RESEARCH ARTICLE

Fatih Sahin[1]
Mustafa Orhan[2]
Ayca Tas Tuna[3]
Onur Palabıyık[3]
Nevcihan Sahutoglu Bal[4]
Sezen Irmak Gozukara[5]
Ozcan Budak[6]
Huseyin Cakiroglu[7]

[1] Sakarya University, Training and Research Hospital, Department of Anesthesiology, Sakarya, Türkiye
[2] Sakarya Private MEDAR Hospital, Department of Anesthesiology, Sakarya, Türkiye
[3] Sakarya University, Faculty of Medicine, Department of Anesthesiology, Sakarya, Türkiye
[4] Yildirim Beyazit University Yeni Mahalle Training and Research Hospital, Department of Anesthesiology, Ankara, Türkiye
[5] Sakarya University Training and Research Hospital, Department of Biochemistry, Sakarya, Türkiye
[6] Sakarya University, Faculty of Medicine, Department of Histology and Embryology, Sakarya, Türkiye
[7] Sakarya University, Faculty of Medicine Experimental Medicine Research and Application Center, Sakarya, Türkiye

Corresponding Author:
Ayca Tas Tuna
mail: aycatas@yahoo.com

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konuralptipdergi@duzce.edu.tr
konuralptipdergisi@gmail.com
www.konuralptipdergi.duzce.edu.tr

The Effect of Different Doses of Amantadine on Lung Tissue in Hepatıc Ischemia Reperfusion Injury in Rats

ABSTRACT

Objective: N-Methyl D-Aspartate (NMDA) receptor blockers have been shown to have protective effects against ischemia/reperfusion (I/R) injury in various tissues. The aim of this study was to investigate the effects of 90 ve 135 mg/kg doses of amantadine on lung in hepatic I/R injury.

Methods: The rats were randomly divided into six groups: Group Sham, Group I/R, Group Amantadine-90, Group Amantadine-135, Group I/R-90 and Group I/R-135. In I/R, an atraumatic vascular clamp was applied to the structures in the left portal triad for 45 minutes and reperfusion period was 2 hours after ischemia. Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) enzyme levels were performed the lung tissue and tissues were examined histopathologically.

Results: A significant difference was found between the groups in terms of MDA, SOD, CAT levels (respectively; $p < 0.001$, $p=0.008$, $p < 0.001$). A significant difference was found between the groups in terms of lung tissue neutrophil/lymphocyte infiltration scores and alveolar wall thickening scores (respectively $p=0.009$, $p=0.002$).

Conclusions: The biochemical and histopathological results of the present study suggested that amantadine, like other NMDA antagonist agents, may have a protective effect on lung tissues against the damage caused by hepatic I/R injury. Although we observed significant improvements after the administration of both doses studied, there was no significant difference between these two doses in terms of their success in protecting against distant organ lung injury. Amantadine appears promising as a therapeutic agent in treatment.

Keywords: Amantadine, Ischemia Reperfusion Injury, Lung Injury, Remote Organ Damage.

Sıçanlarda Hepatik İskemi Reperfüzyon Hasarında Farklı Dozlarda Amantadin'in Akciğer Dokusu Üzerine Etkisi

ÖZET

Amaç: N-Metil D-Aspartat (NMDA) reseptör blokerlerinin çeşitli dokularda iskemi/reperfüzyon (I/R) hasarına karşı koruyucu etkileri olduğu gösterilmiştir. Bu çalışmanın amacı, hepatik I/R hasarında 90 ve 135 mg/kg dozlarındaki amantadinin akciğer üzerindeki etkilerini araştırmaktır.

Gereç ve Yöntem: Sıçanlar her rastgele altı gruba ayrıldı: Grup Sham, Grup I/R, Grup Amantadin-90, Grup Amantadin-135, Grup I/R-90 ve Grup I/R-135. I/R'de sol portal triaddaki yapılara 45 dakika süre ile atravmatik vasküler klemp uygulandı ve iskemi sonrası 2 saat reperfüzyon uygulandı. Akciğer dokusunda malondialdehit (MDA), süperoksit dismutaz (SOD) ve katalaz (CAT) enzim düzeyleri ölçüldü ve dokular histopatolojik olarak incelendi.

Bulgular: MDA, SOD, CAT düzeyleri açısından gruplar arasında anlamlı fark bulundu (sırasıyla; $p < 0,001$, $p=0,008$, $p < 0,001$). Akciğer dokusu nötrofil/lenfosit infiltrasyon skorları ve alveolar duvar kalınlaşma skorları açısından gruplar arasında anlamlı fark bulundu (sırasıyla $p=0,009$, $p=0,002$).

Sonuç: Bu çalışmanın biyokimyasal ve histopatolojik sonuçları, hem 90 hem de 135 mg/kg dozdaki amantadinin, diğer NMDA antagonist ajanları gibi, karaciğer I/R hasarının neden olduğu hasara karşı akciğer dokuları üzerinde koruyucu bir etkiye sahip olabileceğini göstermiştir. Çalışılan her iki dozun uygulanmasından sonra önemli gelişmeler gözlememize rağmen, uzak organ akciğer hasarına karşı korumadaki başarıları açısından bu iki doz arasında anlamlı bir fark yoktu. Amantadin, tedavide terapötik bir ajan olarak umut verici görünmektedir.

Anahtar Kelimeler: Amantadin, İskemi Reperfüzyon Hasarı, Akciğer Hasarı, Uzak Organ Hasarı.

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) injury is a major complication that can occur during liver surgeries, including operations such as liver resection, liver transplantation, and trauma surgery (1). Cellular disorder and organ dysfunction occur alongside the release of various mediators during tissue damage induced by blood deprivation (ischemia) and subsequent blood flow (reperfusion) (2). Further, I/R injury is one of the main underlying causes of post-transplant graft dysfunction (3).

Prolonged liver I/R causes hepatic cell damage and distant organ injury with high morbidity and mortality through the induction of reactive oxygen species (ROS) and pro-inflammatory cytokines (4,5). Damage to the lungs, kidneys, and heart from distant organs has also been shown to occur during liver I/R injury (6-8). Lung injury is an especially important cause of mortality in critically ill patients (8).

Amantadine is an N-methyl-D-aspartate (NMDA)-type glutamate receptor antagonist drug that was approved for use in the United States in 1968 and has been used in the treatment of both influenza and Parkinson's disease. While amantadine accomplishes its antiviral effect by preventing the release of viral RNA, it achieves its effects on the brain by increasing dopamine release, blocking dopamine reuptake, activating microglia, and inhibiting neuroinflammation. Amantadine has been reported to be effective in treating both acute and chronic phases of traumatic brain injury, and its use in different doses has been shown to have a neurorestorative effect on cerebral cortical ischemia (9).

Pharmacological agents that disrupt the reperfusion injury cascade constitute many of the preventative strategies currently under investigation to mitigate I/R injury (10). For example, NMDA receptor antagonist drugs (e.g., ketamine, barbiturates, volatile anesthetics, and morphine) have been previously shown to have protective effects against I/R damage in different tissues (e.g., kidney tissue, myocardium, and skeletal muscle) (11-13). However, no study in the literature has investigated the effects of amantadine on hepatic I/R injury. In our previous study of lower limb I/R, we administered 45 mg/kg amantadine and observed that it could reduce distant lung damage (14). In the present study, our aim was to investigate the protective effects of higher doses of amantadine (90 and 135 mg/kg) on lung tissue, which is a distant organ in hepatic I/R damage.

MATERIAL AND METHODS

This study, which was approved by the Sakarya University Animal Experiments Local Ethics Committee, was carried out at the Sakarya University Experimental Medicine Application and Research Center in January 2019.

Thirty-six adult male Wistar rats weighing 250–330 g were used in this study. These rats were adapted to the environment by being housed in a 12-hr-light:12-hr-dark environment until the beginning of the study. The subjects were examined in an environment of standardized light and temperature. Neither fluid nor feeding restrictions were applied to the animals, which received standard rat food (pellet feed). The weights of the rats were measured before anesthesia was administered, and their abdominal areas were shaved before the surgical incisions were made. All the rats were intraperitoneally (i.p.) to 100 mg/kg ketamine (Ketalar 1 ml: 50 mg, Pfizer, Istanbul, Turkey) and intramuscularly anesthetized injected with 15 mg/kg xylazine (Xylazine Bio 2%, Biovet, Czech Republic), intramuscularly administered 0.01 mg of atropine (Atropine Sulfate 0.5 mg/ml ampoule, Biofarma, Istanbul, Turkey). A heating blanket was used to prevent heat loss and hypothermia, and a rectal thermometer was used for temperature monitoring. The tail vein was cannulated with a 24-G IV cannula for drug administration and hydration.

Amantadine hydrochloride (Sigma A1260-5G) was purchased from Sigma-Aldrich (St. Louis, USA) and dissolved in sterile saline.

The rats were randomly divided into six groups: Group Sham (Group S, n = 6), Group I/R (n = 6), Group Amantadine-90 (Group A-90, n = 6), Group Amantadine-135 (Group A-135, n = 6), Group I/R-90 (n = 6), and Group I/R-135 (n = 6). After the anesthesia was applied, Groups S and I/R waited for 15 minutes without any action. In Groups A-90 and I/R-A-90, 90 mg/kg amantadine hydrochloride was administered with the anesthesia. Groups A 135 and I/R-A-135 were administered 135 mg/kg i.p amantadine. Then, Groups A-90, I/R-A-90, A-135, and I/R-A-135 waited for 15 minutes. In Groups S, A-90, and A-135, a middle abdominal incision was made on each rat but no intervention was applied to the liver. In Groups I/R, I/R-A-90, and I/R-A-135, a mid-abdominal incision was made on each rat to reveal the liver, the ligament attachments of the left lateral and median lobes were carefully separated, and the lobes were freed. The portal circulation of these lobes was separated, and an atraumatic vascular clamp was applied to the portal vein and the hepatic artery feeding the median and left lateral lobes (Figure 1). This procedure induced ischemia in approximately 65–70% of the liver. After 45 minutes of ischemia, the clamp was removed. Reperfusion was applied to the rats in Groups I/R, I/R-A-90, and I/R-A-135 for two hours.

All the rats were sacrificed after 180 minutes, and their lung tissues were obtained. The lung samples were kept at -80 °C for tissue homogenization and cut into small pieces on ice. These samples were weighed and placed in glass tubes, which were then filled with a cold phosphate buffer (pH 7.4, 50 mmol/L) to a final concentration of 100 mg tissue/mL. The homogenization process was carried out in a mechanical homogenizer

(Isolab, Laborgerate GmbH, Germany) on ice with an Isolab homogenization device. The resulting homogenate was centrifuged at 10,000 g and 4 °C for ten minutes and separated from debris and other extraneous particles.

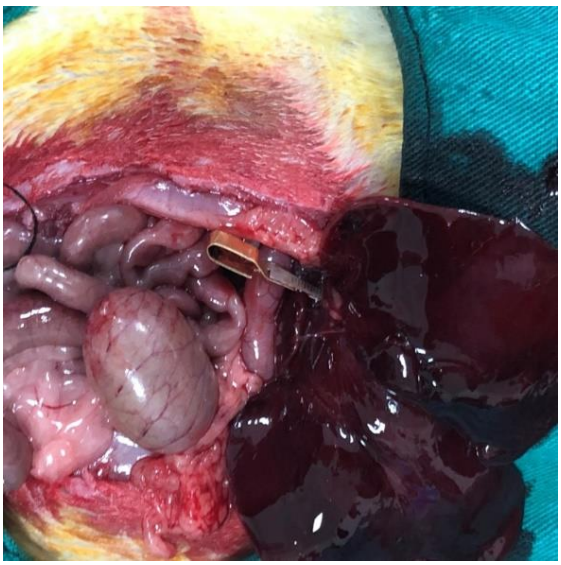

Figure 1. Liver dissection and clamping

All parameters were studied using the supernatants obtained after centrifugation. Catalase (CAT) and superoxide dismutase (SOD) levels were measured through an ELISA (Elabscience Biotechnology Co. Ltd., Wuhan, China). The within-measurement coefficient of variation (CV) of the kit was <10%, and the measurements were made in an automatic ELISA analyzer (Triturus, Grifols, Spain) in accordance with the manufacturer's protocols. The results were calculated by multiplying the obtained results by the dilution factor. Malondialdehyde (MDA) levels were measured and calculated using the same methods in order to determine lipid peroxidation status.

The lung tissue samples were stored in a 10% formaldehyde solution at +4 °C. Then, the tissues were stained with hematoxylin-eosin and evaluated with light microscopy in the laboratory of the Department of Histology and Embryology of the Sakarya University Faculty of Medicine. Ischemia was graded as Grade 0 (no sign of damage), Grade 1 (mild damage), Grade 2 (severe damage), or Grade 3 (severe damage) on the basis of neutrophil/lymphocyte infiltration and alveolar wall thickening scores.

Statistical Analysis: The data were transferred to the IBM SPSS Statistics 23 program for analysis. The study data for the numerical variables were represented as means ± standard deviations (SDs). A Kruskal Wallis test was used to check for differences between more than two groups, and differences between the groups were evaluated with a Mann-Whitney U test. A significance level of $p < 0.05$ was considered significant.

RESULTS

Lung tissue MDA, SOD, and CAT enzyme levels are shown in Table 1. A significant difference was found between the groups in terms of MDA levels ($p < 0.001$). A pairwise comparison of the groups revealed that the MDA levels of Groups A-90, A-135, I/R-A-90, and I/R-A-135 were significantly lower than those of Group I/R ($p = 0.002$, 0.002, 0.002, and 0.002, respectively). However, there was no significant difference between the MDA levels of Group I/R-A-90 and those of Group I/R-A-135 ($p = 0.485$).

Table 1. Oxidant status parameters of rat lung tissue

	Group S (n=6)	Group I/R (n=6)	Group A-90 (n=6)	Group I/R-A-90 (n=6)	Group A-135 (n=6)	Group I/R-A-135 (n=6)	p
MDA (nmol/100mg)	3.14±0.70	2.95±0.18	0.87±0.17*&	0.71±0.12*&	0.67±0.26*&	0.81±0.23*&	**<0.001**
SOD (ng/100mg)	3.75±1.02	3.13±1.33	1.72±0.37*&	1.62±0.27*&	1.69±0.85*&	1.95±0.97*&	**0.008**
CAT (ng/100mg)	4.54±0.84	4.37±1.24	22,81±2,84*&	22.25±1.83*&	22.24±6.03*&	22.02±5.98*&	**<0.001**

*Data are expressed as mean ± SD; *: p<0.05 versus Group I / R; &: p<0.05 versus Group S*

The groups also differed significantly in terms of lung tissue SOD levels ($p = 0.008$). A pairwise comparison of the groups revealed that the SOD levels of Groups A-90 and I/R-A-90 were significantly lower than those of Group I/R ($p = 0.041$ and 0.041, respectively). However, no significant difference was found between the SOD levels of Group I/R-A-90 and those of Group I/R-A-135 ($p = 1.0$).

A significant difference between the groups was also found in terms of CAT levels ($p < 0.001$). A pairwise comparison of the groups revealed that the CAT levels of Groups A-90, A-135, I/R A-90, and I/R-A-135 were significantly higher than those of Group I/R ($p = 0.002$, 0.002 0.002, and 0.002, respectively). No significant difference was found between the CAT levels of Group I/R-A-90 and those of Group I/R-A-135 ($p = 1.0$).

The neutrophil/lymphocyte infiltration and alveolar wall thickening scores determined through the histopathological examination of the lung tissues are shown in Table 2.

A significant difference was found between the groups in terms of lung tissue neutrophil/lymphocyte infiltration scores ($p = 0.009$). Specifically, neutrophil/lymphocyte infiltration was lowest in Group S and highest in Group I/R.

Table 2. Histopathological findings of rat lung tissue

	Group S (n=6)	Group I/R (n=6)	Group A-90 (n=6)	Group I/R-A-90 (n=6)	Group A-135 (n=6)	Group I/R-A-135 (n=6)	p
Neutrophil/lymphocyte infiltration	0.33±0.52	1.83±0.75[&]	1.50±0.55[&]	1.67±0.52[&]	1.50±0.55[&]	1.17±0.41[&]	**0.009**
Alveolar wall thickening	0.33±0.52	2.17±0.75[&]	1.50±0.55[&]	1.83±0.41[&]	1.17±0.41[&]	1.33±0.52[&]	**0.002**

Data are expressed as mean ± SD; &: p<0.05 versus Group S

Further, damage was decreased in Groups I/R-A-90 and I/R A-135 (Figure 2). Similarly, a statistically significant difference was found between the groups in terms of alveolar wall thickening scores (p = 0.002). Specifically, alveolar wall thickening scores were lowest in Group S and highest in Group I/R. Further, both alveolar wall thickening and neutrophil/lymphocyte infiltration scores were decreased in Groups I/RA-90 and I/R-A-135 (Figure 2). When the samples were examined with hemotoxylin eosin staining, capillary congestion, diffuse neutrophil infiltration, inflammation, and alveolar wall thickening were observed after I/R, whereas inflammation, alveolar wall thickness, and neutrophil infiltration were decreased after amantadine administration.

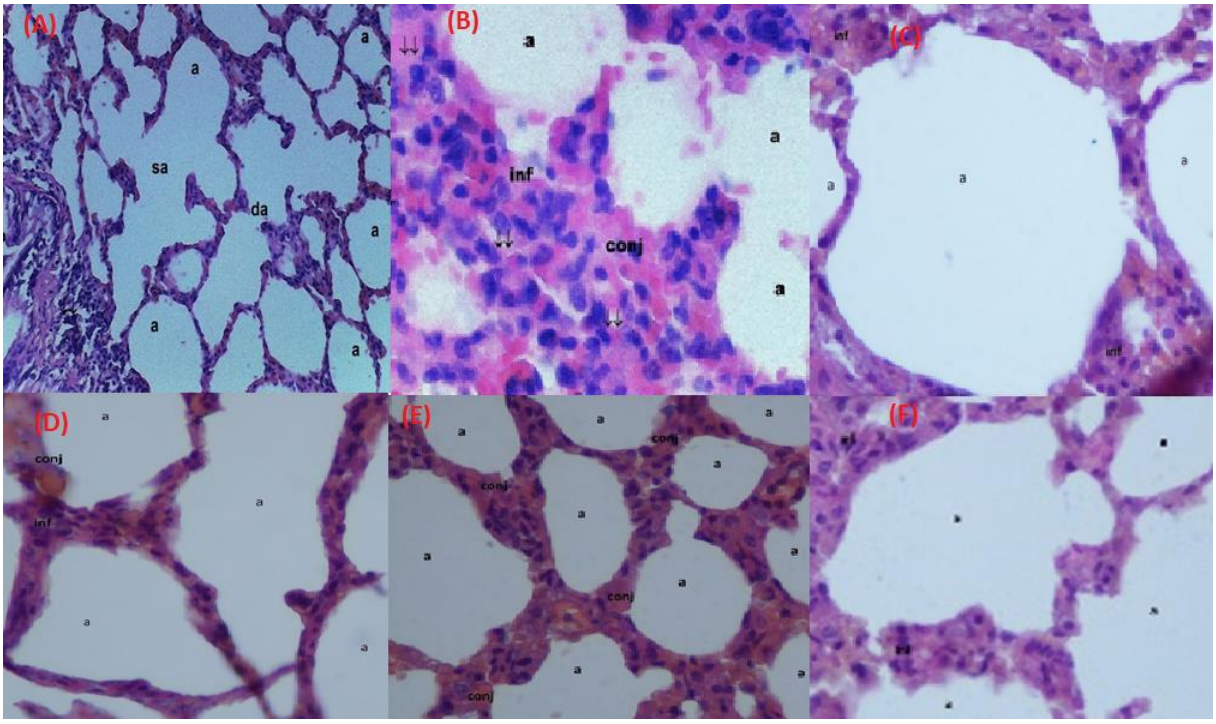

Figure 2. Lung tissue preparations, hematoxylin-eozin, X400: (A) Normal lung tissue parenchyma in Group S; (B). Capillary congestion, diffuse neutrophil infiltration, inflammation, alveolar wall thickening in Group I/R; (C) Inflammation and alveolar wall thickening in Group A-90; (D) Capillary congestion, neutrophil infiltration and alveolar wall thickening in Group-A-135; (E) Reduction on capillary congestion, neutrophil infiltration and alveolar wall thickening in Group I/R-A-90; (F) Reduction on inflammation and alveolar wall thickening in Group I/R-A-135. [s, saccus alveolaris; a, alveoli; d, ductus alveolaris; [↓↓, alveolar septum thickening; inf, inflamation; cong, conjugation]

DISCUSSION

The present study investigated the effects of 90 mg/kg and 135 mg/kg amantadine on lung tissues in a rat hepatic I/R model. The results showed that after I/R, a decrease in histopathological changes and changes in MDA, SOD, and CAT levels in lung tissues could limit lung damage.

Hepatic I/R injury is a complex process involving intrasignaling pathways, media, cells, and pathophysiological messages (15). The damage that develops as a result of this process is not limited to the liver, but causes a systemic response in the whole organism, especially lung and kidney (6-8). Previous studies on I/R injury have reported that NMDA receptor antagonists have protective effects against I/R damage in various organs and tissues (5,16). For example, Tufek et al. reported that administration of NMDA receptor antagonist dexmedetomidine before hepatic I/R injury caused decreases in total oxidant capacity and oxidative stress index as well as increases in total antioxidant capacity and PON-1 activity in the serum, liver, and distant organs (5). In another study, systemic injection of MK-801, an NMDA receptor antagonist, inhibited the activation of tumor necrosis factor-α (TNF-α), attenuated cell

infiltration and demyelination, and induced nitric oxide synthase and nitric oxide activity in a sciatic nerve I/R model. Further, it has been shown to reduce and thus protect against I/R damage (17). Another study reported that in intestinal I/R injury, serum aspartate aminotransferase, lactat dehydrogenase, TNF-α, MDA, and P-selectin levels increased while angiotensin III levels and total antioxidant capacity decreased and severe damage was observed in the intestinal mucosa; however, these changes were significantly improved with ketamine administration (16).

The compound MDA is the end product of lipid peroxidation. An increase in MDA levels is an indicator of free radical formation in postischemic tissue (18), as an increase in free radicals causes MDA to be overproduced. Increased MDA has been found to be associated with hepatic I/R damage and is widely used to determine I/R damage (19,20). Sahin et al. showed that the MDA levels of liver tissues increased after hepatic I/R and decreased with dexmetomidine administration (19). In another study, MDA levels increased after hepatic I/R but decreased with melatonin administration (20). Similar to other studies in the literature, the present study showed that the MDA levels of lung tissues increased after I/R injury and decreased with amantadine administration. However, we did not find any significant differences between 90 mg/kg and 135 mg/kg amantadine administration.

The enzymes SOD and CAT are responsible for cellular antioxidant defense mechanisms. These enzymes remove superoxide anions and hydrogen peroxide (H2O2) and prevent free radical production. The SOD enzyme is the primary defense mechanism against free oxygen radicals and catalyzes the conversion of H2O2- to O2- (21). There are two common opinions in the literature regarding SOD. On one hand, the SOD activity levels of tissue and serum samples have been shown to decrease after I/R damage, in comparison with control groups, due to the dominance of oxidant mechanisms and increased with antioxidant administration (19). In another opinion, it is thought that SOD activity increases with I/R damage to control oxidative stress. One study supporting this latter view showed that SOD levels increased after I/R but decreased with dexmetomidine administration (22). Another study showed that the SOD levels of lung tissues increased with I/R (23). In line with this view, we observed in the present study that SOD levels increased after I/R injury but decreased with the application of amantadine. While SOD levels decreased with the application of both doses (90 mg/kg and 135 mg/kg) of amantadine, we observed a more significant decrease in the group that was administered 90 mg/kg amantadine.

Oxidoreductases, another group of antioxidant enzymes, are among the most important free radical scavenging systems and play a cell protective role. The CAT enzyme is one of these antioxidant enzymes. The CAT enzyme catalyzes H_2O_2 destruction, and high blood levels of CAT indicate antioxidant activity. Kucuk et al. showed that CAT levels decreased after hepatic I/R and increased with the application of dexmetomidine, an NMDA receptor antagonist (22). In another study, CAT levels decreased after hepatic I/R and increased with the administration of different doses of dexmetomidine [19]. As in these other studies, the present study showed that CAT levels decreased after I/R and increased significantly with the application of amantadine in both examined doses (90 mg/kg and 135 mg/kg). However, no differences were observed between these doses.

The lungs are among the organs most affected by distant organ damage during I/R (24-25). Perivascular and peribronchial edema, increases in alveolar wall thickness, and leukocyte infiltration have been observed in lung tissues after hepatic I/R (26). Similarly, another study reported that necrosis, inflammatory cells, bleeding, and microsteatosis significantly increased in the lungs after hepatic I/R injury (27). In the present study, increases in alveolar wall thickness and neutrophil/lymphocyte infiltration rates were observed after I/R, while these symptoms significantly decreased with amantadine treatment. Although the patients who received 135 mg/kg amantadine experienced a greater decrease in these symptoms than those who received 90 mg/kg amantadine, this difference was not significant.

CONCLUSION

The biochemical and histopathological results of the present study suggested that amantadine, like other NMDA antagonist agents, may have a protective effect on lung tissues against the damage caused by hepatic I/R injury. Although we observed significant improvements after the administration of both doses studied, there was no significant difference between these two doses in terms of their success in protecting against distant organ lung injury. Amantadine appears promising as a therapeutic agent in treatment.

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