Oral L-arginine reduces lung injury following reperfusion after ischemia in the lower torso of rats

Oral L-arginin sıçanlarda alt ekstremitelerin iskemi/reperfüzyonu sonrası gelişen akciğer hasarını azaltır

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Background: Lung injury is known to occur after procedures that involve temporary occlusion of the abdominal aorta and subsequent ischemia/reperfusion (IR). In the present study, we evaluated the effect of oral administration of L-arginine in lung injury caused by lower extremity IR.

Methods: Forty rats were grouped as normal control group (n=10), sham group (n=10), IR injury (IRI) group (n=10) and L-arginine group (n=10). In the IRI and L-arginine groups, infrarenal aorta was cross-clamped for 3 hours followed by 2 hours of reperfusion; rats in L-arginine group received L-arginine monohydrochloride (2.25 mg/kg/day) in drinking water for 7 days prior the experiment. Arterial pH and pCO_2 were measured in blood before ischemia, after 90 and 180 minutes of ischemia, and 60 minutes after reperfusion. At the end of the experiment, the right lungs were removed and histologically examined for evidence of lung injury.

Results: There was no significant difference in lung injury score between the normal control and sham groups. IR resulted in a significant increase in lung injury scores in the IRI group and the lungs were less injured in the L-arginine group (3.0±0.6 and 1.5±0.4, respectively, p<0.05). In the blood gas analysis, the IRI group had lower pH than the L-arginine group in reperfusion period (7.28±0.02 and 7.33±0.03 respectively, p<0.05). There was no significant difference in the pCO_2 among all four groups.

Conclusion: These data indicate that transient infrarenal aortic occlusion with subsequent ischemia/reperfusion of the lower extremities caused a significant lung injury and that oral administration of L-arginine significantly reduced this injury.

Key words: Arginine; lung diseases/physiopathology; reperfusion injury/physiopathology.

Amaç: Abdominal aortanın geçici oklüzyonunu gerektiren işlemlerde iskemi/reperfüzyon (İR) sonrası akciğer hasarı geliştiği bilinmektedir. Bu çalışmada, alt ekstremitelerin iskemi reperfüzyonunun yol açtığı akciğer hasarına oral olarak verilen L-argininin etkisi değerlendirildi.

Çalışma planı: Kırk adet sıçan, normal kontrol grubu (n=10), sham grubu (n=10), İR grubu (n=10) ve L-arginin grubu (n=10) olarak gruplandırıldı. İskemi/reperfüzyon grubu ve L-arginin grubunda, infrarenal aorta üç saat süreyle klemplendikten sonra iki saat süreyle reperfüzyon uygulandı; L-arginin grubundaki sıçanlara deney öncesi yedi gün süreyle içme suyu ile L-arginin monohidroklorid (2.25 mg/kg/gün) verildi. İskemi öncesi, iskemiden 90 ve 180 dakika sonra ve reperfüzyondan 60 dakika sonra arter kanında pH ve pCO_2 ölçümleri yapıldı. Deney sonunda sağ akciğerler çıkarılarak histolojik olarak hasar derecesi değerlendirildi.

Bulgular: Normal kontrol ve sham gruplarının akciğer hasar dereceleri arasında anlamlı fark yoktu. İskemi/reperfüzyon grubunda akciğer hasar derecelerinde önemli artış saptanırken L-arginin grubunda hasar derecesi daha azdı (sırasıyla 3.0±0.6 ve 1.5±0.4, p<0.05). Kan gazı analizlerinde, reperfüzyon periyodunda İR grubu, L-arginin grubundan daha düşük pH değerlerine sahipti (sırasıyla 7.28±0.02 ve 7.33±0.03 p<0.05). Tüm gruplar arasında pCO_2 değerleri arasında anlamlı fark yoktu.

Sonuç: Elde edilen bulgular, infrarenal aortanın geçici oklüzyonu sonrası alt ekstremitelerin iskemi/reperfüzyonuna bağlı olarak gelişen akciğer hasarı, oral yolla verilen L-arginin ile azalmaktadır.

Anahtar sözcükler: Arginin; akciğer hastalığı/fizyopatoloji; reperfüzyon hasarı fizyopatoloji.

Extremity ischemia is an unavoidable clinical symptom during peripheral vascular surgery, aortic aneurysm surgery, re-implantation of extremities or during peripheral vascular injury. During ischemia, muscle cells cannot keep their membrane integrity and this causes releasing of calcium, phospholipid A_2 and formation of polyunsaturated fatty acids and fatty acid radicals. If the oxygenation is re-established at that stage of ischemia, fatty acid radicals react with oxygen and perform the lipid peroxidation reaction. This reaction increases the membrane permeability and also stimulates chemotaxis of leukocytes, which can release oxygen-derived free radicals and proteolytic enzymes when activated.[1]

Although the primary process is to recover the blood circulation of the ischemic limbs during resuscitation, there are strong clinical and experimental evidences that the reperfused ischemic tissues can induce pulmonary dysfunction.[2-4]

Prior experiments on ischemia/reperfusion (IR) have shown that leukocytes have an important role in the injury resulting from IR[5] and endothelial dysfunction with reduced release of nitric oxide (NO) occurs after IR. Nitric oxide is a simple, ubiquitous molecule in biological systems, which is a potent vasodilator, controls vascular tone, inhibits platelet aggregation and endothelial-neutrophil interaction.[6] Nitric oxide is formed as a by product of the conversion of L-arginine to L-citrulline by a group of enzymes called the nitric oxide synthases (NOS). NOSs can be broadly categorized into the constitutive isoforms (neuronal NOS and endothelial NOS), which are released at basal level under physiological conditions and an inducible isoform (iNOS), which is expressed when activated by pro-inflammatory cytokines.[7] Endothelial NOS (eNOS) is thought to be protective in early reperfusion,[8] while excessive iNOS has been shown to contribute to IR injury. Nitric oxide is also a source of the peroxinitrite radical, which may cause further tissue injury.[9]

The aim of this study was to investigate acute lung injury following infrarenal aortic cross-clamp induced IR injury. Specifically, we tested the hypothesis that oral administration of L-arginine would reduce lung injury after IR of lower extremities. Although many reports have demonstrated the protective effect of L-arginine supplementation in IR injury, we did not find any studies which specially investigated the effect of oral administration of L-arginine on the injured lungs following limb IR.

MATERIALS AND METHODS

The study was performed at the Experimental Animal Research Laboratory. All rats received human care in compliance with the European Convention on Animal Care.

Animals and grouping. Forty female Wistar Albino rats, weighing between 300-400 g, were employed in this study. The animals were randomly divided into 4 groups: normal control group (n=10), sham group (n=10), IR injury (IRI) group (n=10), L-arginine group (n=10). In the normal control group, animals were sacrificed to evaluate the normal lungs of rats without surgical procedures. In the sham group, animals were anesthetized and subjected to the surgical procedures, aorta was dissected into visibility without aortic occlusion. This group of animals was used for eliciting the effects of anaesthesia and operation on results. In the IRI group, infrarenal aorta was cross-clamped for 3 hours followed by 2 hours of reperfusion. In the L-arginine group, rats received L-arginine monohydrochloride (2.25 mg/kg/day) (Neksim Chemical, Turkey) in drinking water for 7 days prior to the aortic occlusion. The amount of daily ingestion of water and L-arginine was recorded. The dose of L-arginine was given according to previous studies[10,11] and the dose volume for individual animals was calculated based on the body weight measured just before dosing.

Experimental design. Animals were housed individually and allowed free access to standard rat chow and water. Anesthesia was administered at room temperature (20 °C) by 30 mg/kg intramuscular injection of ketamine hydrochloride (Ketalar, Pfizer, Turkey) and 6 mg/kg xylosine hydrochloride (Rompun, Bayer, Turkey) to the left anterior foot. During the surgical procedures, anesthesia was maintained with IM ketamine at every 30-45 minutes and body temperature was maintained with a water-filled heating pad. Carotid arterial catheter (24 gauge) was inserted for arterial blood sample and a jugular venous line (24 gauge) was established for intravenous fluid infusion through the same neck incision. The animals were then given heparin (1000 U/kg) via the right jugular vein. The abdominal aorta was exposed through a midline abdominal incision under aseptic conditions and the infrarenal aorta was cross-clamped for 3 hours followed by 2 hours of reperfusion. A bulldog clamp was used for the infrarenal aortic occlusion. Blood flow was verified by doppler ultrasound (Datascope doppler system 97) at the beginning and the end of the aortic occlusion. Abdominal contents were replaced and covered with a damp swab for the 3-hour period of cross-clamping and abdomen was resutured for the period of reperfusion.

Arterial blood samples were obtained for blood gas (BG) analysis. Blood samples (0.5 ml) were drawn and the blood was replaced with an equal volume of fluid. Blood pH and pCO_2 were measured before ischemia,

after 90 and 180 minutes of ischemia, and 60 minutes after reperfusion with BG analyzer (Medica, USA).

At the end of the experiment, a median sternotomy was performed and right lungs were harvested. The right lung samples were fixed with a 10% formaldehyde solution. The tissues were embedded in paraffin, sectioned in 6 μm thick slices, and stained with routine hematoxylin and eosin. The specimens were examined by light microscopy and evaluated by the same pathologist who was blinded to the study. A lung injury scoring system was used previously described by Tassiopoulos,[12,13] based on PMN infiltration, congestion, interstitial edema and airspace hemorhage, as follows: Grade 0: no changes; grade 1: focal, mild, subtle changes; grade 2: multifocal mild changes; grade 3:

multifocal prominent changes; and grade 4: extensive prominent changes (Fig 1a-e).

Statistical analysis. The parametric data was expressed as mean±standard deviation. Analytic results were evaluated using SPSS 11.5 statistical package program. Control variables were compared among groups with one-way analysis of variance (ANOVA) using with Tuckey's honestly significant difference test. A p-value of less than 0.05 was considered significant.

RESULTS

All animals have completed the study and there was no mortality. In hematoxylin and eosin stained sections, the lungs were normal in the normal control group. In the sham group, mean lung injury score was 0.5±0.5. In
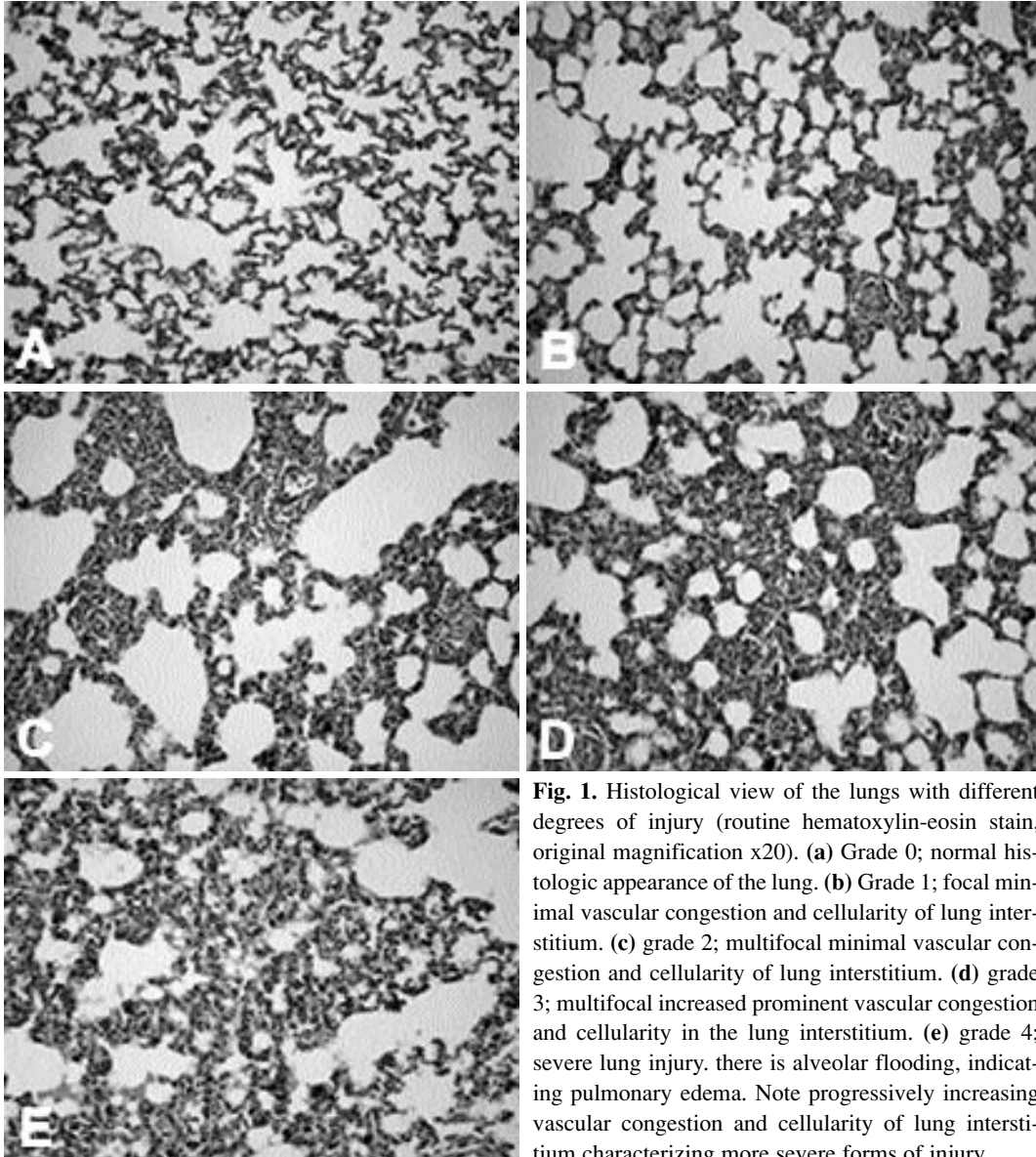

Fig. 1. Histological view of the lungs with different degrees of injury (routine hematoxylin-eosin stain, original magnification x20). **(a)** Grade 0; normal histologic appearance of the lung. **(b)** Grade 1; focal minimal vascular congestion and cellularity of lung interstitium. **(c)** grade 2; multifocal minimal vascular congestion and cellularity of lung interstitium. **(d)** grade 3; multifocal increased prominent vascular congestion and cellularity in the lung interstitium. **(e)** grade 4; severe lung injury. there is alveolar flooding, indicating pulmonary edema. Note progressively increasing vascular congestion and cellularity of lung interstitium characterizing more severe forms of injury.

the IRI group, the lung injury scores were significantly higher (mean 3.0±0.6) than in the normal control and sham group (p<0.05). However, in the group pretreated with L-arginine, the lungs became less injured with less inflammatory infiltration (mean 1.5±0.4) and when compared with IRI group there was a statistically significant difference (p<0.05).

The pH remained unchanged during the experiment in normal control and sham group. In contrast, the pH decreased significantly during ischemia and after reperfusion in IRI and L-arginine groups. However the IRI group had lower pH than the group pretreated L-arginine (respectively, 7.28±0.02 and 7.33±0.03, p<0.05). This was indicating a degree of metabolic acidosis (Fig. 2). The pCO_2 gradually decreased throughout ischemia in all four groups without any significant difference among them during the experiment (Fig. 3).

DISCUSSION

Tissue and cell injury can be aggravated by the reperfusion after the ischemia of organs and tissues.[14] A devastating consequence of tissue reperfusion is the damage in organs uninvolved in the initial ischemic insult.[15] In clinical settings, temporary ischemia of the lower extremities may result in shock and acute lung injury that requires inotropic and ventilatory support. It is still unclear, however, whether the injury process starts during ischemia or whether it is a "washout" phenomenon caused by the release of humoral mediators and activated polymorphonuclear neutrophills (PMNs) from the ischemic region to the systemic circulation during reperfusion.[12]

Nitric oxide, an endothelium derived relaxing factor, is produced from the semiessential amino acid L-argi-nine as it is converted to L-citrulline by NO synthase in endothelial cells.[16,17] The endogenous production of NO plays a vital role in the regulation of physiologic processes such as blood vessel tone, in host defense and immunity, as well as in the modulation of the inflammatory response.[18,19] Nitric oxide has been shown to be an endogenous inhibitor of leukocyte chemotaxis, adherence and activation.[18]

Many studies show that L-arginine alleviates reperfusion injury in kidney,[20] myocardium[21] and liver.[22] The present study suggests that the oral administration of L-arginine can protect rat lung after IR of lower extremities. The mechanisms have not been confirmed and may have several explanations. Firstly, NO may exert a protective effect as an indirect iron chelater by reacting with free coordination sites of iron forming iron-nitrosyl complexes, which may limit iron-dependent electron transfer reaction.[23] Secondly, NO can activate guanylate cyclase and subsequently induce cGMP-dependent effects. In the rats with IR injury, NO inhibits the neutrophils and platelets from aggregation and adhesion to endothelial cells, which is related to the increase of cGMP.[24] Thirdly, NO serves as a potent inhibitor of lipoprotein oxidation by annihilation of lipid radicals, thus terminating the free radical chain propagation reactions.[25] Fourthly, NO may divert the O_2^- mediated toxic reaction to other less toxic oxidation pathways and thus protect O_2^--sensitive target molecules. NO and O_2^- react to yield $ONOO^-$, reduce O_2^- and inhibit the accumulation and reaction of H_2O_2. This reaction is three times faster than the SOD-catalyzed dismutation of O_2^-.[26] And fifthly, NO may lead to the activation of gene expression of antioxidase or inhibition of transcription of proinflammatory mediators. For instance, activation of inducible NOS in human tracheal
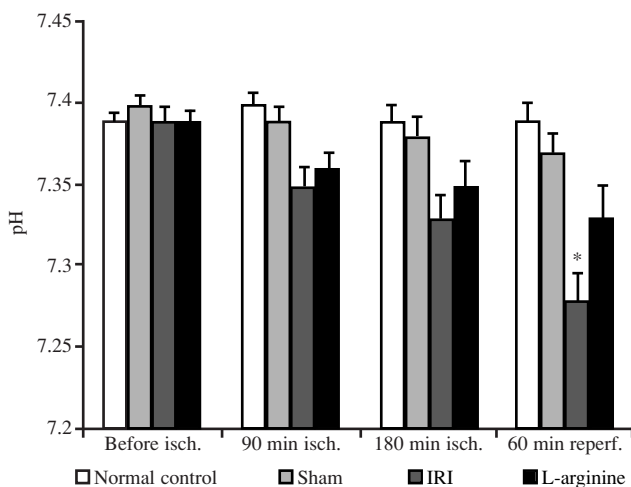

Fig. 2. pH changes during the experiment in the four groups. *p<.0.05 vs. normal control, sham and L-arginine groups. Isch., ischemia; reperf., reperfusion.
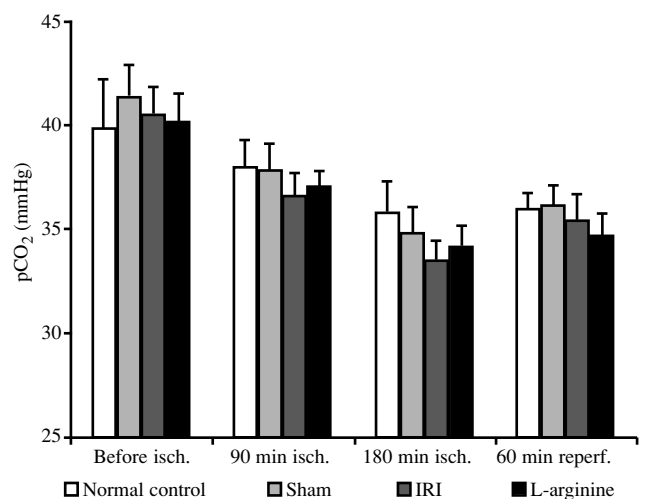

Fig. 3. pCO_2 changes during the experiment in the four groups. Isch., ischemia; reperf., reperfusion.

epithelial cells may induce transcription of c-fos.[27] NO may modulate the expression of adhesion molecules on the neutrophils and intercellular adhesion molecule-1 on endothelial cells to inhibit the neutrophil adhesion to endothelial cells and the neutrophil activation.

In conclusion, we have shown that IR of the lower extremities causes a significant lung injury. This injury can be attenuated by oral administration of L-arginine. Although the exact mechanism or mechanisms remain unclear, oral L-arginine pretreatment appears to be useful to decrease lung injury in elective peripheral vascular and aortic aneurysm surgery. But the clinical application should be studied further.

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