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Original Article

The effects of different doses of amantadine on lung tissue in lower extremity ischemia reperfusion injury in rats

Sıçanlarda alt ekstremite iskemi reper füzyon hasarında farklı amantadin dozlarının akciğer dokusu üzerine etkileri

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Abstract

Aim: Ischemia/reperfusion (I/R) injury is a common complication after abdominal aortic surgery. N-Methyl D-Aspartate (NMDA) antagonists protect many organs against to I/R injury. Therefore, in this study, we aimed to investigate the effects of 135 mg/kg and 90 mg/kg of amantadine, on lung tissue after lower extremity I/R injury in rats.

Material and Methods: Thirty six wistar rats were randomly divided into 6 groups each containing six rats as follows; Sham group (Group S), Amantadine 90 group (Group A-90), Amantadine 135 group (Group A-135), Ischemia/Reperfusion group (Group I/R), Ischemia/Reperfusion + Amantadine 90 group (Group I/R-A 90), Ischemia/Reperfusion + Amantadine 135 group (Group I/R-A 135). At the end of procedure, all rats were sacrificed, and their lung tissues were obtained. Lung tissues were examined biochemical and histopathologically.

Results: The lung tissue catalase, superoxide dismutase activities and malondialdehyde levels were similar between the groups. Lung tissue neutrophil/lymphocyte infiltration score levels were higher in Group I/R than Group S, Group A-90 and Group A-135. Alveolar wall thickening score levels were higher in Group I/R than Group S, Group A-90, Group A-135 and Group I/R-A 135.

Conclusion: Although we could not find a statistically significant difference between lung tissue biochemical values, we observed that lung tissue was histopathologically affected by I/R damage and the damage was less with amantadine use. In the reduction of I/R damage, 135 mg/kg administration of amantadine was more beneficial than 90 mg/kg.

Keywords: amantadine; ischemia reperfusion injury; lower extremity, lung tissue

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Öz

Amaç: İskemi/reperfüzyon (İ/R) hasarı abdominal aort cerrahisi sonrası sık görülen bir komplikasyondur. N-Metil D-Aspartat (NMDA) antagonistleri birçok organı İ/R hasarına karşı korur. Bu nedenle bu çalışmada, sıçanlarda alt ekstremite İ/R hasarı sonrası 135 mg/kg ve 90 mg/kg amantadinin akciğer dokusu üzerindeki etkilerini araştırmayı amaçladık.

Gereç ve Yöntemler: Otuz altı wistar sıçan rastgele her biri altı sıçan içeren 6 gruba ayrıldı; Sham grubu (Grup S), Amantadin 90 grubu (Grup A-90), Amantadin 135 grubu (Grup A-135), İskemi/Reperfüzyon grubu (Grup İ/R), İskemi/Reperfüzyon + Amantadin 90 grubu (Grup İ/R-A 90), İskemi/Reperfüzyon + Amantadin 135 grubu (Grup İ/R-A 135). İşlem sonunda tüm ratlar sakrifiye edilerek akciğer dokuları alındı. Akciğer dokuları biyokimyasal ve histopatolojik olarak incelendi.

Bulgular: Gruplar arasında akciğer dokusu katalaz, süperoksit dismutaz aktiviteleri ve malondialdehit seviyeleri benzerdi. Akciğer dokusu nötrofil/lenfosit infiltrasyon skoru Grup İ/R'de Grup S, Grup A-90 ve Grup A-135'e göre daha yüksekti. Alveolar duvar kalınlaşma skoru Grup İ/R'de Grup S, Grup A-90, Grup A-135 ve Grup İ/R-A 135'e göre daha yüksekti.

Sonuçlar: Akciğer dokusu biyokimyasal değerleri arasında istatistiksel olarak anlamlı bir fark bulamasak da akciğer dokusunun histopatolojik olarak İ/R hasarından etkilendiğini ve amantadin kullanımı ile hasarın daha az olduğunu gözlemledik. İ/R hasarının azaltılmasında 135 mg/kg amantadin uygulaması 90 mg/kg'dan daha faydalı olmuştur.

Anahtar Kelimeler: amantadin; iskemi reperfüzyon hasarı; alt ekstremite, akciğer dokusu

Introduction

Lower extremity ischemia/reperfusion (I/R) injury occurs during temporary cross-clamping of the abdominal aorta in aortic surgery and in unilateral or bilateral acute femoral artery occlusions [1]. In distant organ damage after I/R, the lungs are the target organ, and the determination of the role of free radicals and antioxidant enzymes in the pathogenesis of the damage has brought forward the antioxidant treatment trials [2].

N-Methyl D-Aspartate receptor antagonists (NMDA) have been shown to have protective effects against I/R damage in various tissues (brain, kidney, myocardium, skeletal muscle) [3]. In our previous study, which is the first research on the effects of a NMDA receptor antagonist amantadine on lung tissue in lower extremity I/R injury, we observed that amantadine administered at a dose of 45 mg/kg was protective [4]. In this study, we aimed to investigate the effect of amantadine 135 mg/kg and 90 mg/kg doses on lung tissue after lower extremity I/R injury in rats in order to investigate whether the protective effect is dose-dependent.

Material and Methods

Animals

This experimental study was conducted at Sakarya University Animal Experiments Laboratory in 2019. The study protocol was approved by the Animal Research Committee of Sakarya University, Sakarya, 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 (Ethical committee date and number: 06.02.2019 / 05).

A total of 36 adult male Wistar Albino rats (weighing 250 to 330 g) were used. The rats were kept at 20-21 oC in cycles of 12 hours daylight and 12 hours dark environment and had free access to food until two hours before the initiation of the study.

Experimental design

The rats were randomly divided into six equal groups each containing six rats: Sham group (Group S, n=6), the amantadine-90 group (Group A-90, n=6), the amantadine-135 group (Group A-135, n=6), the ischemia/reperfusion group (Group I/R, n=6), and the ischemia/reperfusion-amantadine-90 group (Group I/R-A-90, n=6), ischemia/reperfusion amantadine-135 group (Group I/R-A-135, n=6).

Anesthesia was provided by intraperitoneally (i.p.) administration of 100 mg/kg ketamine (Ketalar® 1 ml:50 mg, Pfizer, Istanbul, Turkey) and 15 mg/kg xylazine (Xylazinbio® 2%, Bioveta, Czech Republic). Surgical site antisepsis was provided with 10% povidone iodine solution. A heating blanket was used to prevent heat loss and hypothermia and a rectal thermometer was used for temperature monitoring. After providing unresponsiveness to painful stimuli as an adequate anesthesia criterion, cannulation was performed from the tail vein with a 24 Gauge (G) intravenous cannula (IV FlonAr-Es, Izmir, Turkey) to provide hydration. During the maintenance of anesthesia, i.p. ketamine injection was applied to the rats at regular intervals.

Sham Group (Group S): Only midline laparotomy was performed without any additional surgical intervention.

Amantadine-90 group (Group A-90): Fifteen minutes after the administration of 90 mg/kg amantadine i.p., midline laparotomy was performed without any additional surgical intervention.

Amantadine-135 group (Group A-135): Fifteen minutes after the administration of 135 mg/kg amantadine i.p., midline laparotomy was performed without any additional surgical intervention.

Ischemia/Reperfusion group (Group I/R): Midline laparotomy was performed. Infrarenal aorta was clamped for 120 minutes. After removing the clamp, reperfusion was established for 120 minutes.

Ischemia/Reperfusion-Amantadine-90 group (Group I/R-A-90): Fifteen minutes after the administration of 90 mg/kg amantadine i.p., midline laparotomy was performed. Infrarenal aorta was clamped for 120 minutes. After removing the clamp, reperfusion was established for 120 minutes.

Ischemia/Reperfusion Amantadine-135 group (Group I/R-A-135): Fifteen minutes after the administration of 135 mg/kg amantadine i.p., midline laparotomy was performed. Infrarenal aorta was clamped for 120 minutes. After removing the clamp, reperfusion was established for 120 minutes.

Ischemia and reperfusion times of lower limb were performed according to the literature [5]. At the end of the reperfusion period, all rats were sacrificed by exsanguination from the abdominal aorta. Serum samples were drawn for biochemical assays, and the lung tissues were removed for histopathological evaluation.

Biochemical Evaluation

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 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 evaluation

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 [6]. Lung 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

SPSS 23.0 (IBM, Armonk, New York, USA) packet program was used for statistical analysis. Results were presented as mean + Sd where appropriate. 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. P value less than 0.05 was considered statistically significant.

Results

The lung tissue CAT, SOD activities and MDA levels were found to be similar between the groups (p=0.063, 0.400 and 0.209; respectively). Lung tissue CAT and SOD activities was higher in Group I/R, Group I/R-A-90 and I/R-A-135 than Group S. MDA levels was the highest in Group I/R. MDA levels in Group I/R-A-90 and group I/R-A-135 were lower than that of Group I/R and Group S (p>0.05) (Table 1).

Lung tissue alveolar wall thickening scores and neutrophil/lymphocyte infiltration scores were statistically significantly higher in Group I/R than Group S, Group A-90 and Group A-135 (p=0.002, 0.002, 0.002, respectively), and were lower in Group I/R-A 90 and Group I/R-A-135 than that of Group I/R (p>0.05) (Table 2).

Table 1. Oxidative status parametres of lung tissue

	Group S (n=6)	Group A-90 (n=6)	Group A-135 (n=6)	Group I/R (n=6)	Group I/R-A 90 (n=6)	Group I/R-A 135 (n=6)	P value
SOD (pg/mg)	1.8±0.37 (1.4-2.3)	1.24±0.9 (0.3-2.7)	2.14±0.98 (1.4-4)	1.91±0.97 (0.7-2.9)	2.25±0.72 (1.5-3.5)	1.97±0.61 (1.4-3)	0.400
CAT (pg/mg)	18.85±4.63 (14.6-25.4)	16.06±8.78 (1.3-27.5)	23.84±3.44 (21.6-30.3)	19.2±5.6 (10.7-25.4)	25.05±4.99 (18.3-32.8)	24.23±3.73 (17.3-18.3)	0.063
MDA (ng/mg)	0.77±0.11 (0.6-0.9)	0.62±0.13 (0.5-0.8)	0.66±0.27 (0.2-0.9)	0.86±0.19 (0.6-1.1)	0.76±0.25 (0.3-0.9)	0.7±0.17 (0.5-0.9)	0.209

CAT,Catalase; SOD, Superoxide dismutase; MDA,Malondialdehyde
Data areexpressed as mean ± SD (min-max)
Kruskal Wallis Test.

Table 2. Alveolar wall thickening and Neutrophil/Lymphocyte infiltration scores

	GroupSham (n=6)	Group A-90 (n=6)	Group A-135 (n=6)	Group I/R (n=6)	Group I/R-A 90 (n=6)	Group I/R-A 135 (n=6)	P value
Alveolar wall thickening	0.17±0.41* (0-1)	0.33±0.52* (0-1)	0.33±0.52* (0-1)	2.67±0.52 (2-3)	1.33±0.52 (1-2)	0.67±0.52 (0-1)	<0,001
Neutrophil/ Lymphocyte infiltration scores	0.17±0.41* (0-1)	0.33±0.52* (0-1)	0.50±0.55* (0-1)	2.33±0.52 (2-3)	1.50±0.55 (1-2)	1.17±0.41 (1-2)	<0,001

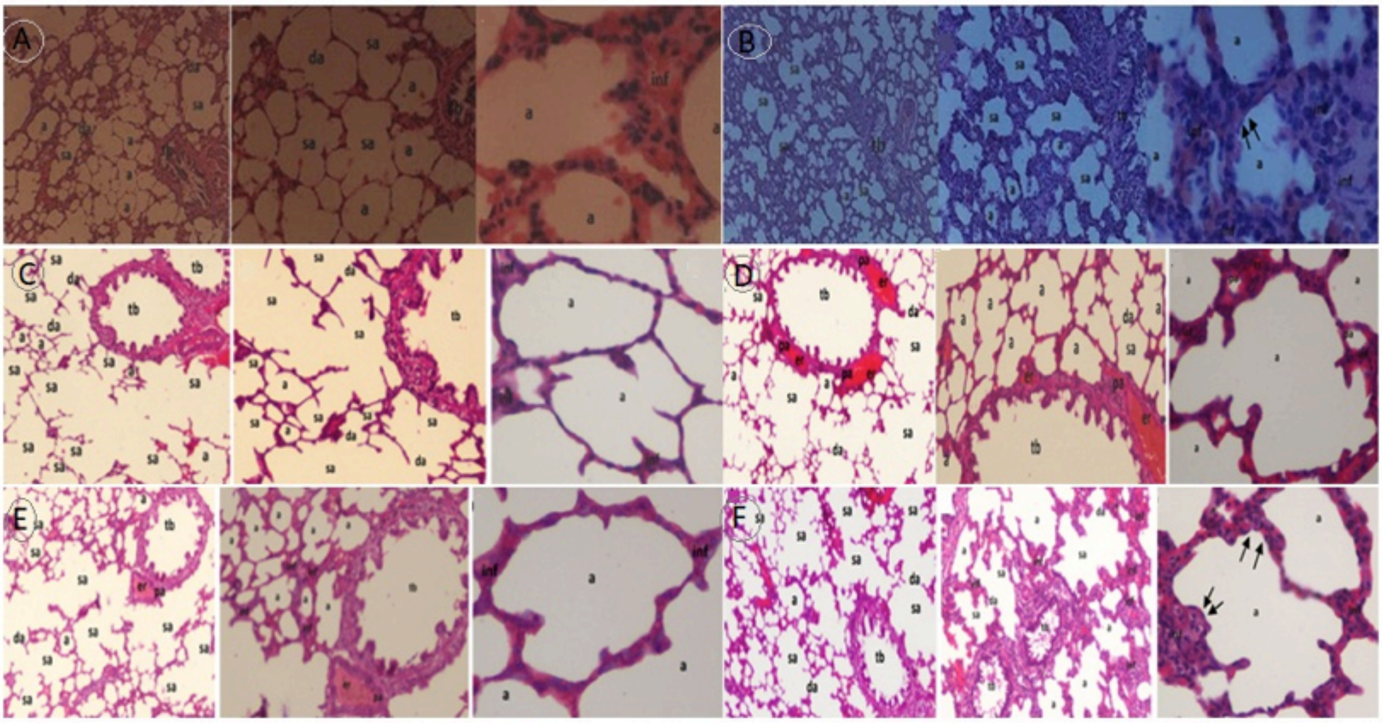
Data are expressed as mean ± SD (min-max)
Mann-Whitney U Test. *: p<0.05 versus Group I/R

The histopathological examination of the lung tissue preparations obtained from the groups are shown in Figure 1. All lung tissue components were observed in normal structure in Group S (Figure 1A). However, intense lymphocyte infiltration was observed in Group I/R and clustered lymphocyte assemblages were more prominent. Degeneration and deterioration of the wall structure of the respiratory bronchioles were evident. It was observed that the ruptures in the epithelium were accompanied by a small amount of cell debris in the respiratory bronchiole lumen. There was marked deterioration in the alveolar lumen. Thickening of the vessel wall was noted (Figure 1B).

Intense lymphocyte infiltration areas were observed very rarely in Group A-90 and Group A-135. The lumen of the terminal bronchioles and alveoli did not contain fragmented cell aggregation. Thickening of alveolar walls was observed in rare regions and alveolar walls were observed to be thinner than Group I/R (Figure 1C,D).

In Group I/R-A 90, regions with intense lymphocyte infiltration were less than in Group I/R, and an increase in smooth muscle surrounding the bronchial tree was observed in these regions. The thickening of the vessels was observed thinner than Group I/R (Figure 1E).

Significant regeneration was noted in Group I/R-A 135 compared to Group I/R and Group I/R-A 90. There was a significant decrease in lymphocyte infiltration compared to these two groups. Terminal bronchioles were found to be close to normal histological appearance. Cellular aggregation was not observed in the bronchiole and alveolar lumen. While the smooth muscle structures of the vessel wall were observed in a normal histological structure, thinning of the vessel wall was more prominent compared to Group I/R and Group I/R-A 90 (Figure 1F).



a, Alveoli; tb, Terminal bronchiole; sa, Saccusalveolaris; da, Ductusalveolaris; inf, Inflamation; pa,Pulmonaryarterywall; er, Erythrocyte; ↓↓, Alveolarseptumthickening

Figure 1. Lung tissue preparations, hematoxylin-eosin (X40; X100; X400): **A-** Normal lung tissue parenchyma in Group S **B-** In Group I/R, dense lymphocyte infiltration was seen and clustered lymphocyt eassemblages were evident; **C-**In Group A-90, thickening of the alveolar walls was observed in rare regions; **D-** In Group A-135,thickening of the alveolar walls was observed in rare regions; **E-**In Group I/R-A 90, regions with intense lymphocyte infiltration were less, and an increase in smooth muscle surrounding the bronchial tree was observed in these regions; **F-**In Group I/R-A 135, significant regeneration and decrease in lymphocyte infiltration were noted when compared to Group I/R and Group I/R-A 90.

Discussion

In the current study that we investigated the effects of the doses of 90 and 135 mg/kg of amantadine on lung tissue in lower extremity I/R injury; when considering the lung tissue CAT, SOD activities and MDA levels, amantadine has a protective effect against I/R injury, similar to other NMDA antagonist agents. Although we could not find a statistically significant difference between the biochemical values of the lung tissue, we observed that the lung tissue was histopathologically affected by I/R damage and this damage could be corrected using amantadine. This situation was more pronounced in subjects given 135 mg/kg amantadine than in subjects given 90 mg/kg amantadine.

N-Metil D-Aspartat receptor; is a member of the glutamate receptor family, originally identified in the central nervous system, that functions as a membrane calcium channel [7]. It has been reported that NMDA antagonists have protective effects against I/R damage in various organs and tissues. These agents increase antioxidant activity and decrease the oxidant effect. In the literature, it has been determined that NMDA antagonists such as MK-801, memantine, and ketamine have protective effects on I/R damage [8].

Amantadine, a NMDA receptor antagonist, was approved in the United States in 1966 as a protective agent against Asian Influenza [9]. In a study conducted in rats with traumatic brain injury, the use of amantadine at doses of 45 and 135 mg/kg/day was shown to be effective in the treatment of depression-like symptoms [10]. Orhan et al. reported that 45 mg/kg amantadine had a protective effect on lung in lower extremity I/R injury [4]. However, we planned this study because there is no another study in the literature that showed the protective effect of higher doses of amantadine on I/R injury.

After acute extremity ischemia, re-bloodification of the extremity and normal circulation, tissue damage and systemic complications may occur. After I/R, both local damage occurs in the ischemic area and damage can occur in distant organs outside the ischemic area. In distant organ damage, the lung is the target organ and is of great clinical importance [11].

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Therefore, in this study, we investigated the effects of I/R on lung tissue and the protective effects of different doses of amantadine on lung tissue.

Many antioxidant substances have been tried to prevent the effect of reactive oxygen species (ROS) [12,13]. These antioxidant substances have a protective effect against distant tissue-organ damage after I/R, either by increasing pulmonary microvascular permeability and preventing neutrophil accumulation or by activating the antioxidant system [13]. ROS scavengers are agents that react with reactive oxygen moieties and convert them into harmless substances. These agents can be listed as SOD, CAT and glutathione peroxidase [12].

Catalase works in combination with SOD in the degradation of hydrogen peroxide [12]. In many of the experimental studies, the authors showed that CAT activity decreased in tissue and serum samples in I/R injury and increased by antioxidant application [14]. On the contrary, some authors showed that CAT and SOD activities increased in tissue and serum samples in I/R injury and a decrease was achieved with antioxidant application. It has been argued that this increase in CAT and SOD activities in I/R injury is a response to suppression of oxidative stress [15]. In our study, the CAT and SOD activities after I/R were higher than the control values. We thought that this response was potentiated by the further increase in CAT and SOD levels after administration of amantadine.

Free radicals formed in I/R initiate lipid peroxidation by attacking lipids in membranes due to their high reactivity. An idea about the degree of lung damage can be obtained by measuring the reaction of lipid peroxidation product MDA, which accumulates during I/R injury in the lungs, with thiobarbituric acid reagents spectrophotometrically [16]. It is known that MDA levels increase in I/R injury, and this increase decreases in antioxidant application [4,17]. In the current study, MDA levels increased after I/R and decreased with amantadine administration, in line with the literature. This reduction was more pronounced in the administration of 135 mg/kg amantadine. This decrease in MDA level suggested that amantadine may have protective effects in I/R and this effect may be more pronounced with dose increase.

Lung tissue is the target organ most affected by lower extremity I/R injury. Although the etiology is unknown, it is thought that some humoral mediators play a role in this target organ damage during reperfusion [11,18]. Damage in the lungs is histopathologically manifested as alveolar wall thickening, infiltration of neutrophils and lymphocytes, and interstitial edema [19]. Polymorphonuclear leukocytes have a major role in lung injury caused by I/R of the lower extremities, and the reduction of these cells has a protective effect on the lungs. Experimentally, it has been reported that histopathological changes in the lung tissue can be significantly reduced with various agents [20,21]. In our study, in the histopathological examination of the lung tissue, amantadine decreased neutrophil infiltration and this was more pronounced with 135 mg/kg amantadine. We observed that there was a significant increase in alveolar wall thickness due to I/R damage and this damage was improved with amantadine. Especially when the alveolar wall thickening scores are examined; we found the changes were significantly reduced with 135 mg/kg amantadine, but this effect was not significant at 90 mg/kg. We think that high-dose amantadine is more effective in histopathological changes caused by I/R injury.

The limitation of our study is the sample size was small.

In conclusion, although we could not find a statistically significant difference between lung tissue biochemical values, we observed that lung tissue was histopathologically affected by I/R damage and the damage was less with amantadine use. In the reduction of I/R damage, 135 mg/kg administration of amantadine was more beneficial than 90 mg/kg.

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