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Original Article

Tocilizumab Reduces Lung Injury in a Rat Lung Ischemia and Reperfusion Model

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Abstract

OBJECTIVE: In this study, the effect of tocilizumab (TCZ) on lung tissue in lung ischemia–reperfusion (I/R) injury in rats was investigated.

MATERIAL AND METHODS: A total of 24 Wistar rats were divided into 4 equal groups, with 6 rats in each group: Left lung I/R was applied to I/R groups. In the I/R groups, the left lung hilum was clamped for 45 minutes, and then the clamp was removed and reperfused for 120 minutes. In the TCZ groups, 4 mg/kg and 8 mg/kg of TCZ were administered intraperitoneally to the rats 30 minutes before surgery.

RESULTS: The tumor necrosis factor-alpha mean value was not statistically significant between the groups (P = .091). Statistically significant results were observed between group I/R-TCZ (8 mg/kg) and group I/R for catalase. (P = .005). Statistically significant results were observed between group I/R-TCZ (8 mg/kg) and group I/R for malondialdehyde. (P = .009). The difference in total ischemia score between group I/R-TCZ (4 mg/kg) and group I/R-TCZ (8 mg/kg) and group I/R was statistically significant (P < .001). In terms of alveolar hemorrhage, there was a statistically significant difference between group I/R-TCZ (4 mg/kg) and group I/R-TCZ (8 mg/kg) and group I/R (P = .01 and P = .002, respectively). There was a statistically significant difference between group I/R-TCZ (8 mg/kg) and group I/R in terms of neutrophil accumulation (P = .01). In terms of interstitial edema, there was a statistically significant difference between group I/R-TCZ (4 mg/kg) and group I/R-TCZ (8 mg/kg) and group I/R (P = .006 and P = .001, respectively). In terms of pulmonary edema, there was a statistically significant difference between group I/R-TCZ (4 mg/kg) and group I/R-TCZ (8 mg/kg) and group I/R (P = .01 and P = .009, respectively).

CONCLUSION: Lung tissue may be affected by I/R injury and this damage can be reversed with the use of TCZ.

KEYWORDS: Ischemia/reperfusion, interleukin 6 blocker, lung, rat, tocilizumab

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INTRODUCTION

Ischemia–reperfusion (I/R) injury in the lung occurs frequently in cardiopulmonary bypass, pulmonary thromboendarterectomy, lung cancer operations, and lung transplantation. Various signs of damage occur in the tissue during the reperfusion period, which occurs with blood supply in the tissue after ischemia.[1-3] This process is called "reperfusion injury." The concept of reperfusion injury was first introduced by Buckberg and later by Braunwald et al.[4] Pulmonary edema after reperfusion, changes in vascular balance due to the exposure of endothelial cells to ischemia–reperfusion, decreased nitric oxide during reperfusion, vascular dysfunction due to cyclic guanosine monophosphate levels, impaired coagulation, vascular permeability, vasomotor tone, leukocyte adhesion, and aggregation increase in function is the most important problem encountered.[4-9]

Tocilizumab (TCZ) is an agent that acts as an interleukin 6 (IL-6) monoclonal antagonist and is used in some autoimmune diseases such as rheumatoid arthritis, juvenile idiopathic arthritis, and giant cell arteritis.[7] Interleukin 6 induces the proliferation and differentiation of T cells as well as terminal differentiation of B cells, including autoantibody-producing cells. Increased IL-6 production also increases autoimmune reactions. Transforming growth factor beta (TGF-β), in the presence of IL-6, was reported to induce the differentiation of pathogenic T helper cells.[8] Tocilizumab has recently emerged as a drug with significant benefits in clinical recovery in severe coronavirus disease 2019 (COVID-19) pneumonia. A study conducted in China on 21 patients with severe COVID-19 pneumonia reported that TCZ treatment had significant clinical benefits. The pathogenesis of severe respiratory failure associated with coronavirus, elevated serum proinflammatory cytokines and chemokines [interleukins 1, 6, 8, and 12 (IL-1, IL-6, IL-8, IL-12), tumor necrosis factor alpha (TNF-α), and interferon gamma] is associated with a cytokine storm. The treatment dose can start with 4 mg/kg and be increased up to 8 mg/kg.[10-12]

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This study aimed to investigate the effectiveness of TCZ in reducing oxidative damage due to lung I/R injury.

MATERIAL AND METHODS

Twenty-four Wistar albino male rats (250 mg ± 25 mg) were used in the study. This experimental study was conducted at İstanbul Bezmialem Foundation University Experimental Medicine Research and Application Center Experimental Animals Laboratory on December 15, 2021. Approval was obtained from the İstanbul Bezmialem Foundation University Experimental Medicine Research and Application Center Experimental Animals Ethics Committee (2021/240). All rats were cared for in accordance with the Principles of Care for Experimental Animals as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health.

Groups

The subjects were divided into 4 groups, each of which had 6 rats. In group Sham, only thoracotomy was performed. In group I/R, thoracotomy was performed and an I/R period was performed. Tocilizumab was applied before ischemia–reperfusion in group I/R/4 mg/kg TCZ and group I/R/8 mg/kg TCZ groups. The rats underwent 45 minutes of ischemia and 120 minutes of reperfusion. Tocilizumab (Actemra) was given intraperitoneally (i.p.) 30 minutes before ischemia. After ischemia, the thorax was closed with sutures. At the end of the experiment, the experimental animals were sacrificed by administering a high dose (150 mg/kg ketamine/30 mg/kg xylazine) of anesthetic substance i.p. The left lung tissue was fixed in 10% formaldehyde and subjected to pathological examination. A blood sample was taken from the heart for biochemical study.

Anesthesia and Surgery

All procedures were performed under sterile conditions. The animals were kept in standard laboratory cages under standard conditions (12 hour light–12 hour darkness cycle). Anesthesia was provided intraperitoneally with 50 mg/kg ketamine (Ketalar vial, Pfizer Pharma GMBH, Germany) and 10 mg/kg xylazine hydrochloride (Alfazyne 2%, Alfasan International, Holland). When necessary, ketamine (half dose, 25 mg/kg) was repeated in order to keep the depth of anesthesia constant, by looking at reflex responses (painful stimulus to the foot with forceps-pedal reflex). Heparin (50 IU) and 0.01 mg atropine were given by IP to the rats before thoracotomy. After the tracheostomy was opened, the patient was mechanically ventilated with a tidal volume of 10 mL/kg, a respiratory frequency of 70 respirations/minute, a peep pressure of 2 cm H_2O, and 100% O_2. Arterial monitoring was performed from the right carotid artery. Drugs were administered i.p. 30 minutes before ischemia. After thoracotomy, the thorax was explored and the inferior ligament was freed by cutting, and the left lung was laterally retracted to expose the left hilar structures. Hilar ischemia was applied.

Main Points

- Ischemia–reperfusion (I/R) injury in the lung occurs frequently in cardiopulmonary bypass, pulmonary thromboendarterectomy, lung cancer operations, and lung transplantation.
- Tocilizumab (TCZ) is an agent that acts as an IL-6 monoclonal antagonist and has recently come to the fore as a drug with significant benefits in clinical recovery in severe coronavirus disease-19 pneumonia.
- Histopathologically poor results due to increased inflammation and oxidative stress after I/R were significantly reversed with TCZ.
- A statistically significant decrease was observed in the blood biochemically measured malondialdehyde and catalase levels in the 8 mg/kg group compared to the I/R group.

Histopathological Examination

After blood samples were taken, samples were taken from the lung tissue.[4-6] Samples were evaluated blindly under a light microscope by a pathologist experienced in cytology. Left lung samples taken from all groups were fixed in 10% formol and underwent routine follow-up procedures. Serial sections of 4 µm thickness were taken from the tissues embedded in paraffin blocks. Sections taken were deparaffinized, stained with hematoxylin–eosin (H-E), and evaluated under a light microscope. The scoring system previously used by Tassiopoulos and Zhou et al was modified to determine the amount of damage in the lungs. Accordingly, 0 points indicate no change; 1 point indicate focal slight changes; 2 points indicate multifocal moderate changes; 3 points indicate diffuse marked changes; 4 points indicate very heavy changes. The tissue damage score was calculated by summing the scores of 4 indexes (perivascular edema, interstitial edema, neutrophil infiltration, and intra-alveolar hemorrhage).

Biochemical Examination

Blood samples from the rats were taken from the heart just before sacrificing.[4-6] Blood samples stored at −80°C were homogenized in phosphate buffer (PBS, 0.01 M, pH 7.4). The homogenate was centrifuged at 1600 *g* for 10 minutes. Malondialdehyde (MDA) and thiobarbituric acid (TBA) composition levels (nmol/milliliter) (µmol), which show lipid peroxidation, were measured by colorimetric method using TBARS Assay kit (Cayman Chemical, Item No: 10009055). Tumor necrosis factor alpha levels (pg/mL) were measured by sandwich ELISA principle using rat TNF-α ELISA Kit (Elabscience Biotechnology Inc., Catalog No: E-EL-R0019). Results are nmol/min catalase (CAT) per milligram (mg) protein (nmol/mg) and nmol MDA composition per milligram (mg) protein (nmol/mg), picogram per milligram (mg) protein (pg) TNF-α (pg/mg). Again, with the measurement methods and kits mentioned above, CAT activity (nmol/mL), MDA (nmol/mL), and TNF-α levels (pg/mL) were measured.

Statistical Analysis

The effect size was taken as 0.0755 and the sample size required to determine the difference between the 4 groups with the help of G-Power 3.1.9.7, was determined by taking a total of 24 rats with .05 type 1 error and a power of 0.70. The data obtained in the experiment were analyzed using the Statistical Package for the Social Sciences 20.0 program for

Windows (IBM Corp., Armonk, NY, USA). One-way ANOVA was used for parameters where the data met the normality assumption for multiple analyses. When the assumption of normality was not met, the Kruskal–Wallis test was used and values with $P < 0.05$ were considered statistically significant. In cases where there was a statistically significant difference between the groups, TUKEY, one of the Post Hoc analyses for normal distribution data, and Tammahane's T2 analysis for non-normally distributed data, were used to examine which group caused the difference between the groups. The Shapiro–Wilk test was analyzed because the sample size was less than 30. Since the CAT and MDA values followed a normal distribution, parametric analyses were performed for these data, while nonparametric analyses were performed for other data that did not normally distributed.

RESULTS

Tumor necrosis factor alpha mean value in the blood was higher in group I/R than in group Sham ($P = < .001$). The mean value for TNF-α in group Sham was 8.6 pg/mL. TNF-α mean value was decreased in group I/R-TCZ 4 mg/kg (57.5 pg/mL) and group I/R-TCZ 8 mg/kg (55.2 pg/mL) compared to group I/R (65.2 pg/mL). However, the difference in TNF-α value was not statistically significant between the groups ($P = 0.091$). Box plot for TNF-α is shown in Figure 1.

Catalase mean value in the blood was higher in group I/R (7.39 nmol/mL) than in group Sham (3.82 nmol/mL). Catalase mean value was decreased in group I/R-TCZ 4 mg/kg (6.50 nmol/mL) and group I/R-TCZ 8 mg/kg (5.54 nmol/mL) compared to group I/R.Statistically significant results were also observed between group I/R-TCZ 8 mg/kg and group I/R for CAT ($P = .005$). There was a statistically significant difference between group Sham and group I/R ($P < .001$). No statistically significant result was observed between group I/R-TCZ 4 mg/kg and group I/R ($P = .268$). The box plot for CAT is shown in Figure 2.

The blood MDA level was higher in group I/R (5.60 nmol/mL) than group S (3.09 nmol/mL). MDA mean value was decreased in group I/R-TCZ 4 mg/kg (4.78 nmol/mL) and group I/R-TCZ 8 mg/kg (4.15 nmol/mL) compared to group I/R (5.60 nmol/mL). Statistically significant results were also observed between group I/R-TCZ 8 mg/kg and group I/R for

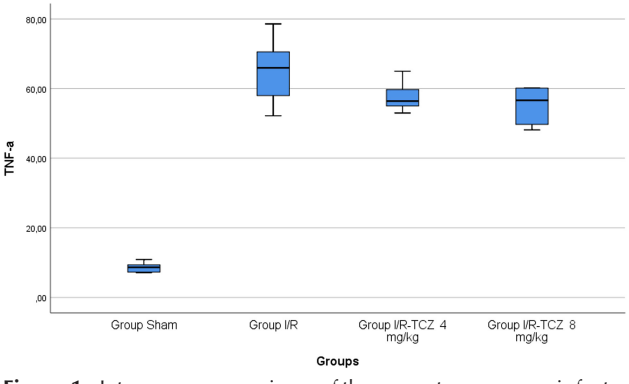

Figure 1. Intergroup comparisons of the serum tumor necrosis factor alpha (TNF-α) levels. Mann–Whitney *U*-test was used to determine statistical differences between groups and $P < .05$ was considered significant. The difference in TNF-α value was not statistically significant between the groups ($P = .091$).

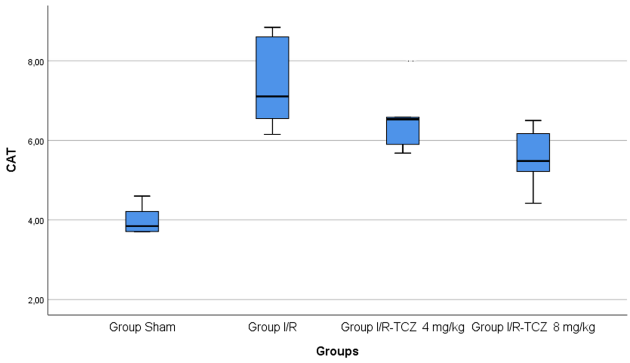

Figure 2. Intergroup comparisons of the serum catalase (CAT) levels. The Mann–Whitney *U*-test was used to determine statistical differences between groups and $P < .05$ was considered significant. Statistically significant results were also observed between group I/R-TZM 8 mg/kg and group I/R for CAT ($P = .005$).

MDA ($P = .009$). There was a statistically significant difference between group Sham and group I/R ($P < .001$). No statistically significant result was observed between group I/R-TCZ 4 mg/kg and group I/R ($P = .217$). The box plot for MDA is shown in Figure 3.

The difference in total ischemia score between group I/R-TCZ 4 mg/kg and group I/R-TCZ 8 mg/kg and group I/R was statistically significant ($P < .001$). In terms of alveolar hemorrhage, there was a statistically significant difference between group I/R-TCZ 4 mg/kg and group I/R-TCZ 8 mg/kg and group I/R ($P = .01$ and $P = .002$, respectively). There was a statistically significant difference between group I/R-TCZ 8 mg/kg and group I/R in terms of neutrophil accumulation ($P = 0.01$). In terms of interstitial edema, there was a statistically significant difference between group I/R-TCZ 4 mg/kg and group I/R-TCZ 8 mg/kg and group I/R ($P = .006$ and $P = .001$, respectively). In terms of pulmonary edema, there was a statistically significant difference between group I/R-TCZ 4 mg/kg and group I/R-TCZ 8 mg/kg and group I/R ($P = .01$ and $P = .009$, respectively).

Histopathological changes of the lung tissue are shown in Figure 4. Lung sections of group Sham stained with H-E showed a normal alveolar histological structure. No infiltration was observed, and alveolar structure was normal

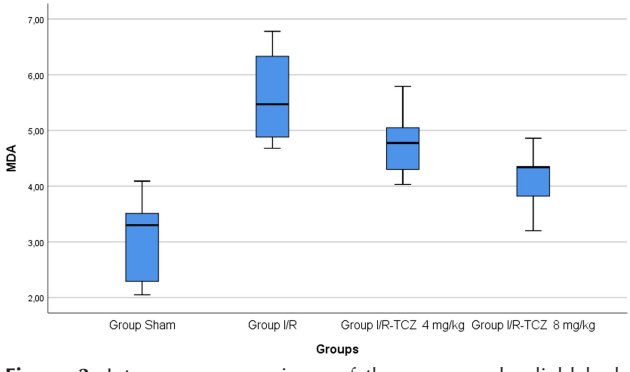

Figure 3. Intergroup comparisons of the serum malondialdehyde (MDA) levels were determined using the Mann–Whitney *U*-test was used to determine statistical differences between groups and $P < .05$ was considered significant. Statistically significant results were also observed between group I/R-TZM 8 mg/kg and group I/R for MDA ($P = .009$).

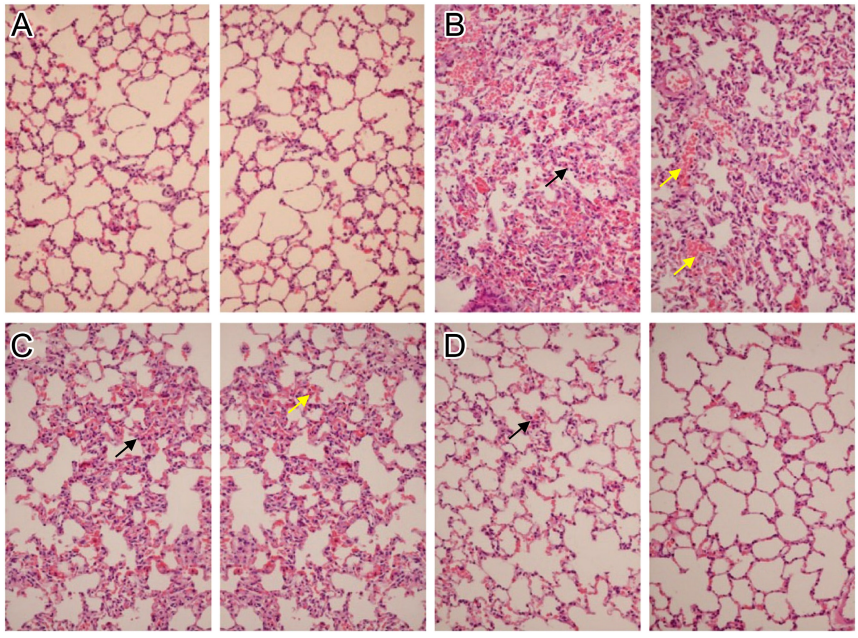

Figure 4. Lung tissue preparations, hematoxylin–eosin (×40; ×100; ×400): (A) Normal lung tissue parenchyma; group Sham; (B) Intense neutrophil infiltration and alveolar hemorrhage, alveolar edema in lung tissue, group I/R (black arrow: neutrophils, yellow arrow: erythrocytes); (C) Neutrophil infiltration, alveolar hemorrhage, alveolar edema, and hemorrhage in group I/R-4 mg/kg TZM (black arrow: neutrophils, yellow arrow: erythrocytes); (D) Neutrophil infiltration, alveolar hemorrhage, alveolar edema, and decreased hemorrhage in group I/R-8 mg/kg TZM (black arrow: neutrophils).

(Figure 4A). However, neutrophil infiltration, alveolar edema, and hemorrhage were present in lung sections on I/R (Figure 4B). Dense neutrophil infiltrates were shown, and alveolar edema, and hemorrhage were severely increased. A decrease in histological changes was observed in group I/R-TCZ 4 mg/kg and group I/R-TCZ 8 mg/kg compared to group I/R (Figure 4C and Figure 4D).

DISCUSSION

In this study, we investigated the extent to which TCZ applied before lung IR prevented histopathological changes in lung tissue. In our study, we observed a significant improvement in I/R-related damage in rats using TCZ, depending on the dose, at the histopathological level.

I/R injury is characterized by the accumulation of free oxygen radicals [Reactive oxygen species (ROS)], endothelial cell damage, increased vascular permeability, activation of the cytokine and complement system in parallel with the increase in neutrophil and platelet cell accumulation.[7,9,13] Acute lung injury in critically ill patients can occur after cardiopulmonary bypass and lung transplantation. Tissue damage occurs especially during the reperfusion period, and it has been observed that this period causes more damage than ischemia. This condition is called I/R or reperfusion injury. This process is a complex pathophysiological process in which vascular, humoral, and cellular factors interact. In the I/R period, microcirculation is also damaged, blood flow homogeneity is impaired, and local tissue damage may occur. Many studies are carried out to prevent the increase in mortality and morbidity resulting from I/R injury.[14-17]

Tocilizumab is a monoclonal IL-6 membrane receptor antagonist that competes selectively and blocks the dimerization of the glycoprotein 130 molecule in the cell membrane and IL-6 signal transmission into the cell. In vivo, the maximum (>90%) saturation saturation of the IL-6 receptor is reached at a serum TCZ concentration >1 µg/mL. At a dose of 4 ug/mL, complete inhibition of the receptors occurs.[11] Its half-life varies depending on the murine antibodies present in the human body and can be up to 240 hours after 3 doses of 8 mg/kg TCZ. Tocilizumab is dose-dependent, has nonlinear pharmacokinetics, and has a long half-life. Free TCZ can be detected in the blood.[10] It has clinical use at doses of 4 and 8 mg/kg. In our study, we used the same doses of drugs that are used clinically. There is a dose-dependent relationship between TCZ therapy and a decrease in neutrophil levels.[11] Interleukin 6 is associated with the pathogenesis of inflammatory diseases such as rheumatoid arthritis and induces the production of some mediators such as CRP. Interleukin 6 is produced from various types of cells in immunological, traumatic, and infection conditions. Interleukin 6 has pro-inflammatory and anti-inflammatory properties in the disease process.[11,12] By accelerating the transformation of T cells and B cells, it provides the expansion and activation of inflammation. Inhibition of the IL-6 receptor results in suppression of cytokine signaling. Inhibition of the IL-6 receptor complex inhibits cytokine generation from B and T lymphocytes via IL-6.

Karataş et al[15] found that 4 mg/kg TCZ administered in spinal cord ischemia had a neuroprotective effect, and the levels of TNF-α, IL-6, IL-10, whose levels were increased in the IR group, decreased significantly in rats given TCZ. They showed that the scores of histopathological symptoms such as neutrophil vacuolization, Nissl bodies, and tissue loss in the ischemia group were significantly lower in those who received TCZ.[15] Wang et al[9] showed that 8 mg/kg TCZ inhibited neuronal cell apoptosis in the hippocampus and cortex

in a rat model of cerebral infarction. In this study, antioxidant enzyme levels such as MDA, TNF-α, and CAT, which are indicators of reperfusion injury, were found to be lower in the I/R + TCZ group than in the I/R group, indicating the free radical scavenging effect of TCZ. Additionally, the decrease in inflammation and degeneration of the lung tissue in the histopathological examination of the lung tissue in the I/R + TCZ group supports the protective effect of TCZ against the reperfusion injury caused by I/R in the lung tissue. In our study, supporting these 2 studies, MDA and CAT levels showed a statistically significant decrease in group I/R-TCZ 8 mg/kg compared to the I/R group.

The release of cytokines and chemokines such as IL-6, IL-1, granulocyte–macrophage colony stimulating factor, and TNF-α from pneumocytes and other cells increases due to COVID-19 increases. Due to the release of these cytokines and chemokines, acute respiratory distress and respiratory failure occur due to increased vascular permeability and exudation in the lung alveoli.[11] Proinflammatory cytokines and IL-6 play a pivotal role in the development of many COVID-19-related complications. A 2.9 times higher concentration of IL-6 was observed in severe COVID-19 patients than in uncomplicated patients.[11] Therefore, a decrease was observed in the length of hospital stay and mortality rates after the use of TCZ in COVID-19 patients. Tocilizumab has been increasingly used in COVID-19 patients. Although the TNF-α level was not statistically significant in our study, it showed a decrease in the mean value depending on the dose in the groups given TCZ compared to group I/R.

Enzymes such as superoxide dismutase, CAT, and glutathione peroxidase play an important role in preventing tissue damage due to ROS, which is important in the pathophysiology of I/R injury. The antioxidant enzyme group, oxidoreductases, plays an important role in scavenging of free radicals and maintaining the cellular structure.[2,13,16] Catalase is one of these enzymes. Studies have shown an increase in I/R groups due to the oxidant mechanism compared to the control group. In the study by Orhan et al,[2] a decrease in the CAT level was observed in the I/R group compared to the control group, and this decrease was more prominent than in the amantadine group.[2] In our study, the CAT level was statistically significantly decreased in the group given 8 mg/kg TCZ compared to group I/R.

In IR, free oxygen radicals appear after lipid peroxidation as a result of high activity in the cell membrane. These radicals cause damage to the cell's deoxyribonucleic acid, protein, and cellular lipid structure. Malondialdehyde level is thought to be an indicator of lipid peroxidation of radicals.[17,18] In previous studies, it was shown that MDA level increased after IR and decreased in cases where agents were administered.[7,18] Orhan et al[2] found that the lung tissue MDA level, which increased in the lower extremity IR, decreased in their group in rats given amantadine. Malondialdehyde is a product of lipid peroxidation and its production increases in parallel with the formation of free oxygen radicals. Malondialdehyde levels are a very good indicator of the level of oxidative stress.[17,19] In our study, the MDA level was statistically significantly decreased in the group given 8 mg/kg TCZ compared to group I/R.

Lung IR can occur as a result of trauma, atherosclerosis, pulmonary embolism, surgery, and cardiopulmonary bypass. The mechanism of I/R damage in the pulmonary parenchyma is similar to the damage in other organs. The ROS system is activated, intracellular calcium increases, endothelial cell damage occurs, leukocyte accumulation increases in the pulmonary circulation, and as a result, the complement system is activated and arachidonic acid metabolites are released into the circulation. Basically, hydrostatic pressure increases in postcapillary venules, and edema formation occurs with increased capillary permeability due to endothelial damage. ROS and other free radicals play a key role in the development of pulmonary damage. Intracellular ROS accumulation has been demonstrated in lung endothelial cells, alveolar type 2, Clara cell, ciliated epithelial cells, and alveolar macrophage cells and alveolar macrophage cells.[13,14,18] Histopathological indicators of lung injury are alveolar wall thickening, interstitial edema, neutrophil, and lymphocyte infiltration. An increase in extravascular albumin accumulation was observed after 30 and 45 minutes of ischemia.[7] Vascular abnormality causes pulmonary hypertension, vascular reality variability, vascular obstruction, intrapulmonary shunt, increased vascular permeability, and ventilation/perfusion change.[17] In our study, an increase in perivascular edema, interstitial edema, and alveolar hemorrhage was observed in group I/R compared to the control group. In these parameters, a dose-related improvement was found in the TCZ groups.

In the study of Eppinger et al,[12] lung reperfusion injury showed a biphasic picture at 30 minutes and 4 hours.[9] In this study, 30 minutes, 1 hour, 2 hours, and 4 hours reperfusion periods were compared after 90 minutes of Lung IR. After 2 hours of reperfusion, an increase was observed in MPO, complement activity, TNF-α mRNA level, and lung permeability.[16] In our study, an increase in lung permeability and TNF-α levels was observed as a result of a 2-hour reperfusion in parallel with the results of Eppinger et al.[12]

Due to the direct contact of the lung with the external environment, it is in the largest reservoir of macrophages, monocytes, and leukocytes. As a result of reperfusion and reexpansion, lipid mediators, polypeptide mediators, and immune complexes increase in the environment. Due to these increased mediators, dysfunction occurs in endothelial cells and monocytes, PNL and macrophages enter the alveolar–capillary membrane. These blood cells that come to the environment initiate a series of reactions that cause the formation of superoxide radical.[9,14] In our study, leukocyte accumulation, which was clearly observed in the tissue in group I/R, showed a dose-dependent decrease in the TCZ groups.

The limited number of animals and our application of clamping not only to the pulmonary artery but also to the hilum in the lung are the limitations of our study. This can limit your statistical power.

In this study, we showed for the first time in the literature that the antioxidant activity of TCZ in lung I/R is particularly evident in histopathological improvement. Histopathologically poor results due to increased inflammation and oxidative stress after I/R were significantly reversed with TCZ. A statistically significant decrease was observed in the blood

biochemically measured MDA and CAT levels in the 8 mg/kg group compared to the I/R group. Future studies will add to the possibility of clinical use of the drug.

Ethics Committee Approval: This study was approved by the İstanbul Bezmialem Experimental Medicine Research and Application Center Experimental Animals Ethics Committee (2021/240).

Peer review: Externally peer-reviewed.

Author Contributions: Concept – Y.Ö.; Design – Y.Ö.; Supervision – Y.Ö.; Resources – Y.Ö.; Materials – Y.Ö., R.I., B.O., B.B.İ., T.Ö., Ş.A., B.Ö.; Data Collection and/or Processing – Y.Ö., R.I., B.O., B.B.İ., T.Ö., Ş.A., B.Ö.; Analysis and/or Interpretation – Y.Ö., R.I., B.O., B.B.İ., Ş.A., B.Ö.; Literature Search – Y.Ö.; Writing – Y.Ö.; Critical Review – Y.Ö.

Declaration of Interests: The authors have no conflict of interest to declare.

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