

The proinflammatory cytokine-mediated protective effects of pentoxifylline, iloprost, and cilostazol on a mitigating lung injury induced by lower limb ischemia and reperfusion in rats

Sıçanlarda alt ekstremite iskemisi ve reperfüzyonu ile tetiklenen akciğer hasarının azaltılmasında pentoksifilin, iloprost ve silostazolün proenflamatuvar sitokin aracılı koruyucu etkileri

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Background: This study aims to elucidate whether pentoxifylline, iloprost and cilostazol mitigates acute lung injury induced by lower limb ischemia-reperfusion (I/R) and their protective effects cover cytokines.

Methods: Forty rats were randomized into five groups: control (group 1), ischemia-reperfusion (group 2), pentoxifylline (group 3), iloprost (group 4), iloprost and cilostazol (group 5). All drugs were administered before ischemia. Samples were obtained for tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6), and total sialic acid (TSA) assays. Findings of lung injury were examined.

Results: Interleukin-6 and TNF levels were increased at 90 minutes and sustained elevated even after 240 minutes. In groups 3 and 4, IL-6 and TNF levels were significantly lower at 90, 180 and 240 minutes compared to group 2. At 180 minutes, TSA levels in groups 2, 3, 4 and 5 were significantly different from baseline and 90 minute levels. At this time point, TSA levels of group 2 and 3 were significantly higher compared to group 4 and 5.

Conclusion: In this acute lung injury model induced by I/R of the lower limbs, pretreatment with pentoxifylline, iloprost and cilostazol significantly attenuated pro-inflammatory activities and parenchymal lung damage.

Key words: Cilostazol; iloprost; ischemia-reperfusion injury; lung injury; pentoxifylline.

Amaç: Bu çalışmada, pentoksifilin, iloprost ve silostazolün alt ekstremite iskemisi-reperfüzyonu ile tetiklenen akut akciğer hasarını azaltıp azaltmadığı ve bu koruyucu etkilere sitokinlerin dahil olup olmadığı araştırıldı.

Çalışma planı: Kırk sıçan rastgele beş gruba ayrıldı: kontrol (grup 1), iskemi-reperfüzyon (grup 2), pentoksifilin (grup 3), iloprost (grup 4), iloprost ve silostazol (grup 5). Tüm ilaçlar iskemi öncesi uygulandı. Tümör nekroz faktör-alfa (TNF- α), interlökin-6 (IL-6) ve total sialik asit (TSA) incelemeleri için numuneler alındı. Akciğer hasarı bulguları incelendi.

Bulgular: İnterlökin-6 ve TNF düzeylerinde 90. dakikada artış olduğu ve hatta 240. dakikadan sonra da yüksek seyrettiği saptandı. Grup 3 ve 4'de, IL-6 ve TNF düzeyleri, grup 2 ile karşılaştırıldığında 90, 180. ve 240. dakikalarda anlamlı olarak düşüktü. Total sialik asit düzeyleri 180. dakikada grup 2, 3, 4 ve 5'te bazal ve 90. dakikadaki değerlerden anlamlı olarak farklı idi. Bu zaman noktasında TSA düzeyleri grup 2 ve 3'te grup 4 ve 5 ile karşılaştırıldığında anlamlı derecede yüksekti.

Sonuç: Alt ekstremite İ/R ile tetiklenmiş bu akut akciğer hasarı modelinde, pentoksifilin, iloprost ve silostazol ile ön tedavinin uygulanması, proenflamatuvar aktiviteyi ve parankimal akciğer hasarını anlamlı düzeyde azaltmıştır.

Anahtar sözcükler: Silostazol; iloprost; iskemi-reperfüzyon hasarı; akciğer hasarı; pentoksifilin.



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Ischemia/reperfusion (I/R) injury is involved in the pathophysiology of many clinical disorders, including myocardial infarction (MI), stroke, mesenteric ischemia, peripheral vascular disease, and acute respiratory distress syndrome (ARDS). Acute ischemia and reperfusion of an extremity initiates an inflammatory turnover, leading to injury and damage to the extremity itself and some target organs as well. This process causes an imbalance that results in the massive secretion of systemic inflammatory mediators, such as interleukins (ILs), tumor necrosis factor alpha (TNF- α), and sialic acid (SA).^[1-4] Severe and sometimes fatal pulmonary complications secondary to this inflammatory process have also been described.^[4,5] Pentoxifylline is a xanthine derivative which improves the flexibility of erythrocytes and reduces blood viscosity. In addition to its effects on erythrocyte deformability, pentoxifylline affects the inflammatory cascade in multiple ways.^[6,7] Iloprost, a synthetic prostacyclin (PGI₂) analogue, mimics the pharmacodynamic properties of this compound, for example the potent inhibition of platelet activation and aggregation, vasodilation, and the ill-defined direct cytoprotection.^[8-10] Cilostazol, a selective inhibitor of phosphodiesterase III, suppresses cyclic adenosine monophosphate (cAMP) degradation, thereby increasing the intracellular cAMP levels in platelets and blood vessels. This leads to the inhibition of platelet aggregation and vasodilatation.^[11,12]

We previously employed an animal model to investigate I/R injury and demonstrated that pentoxifylline reduces this type of lung injury by altering TNF- α and SA production.^[4] The aim of this study was to determine whether pretreatment with pentoxifylline, iloprost, and cilostazol attenuates I/R-induced acute lung injury in a rat model via aortic cross-clamping and reperfusion of the lower limbs.

MATERIALS AND METHODS

Our experimental animal study was conducted with humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH publication 86-32, revised 1985). In addition, the study was approved by the Animal Care and Use Committee of Sivas Cumhuriyet University Medical Faculty.

Model

The experiments were performed on a previously described rat model in the study conducted by

Tassiopoulos et al.^[2] and Berkan et al.,^[4] and we referred to these to plan our experiments. In this study, the infrarenal aorta was cross-clamped for three hours followed by one hour of reperfusion to form a well characterized acute limb I/R model.

Experimental protocol

Forty adult male Sprague-Dawley rats, weighing from 420 to 480 g, were left fasting overnight. Intramuscular injections of ketamine (75 mg/kg) together with xylazine (5 mg/kg) were given for sedation and anesthesia, and the animals remained anesthetized throughout the entire procedure with injections of one-fourth of the initial dose administered every 20-30 minutes. Carotid arterial catheters were inserted for blood sample analysis, and a jugular venous line was established for intravenous fluid infusion through the same neck incision. Next, the animals were given heparin (1000 U/kg), and the infrarenal aorta was exposed through a midline abdominal incision. The rats were randomized into the following five study groups composed of eight rats each:

- In group 1 (control), the aorta was exposed but not cross-clamped, and the animal was observed for 240 minutes.
- In group 2, the aorta was cross-clamped just above the bifurcation with vascular clips for three hours followed by 60 minutes of reperfusion with the removal of the vascular clip.
- In group 3, the animals were pretreated with 50 mg/kg pentoxifylline, before the aortic cross-clamping.
- In group 4, the animals were pretreated with 1 mg/kg iloprost before the aortic cross-clamping.
- In group 5, the animals were pretreated with 1 mg/kg iloprost and 10 mg/kg intravenous cilostazol before aortic cross-clamping.

The animals in groups 3, 4, and 5 were subjected to the same I/R time as the animals in group 2, and reperfusion was achieved with the removal of the vascular clip. Arterial blood samples were obtained for total SA (TSA), IL-6, and TNF- α , and the data was measured at the baseline (prior to aortic clamping), 90th, and 180th minutes of ischemia as well as at the 30th minute after reperfusion in the study groups. In the control group, the baseline samples were taken when the aorta was exposed and at the 90th, 180th, and 210th minutes afterwards. The blood samples (0.5 ml) were

taken for measuring, and the blood was replaced with an equal volume of saline.

The arterial blood samples obtained for the serum TSA assay were centrifuged immediately at 4,000 rpm, and the serum was collected and stored at -20 °C until it was assayed. An enzymatic assay was used for the assessment, and the results were analyzed spectrophotometrically and expressed as mg/dl. Furthermore, all of the TSA determinations were performed at the same time by an independent investigator who was blinded to the study.

The arterial blood samples obtained for the TNF- α assay were centrifuged immediately at 10,000-12,000 rpm for 60 seconds, and the serum was collected and stored at -70 °C until it was assayed. As with the serum TSA, all of the TNF- α determinations were performed at the same time by an independent investigator who was blinded to the study. A TNF- α immunoreactive assay featuring an enzyme-linked immunoabsorbent system containing a hamster antimouse TNF- α antibody was used for the evaluation, and the results were analyzed spectrophotometrically and expressed as pg/ml.

The biologically active IL-6 was measured using a bio-assay on the basis of the proliferation of IL-6-dependent B9 hybridoma cells (a generous gift from Professor Lucien Aarden, Sanquin Research, Department of Autoimmune Diseases, Amsterdam, The Netherlands). The samples were serially diluted with an IL-6-free growth medium and dispensed in duplicate into 96-well microtiter plates. Similarly, a standard curve ranging from 0-500 pg/mL was generated by the use of recombinant human IL-6 (British Biotech, Oxford, United Kingdom) and also plated out in duplicate. The B9 cells were then washed free of the IL-6 and resuspended in an IL-6-free B9 growth medium. Next, a standard cell suspension (2.5×10^4 /mL) 100 μ L was plated into the wells and incubated at 37 °C for four days, and 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) in a phosphate-buffered saline solution (0.5 mg/mL) was added to each well followed by sodium dodecyl sulfate 50 mL (20% in 0.01 mol/L hydrochloric acid) five hours later. Then the plates were incubated for another 24 hours. Absorbance was read at 570 nm, and the amount of IL-6 in each sample was computed from the standard curve. In the end, we determined that the inter-assay and intra-assay coefficients of variation were less than 10%.

At the end of the experiment, the animals were euthanized via a 100 mg/kg intravenous bolus of pentobarbital sodium, which is the lowest dose that does

not induce pulmonary congestion in rats. The lungs were then removed and immersion-fixed in formalin. After this, the fixed specimens were embedded in paraffin, sectioned into 4 μ m pieces, and stained with routine hematoxylin-eosin (H-E) stain. Blinded pathologists examined all of the specimens, and at least two different sections of each specimen were examined to accurately determine the degree of injury. The lung injury was rated using semi-quantitative scores based on congestion, interstitial edema, polymorphonuclear leucocytes (PMNL) infiltration, and airspace hemorrhage, with 0 representing no change, 1+ signifying focal, mild, subtle changes, 2+ indicating multifocal, mild, prominent changes, and 3+ representing extensive, prominent changes.

Statistical analysis

Analyses were performed using the SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL, USA) software package. All of the measured values were expressed as mean \pm standard deviation (SD), and Student's t-test and one-way analysis of variance (ANOVA) tests were applied to determine the differences between the groups. For multiple comparisons, Bonferroni corrections were performed via post-hoc tests. A *p* value of <0.05 was accepted as being significant.

RESULTS

The IL-6 levels are given in Figure 1. At the 90th minute, there were significant increases in all of the groups compared with the baseline, with the highest levels being in group 2. In addition, groups 2, 3, 4, and 5 had significantly higher levels of IL-6 compared with group 1 (*p*<0.0001), and groups 2 and 3 had significantly higher levels of IL-6 than groups 4 and 5 (*p*<0.001). Although the IL-6 levels in group 4 were higher than in group 5, they did not reach statistical significance.

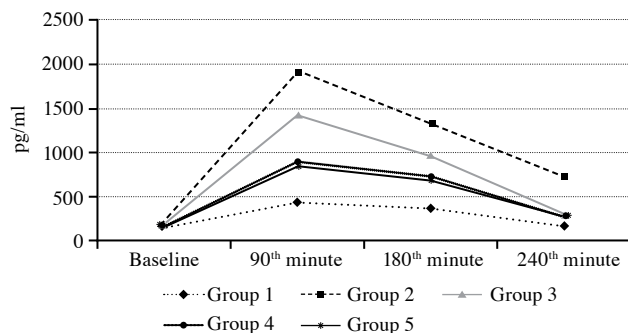


Figure 1. Interleukin-6 levels (pg/ml) in the groups. Group 1: Control group; Group 2: Ischemia/reperfusion group; Group 3: Pentoxifylline group; Group 4: Iloprost group; Group 5: Iloprost + cilostazol group.

At the 180th minute of ischemia, the IL-6 levels decreased significantly compared with the levels at the 90th minute, but they were still significantly higher than the baseline levels. The increase was again the highest in group 2. At this time point, the IL-6 levels of groups 2 and 3 were significantly higher than for groups 4 and 5 ($p < 0.001$). Furthermore, the IL-6 levels in group 3 were significantly higher than in groups 4 and 5, with groups 4 and 5 having almost the same measurements. After 60 minutes of reperfusion, the IL-6 levels continued to decrease, and in groups 3, 4, and 5, they had nearly returned to the baseline levels. However, group 2 had significantly higher levels when compared with groups 1, 3, 4, and 5. Moreover, there was a difference between the baseline and 240th minute IL-6 levels in groups 3, 4 and 5, but these groups still had higher levels than group 1.

The IL-6 levels increased at the 90th minute and remained relatively high even after the 240th minute, despite a small decrease detected at the 180th minute. In group 3, similar changes were also observed; however, the IL-6 levels were significantly lower at the 90th, 180th, 240th minutes compared with the other groups. Even more prominent differences were observed in group 4, and these were significantly lower than those in group 3. Finally, group 5 yielded the lowest levels, but they were similar to those of group 4.

The TNF- α levels are presented in Figure 2. At the 90th minute, there were significant increases in all of the groups compared with the baseline values, and this increase was the highest in group 2. Groups 2, 3, 4, and 5 had significantly higher levels of TNF- α compared with group 1 ($p < 0.0001$) while groups 2 and 3 had significantly higher levels than groups 4 and 5 ($p < 0.001$). Although the level in group 4 was higher than in group 5, it did not reach statistical significance.

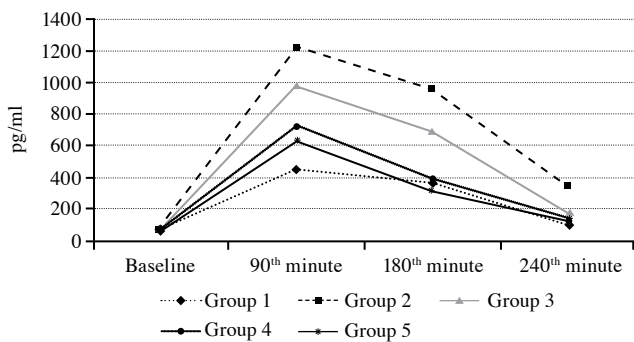


Figure 2. TNF- α levels (pg/ml) in the groups. Group 1: Control group; Group 2: Ischemia/reperfusion group; Group 3: Pentoxifylline group; Group 4: Iloprost group; Group 5: Iloprost + cilostazol group.

At the 180th minute of ischemia, the TNF- α levels decreased significantly compared with the levels at the 90th minute, but they still were significantly higher than the baseline levels. Again the increase was the highest in group 2. At this time point, the TNF- α levels of groups 2 and 3 were significantly higher than those in groups 4 and 5 ($p < 0.001$), which had the same levels. After 60 minutes of reperfusion, the TNF- α levels continued to decrease, with group 3, 4 and 5 almost returning to the baseline levels. However, group 2 had significantly higher levels of TNF- α than groups 1, 3, 4, and 5. The differences between the baseline and 240th-minute TNF- α levels did not differ in groups 3, 4, and 5, but these groups still had higher levels than group 1.

The changes in TSA levels during the experiment for all of the groups are shown in Figure 3. When compared with the baseline levels, those at the 90th minute were significantly higher in all of the groups except for group 1. At this time point, the differences between all of the groups and group 1 were significant ($p < 0.001$, respectively), but there were no statistically significant differences between groups 2, 3, and 4.

At the 180th minute, the increases in the TSA levels in groups 2, 3, 4, and 5 were significantly different from the baseline levels and the levels at 90 minutes, and these increases were significantly different from group 1. The increase was once again the highest in group 2, and the TSA levels for groups 2 and 3 were significantly higher than for groups 4 and 5 ($p < 0.001$). Groups 4 and 5 again had similar levels.

A histological evaluation revealed significant differences in the degree of lung injury in the five groups. There were very advanced histological differences in the lung tissue samples of group 2

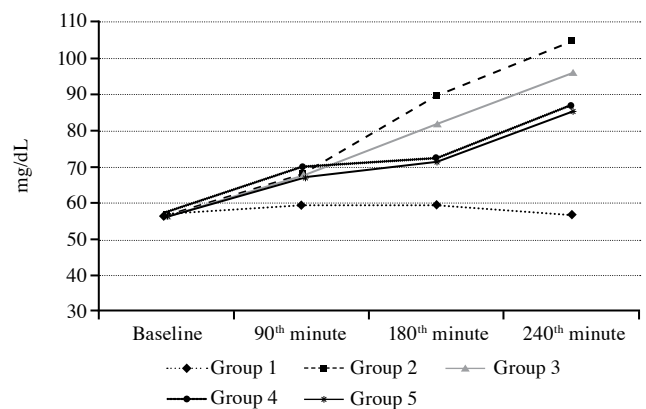


Figure 3. Total sialic acid levels (mg/dl) in the groups. Group 1: Control group; Group 2: Ischemia/reperfusion group; Group 3: Pentoxifylline group; Group 4: Iloprost group; Group 5: Iloprost + cilostazol group.

(the I/R group). Groups 1 and 5 had lesions ranging from 0 to 1+, with average injury scores of 0.65 and 0.87, respectively. Groups 3 and 4 exhibited extensive, prominent histological changes that ranged from 1+ to 2+, with average injury scores of 1.2 and 1.1, respectively, and group 2 had lesion scores ranging from 0 to 3, with an average injury score of 2.71. Although there was no difference between groups 3 and 4, the acute lung injury did significantly improve in group 5 ($p < 0.05$).

DISCUSSION

Many researchers have sought to prevent I/R injury via pharmacological interventions prior to ischemia or at the time of reperfusion,^[2,13,14] and recent studies have determined that pentoxifylline, iloprost, and cilostazole offer protective effects against this type of injury in various tissues and organs in animal experiments.^[4,11,14] Pentoxifylline has also been shown to dramatically reduce the production of cytokines, including TNF- α , and this may be related to its ability to increase c-AMP.^[2,4] Prostaglandins are agents that should be tested in clinical studies for the attenuation of the intensity of I/R injury, and PGI₂ and PGE₁ are especially known to induce vasodilatation, inhibit platelet and leukocyte aggregation, and exhibit anti-inflammatory activity, such as the suppression of TNF- α production. In addition, they probably have direct cytoprotective effects.^[1,3,8,14,15] To our knowledge, no studies in the literature have investigated whether the administration of pentoxifylline, iloprost, and cilostazol ameliorates lung I/R injury. In our study, pretreatment with these agents before ischemia attenuated reperfusion-induced lung injury, which is generally characterized by impaired proinflammatory cytokines.

Other studies have previously reported that aortic cross-clamping followed by reperfusion is associated with pulmonary injury and neutrophil activation, and our study came to the same conclusion.^[1-4] In addition, neutrophil activation has been implicated as one of the key mediators of distant organ damage and dysfunction caused by reperfusion injury. Furthermore, reperfusion of post-ischemic tissue initiates a systemic inflammatory response syndrome characterized by the production of proinflammatory mediators, neutrophil and monocyte activation, and hemodynamic and metabolic derangement.^[15-17] After ischemia, local tissue reperfusion generates a number of inflammatory mediators that activate circulating PMNs and cause remote endothelial damage. Proinflammatory cytokines, including TNF- α , IL-1, and IL-6, are crucial to the initiation and propagation of the inflammatory

response that leads to pulmonary injury^[3,18-20] since they mediate the bidirectional interaction between leukocytes and endothelial cells. Additionally, IL-1 and TNF- α modulate the extravasation of leukocytes and their localization at inflammatory sites. This involves the adhesion of the leukocytes to vessel walls and their passage through the endothelial lining in response to tissue-derived signals, which are associated with significantly increased levels of IL-6.^[17-19]

The concentration of IL-6, a pleiotropic cytokine, correlates well with the degree of systemic inflammation, severity of injury, and prognostic scores.^[2,3,19,21] We measured the levels of IL-6 because they indicate global inflammation and are connected with patient outcomes. The IL-6 levels have also been associated with the activation of circulating neutrophils and delayed apoptosis, thereby prolonging functional longevity and potential tissue injury. A reduced IL-6 response may also be representative of a general decrease in inflammation and cytokine production. In this study, pentoxifylline, iloprost, and cilostazol significantly diminished the IL-6 response to lower limb I/R.

In a previous report, we found elevated TNF- α levels early in the reperfusion period in a survival model of a rat with I/R injury,^[4] and TNF- α , an early proinflammatory mediator, partially controls the release of IL-6 and has been implicated in the development of acute lung injury. Two of the most frequently tested cytokines in acute inflammatory injury models are TNF- α and IL-6. These two proteins have a variety of proinflammatory activities that have led to both scientific and clinical investigations of their functional roles, including leukocyte chemotaxis, phagocyte stimulation, the enhancement of downstream cytokine and chemokine production, and variable effects on cell growth and death.^[16,19,22-24] Seekamp et al.^[3] reported that the plasma levels of TNF- α were undetectable after four hours of ischemia but saw an increase after reperfusion, with peak levels being detected after 60 minutes. However, our previous findings along with those in this study are consistent with those of Tassiopoulos et al.,^[2] who reported a significant increase in serum TNF- α levels during ischemia in a rat lower-torso I/R model. Furthermore, the increased TNF- α levels in our study paralleled the IL-6 levels, with the TNF- α levels rising significantly during the ischemia. They were also significantly above the baseline levels, even during the reperfusion period. Similar to the IL-6 levels, we found that iloprost and cilostazol were more successful at decreasing the TNF- α levels than

pentoxifylline, and Berkan et al.^[4] also detected no significant differences between the IL-6 and TNF- α groups in their study.

We hypothesized that reperfusion of the ischemic limbs would result in the release of a variety of proinflammatory mediators, including TNF- α and SA, an acetylated family of neurominic acid. The levels of SA correlate with the acute phase reactants that appear with acute inflammatory injury, and recent studies have identified a close relationship between TSA and cardiovascular diseases. Furthermore, a correlation has also been found between plasma TSA levels and acute phase reactants that appear in acute inflammatory conditions.^[4,5,25] Sialic acid, which carries a negative electrical charge, plays a major role in the preservation of cell membrane tension. This negative electrical charge is hemorheologically and hemodynamically important for red blood cell membranes. In addition, the changes in plasma SA levels reflect the changes in the integrity, permeability, and viability of red blood cells, which may cause cell injury and various other complications.^[4,26,27]

In the rats with I/R in this study, the increased TSA levels at the 90th minute continued to increase at the 180th minute and the proceeding reperfusion period. We had previously demonstrated that pentoxifylline lowered the TSA levels, and we were able to confirm this. Except for group 1, no difference was observed between the groups at the 90th minute, but the I/R group significantly differed from the other four groups at the 180th minute.

Conclusion

Ischemia/reperfusion of the lower limb is also associated with acute lung injury, which is characterized by increased proinflammatory activities. In this study, pretreatment with pentoxifylline, iloprost, and cilostazol significantly attenuated the proinflammatory activities and parenchymal lung damage, which could prove to be a novel approach for diminishing acute lung injury due to reperfusion damage in humans. However, large-scale, randomized, *in vivo* trials are still needed to confirm this theory.

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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