Experimental Study / Deneysel Çalışma

Protective effects of cilostazol and levosimendan on lung injury induced by lower limb ischemia-reperfusion

Alt ekstremite iskemi reperfüzyonuna bağlı akciğer hasarında silostazol ve levosimendanın koruyucu etkisi

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Background: In this study, we aimed to investigate the effects of cilostazol and levosimendan, and the combination of these agents on the lung remote organ damage after ischemia/reperfusion (I/R) following abdominal aortic surgery.

Methods: The experiments were performed on 35 male Wistar albino rats weighing mean 219±26 g. The rats were randomly assigned into five groups, including each of seven rats. Rats were pretreated with cilostazol and levosimendan, alone or in combination, and then lower extremities were subjected to I/R induced by a infrarenal aortic occlusion for duration of 120 minutes, followed by a-60 minute- reperfusion. The rats were sacrificed under deep anesthesia and the lung tissues were removed. Malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and glutathione (GSH) levels were measured in the lung tissues. The tissue samples were further examined histopathologically under light microscopy.

Results: It was found that I/R elevated MDA levels accompanied by a reduction in SOD activities and GSH levels (p<0.05). Cilostazol and levosimendan, and their combination restored MDA levels, SOD activity, GSH levels and lung injury scores (p<0.05). There was no significant difference among individual or combined treatment of these agents (p>0.05).

Conclusion: In light of these findings, cilostazol and levosimendan may be useful for protecting the lung tissue from I/R injury, before limb ischemia in vascular surgery. However, the combined use of these agents does not further increase the protection from I/R injury.

Key words: Abdominal aorta; cilostazol; ischemia/reperfusion; levosimendan; lung injury.

Amaç: Bu çalışmada, abdominal aort cerrahisinde iskemi/reperfüzyon (İ/R) sonrası uzak organ hasarı olarak akciğer üzerine silostazol, levosimendan ve bu ilaçların kombinasyonunun etkileri araştırıldı.

Çalışma planı: Çalışma ortalama 219±26 g ağırlığında 35 erkek Wistar albino türü sıçan ile gerçekleştirildi. Sıçanlar her bir grupta yedi sıçan olacak şekilde randomize olarak beş gruba ayrıldı. Sıçanlara silostazol, levosimendan ve bu iki ilacın kombinasyonu ile tedavi uygulandıktan sonra, infrarenal aortik oklüzyon ile alt ekstremitelere 120 dakika iskemi ve sonrasında 60 dakika reperfüzyon uygulandı. Sıçanlar derin anestezi altında sakrifiye edildi ve akciğer dokuları çıkarıldı. Akciğer dokularında malondialdehid (MDA) düzeyleri, süperoksit dismutaz (SOD) aktiviteleri ve glutatyon (GSH) düzeyleri ölçüldü. Doku örnekleri ayrıca ışık mikroskobu ile histopatolojik olarak incelendi.

Bulgular: Çalışmada İ/R'nin akciğer dokusunda MDA düzeylerini yükseltirken, SOD aktivitesini ve GSH düzeylerini azalttığı belirlendi (p<0.05). Silostazol, levosimendan ve bu ilaçların kombinasyonu ile MDA düzeyleri, SOD aktiviteleri, GSH düzeyleri ve akciğer hasarı skorunda iyileşme gözlendi (p<0.05). Bu ilaçların tek veya kombine kullanılması arasında anlamlı farklılık yoktu (p>0.05).

Sonuç: Bu bulgular ışığında, vasküler cerrahi sırasında ekstremite iskemisi öncesi silostazol ve levosimendan kullanımı akciğer dokusunu İ/R hasarından korumakta faydalı olabilir. Bununla birlikte, bu iki ilacın kombine kullanımı, İ/R hasarına karşı koruyuculuğu artırmamaktadır.

Anahtar sözcükler: Abdominal aort; silostazol; iskemi-reperfüzyon; levosimendan; akciğer hasarı.

Skeletal muscle injury is a potential complication of abdominal aortic surgery. During ischemia, the muscle cells are not able to keep their membrane integrity, causing the release of calcium as well as phospholipid A2 and the formation of polyunsaturated fatty acids and fatty acid radicals. If the oxygenation is reestablished at that stage of ischemia, the fatty acid radicals react with the oxygen and perform the lipid peroxidation reaction. This reaction increases the membrane permeability and also stimulates the chemotaxis of leukocytes, which can release the oxygen-derived free radicals and proteolytic enzymes when activated.[1] A systemic inflammatory response after ischemia/reperfusion (I/R) causes both local and remote organ injuries. Lung injury is one of the most important complications of remote organ injuries after surgical procedures involving transient aortic occlusion with subsequent I/R of the lower extremities.[2]

Cilostazol, 6-[4-(1-Cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone is an antiplatelet agent that inhibits platelet aggregation induced by collagen, 5'-adenosine diphosphate (ADP), epinephrine, and arachidonic acid.[3] It has been demonstrated to inhibit phosphodiesterase type 3, thus increasing the intracellular level of cyclic adenosine monophosphate (cAMP) and activating protein kinase A.[4] This agent has been approved in the United States for the treatment of intermittent claudication[5] and in Japan for the reduction in the recurrence of cerebral thrombosis and lacunar stroke.[6] It was reported that cilostazol inhibits the cytokine-induced expression of various pro-inflammatory and adhesion molecule genes.[7] The myofilament calcium sensitizer levosimendan is a positive inotropic drug that improves myocardial contraction in a stunned myocardium and also has a vasodilating effect by opening adenosine triphosphate-sensitive potassium channels.[8] It has been reported that levosimendan exerts a beneficial anti-inflammatory, antioxidant, and anti-apoptotic effect by reducing the circulation of pro-inflammatory cytokines and soluble apoptosis mediators.[9]

While there are limited reports concerning the effects of levosimendan on lung injury,[10,11] at present there is no information in the literature regarding how cilostazol affects the same type of injury. In the present study, we investigated the protective effects of cilostazol and levosimendan, both indivu. dually and in combination, on remote organ damage of the lungs in a rat model with transient infrarenal aortic cross-clamp-induced I/R injury.

MATERIALS AND METHODS

Animal preparation

Our experimental protocol was approved by the Animal Care and Use Committee of the Pamukkale University Medical Faculty Research Hospital. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" (National Institute of Health publication No. 85-22, revised in 1985) and the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council). The experiments were performed on 35 male Wistar albino rats weighing 219±26 g (mean±SD). The animals were housed at a temperature of 22±1 °C with a 12-hour dark/light cycle. Standard rat chow pellets and water were allowed ad libitum. During the surgical procedures, anesthesia was induced and then maintained with intramuscular ketamine (30 mg/kg) and xylazine (2 mg/kg), as needed. No animal received hemodynamic or ventilatory support. During the surgical procedures, the body temperature was maintained with a water-filled heating pad. The animals were placed in a nose cone to breath oxygen (O_2) at a rate of 0.5 liter/minute.

Experimental groups and surgical procedures

The rats were randomly allocated into five groups, each with seven rats. After the surgical preparation using aseptic techniques, a jugular vein was dissected and catheterized. A 24-gauge catheter was inserted into the jugular vein and used for the administration of the saline or drug-containing solutions. The animals were than given heparin of 1000U/kg (Liquemine, Roche, İstanbul, Turkey) via the catheter. The abdominal aorta was exposed through a midline abdominal incision. After the exploration of the abdominal aorta, a microvascular clamp was placed on the infrarenal abdominal aorta. Reperfusion was confirmed visually and by Doppler assessment in the femoral region.

In the group 1, sham-operated rats, 1 ml vehicle (0.9% NaCl) was given via the jugular vein 20 minutes before the beginning of the experiment. The laparotomy incision was left open for three hours, but the abdominal aorta was not occluded. This group of animals was used for determining the effects of the anesthesia and the operation on the results. In the group 2 rats with ischemia-reperfusion (I/R), 1 ml vehicle (0.9% NaCl) was given 20 minutes before the beginning of the experiment, and the infrarenal abdominal aorta was occluded with a microvascular clamp for two hours, followed by one hour of reperfusion. The cessation of the blood flow was verified by Doppler ultrasound. The group 3 animals received oral administration of

30 mg/kg cilostazol (Pletal, Abdi Ibrahim, Turkey) two hours before the aortic occlusion. The drug was dissolved in 30% dimethyl sulfoxide. The group 4 animals received a loading dose of 20 μg/kg levosimendan (Simdax, Orion Pharma, Finland) over the course of 15 minutes, followed by a continuous infusion of 0.1 μg/kg/min. This was ceased after declamping during the ischemic period. In group 5, a combination of cilostazol and levosimendan (as above) was given in the same fashion. Following these procedures, all animals were sacrified by cervical dislocation. The malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and glutathione (GSH) levels were measured in the right lungs, and the samples also underwent a histopathological evaluation under light microscopy.

Measurement of MDA levels in the lung tissue

The lung tissue was placed into petri dishes after being washed with cold water and then stored at -70 °C until it was assayed for MDA levels by the procedure of Ohkawa et al.[12] After thawing, each sample was briefly weighed and homogenized in 10 volumes (w/v) 150 mM KCl solution. Aliquots of the resultant homogenates (0.4 ml) were diluted to 3.6 ml in 0.33% thiobarbituric acid, 8.330% acetic acid (pH 3.5), and 0.45% sodium dodecyl sulfate. After mixing, all of the samples and standards were heated to 100 °C for one hour. The absorbance was recorded at 532 nm and compared with that which was obtained from the MDA standards.

Measurement of SOD activity in the lung tissue

The SOD activities were determined according to the method of Winterbourn et al.[13] The tissue was homogenized briefly in 0.02 M phosphate buffer (pH 7.8), and 0.1 ml of the resulting homogenate was added to a final volume of 3 ml containing 3.33 mM ethylenediaminetetraacetic acid (EDTA), 1.02 μM sodium cyanide (NaCN), 0.1 mM nitro-blue tetrazolium (NBT), 2 μM riboflavin, and 16.33 mM phosphate buffer. The samples were shaken and left for 15 minutes under fluorescence at room temperature. The SOD activity was assayed spectrophotometrically (560 nm) as an inhibition of the photochemical reduction of NBT.

Measurement of GSH levels in the lung tissue

Glutathione levels were determined by a modification of the procedure described by Moron et al.[14] After homogenization of the tissue samples in 150 mM KCl, 0.5 ml of the resulting homogenate, it was mixed for a short time with 3 ml deproteinization solution (NaCl, metaphosphoric acid, EDTA in distilled water) and

1.5 ml 150 mM potassium chloride (KCl) solution. Each sample was centrifuged at 1.000 x g for five minutes, and 0.5 ml of the supernatant was added to 2 ml disodium hydrogen phosphate (Na_2HPO_4) and 0.5 ml Ellman solution (DTNB; dithiodinitrodibenzoic acid, sodium citrate, distilled water). The absorbance of these supernatants was recorded at 412 nm and converted through those obtained from the GSH standards.

Histopathological examination

The right lungs were removed, fixed with 10% buffered formalin solution, and stored for 24 hours. A sagittal section was obtained at the level of hilus. The specimens were fixed again with 10% formalin for two days and stained with hematoxylin-eosin. They were then examined with light microscopy (Olympus BX 51, Japan) by the same histologist who was blinded to the study. The lung injury was scored according to inflammatory cell infiltration, alveolar edema, congestion, and preservation of the alveolar septum as grade 0, normal; grade 1, mild; grade 2, moderate; and grade 3, severe.

Statistical analysis

The parametric data was expressed as mean ± standard deviation (SD). The analytical results were evaluated using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA) version 11.0 software program. Control variables were compared among the groups by a one-way analysis of variance (ANOVA) using Tukey's Honestly significant difference test. A p value of less than 0.05 was considered significant. The Kruskal-Wallis test was used to compare group medians for histopathological scores. Probabilities of 0.05 or less were considered statistically significant.
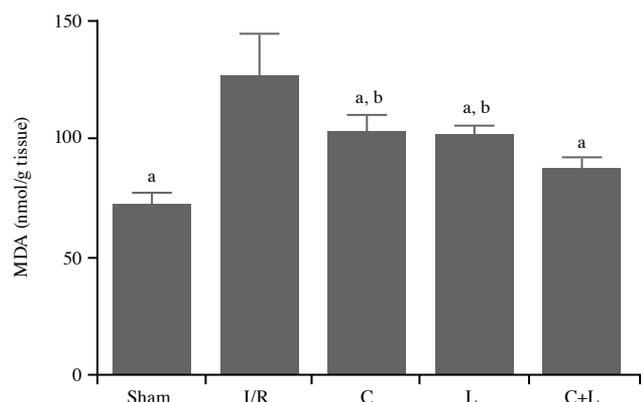

Figure 1. The graph shows the MDA level of different groups. Each group contains mean ± SD of seven animals. Sham: Sham-operated group (group 1); I/R: Control ischemia/reperfusion group (group 2); C: Cilostazol-treated group (group 3); L: Levosimendan-treated group (group 4); C+L: Cilostazol + levosimendan-treated group (group 5). a Significantly different (p<0.05) from group 2. b Significantly different (p<0.05) from group 1.
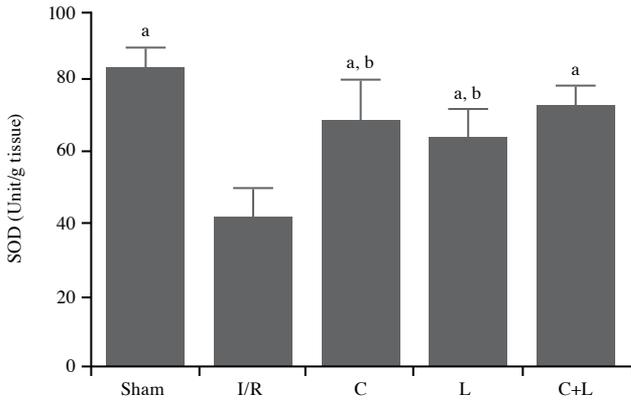
Figure 2. The graph shows the SOD activity of different groups. Each group contains mean ± SD of seven animals. Sham: Sham-operated group (group 1); I/R: Control ischemia/reperfusion group (group 2); C: Cilostazol-treated group (group 3); L: Levosimendan-treated group (group 4); C+L: Cilostazol + levosimendan-treated group (group 5). a Significantly different (p<0.05) from group 2. b Significantly different (p<0.05) from group 1.
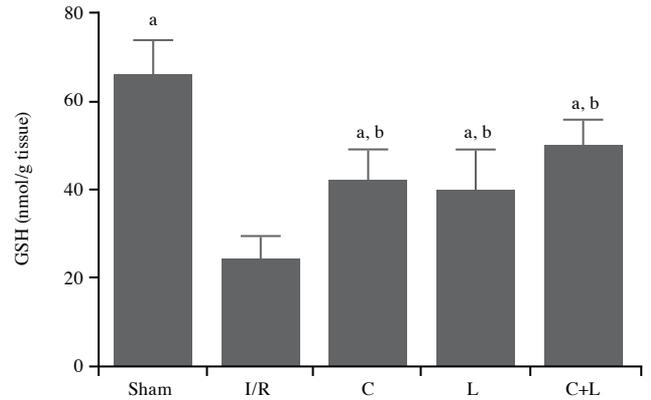

Figure 3. The graph shows the GSH level of different groups. Each group contains mean ± SD of seven animals. Sham: Sham-operated group (group 1); I/R: Control ischemia/reperfusion group (group 2); C: Cilostazol-treated group (group 3); L: Levosimendan-treated group (group 4); C+L: Cilostazol + levosimendan-treated group (group 5). a Significantly different (p<0.05) from group 2. b Significantly different (p<0.05) from group 1.

RESULTS

Changes in MDA levels in lung tissues

As shown in Figure 1, in the group 2 control I/R group, the MDA levels were significantly higher than in all of the other groups (p<0.05). In group V, the MDA levels were much lower than those in group 3 and 4 while there were no statistically significant differences between the MDA levels of groups 3, 4, and 5.

Changes in SOD activity in lung tissues

As seen in Figure 2, the SOD activities in group 2 were much lower than those in group 1. Cilostazol, levosimendan, and combined treatment significantly enhanced the SOD activity. Although the highest SOD activity was found in group 5, there were no statistically significant differences between this and that of groups 3 and 4.

Changes in GSH levels in lung tissues

After the limb I/R (groups 2-5), the GSH levels were significantly lower when compared with the group 1 rats (Figure 3). The GSH levels were significantly higher in groups 3, 4, 5 when compared with group 2. The GSH levels were higher in group 5 than in groups 3 and 4, and there were no significant differences between these groups.

Histopathologic evaluation

The histopathological lung scores are shown in Figures 4 and 5. The lungs in group 2 were found to markedly increase perivascular interstitial inflammatory cell infiltration when compared with the other groups. There were also several signs of interstitial edema and septal swelling in group 2. The scores of the pretreated

groups 3, 4, and 5 were significantly lower than that of group 2. There was no difference between groups 3, 4 and 5 according to lung injury scores.

DISCUSSION

The data from the present study is similar to the clinical setting for abdominal aortic surgery. It is known that there is high-grade oxidative stress during abdominal aortic surgery. The results here demonstrate that both cilostazol and levosimendan can reduce the oxidative stress on the lung tissue in this rat model with transient infrarenal aortic occlusion.

Ischemic tissue injury is not limited to the damage that occurs during the period of hypoperfusion. Additional injury with redelivery of molecular oxygen
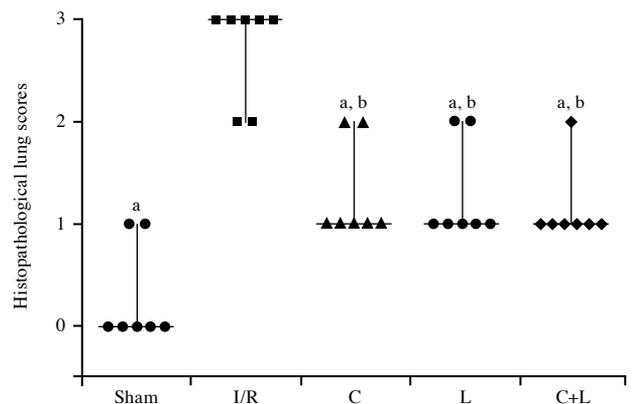

Figure 4. The graph shows the lung injury scores of animals and the median scores of each group. Sham: Sham-operated group (group 1); I/R: Control ischemia/reperfusion group (group 2); C: Cilostazol-treated group (group 3); L: Levosimendan-treated group (group 4); C+L: Cilostazol + levosimendan-treated group (group 5). a Significantly different (p<0.05) from group 2. b Significantly different (p<0.05) from group 1.
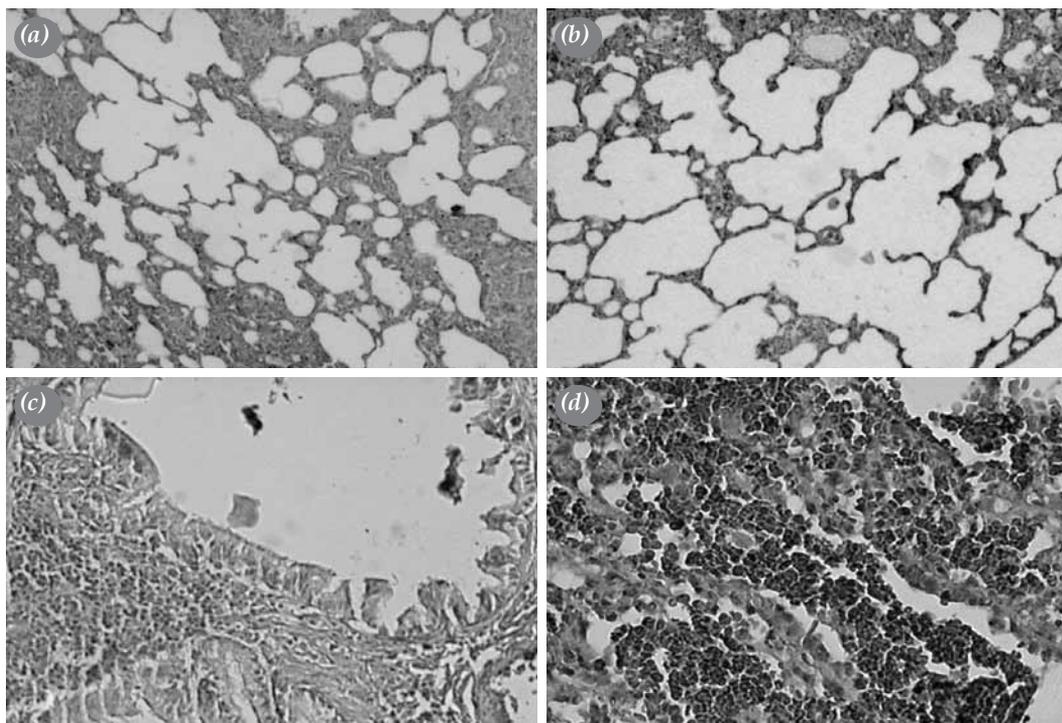
Figure 5. Histological view of the lung with different degrees of injury (H-E x 10). **(a)** Grade 0: Normal histological appearance of the lung **(b)** grade 1: Focal minimal vascular congestion and cellularity of lung interstitium **(c)** grade 2: Multifocal prominent vascular congestion and cellularity in the lung interstitium **(d)** grade 3: Severe lung injury with alveolar flooding, indicating pulmonary edema. Note the progressively increasing vascular congestion and cellularity of the lung interstitium characterizing more severe forms of injury.

to the ischemic tissue can arise from the activation of leukocytes, systemic inflammatory response, and overproduction of reactive oxygen species (ROS), all of which have detrimental effects on cell structure and function.[15] A devastating consequence of tissue reperfusion is the damage to organs which are uninvolved in the initial ischemic insult. It has been demonstrated that acute ischemia of the lower extremities in rats results in a significant lung injury as a remote organ.[11] Our results confirm that transient infrarenal aortic occlusion can cause this type of lung injury.

Reactive oxygen species can cause cellular damage by oxidizing membrane lipids, essential cellular proteins, and DNA. Malondialdehyde is one of the end productions of lipid peroxidation, and it is accepted as a marker of ROS-mediated lipid peroxidation of cell membranes.[16] The plasma and tissue MDA levels are good markers for increased systemic oxidative stress. This is confirmed in the present study by the increase in the MDA levels of the lung tissue after the limb I/R. Increased lipid peroxidation can also result in the release of proteolytic lysosomal enzymes and mitochondrial matrix enzymes into the cytoplasm,

which gives rise to intracellular proteolysis and cellular destruction.[17] Under normal circumstances, ROS is counteracted by the defense system of the body, such antioxidant enzymes like SOD and GSH.[18] This is also seen in terms of the elevation in MDA levels in the present study which were accompanied by a decrease in the SOD activity and GSH levels in the lung tissue after the limb I/R.

Cilostazol is known to increase the intracellular cAMP by blocking its hydrolysis by phosphodiesterase type 3.[4] Experimental studies suggest that cAMP modulates the inflammatory response.[19] Recently, cilostazol was demonstrated to scavenge the hydroxyl and peroxyl radicals and to inhibit apoptotic cell death.[20] Lee et al.[21] reported that cilostazol has a cell-protective effect by reducing increased DNA fragmentation via suppressing nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase-dependent superoxide production and the release of cytokines, consequently resulting in the suppression of monocyte recruitments and macrophage accumulation. Aoki et al.[3] also demonstrated that cilostazol protects the endothelial cells from the inflammation of pro-inflammatory and

adhesion molecule genes. In addition to the direct effect of lipid peroxidation on the cell wall, ROS can cause indirect damage by activating leucocytes and platelets. Cilostazol has been shown to have beneficial effects on this activation in animal and case-controlled human studies.[22] This study demonstrated that cilostazol treatment decreases the cellularity of the lung interstitium, pulmonary edema, and MDA levels in the lung tissue after limb I/R. This is thought to indicate a reduction in lipid peroxidation and cellular injury.

The positive inotropic effect of levosimendan is well known in human studies,[8] but it has not been studied much with regard to remote organ injury after limb I/R. Levosimendan has been shown to induce arteriolar and venous dilatation because of its ability to open ATP-sensitive potassium channels in vascular smooth muscle cells.[23] Levosimendan may also affect vascular tone via its phosphodiesterase inhibitory effect, leading to an increase of cAMP in vascular smooth muscle cells and vasorelaxation.[24] Experimental data suggests that levosimendan has antioxidant properties and seems to be a potent inhibitor of hydrogen peroxide (H_2O_2).[9] Furthermore, studies have shown that levosimendan administration causes a reduction of the circulating pro-inflammatory cytokines and apoptosis mediators.[25] Our study reveals that levosimendan has a protective effect on the lung after transient limb I/R. Therefore, it can be concluded that levosimendan decreased the lipid peroxidation after I/R injury.

In the present study, there were no significant differences in the MDA levels, SOD activity, GSH levels, or lung injury under the combined treatment with cilostazol and levosimendan when compared with either agent alone. On the other hand, a combination of these medications showed similar biochemical results when compared with the sham group. However, there was a significant difference between the combined treatment and sham group in the lung injury scores. In our opinion, since the inhibition of ROS might have a limited value in the I/R model, other alternative mechanisms should be considered.

In conclusion, we have shown that I/R of the lower extremities causes a significant lung injury. The results of the study confirmed the protective effects of cilostazol and levosimendan in the I/R insult. These effects may be, at least in part, due to the inhibition of ROS production. To the best our knowledge, the effects of these two substances were compared for the first time in this study, and the antioxidant properties of cilostazol were comparable to those of levosimendan. However, no additional effect was observed microscopically in rat

lungs when these two agents were used together. More targeted research is needed to determine the clinical importance of cilostazol and levosimendan treatments, especially regarding other possible mechanisms beside the ROS scavenging. This might prove to be effective for improving the protection of the lung after transient aortic occlusion.

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