MDA levels in the lung brain and kidneys [21–37].

With the attack of free oxygen radicals lipid peroxidation increase and fail the Na+/K+-ATPase activity [38]. Na+/K+-ATPase is the other target of cellular oxidative tissue damage [33]. In our study, MTX significantly increased MDA levels in rat lung tissue. On the other hand, following MTX administration, treatment with ALA was significantly decreased the MDA levels and increased the Na+/K+-ATPase enzyme activity, while lung tissue has normal histological appearance. Increased lipid peroxidation may be induced by the direct or indirect effects of elevated ROS following MTX-induced injury.

GSH has quite important function in the maintenance and regulation of the cell thiol-redox status [39]. In our study, tissue GSH levels in MTX group were lower than control group and treatment with ALA increased the levels.

The progression and control of inflammation are significantly affected by mediators produced by leukocytes accumulated at the site of inflammation [40]. One of them is MPO, an abundant hemoprotein of neutrophils, which is typically perceived to primarily mediate host defense reactions [41, 42]. MPO accumulated in the lungs could significantly modulate redox-sensitive cellular signaling pathways controlling inflammatory processes among others through the catabolism of nitric oxide (NO), induction of wide range of posttranslational modification of proteins, and modulation of metabolism of arachidonic and linoleic acid derived mediators [43, 44]. In our study MPO level which is an indicator of polimorphonuclear leukocyte infiltration was higher in MTX group. High levels of MPO show that neutrophil accumulation contributes to MTX-induced oxidative injury in lung tissues. Treatment with ALA decreased the MPO activity.

IL-1β and TNF-α are primary inflammatory cytokines produced by monocytes and macrophages in response to a range of stimuli including various microbial products, activated T cells, immune complexes, and the combined action of other cytokines. They cause leucocytes to move out of capillaries and accumulate at sites of injury or infection. This is because of their stimulating production of chemotactic factors and inducing adhesion molecules for leucocytes on vascular endothelium. The up-regulation of surface molecules such as E-selectin and intercellular adhesion molecule 1 (ICAM-1) causes leucocytes to attach to the endothelium, and then they move out into the extravascular space in response to chemotactic stimuli. Systemic inflammatory response indicators; IL-1β and TNF-α levels were also found increased in the serum of MTX group. Treatment with ALA decreased the level of plasma IL-1β and TNF-α.

Glucocorticoids are important in the treatment of many inflammatory, allergic, immunologic, and malignant disorders and also MILI. But the toxicity of glucocorticoids such as Cushing syndrome, osteonecrosis, cataracts, heart failure and glaucoma restrict the use of these drugs. In case of development of the side effects of course new treatment strategies should be investigated for using instead of glucocorticoids. The number of research on natural antioxidants, especially those that originate from plants should be increased as certain synthetic antioxidants are suspected to be carcinogenic.

In conclusion, this study showed that ALA has substantial protective effect on oxidative damage of MILI in rats, both histopathologically and biochemically. Hence ALA can be used in treatment and prophylaxis of MILI.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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