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Febuxostat ameliorates methotrexate-induced lung damage

Authors: S. M. Zaki, G. H.A. Hussein, H. M.A. Khalil, W. A. Abd Algaleel

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S.M. Zaki[1, 2], G.HA. Hussein[3], H.M.A. Khalil[4], W.A. Abd Algaleel[1]

[1]Department of Anatomy and Embryology, Faculty of Medicine, Cairo University, Egypt

[2]Fakeeh College for Medical Sciences, Jeddah, Saudi Arabia

[3]Department of Anatomy and Embryology, Faculty of Medicine, Beni Suef University, Egypt

[4]Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Egypt

Address for correspondence: Sherif Mohamed Zaki, Fakeeh College for Medical Sciences, Jeddah, Saudi Arabia, e-mail: zakysherif1@gmail.com

Abstract

Background: The intention of the present study was to study the structural affection of the lung following Methotrexate (MTX) overdose. The proposed underlying mechanisms involved in lung affection were studied. The possible modulation role of febuxostat over such affection was studied.

Materials and methods: 24 rats were divided into three groups: control, MTX-treated, febuxostat-treated. The study was continued for 2 weeks. Lung was processed for histological and immunohistochemical {Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2)} studies. Inflammatory markers (TNF-α, IL-1), Western blot evaluation of NF-κB and oxidative/antioxidative markers were done.

Results: MTX-treated group exhibited inflammatory cellular infiltrations, thickened interalveolar septa, dilated congested blood vessels, extravasated blood, and apoptosis. The collagen fibers content increased 3-fold. MTX induced lung affection through oxidative stress (increase MDA /decrease GSH, SOD) and apoptosis. It induced sterile inflammation through increase NF-κB (two -fold), IL-1 (three-fold) and TNF-α (three-fold), COX-2 cells (2 ½-fold) and iNOS (6-fold). With the use of febuxostat, the normal lung architecture was observed with a pit thickened interalveolar septum and extravasated blood. The collagen fibres content was

minimal. Decrement of oxidative stress and sterile inflammation (COX-2 cells and iNOS were comparable to the control group. NF-κB, IL-1 and TNF-α became higher by 34, 64% and 100%). **Conclusions:** The overdose of MTX displays inflammatory lung affection with residual fibrosis. It induces lung affection through oxidative stress, apoptosis and sterile inflammation. With the use of febuxostat, the normal lung architecture was preserved with a little structural affection or fibrotic residue. Febuxostat exerts its lung protection through its anti-inflammatory and antioxidant features.

Key words: febuxostat, methotrexate, lung

INTRODUCTION

Methotrexate (MTX) has displayed effectiveness in treating several diseases, including rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, inflammatory bowel disease, psoriasis, and small- vessel vasculitis [11, 32]. It is commonly used as a treatment for some types of cancer, including breast, ovaries, brain, and leukemia [26]. It is used as an alternative to surgical management of ectopic pregnancy in selected patients with small, unruptured tubal pregnancies [34]. Adding, it is recommended for induction and maintenance of remission in Crohn's disease [13].

The therapeutic application of MTX is usually limited by its severe toxicity [38]. It is involved as a causative agent in lung toxicity [9, 33]. The prevalence of MTX- related lung disease and MTX- related interstitial lung disease (in rheumatoid arthritis) are 7.6% and 11.6% respectively [4, 9].

Several mechanisms have been studied to recognize the mechanism of MTX-induced lung damage. One of these mechanisms is oxidative stress [2]. MTX interferes with the antioxidant defense enzymes, depletes glutathione content, and causes lipid peroxidation (Arpag *et al.*, 2018). Diminution of antioxidant defenses enhances the production of reactive oxygen species (ROS) that result in parenchymal lung injury and interstitial, alveolar fibrosis [24]. Other possible mechanism of MTX-induced lung damage is inflammatory reaction of MTX. MTX increases the levels of IL-1β, and TNF-α, which are indicators of inflammatory response [1]. Adding, overdose of MTX can lead to proinflammatory cytokine release [19].

Febuxostat is a selective xanthine oxidase inhibitor that is used to reduce urate levels in diseases that involve hyperuricemia such as gout and tumor lysis syndrome [28, 31]. The focus on xanthine oxidase inhibitors has increased due to their anti-inflammatory, antioxidant and immune-modulatory features which might be beneficial in the treatment of different auto-inflammatory diseases [27]. Febuxostat showed anti-inflammatory effects in different experimental models [15]. It has an antioxidative stress effect and suppressed ROS production in the rat model of renal ischemia-reperfusion injury [36].

The intention of the present study was to study the structural affection of the lung following MTX overdose. The proposed underlying mechanisms involved in lung affection were studied. The possible modulation role of febuxostat over such affection was studied.

MATERIALS AND METHODS

Animals

Twenty-four Sprague-Dawley adult albino rats were used. The sample size was based according to resource equation method [10, 16]. The rats were housed in a temperature (22 ± 2°C), relative humidity (55% ± 5%), and light/dark cycle (12/12-h). The study was conducted in Experimental Animal Centre, Cairo University. The experiment was carried out in accordance to ARRIVE guidelines (Animal Research: Reporting of In-Vivo Experiments) with the approval of the local IACUC Research Ethics Committee and according to ethical standards of National Institutes of Health guide for care and use of Laboratory Animals (8^{th} edition, revised in 2011).

Experimental design

The rats were divided into three groups. Each group consists of 8 rats:

Control group: 100 mg/kg physiological saline/ intraperitoneally.

MTX-treated group

Febuxostat-treated group (concomitant MTX + febuxostat).

Test materials

MTX was obtained from Pfizer Pharmaceutical Company and Chemical industries (Egypt) and given a single dose 20 mg/kg, intraperitoneally [2]. The used dose MTX was a high dose to evaluate the role of ROS formation and apoptosis [21].

Febuxostat was obtained from Hikma Pharmaceutical Co. (Egypt). It was dissolved in 0.9% saline and given 15 mg/ kg, oral: 2 drops of Tween 80 [15] for 14 successive days.

General toxicological profile

The general toxicological data were recorded including food and water consumption, health status, body weight (BW) and behavioral measures.

Behavioral measures (Anxiety like behavior)

Two days before the end of the experiment, the rats were subjected to behavioral observation to measure anxiety like behavior using two behavioral tests. The rats were accustomed to the presence of the experimenter before the behavioral observation. The tests were carried out between 9 AM and 12 PM for two days.

Light/Dark box. This test based on the innate fear of the rat to bright spaces. It is composed of a box divided into equal compartment, bright and dark compartments separated by a partition with a door. The rat was placed at the bright compartment and allowed to explore for 5 min. Frequency of entry into dark & light compartments and the time spent in these compartments were measured [7].

Elevated plus maze [37]. This test is based on the rat`s conflict between exploring novel places, and avoiding heights and dangerous places. It is composed of wooden two open and enclosed arms connected by central platform. The apparatus elevated 60 cm above the ground. The rats were placed in the open arm and allowed to explore for 5 min. Frequency of entry to the open and closed arms as well as the time spent in each arm were measured.

Tissue sampling

The lung was dissected and fixed immediately in 10% formalin saline. To randomize selection, the entire lung was cut starting at the superior border, every 10^{th} section (5 μm thick) was put aside for staining.

Preparation of tissue extracts

A portion of lungs was homogenized in 10 volumes (1:10; w/v) of ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4) in an Ultra Turrax tissue homogenizer for 30 seconds.

Homogenates were centrifuged at 10.000 rpm for 10 min at 4 °C. The supernatant was pipetted into clean centrifuge tubes and stored in aliquots (- 80 °C) until analysis [12].

Light microscopic study

Hematoxylin and eosin stain.

Masson's trichrome stain [35]

The paraffin sections were dewaxed, rehydrated then stained in acid fuchsin solution for 5 min, rinsed in distilled water, placed in phosphomolybdic acid solution for 3 min, washed in distilled water, stained with methyl blue solution for 2-5 min, rinsed in distilled water, and treated in acetic acid for 2 min. Finally, the sections were dehydrated in absolute alcohol, cleared in xylol, and mounted in Canada balsam. The nuclei appeared dark red, the cytoplasm appeared pale red and, the collagen fibers appeared blue.

Immunohistochemistry [30]

Immunohistochemical staining using the streptavidin-biotin-peroxidase technique for iNOS, and cyclooxygenase (COX)-2 (markers of inflammation). Briefly, 10 sections/ group were deparaffinized, rehydrated, and rinsed in tap water, treated with 3% hydrogen peroxide for 10 min then, immersed in antigen retrieval solution. Non-specific protein binding was blocked by incubating the sections in10% normal goat serum in phosphate buffer solution (PBS). Then, the sections were incubated in a humid chamber at 4◦C with primary anti-iNOS antibody (rabbit polyclonal antibody,1:100dilution, ab15323, Abcam, Cambridge, Massachusetts, USA), anti-NF-kB antibody (rabbit polyclonal anti-rat antibody against P65 subunit of NF-kB; 1:20 dilution, ab86299, Abcam, Cambridge, Massachusetts, USA) and anti-COX-2 (rabbit polyclonal antibody,1:100 dilution, ab15191, Abcam, Cambridge, Massachusetts, USA) overnight. After washing in PBS, the corresponding biotinylated secondary antibody was added to lung sections for one hour at room temperature. Streptavidin peroxidase was added for 10 min and then washed in PBS. Finally, the sections were counterstained by Mayer’s hematoxylin. For negative control sections, the primary antibodies were excluded. All the slides were assessed in triplicates to confirm the accuracy of the obtained results.

Oxidative/antioxidative markers

Lung lipid peroxidation. Malondialdehyde (MDA), a marker for lipid peroxidation, was measured by monitoring thiobarbituric reactive substances (TBARS) formation. Briefly, 500 mL of lung homogenate was added to 200 mL of phosphate buffered saline (PBS, 10 Mm, pH 7.4) and 500 µL of heat trichloroacetic acid-butylated hydroxytoluene (20% TCA, 1% BHT) solution. The resultant was mixed and centrifuged at 3000 rpm for 10 min 4°C. To 800 mL of supernatant, 160 µL of 0.6 M HCL and 640 µL of 1.73% thiobarbituric acid (TBA) were added. This suspension was mixed and heated in a boiling water bath for 15 minutes. After cooling, the thiobarbituric reactive substances (TBARS) were measured in the supernatant at 530 nm against a blank containing all reagents except the tissue homogenate. The concentration of MDA was calculated and expressed in Nano-moles per milligram of protein.

Lung SOD activity. Superoxide dismutase activity (SOD) was determined at room temperature (RT) according to the modified Misra and Fridovich's method [23]. Five microliters of 10% lung homogenate was added to 1965 µl of sodium carbonate buffer and to 10 microliters of bovine catalase. Twenty microliters of 30 mM epinephrine (dissolved in 0.05% acetic acid) was added to the mixture. Superoxide dismutase activity was measured at 480 nm for 5 min on a spectrophotometer. The activity was expressed as the amount of the enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 U per milligram of protein.

Lung glutathione (GSH). Glutathione was measured spectrophotometrically in the bronchoalveolar lavage fluid (BALF) and lung tissue [29]. GSH was measured by adding the standard or sample to 100 µL of a 1:1 mixture of 3 units/mL glutathione reductase with 0.67 mg/mL 5,5'-Dithiobis-2 nitro benzoic acid) (DTNB). The reaction was initiated by the addition of 20 µL of 0.67 mg/mL NADPH and the increase in absorbance at 412 nm was monitored by using a commercial kit (Biodiagnostic, Cairo, Egypt). Values measured in BALF were normalized to urea, values in lung tissue were normalized to protein content. For in vitro samples both the media and lysate were normalized to the lysate protein. The limit of detection for GSH was 0.2 µM. The concentration of the lung GSH was calculated using the standard curve and expressed per mg of protein. The level of epithelial lining fluid GSH (ELF GSH) was expressed µM.

Western blot assay

NF-κB was estimated by the ELISA, according to the constructor's guide (R&D system Inc.). Lung tissue was standardized (1:10) in saline and stockpiled at -80°C. Models were preserved with cell lysis buffer, adjusted with PMSF and PIC former to the measures. Samples were extra watered down in assay buffer. Later to pipetting 100 µl assay buffer, 100 µl of sample/standard were supplemented o the pits.

The plate was incubated at RT for 1 h. The wells were splashed 5 times with rinse buffer. After 1 h incubation, the plate was splashed 5 times with shower buffer and 100 µl of conjugate were supplemented to all wells. The plate was vacuum-packed and incubated for 30 min at RT. Well matters were let down and rinsed 5 times in buffer. 100 µl of substrate was auxiliary to all wells and color endorsed to progress. 30 min later, halt solution was substituted. The plate was delivered on an ELISA Plate Reader (OD 450 nm). Rectilinear average curves were created in assay buffer to estimate the values of NF-κB.

Image analysis and morphometric measurements

The area percent of collagen fibers and immune expression of iNOS, and COX-2 were done using Leica LAS V3.8 image analyzer computer system (Switzerland). The measurements were obtained by an independent blinded observer. The data was obtained in ten non-overlapping microscopic fields taken randomly from each slide and were examined within the standard measuring frame.

Biochemical assay

The serum level of the inflammatory markers TNF-α, and IL-1 were assayed by the commercially Enzyme-linked immunosorbent assays (ELISA) kits supplied by Biopsies, China according to manufacturer instructions.

Protein assay

The protein concentration in the lung homogenate was measured by Bradford's method using bovine serum albumin as standard [8].

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 21.0 (IBM Corporation, Somers, NY, USA) statistical software. Data were expressed as means ± standard deviation (SD). Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons. Significance was considered when the p-value was less than 0.05.

The percentage of increase or decrease (difference) of all study parameters were calculated with the following formula: Percentage of difference = (Mean difference value between two groups)/(Value of the compared group) x100.

RESULTS

The general toxicological data

No mortalities were noticed in the studied groups. The food and water intake and health status were relatively excellent.

At the beginning of study, the BW was 150±3.6 g. By the end of study, the BW of MTX-treated group decreased by 22% compared to the control group. With use of febuxostat, the BW became 12 % less than the control group (Table 1).

Behavioral measures (Anxiety like behavior).

Light/dark box. There was non-significant difference among the different groups concerning time spent in the dark or light compartments. A significant increase (130%) in the number of entries of the MXT-treated rats into the dark compartment as compared to the control rats. Non-significant entry difference was detected in febuxostat-treated group into dark and light compartments as compared to the control group (Fig 1).

Elevated plus maze. As comparted to the other groups, MXT-treated rats entered the open arm of the elevated plus maze less frequent and spent less time. They entered more frequent to the closed arm of the elevated plus maze and spent more time (Fig 2a, b).

Febuxostat-treated rats entered more frequent and spent more time in the open arm (Fig 2c, d).

Structure of lung as revealed by H&E staining

The control group presented normal lung morphology. MTX-treated rats exhibited inflammatory cellular infiltrations, thickened interalveolar septa (with edema), dilated congested blood vessels, extravasated blood, and apoptotic pneumocytes. With the use of febuxostat, normal lung architecture was observed with a pit thickened interalveolar septum and extravasated blood (Fig 3, 4).

The content of collagen fibres

The collagen fibres were minimum in the control group. The fibres content increased three-fold in MTX-treated group when compared to the control group. Much improvement was noticed in febuxostat-treated group as its fibres contents were comparable to the control group (Fig 5, Table 2).

Immunohistochemical evaluation

The COX-2 (cells/mm^2) in the control and febuxostat-treated groups were comparable. The immunostained COX-2 cells increased in MTX-treated rats by > 2½-fold (275%) compared to the control group (Fig 6, Table 2).

The immunohistochemical reaction of iNOS was poor in both control and febuxostat-treated groups. With use of MXT, the reaction in MXT-treated group became 6-fold higher than the control group (Fig 7, Table 2).

Western blot assay

NF-κB was two-fold higher than the control group. With use of febuxostat, NF-κB became 34% higher than the control group (Fig 8, Table 3).

Inflammatory and oxidative/antioxidative markers evaluation (Table 3):

IL-1 and TNF-α were three-fold higher in MTX-treated group than the control group. Improvement was noticed with use of febuxostat as IL-1 and TNF-α were 64% and 100% higher than the control group.

MDA was two and half-fold higher than the control group. With use of febuxostat, MDA became half fold higher than the control group.

GSH and SOD decreased by 60% compared to the control group. Both markers in the control and febuxostat-treated groups were alike.

DISCUSSION

Body weight of MTX-treated rats decreased by 22%. The loss of weight in rats' model of MTX is due anorexia, cachexia, intestinal mucositis, impairment of absorption and digestive functions, alteration of the gut barrier, and diarrhea [6, 22].

MTX-treated rats showed a significant increase in the anxiety like behavior expressed by an increase in the number of entries to the dark compartments of the light/dark box and an increase in the frequency and duration of closed arm entries in the elevated plus maze. The anxiety like behavior is explained in mice by the association of MTX with acute brain toxicity and by its ability to induce folate depletion [17]. The anxiety like behavior was reported with MTX administration in cancer patients [14]. Much improvement was in febuxostat-treated group which attributed to the antidepressant activity of the febuxostat [20].

MTX-treated rats exhibited an acute inflammatory process (cellular infiltrations, dilated congested blood vessels, thickened interalveolar septa with alveolar edema). Because of severe congestion, blood extravasation occurred. Adding, apoptotic pneumocytes were detected.

The cause of lung affection in MTX-treated group is multifactorial. The major cause of is the oxidative stress [21]. Oxidative stress is defined as the shift in the balance between oxidants and antioxidants in favor of oxidants [5]. The oxidant marker (MDA) was higher, while the antioxidant markers (SOD and GSH) were lower in MTX-treated rats. It is used for measuring oxidative damage to lipids resulting from free radicals [3]. Analysis of MDA is sufficient to proof and assess the oxidative stress [21]. The elevated MDA is a consequence of the higher level of IL-1 and the toxic dose of MTX [21].

The sterile inflammation in MTX-treated group was caused by increase NF-κB (two-fold), IL-1 (three-fold) and TNF-α (three-fold), COX-2 cells (2½ -fold) and iNOS (6-fold). The present neutrophils results in ROS formation and tissue damage through release of chemical mediators [21]. The higher level of IL-1 and higher dose of MTX increase the secretion of proinflammatory cytokines TNF-α [21]. TNF-α is crucially responsible for the pathogenesis of oxidative stress and increases ROS [21]. It regulates growth, proliferation, differentiation, and viability of activated leukocytes [25]. TNF-α also provokes the cellular release of other

cytokines, chemokines, or inflammatory mediators [25]. So, excessive TNF-α secretion leads to lung damage by inducing oxidative stress and directly inducing apoptosis.

Fibrosis was observed in MTX-treated group (the collagen fibres content increased 3-fold) as a consequence to oxidative stress and sterile inflammation.

The third major cause of lung damage in the overdose MTX-treated group is apoptosis. Apoptosis is multifactorial. It is induced by higher level of TNF-α which activates the caspase enzyme system [21]. Apoptosis is also induced by lipid peroxidation (higher MDA) as MDA is the end product of the lipid peroxidation process [5]. Lipid peroxidation results in organ and cell damage [21]. The increased observed apoptosis results in excessive release of cytokines and enhanced ROS which finally damage the lung tissue [21].

Another possible cause of lung injury in MXT-treated group is the elevation of iNOS. The NO-derived from iNOS appears to induce inflammatory infiltration and oxidative stress. These events create a deleterious environment that ultimately leads to cellular damages. It seems that disruption of COX-2 activity aggravates the resultant lung damage [18].

With the use of febuxostat, the normal lung architecture was observed with a pit thickened interalveolar septum and extravasated blood. The collagen fibres content was minimal. Decrement of oxidative stress and sterile inflammation (COX-2 cells and iNOS were comparable to the control group. NF-κB, IL-1 and TNF-α became higher by 34, 64% and 100%). The protective role of febuxostat is due to its anti-inflammatory and antioxidant features [27]. Febuxostat showed anti-inflammatory effects in different experimental models [15]. Its antioxidative stress effect was proved in in the rat model of renal ischemia-reperfusion injury [36].

In conclusion, the overdose of MTX displays inflammatory lung affection with residual fibrosis. It induces lung affection through oxidative stress, apoptosis and sterile inflammation. With the use of febuxostat, the normal lung architecture was preserved with a little structural affection or fibrotic residue. Febuxostat exerts its lung protection through its anti-inflammatory and antioxidant features.

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Table 1. Body weight in the different groups at the end of study

Group	**BW (g) Mean ± SD**	**Versus group**
Control	185±5	
MTX-treated	145±5	Control*
		Febuxostat-treated*
Febuxostat-treated	162.6±2.5	Control*
		MTX-treated*

The BW at the beginning of the study was 150±3.6 g.

*P-value significant

Table 2. Area % of collagen fibres, iNOS and COX-2; *p-value significant

Group		**Area percent of collagen fibres**	**COX-2 (cells/mm^2)**	**iNOS**
Control	Mean ±SD	1.47±0.54	40 ±5	3.1±0.8
MTX-treated	Mean ±SD	6.0±0.55	150±30	18.0±2.0
	Versus control	*	*	*
	Versus febuxostat-treated	*	*	*
Febuxostat-treated	Mean ±SD	2.5±0.38	55±8	5.1±1.7
	Versus control	NS	NS	*
	Versus MTX-treated	*	*	*

Table 3. Inflammatory and oxidative/antioxidative markers**;** *p-value significant

Group		**NF-κB**	**IL-1 (pg/mg protein)**	**TNF-α (pg/mg protein)**	**MDA (nmol/mg protein)**	**GSH (μg/mg protein)**	**SOD (nmol/mg protein)**
Control	Mean ±SD	1.17±0.05	149.0±22.8	123.3±43.1	1.48±0.17	1.65±0.07	8.02±0.39
MTX-treated	Mean ±SD	3.80±0.06	604.0±17.6	506.6±28.4	5.22±0.38	0.66±0.10	3.07±0.24
	Versus control	*	*	*	*	*	*
	Versus febuxostat-treated	*	*	*	*	*	*
Febuxostat-treated	Mean ±SD	1.57±0.15	245.0±21.7	256.6±19.0	2.22±0.07	1.69±0.13	7.10±0.72
	Versus control	*	*	*	*	NS	NS
	Versus MTX-treated	*	*	*	*	*	*

Figure 1 Anxiety like behavior in the light/dark box test. **a:** Frequency of entry into the light compartment. **b:** Time spent in the light compartment. c**:** Frequency of entry into the dark compartment. **d:** Time spent in the dark compartment. * Significant from control group, p=0.006.

Figure 2. Anxiety like behavior in the elevated plus maze teat. **a:** Open arm entry. **b:** Open arm duration. **c:** Close arm entry **d:** Close arm duration. * Significant from control group, p=0.000.

Figure 3. Lung morphology of the different studied groups. Note respiratory bronchiole (rb), terminal bronchiole (b), alveolar sac (s), alveoli (v), interalveolar septa (sp) and blood vessels (bv). **a, b:** Normal lung architecture of the control rats. **c, d:** inflammatory cellular infiltrations (arrows), thickened interalveolar septa, dilated congested blood vessels and extravasated blood of MTX-treated rats. **e, f:** Normal lung architecture with a pit thickened interalveolar septum and extravasated blood of febuxostat-treated rats. H & E, scale bar, 200 μm (a-f).

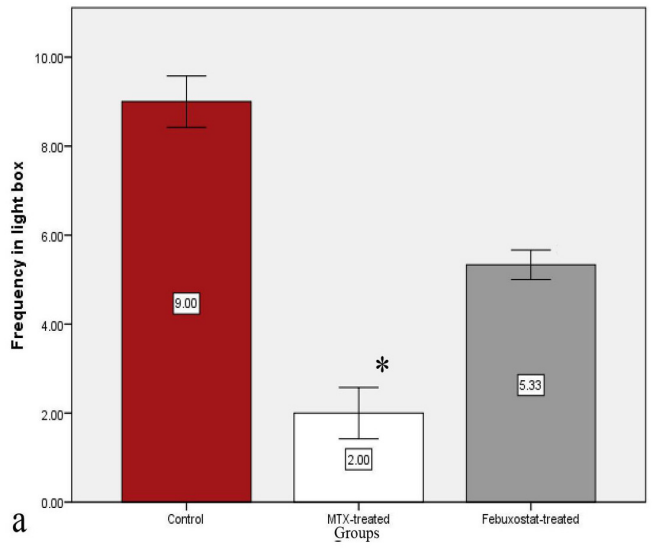
Figure 4. a: Normal lung architecture of the control rats. B**:** inflammatory cellular infiltration, extravasated blood (arrowheads), edema (ed), macrophages (thick arrows) and apoptotic pneumocytes (thin arrows) of MTX-treated rats. c, Normal lung architecture with a pit congestion (arrowheads), macrophages (thick arrows) and apoptotic pneumocytes (thin arrows) of febuxostat-treated rats. H & E, scale bar, 50 μm (a-c).

Figure 5. a: The collagen fibres (arrows) in the control rats. **b:** Increased collagen fibres (arrows) in MTX-treated rats. **c:** Minimal collagen fibres (arrows) in febuxostat-treated rats. Masson's trichrome, scale bar, 200 μm (a-c).

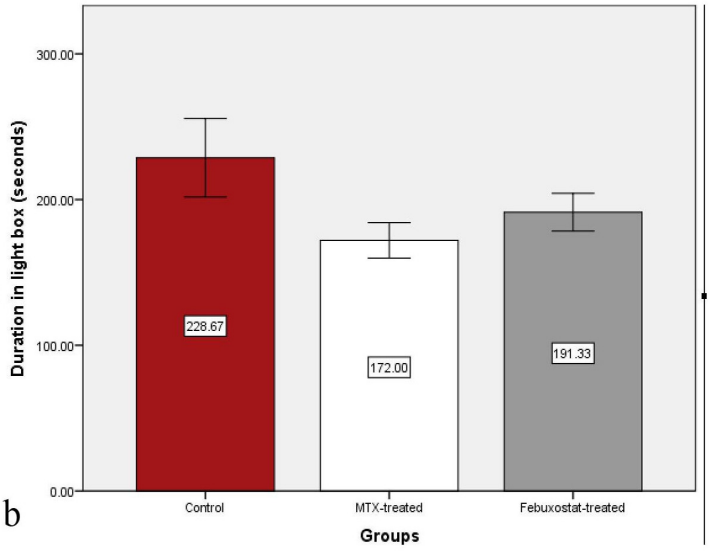
Figure 6. a: Minimal immunostaining of COX-2 (arrows) in the control rats. **b, c:** Increased staining (arrows) in MTX-treated rats. **d:** Minimal immunostaining of COX-2 (arrows) in febuxostat-treated rats. COX-2 immunostaining, scale bar, 200 μm (a-d).

Figure 7. a: Poor immunohistochemically reaction of iNOS (arrows) in the control rats. **b, c:** increase immunohistochemically reaction of iNOS (arrows) in MTX-treated rats. **d:** immunohistochemically reaction (arrows) in febuxostat-treated rats. iNOS immunostaining, scale bar, 50 μm (a-d).

Figure 8. Western blot assay of NF-κB in the different groups. NF-κB was higher in MXT-treated group than the control group. NF-κB was a pit higher in febuxostat-treated rats than the control rats. Beta actin was used as internal control to measure the relative quantitation of the expression of the target gene.


Frequency in light box
10.00
8.00
6.00
4.00
2.00
0.00
9.00
2.00
5.33
*
Control
MTX-treated
Febuxostat-treated
Groups

a


Duration in light box (seconds)
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200.00
100.00
0.00
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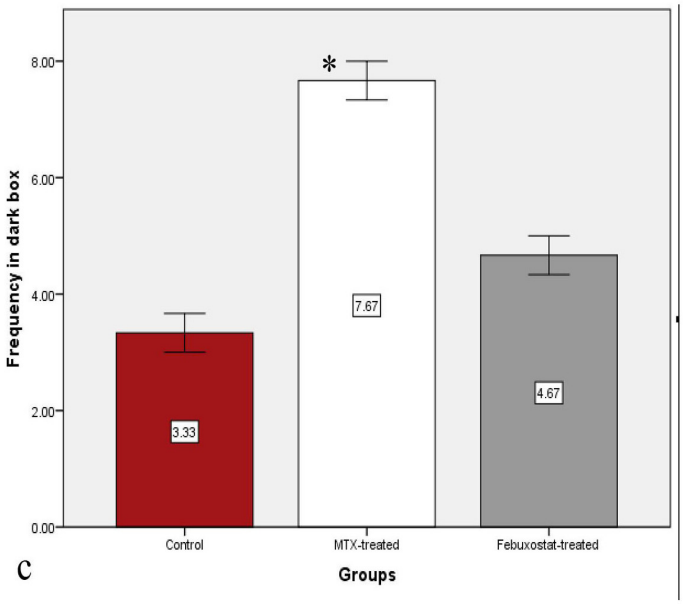
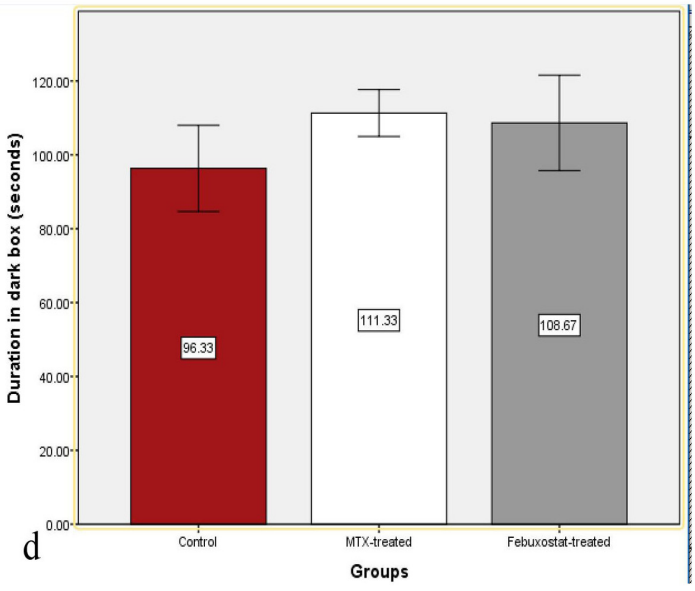
172.00
191.33
Control
MTX-treated
Febuxostat-treated
Groups

b


Frequency in dark box
8.00
6.00
4.00
2.00
0.00
3.33
7.67
4.67
*
Control
MTX-treated
Febuxostat-treated
Groups

c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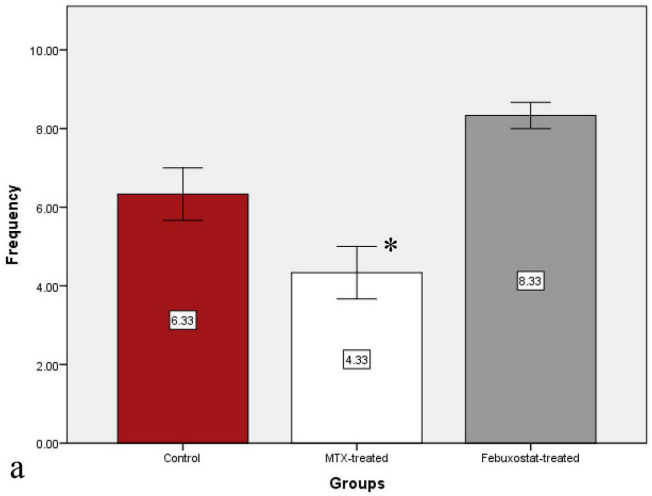
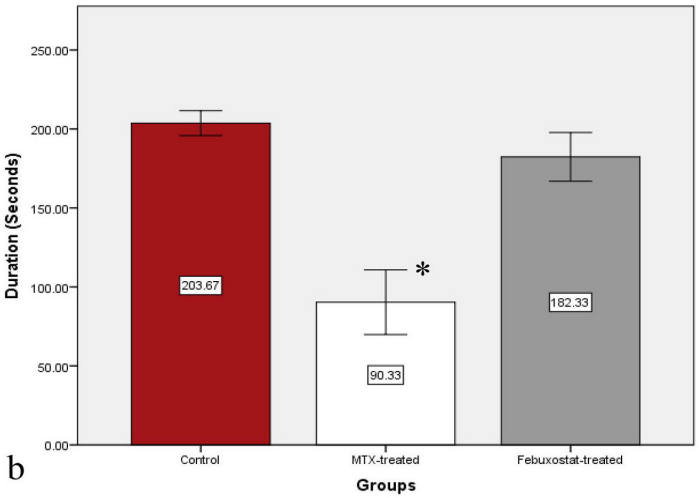
100.00
80.00
60.00
40.00
20.00
0.00
96.33
111.33
108.67
Control
MTX-treated
Febuxostat-treated
Groups

d


Frequency
10.00
8.00
6.00
4.00
2.00
0.00
6.33
4.33
8.33
*
Control
MTX-treated
Febuxostat-treated
Groups

a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200.00
150.00
100.00
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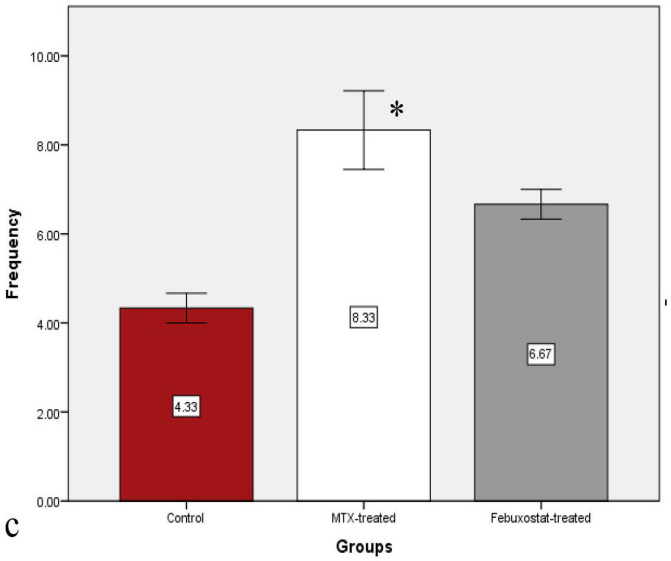
0.00
203.67
90.33
182.33
*
Control
MTX-treated
Febuxostat-treated
Groups

b


Frequency
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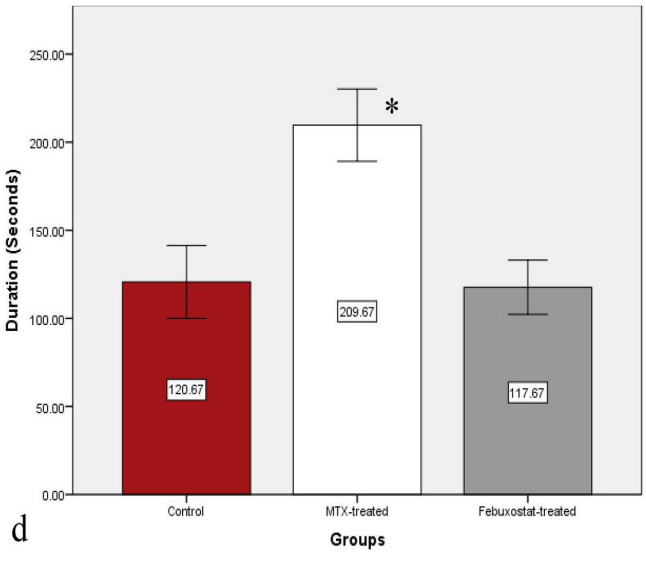
6.00
4.00
2.00
0.00
4.33
8.33
6.67
*
Control
MTX-treated
Febuxostat-treated
Groups

c


Duration (Seconds)
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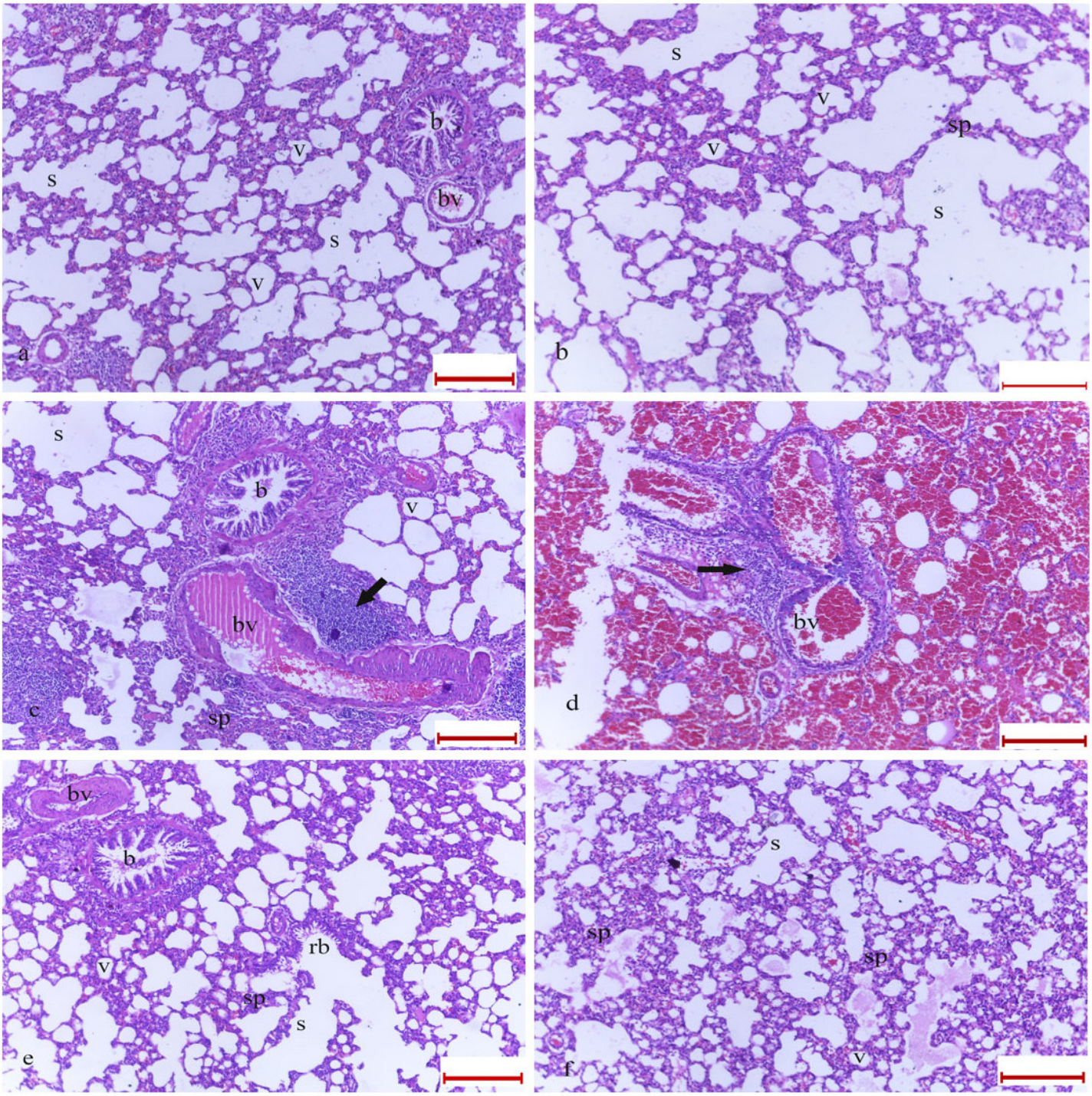
200.00
150.00
100.00
50.00
0.00
120.67
209.67
117.67
*
Control
MTX-treated
Febuxostat-treated
Groups

d


a
b
v
bv
s
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v
sp
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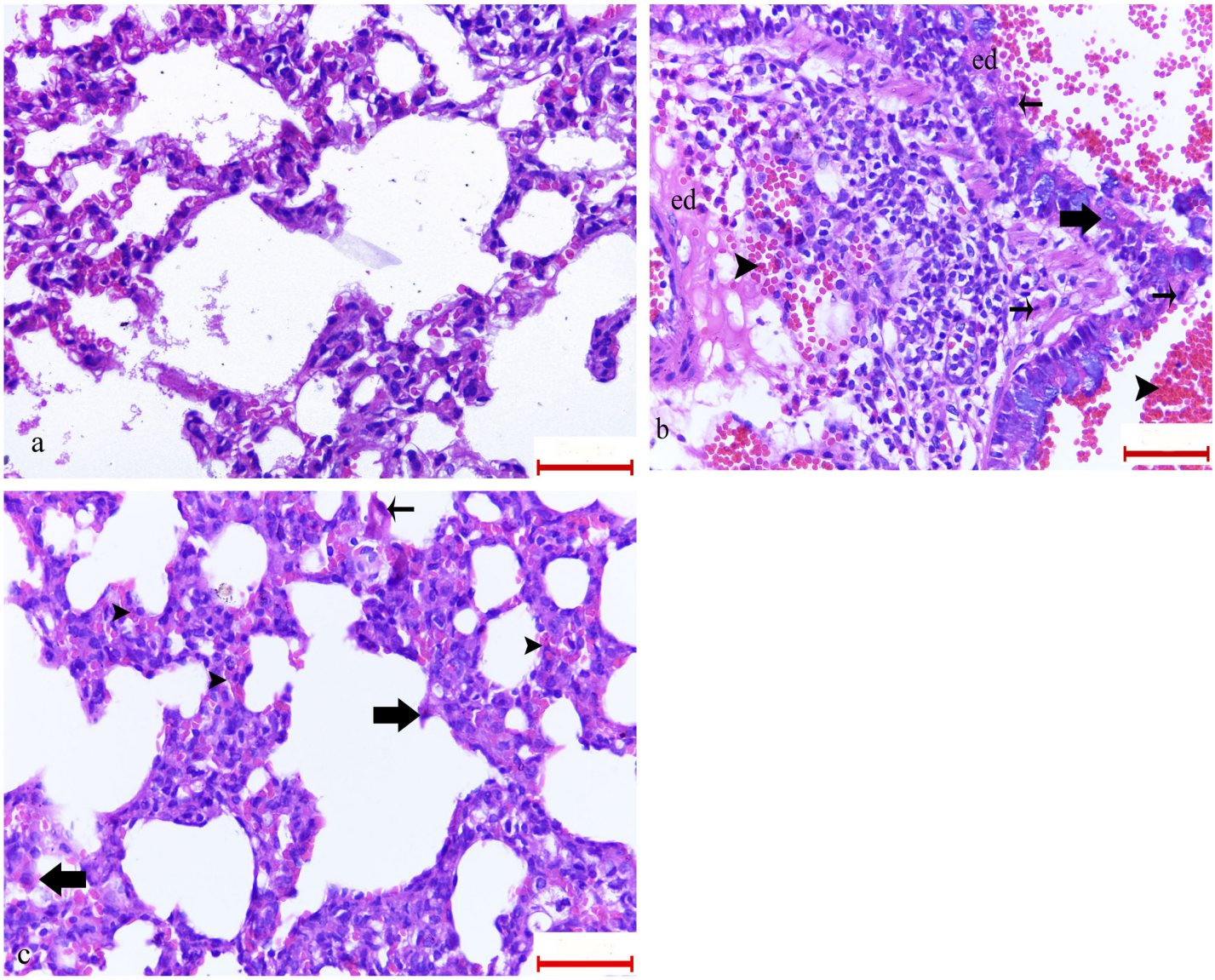
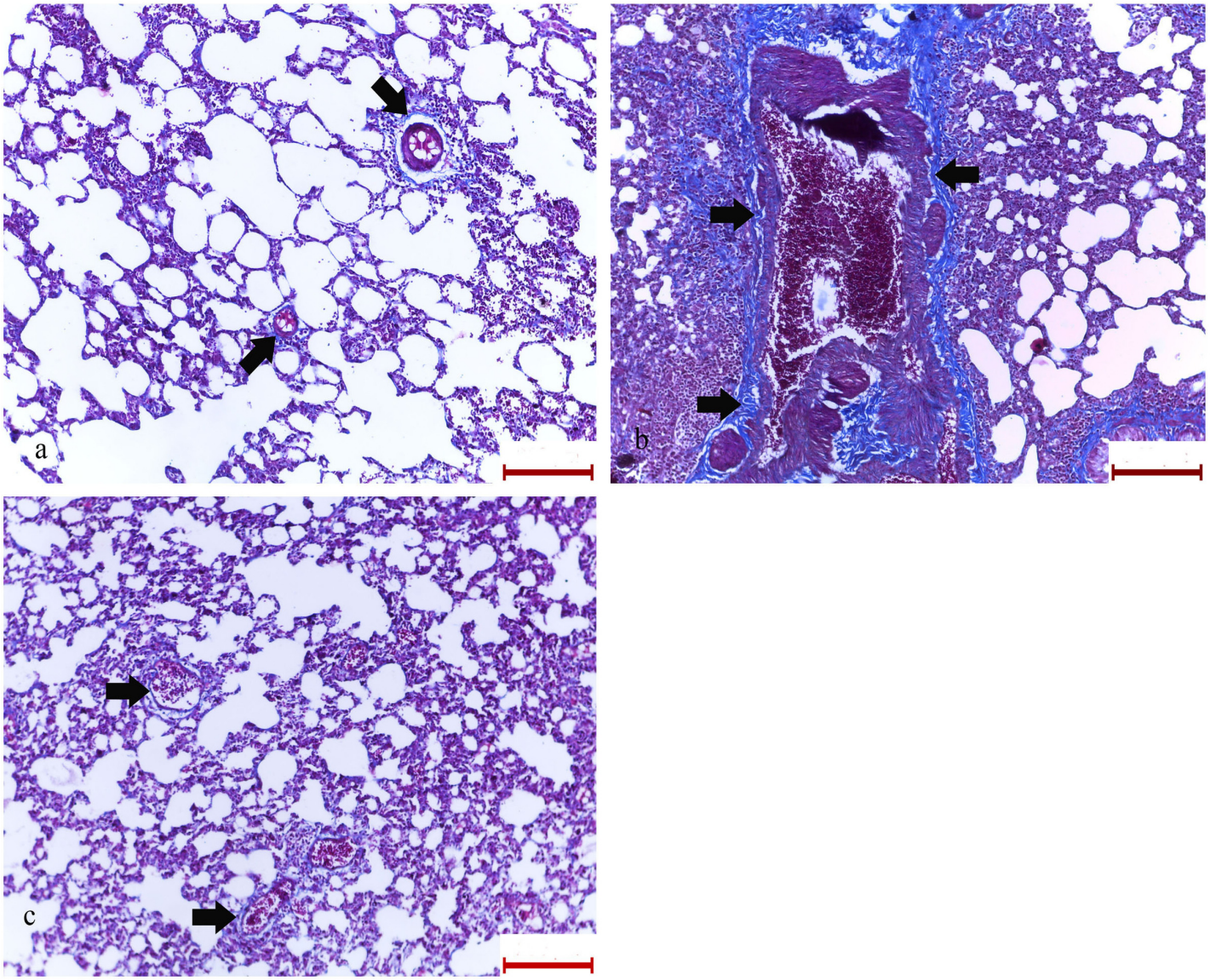
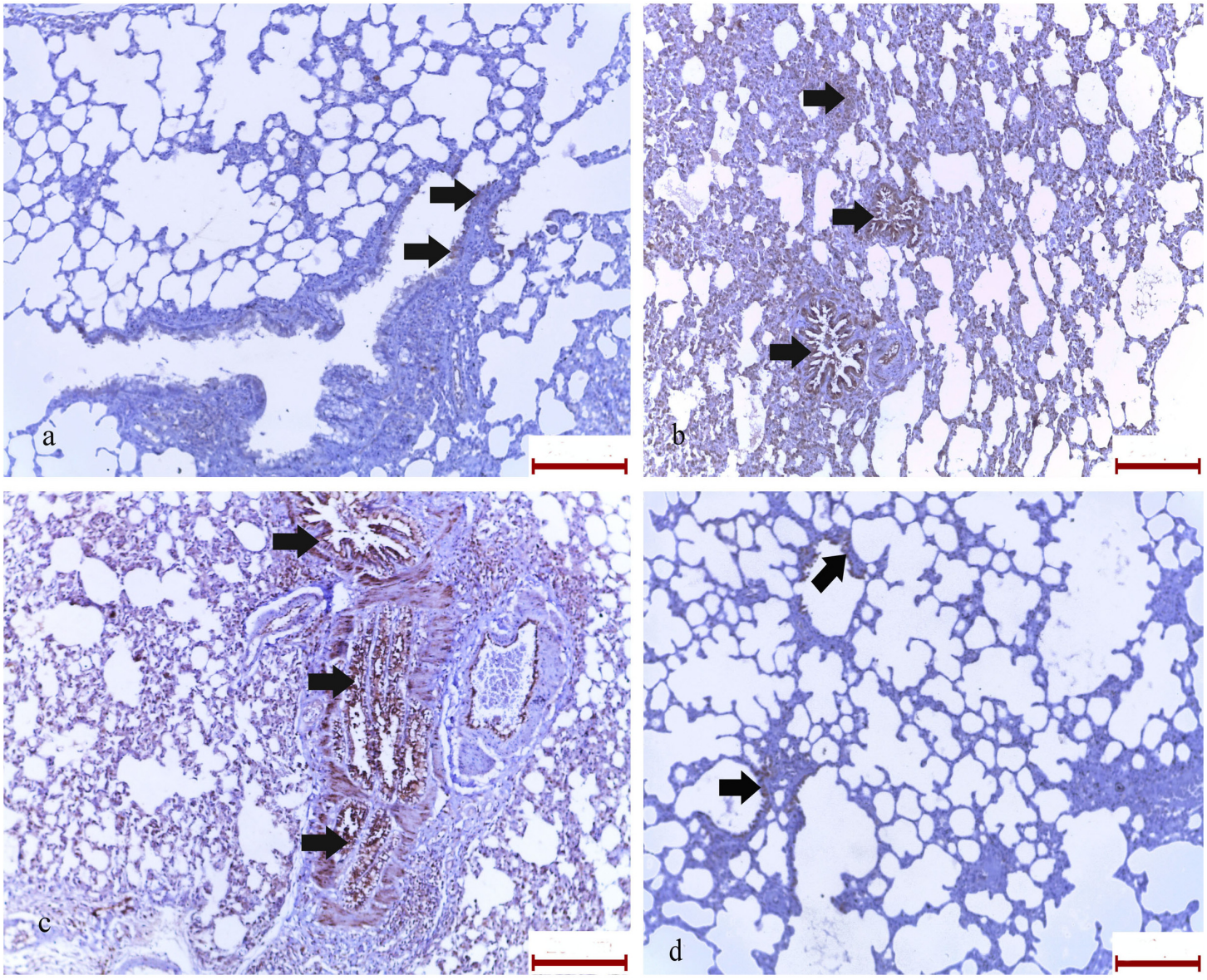
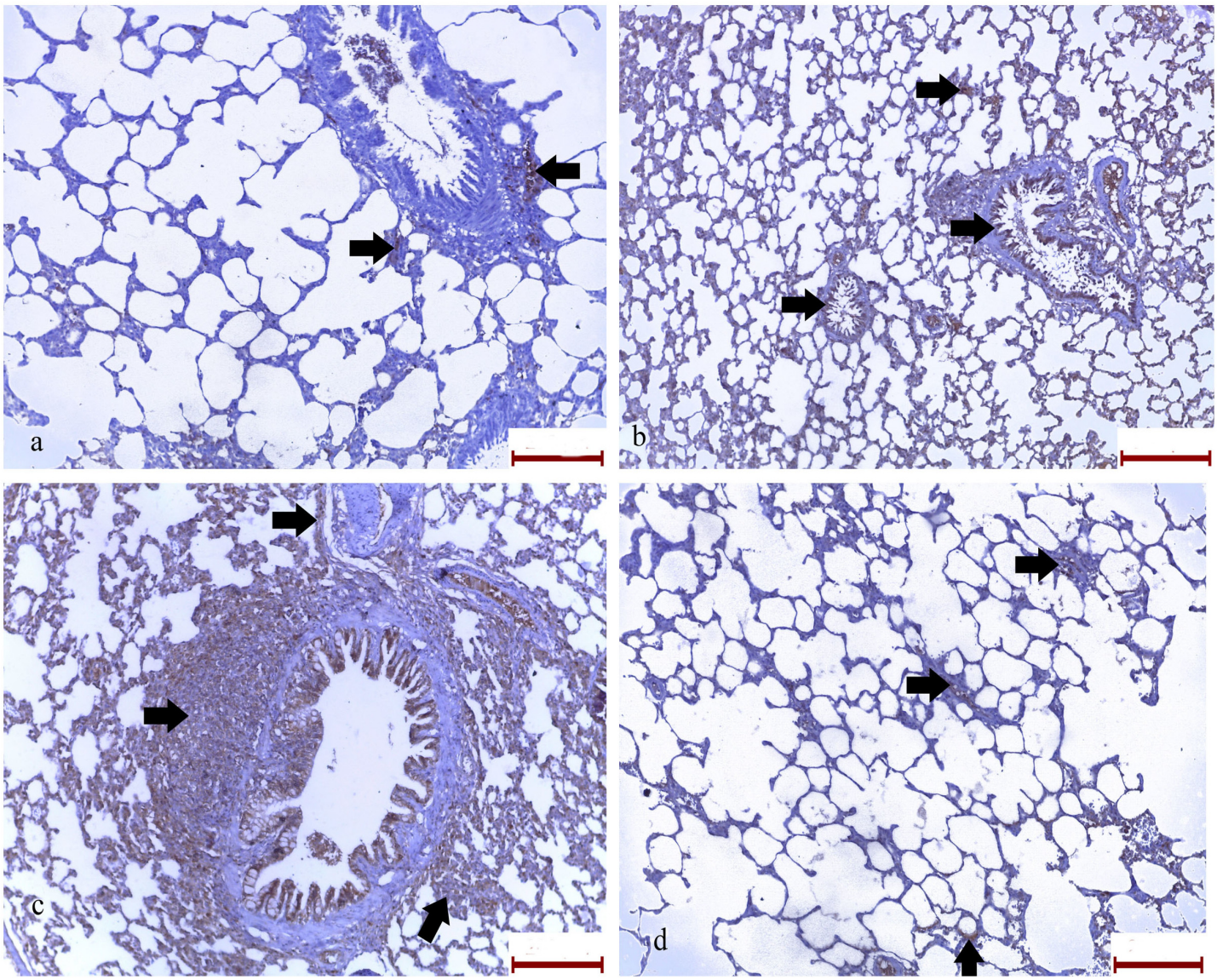
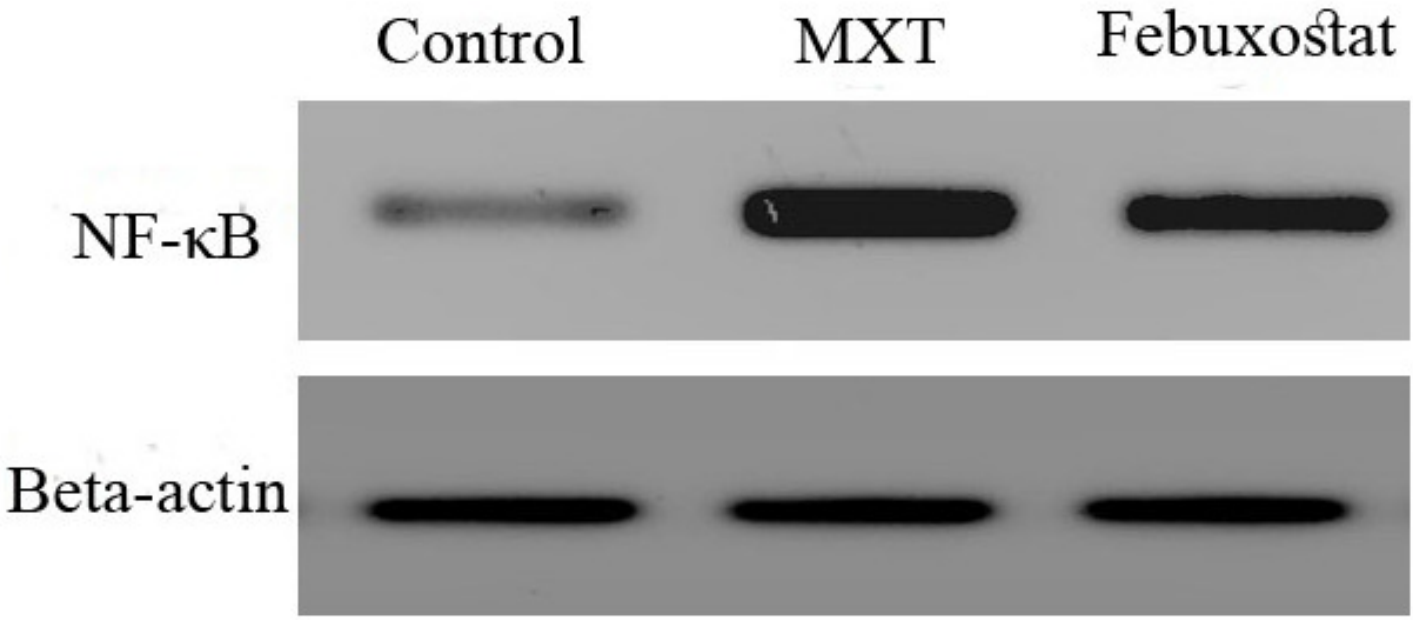
sp
v


a
ed
ed
b
c


a
b
c


a
b
c
d


a
b
c
d


Control
MXT
Febuxostat
NF-κB
Beta-actin