*Research Article*

# Changes Induced by Methotrexate on the Lung of Adult Albino Rat

**Ahmad F. Nassr*, Abd-Elmawla E. Aidaros**, Joseph A. Aziz**, Mohamed A. El Nabtiti***,**

* Faculty of medicine, ALjabal Algharbi university, Libya
** Department of Anatomy and embryology, Faculty of medicine, Zagazig university
*** Department of Anatomy, Faculty of medicine, Zagazig university

**Abstract**

**Background:** Methotrexate (MTX) is widely used in medicine owing to its extended therapeutic effects as one of the antineoplastic and antimetabolite drugs. It is given at high doses to treat tumors. However, in diseases of the immune system, MTX is prescribed at low doses (Armagan et al., 2015). **Aim of the work:** This study was performed to assess the biochemical and histopathological changes that take place in the rat lungs after exposure to methotrexate. **Materials & Methods:** This study was done using thirty-two male Wistar albino rats (180–220 g). The animals were divided into two equal groups. The control group: received distilled water by oral gavage for ten days and injected intraperitoneally (i.p.) with1 ml of physiologic saline (0.09% NaCl) solution on the fourth day.. Methotrexate (MTX) group: injected with a single dose of 20mg/kg MTX i.p. on the fourth day of the study. By the end of the experiment, all the animals were anaesthetized using phenobarbital 3%. Blood samples were collected to measure c-reactive protein (CRP). The lungs were dissected out and prepared for tissue homogenates, routine histological study and caspase-3 immunohistochemical evaluation. **Results:** In the MTX group, there were high levels of CPR, glutathione (GSH) and malondialdehyde (MDA) with reduction of superoxide dismutase (SOD) and catalase (CAT) activities. Lung sections showed marked inflammatory cells infiltration, thickening of interalveolar septa, congested blood vessels, intrapulmonary hemorrhages, abundant collagen fibers and significant expression of caspase-3. **Conclusion:** administration of MTX can induce significant changes on the structure of the lungs of adult albino rat.

**Keywords:** Methotrexate, Rat, Lung, Caspase-3

## Introduction

Initially, methotrexate (MTX) has been used in haematological malignancies of childhood; currently, its therapeutic benefits cover a wide range of neoplastic and inflammatory conditions[1].Yet, clinical and experimental studies have demonstrated adverse effects of MTX on the brain[2], kidney[3], liver[4], heart[5], intestine[6], hematopoietic tissue[7] and lungs[8]. The pulmonary complications comprise hypersensitivity pneumonitis, interstitial fibrosis and pulmonary nodules[9]. Hypersensitivity, immunomodulation, idiosyncrasy, and direct lung toxicity have been implicated in the development of such complications[10,11]. Oxidative stress and lipid peroxidation have been suggested to play the key role in methotrexate-induced lung injury (MILI)[12]. MTX administration resulted in oxidative-induced injury in the kidney[13], liver[14] and testis[15].

This study was designed aiming at clarifying the effect of MTX on lung of the albino rat.

## Materials and methods

### Chemicals

Methotrexate (Mylan) vial (50 mg/2ml) was purchased from Al-Gomhoria pharmaceutical company, Cairo, Egypt.

**Animals**

Thirty-two male Wistar albino rats (180–220 g) obtained from the animal house of Zagazig University were selected for this study. The animals were kept in a room illuminated for 12 hours by daylight at a temperature of 25-30°C, fed a standard rat chow diet and had access to water ad-libitum. Animal care followed the guidelines of the Ethical Committee of Zagazig University and the protocol of the study was approved by the institutional animal care and uses committee of the Zagazig University (ZU-IACUC); number ZU-IACUC\3\F\49\2019.

**Experimental design**

Animals were randomly allocated into four groups; 16 animals in each one. Control group: received distilled water by oral gavage for ten days and a single i.p. injection with1 ml of physiologic saline (0.09% NaCl) solution on the same day of MTX injection. Group II, (MTX group): The rats in this group were injected with a single dose of 20mg/kg MTX i.p. on the fourth day of the study[16].

After10 days, all rats were weighed and anaesthetized using sodium thiopental (75mg/ kg) injected intraperitoneally. Blood samples were taken from the retro-orbital venous plexus to assess the level of CRP. Then a midline thoracic incision was done, the lungs were carefully excised, washed in cool saline, dried and weighed. Lung specimens were prepared for tissue homogenates, histopathological evaluation.

**Serum C - reactive protein (CRP) assay**

Blood samples were centrifuged at 2000 pm. The separated serum kept at -80°C. CRP was measured using enzyme-linked immunosorbent assay kits (Biosource International, Nivelles, Belgium).

**Tissue biochemical analysis**

The lung specimens were weighed, homogenized in ice-cooled Tris-HCl buffer (pH 7.4) using Teflon–glass homogenizer. The homogenates were centrifuged at 4.000 rpm for 10 minutes at 4°C. The obtained supernatant was kept at -80°C till the time of analysis. Oxidative stress parameters: MDA and GSH were evaluated using colourimetric kits purchased from Bio-diagnostic, Giza, Egypt.

Malondialdehyde (MDA)[17]: Lung homogenate is mixed with equal volumes of thiobarbituric (TBA) and trichloroacetic acids and heated at 95°C for 30 minutes. MDA, if present, reacts with TBA giving pink thiobarbituric acid reactive products (TBARPs). TBARPs color was assayed at 534 nm. Values were presented as nmol/ g tissue.

Reduced glutathione (GSH)[18]: The method based on the reduction of 2 - nitrobenzoic acid (DTNB) with glutathione (GSH) to produce a yellow compound which can be measured at 405 nm. GSH concentration is directly proportional to the reduced chromogen and is expressed as nmol/g of tissue.

**Histopathological Examination**

After being fixed in 10% formalin, the lung tissues were dehydrated using ethyl alcohol in ascending grades (70%, 80%, 90%, 95%, and 100%) then cleared in xylene, and embedded in paraffin wax. Sections of 4-5 micrometers (µm) thickness were cut and stained with hematoxylin and eosin[19]. Stained slides were used for histopathological and morphometric evaluations. Photographs were taken using an optical microscope (LeicaICC50W) at the image analysis unit of anatomy and embryology department.

## Results

Serum CPR level: the level of CRP in MTX group was significantly higher than it in the control group ($P$<0.001). (Table 1).

Tissue biochemical results: MTX induced remarkable changes in oxidative stress markers; it significantly reduced the GSH level ($P$<0.001). On the other hand, MTX markedly increased MDA level ($P$<0.001). (Table 1).

**Light microscopic observations:**

Haematoxylin and Eosin: study of lung slides stained with Hx&E demonstrated that the control (Figs. 1 & 2) had normal architecture. Bronchioles of different sizes appeared regular with intact folded mucosa

and surrounded by regularly arranged smooth muscle fibers and outer adventitia. Lung parenchyma consisted of alveolar ducts, alveolar sacs and alveoli of different sizes with thin septa. The alveoli were lined by a single layer of two types of cells; squamous, thin cells with flat nuclei (type I pneumocyte) and cuboidal cells with rounded nuclei (type II pneumocyte). The lung exposed to MTX showed destruction of the epithelial lining of the bronchioles and disorganization of the surrounding musculosa (Fig. 3). Apparent thickening of the interalveolar septa with mononuclear cells and predominance of type II pneumocytes was also noticed (Fig. 4,5). Massive interstitial and alveolar hemorrhage was detected in some specimens (Fig. 6,7). Also The interstitial tissue was infiltrated by different inflammatory cells mainly around the bronchovascular bundle and penetrating the bronchial wall to the mucosa (Fig. 8).

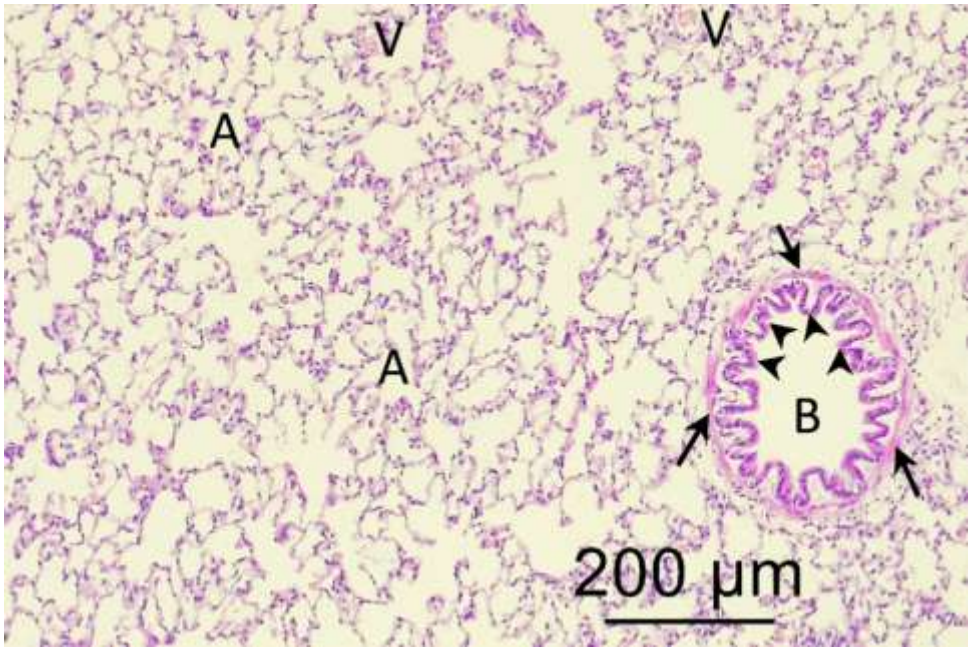


**Figure (1):** A photomicrograph of section in the lung of the control group showing lung tissue with alveoli (A) and the lining respiratory epithelium with folded mucosa (arrow heads) due to contraction of the regularly arranged surrounding smooth muscles (arrows). In addition, medium sized bronchiole(B) and small vessels intervening (V).
(H&E X 100)

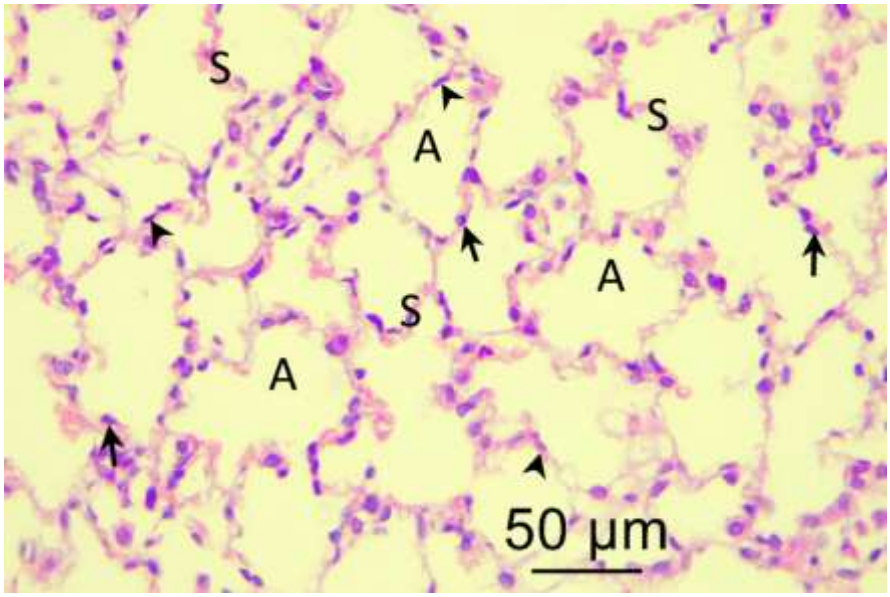


**Figure (2):** A photomicrograph of section in the lung of the control group showing multiple alveoli (A) with thin interalveolar septa (S) formed by single layer of cells. The alveoli showing different types of alveolar cells: squamous type I alveolar cells (arrow heads), which line almost the entire alveolus surface and type II alveolar cells bulging into the alveolus (arrows).
(H&E X 400)

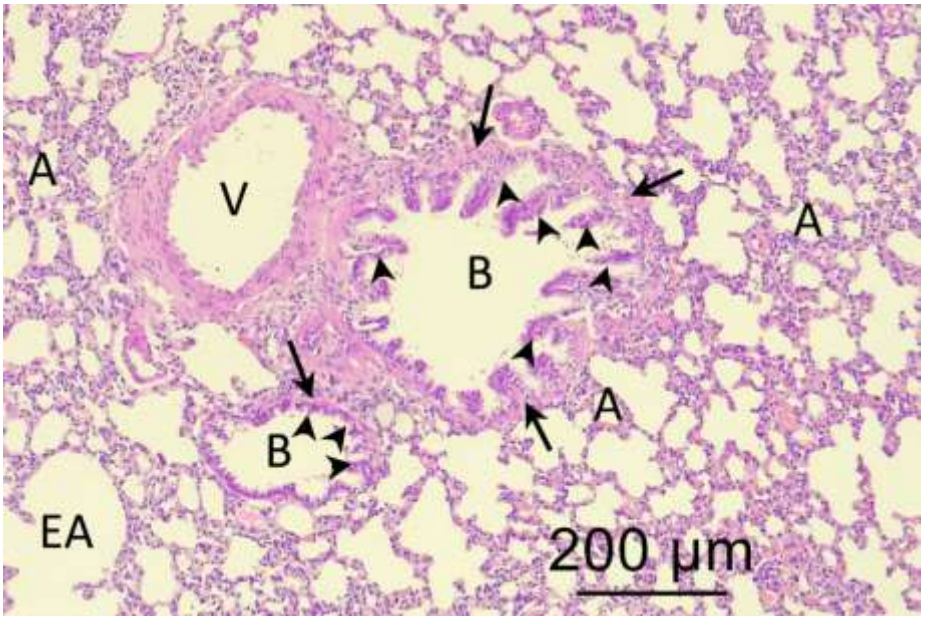


**Figure (3):** A photomicrograph of section in the lung of the methotrexate treated group showing lung tissue with alveoli (A) with abundant destruction of the mucosal lining (arrow heads). As well as, the surrounding smooth muscles (arrows) are disorganized. In addition different sized bronchioles (B), widely dilated emphysematous alveolus (EA) and blood vessel (V) with disturbed intima.
(H&E X 100)

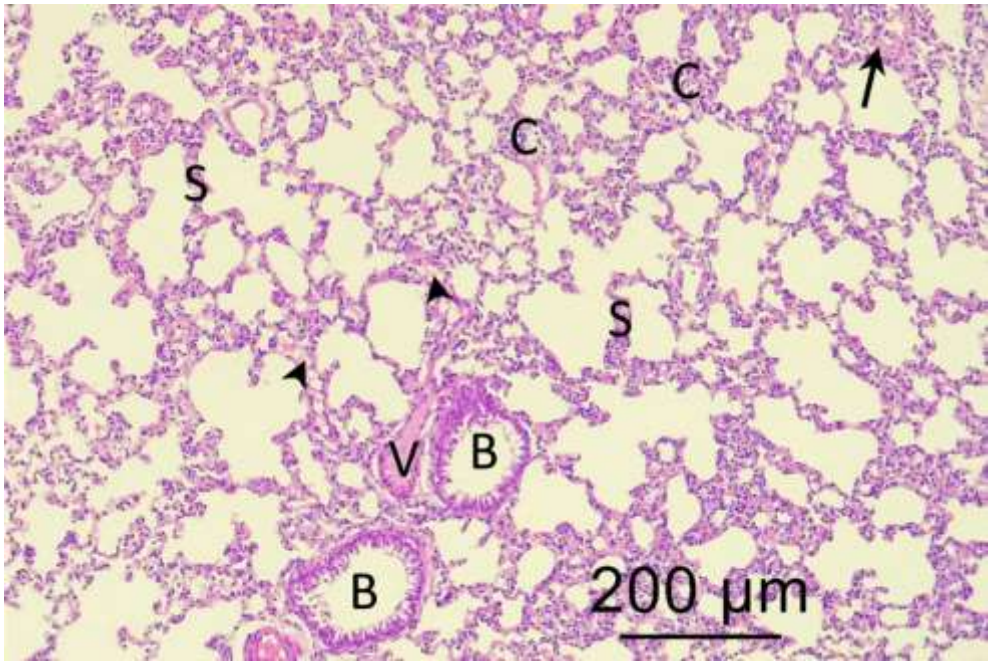


**Figure (4):** A photomicrograph of section in the lung of the methotrexate treated group showing bronchioles (B) with destroyed mucosal lining and small bronchiolar vessel (V) with nearby exudate (arrow heads). In addition, lung tissue with thick interalveolar septa (S), multiple foci of cellular infiltration (C) and extravasation of blood (arrow).
(H&E X 100)

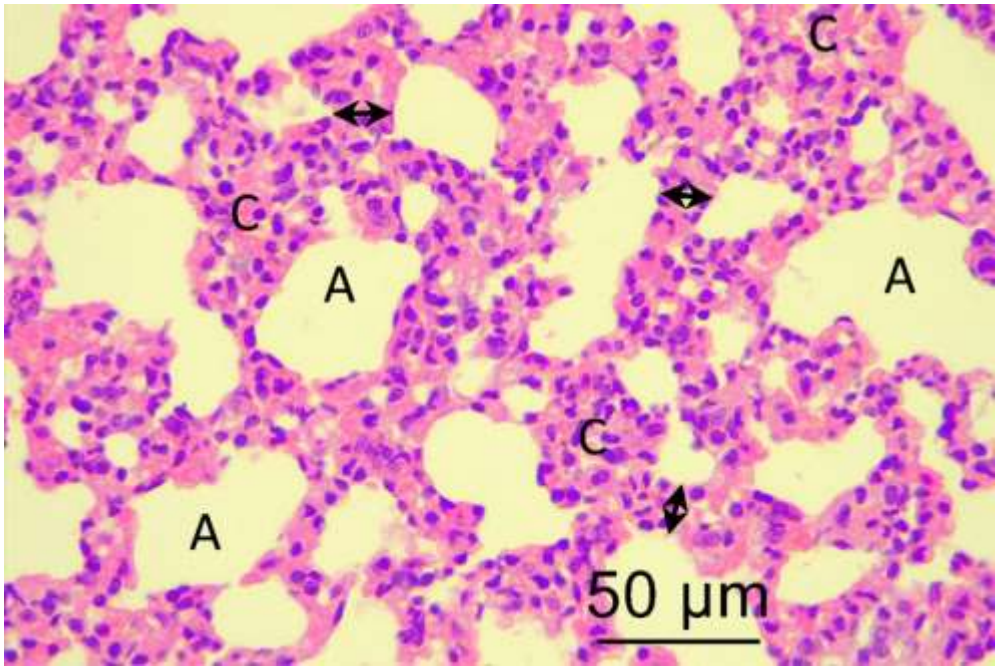


**Figure (5):** A photomicrograph of section in the lung of the methotrexate treated group showing multiple alveoli (A) with thick prominent interalveolar septa (arrows) formed by perminant cellular infiltration (C).
(H&E X 400)

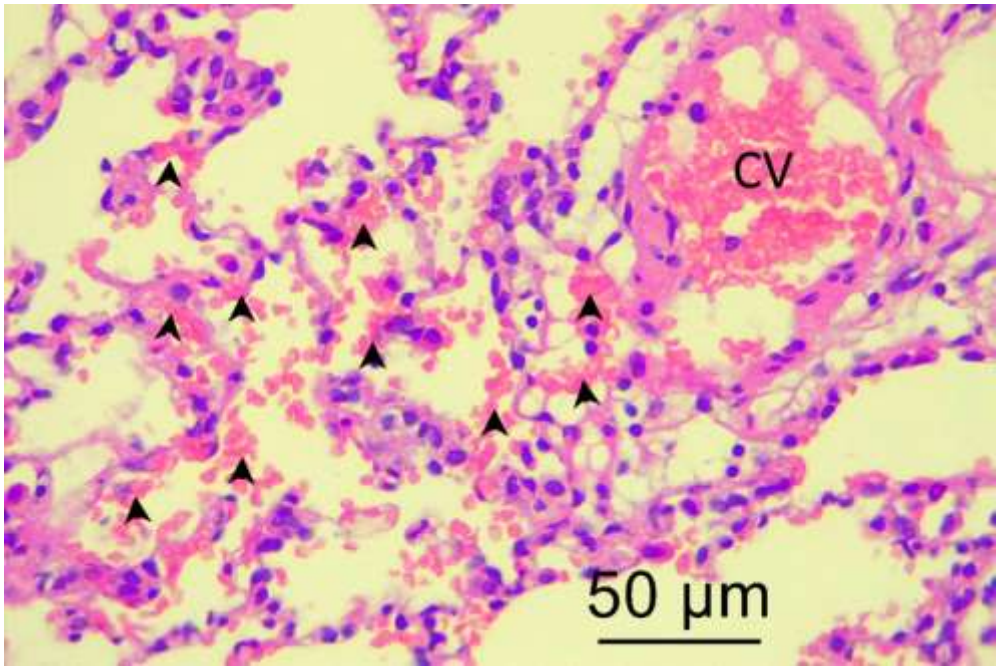


**Figure (6):** A photomicrograph of section in the lung of the methotrexate treated group showing massive extravasation of blood (arrow heads) and highly congested blood vessel (CV).
(H&E X 400)

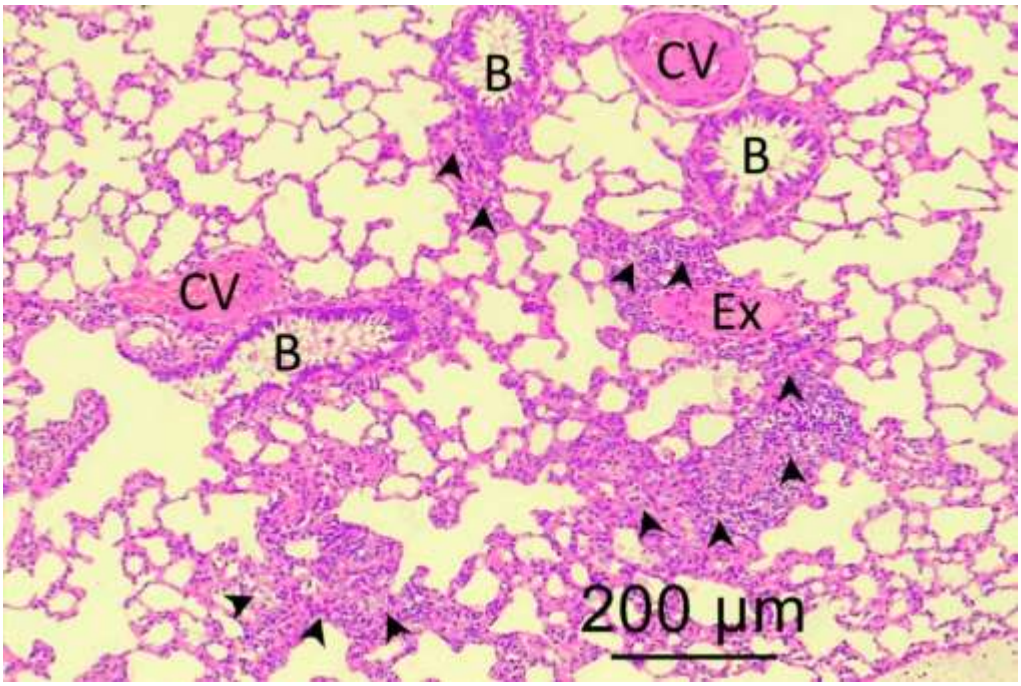


**Figure (7):** A photomicrograph of section in the lung of the methotrexate treated group showing small bronchioles filled with secretions (B) with destroyed mucosal lining and small congested vessels (CV) with nearby localized focus of intra-alveolar exudate (Ex). In addition, abundant lung infiltration nearby bronchioles and vessels (arrow heads).
(H&E X 100)

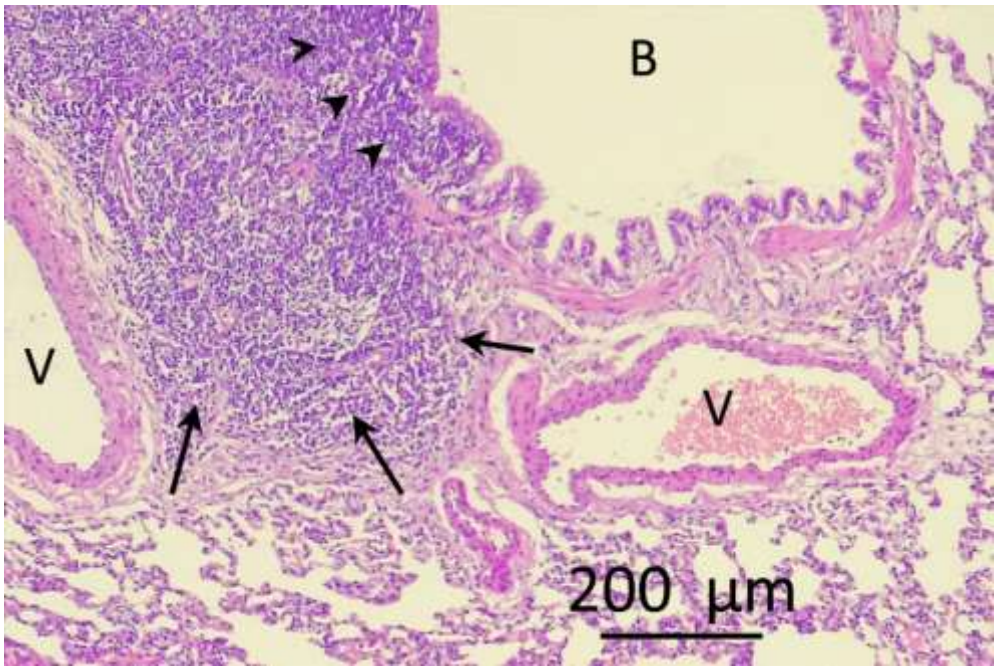


**Figure (8):** A photomicrograph of section in the lung of the methotrexate treated group showing large bronchiole (B) and large bronchial vessels (V). In between, huge mass of infiltration (arrows) destroying the wall of the bronchiole (arrow heads).
(H&E X 100)

**Table (1) Comparison between the studied groups regarding biochemistry data:**

| Parameter | Control Means ± SD | Mtx Means ± SD | ANOVA | (Tukey's test) | |
|---|---|---|---|---|---|
| | | | | Control vs Mtx | |
| **CRP** | 0.279 ± 0.065 | 0.596 ± 0.183 | < 0.001 ** | ** | |
| **MDA (nmol/ gm)** | 10.980 ± 0.926 | 21.730 ± 1.036 | < 0.001 ** | ** | |
| **GSH (mmol/ gm)** | 2.429 ± 0.252 | 1.173 ± 0.139 | < 0.001 ** | ** | |

F one was ANOVA test
**p≤0.001 is statistically significant * significant difference p<0.05
NS statistically non-significant
Mtx: Methotrexate group

## Discussion

MTX is an efficacious chemotherapeutic and immunosuppressive medication. In the vast majority, MTX is well-tolerated. However, it is potentially toxic to an array of organs including liver, intestine, kidneys, bone marrow and lungs[22-24]. Pulmonary adverse sequelae of MTX can take place in up to7% of patients even they are on a low-dose regimen[8].

Since the clinical, laboratory, radiological and even pathological findings are not pathognomonic, lung lesions due to MTX are problematic for clinicians whereby diagnosis is feasible only after the exclusion of other etiologies.[10,25].

It has been suggested that in humans pulmonary toxicity is dose-independent and can occur after MTX intake for once[9]. In the animal studies, a single dose of 20mg/kg of MTX injected into the peritoneal cavity has been commonly used in previous studies of[14 and, 15, 18, 26].

CRP is a classical biomarker in inflammatory conditions. IL-6 and other pro-inflammatory cytokines stimulate the synthesis of such ideal indicator in the hepatic cells.[27,28]. Although MTX chronotherapy effectively lowered CPR and tumour necrosis factor-α (TNF-α) in rats with collagen induced-arthritis[41], the highest level of CPR in the present study was in the MTX group. In harmony with our results, CRP and TNF-α were expressed immunohistochemically in the tubular epithelium of MTX-treated rats kidney[13]. According to Famurewa, Folawiyo[12], the activation of macrophages nuclear factor kappa B(NF-ƙB) seems to play a role in CRP and IL-6 elevation associated with MTX.

Methotrexate-induced lung injury (MILI) most probably results from flared oxidative stress wherein there is overwhelming of peroxides, superoxide, hydroxyl radical and other reactive oxygen species (ROS) and concomitant depletion of reduced glutathione and different antioxidant enzymes[13,23].

Peroxidation of lipid has been claimed to be a direct impact of MTX[30]. MDA is an end product of fat breakdown, thus itis elevated when cellular wall is damaged[16]. Following the results of Kurt, Tumkaya[26], MDA level in the present study was significantly elevated in the lung homogenates of MTX injected rats. High levels of MDA were also detected in the cerebellum[30], liver, kidney[18] and testis[15] of MTX-treated rats.

In the MTX group of this study, the concentration of GSH diminished greatly in the pulmonary tissues. Similar alterations in oxidative stress biomarkers have been reported in hepatic tissue[31], and the sera[12] of rats received the same dose of MTX used in the present study.

The microscopic findings in lung sections of the MTX group in the present work were similar to those observed in lung biopsies taken from patients complaining from MTX-induced pulmonary toxicity[8,11,33]. Lung infiltration and septal thickening are common findings in the imaging studies of these patients[9,11]. In agreement with Arpag, Gül[17], the infiltration of inflammatory cells together with predominance of type II pneumocytes resulted in remarkable thickening of the interalveolar septa which was morphometrically confirmed.
According to Kalemci, Akpınar[32] the toxicity of MTX to the lung is mediated through activation of P38 mitogen-activated protein kinases (MAPK).Yang, Kim[34] suggested that P38-MAPK plays a central role in inflammatory processes and the production of inflammatory mediators.

Additionally, we noticed congestion and thickening in the wall of blood vessels along with interstitial and intra-alveolar hemorrhages. In the experimental study of Kurt, Tumkaya[26], the main two histopathological findings in the lungs of MTX-treated rats were: congestion and inflammatory cells infiltration.
Hemorrhage, congestion, cellular infiltration and cellular degenerations were prominent features in the livers and kidneys of MTX-treated rats[13,18].

In healthy lungs, collagen fibers in the alveolar walls are quite thin, and can only be seen by electron microscopy. Therefore, it is abnormal to detect collagen fibers in routine light microscopy[35]. Data from systematic review revealed that interstitial fibrosis comes next after lymphoproliferative disorders as the most common pulmonary complications of MTX[8]. In this study, and in concurrence with Kurt, Tumkaya[26] and Kalemci, Akpınar[32].

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**Conflict of interest:** The authors declare that they have no conflict of interest

## References

1. Conway, R. and J.J. Carey, Methotrexate and lung disease in rheumatoid arthritis. Panminerva Med, 2017. **59**(1): p. 33-46.
2. Phillips PC, Dhawan V, Strother SC, Sidtis JJ, Evans AC, Allen JC, et al., Reduced cerebral glucose metabolism and increased brain capillary permeability following high-dose methotrexate chemotherapy: a positron emission tomographic study. Ann Neurol, 1987. **21**(1): p. 59-63.
3. Nowicki TS, Bjornard K, Kudlowitz D, Sandoval C, Jayabose S. Early recognition of renal toxicity of high-dose methotrexate therapy: a case report. J Pediatr Hematol Oncol, 2008. **30**(12): p. 950-2.
4. Sotoudehmanesh R, Anvari B, Akhlaghi M, Shahraeeni S, Kolahdoozan S. Methotrexate hepatotoxicity in patients with rheumatoid arthritis. Middle East journal of digestive diseases, 2010. **2**(2): p. 104-109.
5. Perez-Verdia A, Angulo F, Hardwicke FL, Nugent KM. Acute cardiac toxicity associated with high-dose intravenous methotrexate therapy: case report and review of the literature. Pharmacotherapy,2005.**25**(9):p.1271-6.
6. Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran A, Sukhotnik I. Dietary L-arginine supplementation reduces Methotrexate-induced intestinal mucosal injury in rat. Vol.12.2012. 41.
7. Howard SC, McCormick J, Pui C-H, Buddington RK, Harvey RD. Preventing and Managing Toxicities of High-Dose Methotrexate. The Oncologist, 2016.**21**(12):p. 1471-1482.
8. Thaniyan A, Ayman FFA, Mirghani HO, Al-Sayed BA, Merghani TH. Histopathological Features of Methotrexate Induced Pulmonary Lesions in Rheumatoid Arthritis Patients: A Systematic Review of Case Reports. Open access Macedonian journal of medical sciences, 2017.**5**(2):p.266-270.
9. Chhabra P, Law AD, Suri V, Malhotra P, Varma S. Methotrexate induced lung injury in a patient with primary CNS lymphoma: a case report. Mediterranean journal of hematology and infectious diseases, 2012. **4**(1): p. e2012020-e2012020.
10. Jakubovic BD, Donovan A, Webster PM, Shear NH. Methotrexate-induced pulmonary toxicity. Canadian respiratory journal, 2013. **20**(3): p. 153-155.
11. Karadag AS, Kanbay A, Ozlu E, Uzuncakmak TK, Gedik C, Akdeniz N. Pulmonary fibrosis developed secondary to methotrexate use in a patient with psoriasis vulgaris. Northern clinics of Istanbul, 2015. **2**(2): p. 159-161.
12. Famurewa AC, Folawiyo AM, Enohnyaket EB, Azubuike-Osu SO, Abi I, Obaje SG, et al., Beneficial role of virgin coconut oil supplementation against acute methotrexate chemotherapy-induced oxidative toxicity and inflammation in rats. Integr Med Res, 2018. **7**(3): p. 257-263.
13. Asci H, Ozmen O, Ellidag HY, Aydin B, Bas E, Yilmaz N. The impact of gallic acid on the methotrexate-induced kidney damage in rats. J Food Drug Anal, 2017. **25**(4): p. 890-897.
14. Yucel Y, Oguz E, Kocarslan S, Tatli F, Gozeneli O, Seker A, et al., The effects of lycopene on methotrexate-induced liver injury in rats. Bratisl Lek Listy, 2017. **118**(4): p. 212-216.
15. Pınar N, Çakırca G, Özgür T, Kaplan M. The protective effects of alpha lipoic acid on methotrexate induced

testis injury in rats. Biomedicine & Pharmacotherapy, 2018. **97**: p. 1486-1492.
16. Cakir T, Basturk A, Polat C, Aslaner A, Durgut H, Sehirli AO, et al. Does alfa lipoic acid prevent liver from methotrexate induced oxidative injury in rats? Acta Cir Bras, 2015. **30**(4): p. 247-52.
17. Arpag H, Gül M, Aydemir Y, Atilla N, Yiğitcan B, Cakir T, et al., Protective Effects of Alpha-Lipoic Acid on Methotrexate-Induced Oxidative Lung Injury in Rats. Journal of Investigative Surgery, 2018. **31**(2): p. 107-113.
18. Armagan I, Bayram D, Candan IA, Yigit A, Celik E, Armagan HH, et al., Effects of pentoxifylline and alpha lipoic acid on methotrexate-induced damage in liver and kidney of rats. Environ Toxicol Pharmacol, 2015. **39**(3): p. 1122-31.
19. Draper, H.H. and M. Hadley, Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol, 1990. **186**: p. 421-31.
20. Beutler, E., O. Duron, and B.M. Kelly, Improved method for the determination of blood glutathione. J Lab Clin Med, 1963. **61**: p. 882-8.
21. Bancroft, J. and M. Gamble, Theory and practice of histological technique. 2008, 6th ed. Churchill Livingston: London.
22. Jariwala P, Kumar V, Kothari K, Thakkar S, Umrigar DD. Acute Methotrexate Toxicity: A Fatal Condition in Two Cases of Psoriasis. Case Reports in Dermatological Medicine, 2014. **2014**: p. 3.
23. Samdanci ET, Huz M, Ozhan O, Tanbek K, Pamukcu E, Akatli AN, et al., Cytoprotective effects of molsidomine against methotrexate-induced hepatotoxicity: an experimental rat study. Drug design, development and therapy, 2018. **13**: p. 13-21.
24. Zhou B, Xia X, Wang P, Chen S, Yu C, Huang R, et al., Induction and Amelioration of Methotrexate-Induced Gastrointestinal Toxicity are Related to Immune Response and Gut Microbiota. EBioMedicine, 2018. **33**: p.122-133.
25. Kileci, J.A., O. Charran, and J. Filopei. Severe Methotrexate-Induced Pulmonary Toxicity, in D34. LUNG TRANSPLANT AND DRUG INDUCED LUNG DISEASE: CASE REPORTS. 2018, American Thoracic Society. p. A6595-A6595.
26. Kurt A, Tumkaya L, Turut H, Cure MC, Cure E, Kalkan Y, et al., Protective Effects of Infliximab on Lung Injury Induced by Methotrexate. Archivos de Bronconeumología (English Edition), 2015. **51**(11):p.551-557.
27. O'Doherty MG, Gilchrist SECM, Young IS, McKinley MC, Yarnell JWG, Gey KF, et al., Effect of supplementation with B vitamins and antioxidants on levels of asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP): a double-blind, randomised, factorial design, placebo-controlled trial. European Journal of Nutrition, 2010. **49**(8): p. 483-492.
28. Sugitharini, V., A. Prema, and E. Berla Thangam, Inflammatory mediators of systemic inflammation in neonatal sepsis. Inflammation Research, 2013. **62**(12): p. 1025-1034.
29. Wang X, Yan X, Wang F, Ge F, Li Z. Role of methotrexate chronotherapy in collagen-induced rheumatoid arthritis in rats. Zeitschrift für Rheumatologie, 2018. **77**(3): p. 249-255.
30. Uzar E, Koyuncuoglu HR, Uz E, Yilmaz HR, Kutluhan S, Kilbas S, et al. The Activities of Antioxidant Enzymes and the Level of Malondialdehyde in Cerebellum of Rats Subjected to Methotrexate: Protective Effect of Caffeic Acid Phenethyl Ester. Molecular and Cellular Biochemistry, 2006. **291**(1): p. 63-68.
31. Mehrzadi S, Fatemi I, Esmaeilizadeh M, Ghaznavi H, Kalantar H, Goudarzi M. Hepatoprotective effect of berberine against methotrexate induced liver toxicity in rats. Biomed Pharmacother, 2018. **97**: p. 233-239.
32. Kalemci S, Akpınar O, Dere Y, Sarıhan A, Zeybek A, Tanriverdi Ö. Efficacy of clarithromycin as a protective agent in the methotrexate-

induced pulmonary fibrosis model. Kardiochirurgia i torakochirurgia polska= Polish journal of cardio-thoracic surgery, 2018. **15**(4): p. 209-212.

33. Imokawa S, Colby TV, Leslie K, Helmers RA. Methotrexate pneumo-nitis: review of the literature and histopathological findings in nine patients. Eur Resp J 15: 373-381. European Respiratory Journal, 2000. **15**: p. 373-381.
34. Yang Y, Kim SC, Yu T, Yi Y-S, Rhee MH, Sung G-H, et al., Functional Roles of p38 Mitogen-Activated Protein Kinase in Macrophage-Mediated Inflammatory Responses. Mediators of Inflammation, 2014. **2014**: p. 13.
35. Tomashefski, J.F., C. Farver, and A.E. Fraire, Dail and Hammar's Pulmonary Pathology: Volume I: Nonneoplastic Lung Disease. 3rd ed. 2009: Springer New York.