

## The effects of amantadine on lung tissue in lower limb ischemia/reperfusion injury model in rats

Amantadinin alt ekstremite iskemi/reperfüzyon sıçan modelinde akciğer dokusu üzerindeki etkileri

Mustafa Orhan<sup>1</sup>, Ayça Taş Tuna<sup>1</sup>, Yusuf Ünal<sup>2</sup>, Mustafa Arslan<sup>2</sup>, Hayrullah Yazar<sup>3</sup>, Şaban Cem Sezen<sup>4</sup>, Sezen Irmak Gözükara<sup>5</sup>, Onur Palabıyık<sup>1</sup>

Institution where the research was done:  
Gazi University, Faculty of Medicine, Ankara, Turkey

### Author Affiliations:

<sup>1</sup>Department of Anesthesiology of Reanimation, Sakarya University, Faculty of Medicine, Sakarya, Turkey

<sup>2</sup>Department of Anesthesiology of Reanimation, Gazi University, Faculty of Medicine, Ankara, Turkey

<sup>3</sup>Department of Biochemistry, Sakarya University, Faculty of Medicine, Sakarya, Turkey

<sup>4</sup>Department of Histology and Embryology, Kırıkkale University, Faculty of Medicine, Kırıkkale, Turkey

<sup>5</sup>Biochemistry Clinic, Sakarya University, Training and Research Hospital, Sakarya, Turkey

### ABSTRACT

**Background:** This study aims to evaluate the effect of amantadine on lung tissue of after lower limb ischemia/reperfusion injury in rats.

**Methods:** A total of 24 Wistar rats were divided into four equal groups including six rats in each: sham group (Group S), amantadine group (Group A), ischemia/reperfusion group (Group I/R), and ischemia/reperfusion + amantadine group (Group I/R-A). All groups underwent a midline abdominal incision. In Groups I/R and I/R-A, the infrarenal abdominal aorta was clamped for 120 min and, then, reperused for 120 min after removal of the clamp. Amantadine hydrochloride 45 mg/kg was administered intraperitoneally to the rats of Groups A and Group I/R-A 15 min before surgery. At the end of reperfusion period (240 min), all rats were sacrificed, and their lung tissues were obtained. Lung tissue catalase and superoxide dismutase activities and glutathione S-transferase and malondialdehyde levels were analyzed. Lung tissues were examined histopathologically.

**Results:** Catalase activity was lower in Groups A, I/R, and I/R-A compared to Group S. Superoxide dismutase activity was higher in Group I/R than Group S. Superoxide dismutase activity in Groups I/R-A and A decreased, compared to Groups S and I/R. Glutathione S-transferase levels decreased in Groups I/R and A, compared to Group S. Glutathione S-transferase levels in Group I/R-A were higher than Groups I/R and A. The highest level of malondialdehyde was found in Group I/R and the lowest level was found in Group I/R-A. According to histopathological examination, infiltration scores were significantly lower in Group S than Groups I/R and I/R-A ( $p=0.009$  and  $p=0.011$ , respectively). The alveolar wall thickening scores in Group I/R were also significantly higher than Groups S and Group A ( $p=0.001$  and  $p=0.001$ , respectively).

**Conclusion:** Lung tissue can be affected histopathologically by ischemia/reperfusion injury and this injury can be reversed by amantadine administration.

**Keywords:** Amantadine, ischemia/reperfusion, lower limb, lung.

### ÖZ

**Amaç:** Bu çalışmada, sıçanlarda alt ekstremite iskemi/reperfüzyon hasarından sonra amantadinin akciğer dokusu üzerindeki etkisi araştırıldı.

**Çalışma planı:** Toplam 24 adet Wistar cinsi sıçan her grupta altı sıçan olacak şekilde dört eşit gruba ayrıldı: Sham grubu (Grup S), amantadin grubu (Grup A), iskemi/reperfüzyon grubu (Grup I/R) ve I/R+amantadin grubu (Grup I/R-A). Tüm gruplara orta abdominal insizyon uygulandı. Grup I/R ve I/R-A'da infrarenal abdominal aorta 120 dk. süreyle kleplendi ve ardından klemp kaldırılarak 120 dk. süreyle reperfüze edildi. Grup A ve I/R-A'daki sıçanlara 45 mg/kg amantadin hidroklorür cerrahiden 15 dk. önce intraperitoneal olarak uygulandı. Reperfüzyon periyodu sonunda (240 dk.) tüm sıçanlar kurban edildi ve akciğer dokuları alındı. Akciğer dokusunda katalaz ve süperoksit dismutaz aktiviteleri ve glutatyon S-transferaz ve malondialdehit düzeyleri çalışıldı. Akciğer dokuları histopatolojik olarak incelendi.

**Bulgular:** Katalaz aktivitesi Grup A, I/R ve I/R-A'da, Grup S'ye kıyasla daha düşüktü. Süperoksit dismutaz aktivitesi Grup I/R'de, Grup S'ye kıyasla daha yüksekti. Grup I/R-A ve A'da, Grup S ve I/R'ye kıyasla, süperoksit dismutaz aktivitesi azaldı. Glutatyon S-transferaz düzeyleri Grup I/R ve A'da, Grup S'ye kıyasla azaldı. Glutatyon S-transferaz düzeyleri, Grup I/R-A'da, Grup I/R ve A'ya kıyasla daha yüksekti. Malondialdehit düzeyleri, Grup I/R'de en yüksek ve Grup I/R-A'da en düşük izlendi. Histopatolojik incelemeye göre, Grup S'de infiltrasyon skoru Grup I/R ve Grup I/R-A'ya kıyasla, anlamlı düzeyde daha düşüktü (sırasıyla,  $p=0.009$  ve  $p=0.011$ ). Grup I/R'de alveol duvar kalınlaşması skoru, Grup S ve Grup A'ya kıyasla, anlamlı düzeyde yüksekti (sırasıyla,  $p=0.001$  ve  $p=0.001$ ).

**Sonuç:** Akciğer dokusu iskemi/reperfüzyon hasarından etkilenebilir ve bu hasar amantadin kullanımı ile geri döndürülebilir.

**Anahtar sözcükler:** Amantadin, iskemi/reperfüzyon, alt ekstremite, akciğer.

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**Correspondence:** Mustafa Arslan, MD, Gazi Üniversitesi Tıp Fakültesi Anesteziyoloji ve Reanimasyon Anabilim Dalı, 06560 Yenimahalle, Ankara, Türkiye.  
Tel: +90 312 - 202 53 13 e-mail: mustarslan@gmail.com

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Ischemia is the lack of oxygen caused by insufficient perfusion of organs and tissues due to decreased or complete ablation of arterial or venous blood flow. Ischemia leads to cell death resulting from the depletion of cellular energy stores and the accumulation of toxic metabolites. In the ischemic period, several metabolic and structural changes occur in the cell. Cellular oxidative phosphorylation and high-energy phosphate synthesis, such as adenosine triphosphate and phosphocreatine, are reduced by discontinuation of blood flow to the tissue.<sup>[1]</sup> Ischemic tissue requires blood circulation again for both self-renewal of cells and removal of accumulated toxic substances. However, reperfusion of the ischemic tissue causes more severe injury than tissue injury caused by ischemia, which is called ischemia/reperfusion (I/R) injury.<sup>[2]</sup> This injury is a pathological condition which continues with a sterile inflammatory response in which neutrophils and free oxygen radicals play a role with insufficient oxygen presentation.<sup>[3-6]</sup>

Lower limb I/R injury occurs, particularly in aortic surgery by temporarily clamping the aorta and by acute or double-sided acute femoral artery occlusion. After I/R, local injury occurs in the ischemic area and injury to distant organs outside the ischemic area may also occur. The lung is the target organ in distant organ injury.<sup>[7-9]</sup>

N-methyl D-aspartate (NMDA) receptor is a member of the glutamatergic receptor types.<sup>[10,11]</sup> The NMDA antagonists have been shown to have protective effects against I/R injury in various organs and tissues by increasing antioxidant activity and reducing oxidative effects.<sup>[12]</sup> Amantadine, is a NMDA receptor antagonist, has direct and indirect glutaminergic and dopaminergic signaling effects.<sup>[12-14]</sup> Therefore, in the current study, we aimed to investigate the effect of amantadine, an NMDA antagonist, on lung tissue following lower limb I/R injury in rats.

## MATERIALS AND METHODS

This experimental study was conducted at Gazi University Animal Experiments Laboratory on 20<sup>th</sup> June 2017. The study protocol was approved by the Animal Research Committee of Gazi University, Ankara, Turkey. All animals were maintained in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

A total of 24 adult male Wistar rats (10-12 week-old, weighing 250 to 330 g) were used in the study. The rats were housed in a 12-h luminous, 12-h dark environment, until the initiation of the study. The

subjects were examined in a light- and temperature-standardized environment. Animals did not undergo fluid and food restriction and were fed with standard rat food (pellet feed).

The rats were randomly divided into four equal groups: sham group (Group S, n=6), the amantadine group (Group A, n=6), the I/R group (Group I/R, n=6), and the I/R + amantadine group (Group I/R-A, n=6). In Groups A and I/R-A, amantadine 45 mg/kg (amantadine hydrochloride, A1260-5G, Sigma-Aldrich, St. Louis, USA) was administered intraperitoneally at the same time as anesthetic drugs. A total of 15 min after the anesthesia induction, surgical procedure was performed.

### Experimental procedure

The abdominal areas of the rats whose weights were measured before anesthesia were shaved before the surgical incision. For anesthesia induction, all rats were administered ketamine 100 mg/kg (Ketalar 1 mL: 50 mg, Pfizer, Istanbul, Turkey) and xylazine 15 mg/kg (Xylazine bio 2% Biovet, Czech Republic) intraperitoneally, and atropine 0.01 mg (Atropine Sulfate 0.5 mg/mL, Biofarma, Istanbul, Turkey) intramuscularly. A heater blanket was used to prevent heat loss and to avoid hypothermia and a rectal thermometer was used for heat monitoring. As the criterion for adequate anesthesia, unresponsiveness to painful stimuli was achieved and, then, tail vein cannulation was performed with a 24-gauge intravenous cannula (R-ES Neo IV, Izmir, Turkey) to ensure hydration. Anesthesia was maintained with an intermittent ketamine and xylazine injection.

In Groups S and A, a mid-abdominal incision was performed, but no I/R was applied. In Groups I/R and I/R-A, a mid-abdominal incision was performed. After removal of intestines from the surgical field, the infrarenal abdominal aorta was explored, and aorta was clamped by an atraumatic vascular clamp. The clamp was removed after 120 min, and reperfusion was performed for 120 min. Ischemia and reperfusion times of lower limb were performed according to the literature.<sup>[15]</sup> At the end of reperfusion period (240 min), all rats were euthanized for blood and organ sampling.

### Biochemical investigations of rat lung tissues

The lung tissues were collected and stored at -80°C. The lung samples were separated into small pieces by removing fat and connective tissues on ice. The samples were weighed and placed in glass tubes containing a cold phosphate buffer (pH 7.4, 50 mmol/L) with a final concentration of 100 mg tissue/mL. A homogenization process was performed

on ice homogenizer (ISOLAB, Laborgerate GmbH, Germany), and an ISOLAB homogenization device was used. The obtained homogenate was separated from the debris and other particles by centrifugation at 10,000 g, 4°C, for 10 min. All parameters were studied from the supernatants obtained after centrifugation.

The lung tissue catalase (CAT) and superoxide dismutase (SOD) enzyme activities, and glutathione-S-transferase (GST) levels were measured by enzyme-linked immunosorbent assay (ELISA; Elabscience Biotechnology Co. Ltd., Wuhan, China). The coefficient of measurement within the kit was <10%. Measurements were made on the automated ELISA analyzer (Triturus, Grifols, Spain), following the manufacturer's protocols. The results were multiplied by the dilution factor, and the results were calculated. Malondialdehyde (MDA) levels were determined to examine the lipid peroxidation status. The MDA levels were also measured and calculated by the same methods.

### Histopathological examination

Histopathological evaluation was performed at Kirikkale University, Medical Faculty, Histology and Embryology Department. For histopathological examination, lung tissue samples were kept at +4°C in 10% formaldehyde solution. Tissues were stained with hematoxylin-eosin (H-E) and examined under light microscopy and findings were scored using a scoring system.<sup>[16]</sup> Pulmonary injury was graded into four categories as follows: Grade 0, no diagnostic change;

Grade 1, mild neutrophil leukocyte infiltrations and mild to moderate interstitial congestion; Grade 2, moderate neutrophil leukocyte infiltrations, perivascular edema formation, and partial destruction of pulmonary architecture; and Grade 3, dense neutrophil leukocyte infiltration and complete destruction of the pulmonary architecture.

### Statistical analysis

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Data were expressed in median (min-max) values for the numerical variables. The Kruskal-Wallis test was used for intergroup data comparisons. The Bonferroni-corrected Mann-Whitney U test was used to examine which group differs from the other. A *p* value of <0.05 was considered statistically significant.

## RESULTS

The CAT, SOD activities and GST and MDA levels of four groups are shown in Table 1. The CAT activity decreased in Groups A, I/R, and I/R-A compared to Group S. The CAT activity in Group I/R-A was lower than Groups S, A, and I/R. However, the difference in the CAT activity was not statistically significant among the groups (*p*=0.064). The SOD activity of the lung tissue was higher in Group I/R than Group S. The SOD activity in Groups I/R-A and A decreased, compared to Groups S and I/R. However, the difference in the SOD activity was not statistically

**Table 1. Oxidative status of lung tissue**

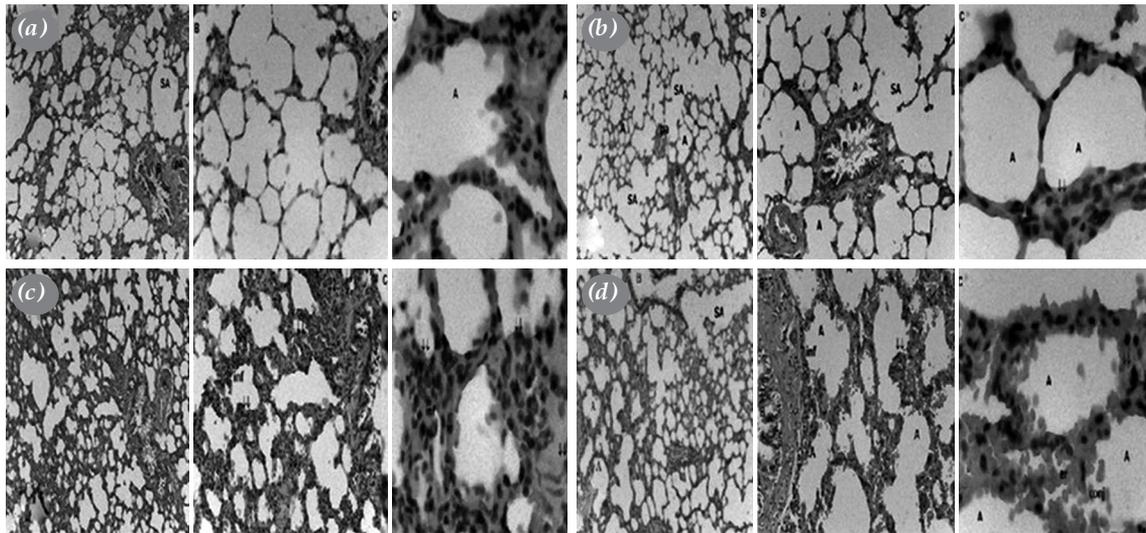
	Group S (n=6)		Group A (n=6)		Group I/R (n=6)		Group I/R-A (n=6)		<i>p</i>
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max	
CAT (pg/mg)	4.84	3.78-5.63	4.45	3.46-4.80	4.66	3.89-4.81	3.68	3.55-4.57	0.064
SOD (pg/mg)	3.06	2.10-3.40	2.68	2.12-2.90	3.22	2.35-4.61	2.18	1.70-2.92	0.052
GST (pg/mg)	25.05	21.00-27.41	22.56	20.47-24.00	22.64	19.66-26.54	25.66	23.90-28.00	0.090
MDA (ng/mg)	2.90	2.71-3.14	3.01	2.68-3.47	3.11	2.37-3.50	2.49	2.36-3.84	0.193

Min: Minimum; Max: Maximum; CAT: Catalase; SOD: Superoxide dismutase; GST: Glutathione-S-transferase; MDA: Malondialdehyde.

**Table 2. Alveolar wall thickening and infiltration scores**

	Group S (n=6)		Group A (n=6)		Group I/R (n=6)		Group I/R-A (n=6)		<i>p</i>
	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max	
Infiltration scores	0.00	0-1	0.50	0-1	1.00	1-3*	1.00	1-2*	0.010
Alveolar wall thickening	0.00	0-1†	0.00	0-1†	2.50	1-3	1.00	1-2	0.001

Min: Minimum; Max: Maximum; \* *p*<0.05; compared to Group S; † *p*<0.05; compared to Group I/R.



**Figure 1.** Pulmonary tissue preparations, hematoxylin-eosin ( $\times 40$ ;  $\times 100$ ;  $\times 400$ ): (a) Normal lung tissue parenchyma in Group S; (b) Neutrophil infiltration in lung tissue in Group A; (c) Intense neutrophil infiltration and severe increase in alveolar wall thickness in Group I/R; (d) Slight capillary congestion, neutrophil infiltration and slight increase in alveolar wall thickness in Group I/R-A.

significant among the groups ( $p=0.090$ ). The GST levels of the lung tissue decreased in Groups I/R and A, compared to Group S. The GST levels in Group I/R-A were higher than Groups I/R and A. However, the GST levels were not statistically significant among the groups ( $p=0.052$ ). In addition, there were no significant differences in the lung tissue MDA levels among the groups ( $p=0.193$ ). The highest and lowest level of MDA was found in Group I/R and Group I/R-A, respectively.

As shown in Table 2, lung tissue neutrophil/lymphocyte infiltration score levels were significantly lower in Group S than Groups I/R and I/R-A ( $p=0.009$  and  $p=0.011$ , respectively). The lung tissue alveolar wall thickening scores were significantly higher in Group I/R than Groups S and A ( $p=0.001$  and  $p=0.001$ , respectively). In Groups A and S, the results were similar ( $p>0.05$ ). Additionally, the lung tissue alveolar wall thickening scores in Group I/R-A was lower than Group I/R, although it did not reach statistical significance ( $p>0.05$ ).

The histopathological changes of lung tissue are shown in Figure 1. The H-E-stained lung sections of Group S showed a normal alveolar histological structure. No infiltration was shown, and alveolar wall thickness was normal (Figure 1a). However, there was neutrophil infiltration in the lung sections of Group A (Figure 1b). On the other hand, Group I/R showed some structural changes. Intense neutrophil infiltrations were shown, and alveolar wall thickness increased

severely. In addition, there were macrophage and erythrocyte accumulation and edema in some parts of bronchioles and alveolar lumens. Hyperemia, bleeding, and inflammatory cell infiltration in capillaries in the interalveolar septa were observed (Figure 1c). In Group I/R-A, hyperemia, hemorrhage, edema, inflammatory cell infiltration, and partial enlargement in the interalveolar septum were observed (Figure 1d). However, those findings were quite mild in terms of general appearance, compared to Group I/R.

## DISCUSSION

In this study, we evaluated that the histopathological changes in the lung tissue as a distant organ, after the lower limb I/R injury can be limited by amantadine before I/R injury.

The protective effects of NMDA antagonists on lung tissue have been shown in previous studies.<sup>[17,18]</sup> In a study investigating the effects of dexmedetomidine-ketamine combination on ventilator-associated lung injury in endotoxemic rats, the combination of dexmedetomidine-ketamine has been shown to have protective effects on ventilator-associated lung injury and reduce the degree of inflammation.<sup>[17]</sup> In the literature, however, there is no study available regarding the effects of amantadine on I/R injury. Nevertheless, it has been reported that amantadine decreases oxidative stress in abnormal glutamatergic synaptic transmission of corticosterone-induced

CA3-CA1 pathway in hippocampal sections in rats, possibly related to the mechanism of neuroprotective effect of amantadine, and it is formed by regulating antioxidant SOD and CAT activities.<sup>[19]</sup> Some authors suggested that amantadine exerts its effect by reducing Ca<sup>2+</sup> influx into the cell and prevents apoptosis.<sup>[19]</sup> Furthermore, recent studies have shown how amantadine has been used extensively in their clinical practice of patients with severe traumatic brain injury.<sup>[20]</sup> In another study in rats with traumatic brain injury, amantadine had a potential value in antidepressant treatment by using a dose of 45 or 135 mg/kg/day.<sup>[21]</sup> According to this study, we used 45 mg/kg dose of amantadine to evaluate whether it had a protective effect against I/R injury.

The antioxidant enzyme group, oxidoreductases, represents one of the most important free radical scavenging systems and plays a cell-protective role beyond the antioxidant function. The CAT is one of these antioxidant enzymes. In experimental I/R injury studies, the CAT activity in tissue and serum samples decreased after I/R, compared to control groups, and this reduction was caused by oxidant mechanisms.<sup>[19,22]</sup> In contrast, other studies showed that CAT activity in tissue and serum samples increased after I/R injury, compared to control groups, and this rising was due to the elimination of hydrogen peroxide caused by elevated SOD activity.<sup>[23,24]</sup> In our study, the CAT level was decreased after I/R compared to the control group and the CAT activity with amantadine was further decreased. However, we observed no statistically significant difference among the groups in terms of CAT enzyme activity.

Glutathione-S-transferase catalyzes the conjugation of various endogenous and exogenous compounds with glutathion. Glutathion is the most important antioxidant compound in the cell that protects cells from oxidant injury.<sup>[25]</sup> El-Sayyad *et al.*<sup>[26]</sup> reported that GST level decreased after I/R and increased in liver tissue, when mangiferin was administered in intestinal I/R injury in rats. Similar to this study, we observed that GST level of the lung tissue decreased after lower limb I/R injury and increased after amantadine administration. The increase in the GST level suggests that amantadine may have protective effects in I/R. However, GST levels among the groups were not statistically significant.

Antioxidant enzymes play an important role in the removal of free oxygen radicals. The SOD, one of these enzymes, exerts its antioxidant effect by catalyzing the conversion of superoxide to hydrogen peroxide. According to some experimental I/R

injury studies, the SOD activity in tissue and serum samples decreased and this reduction might be due to predominance of oxidant mechanisms.<sup>[27-29]</sup> In contrast, in other studies, the SOD activity increased in tissue and serum samples after I/R and this increase was due to oxidative stress suppression response.<sup>[15,24]</sup> In our study, we observed that the SOD activity of the lung tissue increased after lower limb I/R and decreased when amantadine was applied. We believe that the reduction in the SOD activity may be related to the amantadine administration.

Free oxygen radicals that formed in I/R injury initiate lipid peroxidation by attacking lipids in membranes due to their high activity. The radicals cause cellular injury by oxidizing membrane lipids, cellular proteins and deoxyribonucleic acid. The MDA is considered as an indicator of lipid peroxidation mediated by free oxygen radicals.<sup>[30]</sup> Previous studies showed that MDA levels increased after I/R injury and decreased by antioxidant agent application.<sup>[24,31-33]</sup> Similar to these studies, we observed that MDA levels of the lung tissue increased after I/R. In addition, we found that MDA levels decreased, when we administered amantadine before I/R. This decrease in MDA level suggests that amantadine may have protective effects in I/R.

In the underlying cause of lung injury following lower limb I/R injury, the development of neutrophil sequestration by the inflammatory mediators stimulated by ischemic tissue and the accumulation of activated neutrophils into the lung through the bloodstream and accumulating in the pulmonary bed play an important role. Degranulation of the sequestered neutrophils leads to injury to the lung tissue.<sup>[34-36]</sup> Injury of the lung is histopathologically manifested by alveolar wall thickening, interstitial edema, neutrophils, and infiltration of lymphocytes.<sup>[33]</sup> It has been reported that the histopathological changes in the lung tissue due to I/R injury of lower limb can be significantly reduced with various agents.<sup>[33,37,38]</sup> In our study, neutrophil/lymphocyte and alveolar wall thickening scores were significantly increased after I/R. Although not statistically significant, there was a reduction in the alveolar wall thickening due to I/R injury through amantadine administration.

The main limitation of our study is its relatively small sample size; however, the Animal Research Committee allowed up to six rats per group per protocol.

In conclusion, amantadine as an NMDA antagonist with its neuroprotective effects may have a protective effect against I/R injury, similar to other NMDA

antagonists. Based on our study results, the lung tissue can be affected histopathologically by I/R injury and this injury can be reversed by amantadine administration.

#### Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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