VASCULAR SURGERY

Vitamin C and iloprost attenuate skeletal muscle injury caused by ischemia-reperfusion of the lower extremities

Vitamin C ve iloprostun alt ekstremite iskemi reperfüzyonuna bağlı iskelet kası hasarında koruyucu etkisi

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Background: We evaluated the effect of iloprost and vitamin C on the reperfusion injury to the skeletal muscle, using an ischemic revascularized hindlimb model in rats.

Methods: Thirty-four Wistar albino rats were divided into five groups. Three groups were assigned to ischemic/reperfusion (I/R) injury via cross-clamping of the abdominal aorta just below the renal arteries for three hours, followed by one hour of reperfusion. Of these, one group (n=8) of rats received 100 mg/kg ascorbic acid via the left jugular vein before cross-clamping, while another group (n=8) received 20/ng/kg/min iloprost by constant intravenous infusion via the left jugular venous cannula during cross-clamping. In the sham group (n=6), the rats were anesthetized, a left jugular venous cannula was inserted and the abdomen was left open during the same period. In the control group (n=6), the soleus muscles were removed and blood samples were taken immediately after sternotomy without any further treatment.

Results: Compared to the I/R group, arterial blood pO_2 and HCO_3 levels were significantly high ($p<0.05$) and skeletal muscle malondialdehyde (MDA), plasma MDA, lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) levels were significantly low ($p<0.05$) in both the iloprost and vitamin C groups. Vitamin C-treated rats had significantly lower skeletal muscle MDA levels than those of the iloprost group ($p<0.05$). No significant differences were found between the iloprost and vitamin C groups with regard to pO_2, HCO_3, plasma MDA, LDH and CPK ($p>0.05$).

Conclusion: Ischemia and reperfusion of the lower extremity result in significant skeletal muscle injury. This injury can be attenuated by both vitamin C and iloprost pretreatment.

Key words: Ascorbic acid/therapeutic use; disease models, animal; iloprost/therapeutic use; ischemia; muscle, skeletal; peripheral vascular diseases; rats; reperfusion injury.

Amaç: Sıçanda uygulanan alt ekstremite iskemi-reperfüzyonuna (İ/R) bağlı gelişen iskelet kası hasarı üzerine iloprost ve C vitaminin etkisi değerlendirildi.

Çalışma planı: Otuz dört adet Wistar albino cinsi sıçan beş gruba ayrıldı. Bir grupta (I/R, n=6), renal arterlerin altından abdominal aorta kros-klemp kondu; üç saatlik iskemi süresini bir saatlik reperfüzyon izledi. Bir gruba (n=8), kros-klemp konmadan önce juguler ven yoluyla 100 mg/kg askorbik asit verildi. Bir başka gruba (n=8) kros-klemp süresince juguler ven yoluyla 20/ng/kg/min dozunda iloprost infüzyonu uygulandı. İloprost ve vitamin C gruplarında üç saatlik iskemi ve bir saatlik reperfüzyon uygulandı. Sham grubunda (n=6) sıçanlara anestezi uygulandı, batın ve juguler venöz hat açıldı; sıçanlar aynı süre boyunca izlendi. Kontrol grubunda (n=6), sternotomi sonrasında kan örnekleri alındı ve ardından soleus kasları çıkartıldı.

Bulgular: İloprost ve C vitamini alan gruplarda arteryel pO_2 ve HCO_3 düzeyleri I/R grubuna göre anlamlı derecede yüksek iken, iskelet kası malondialdehit (MDA), plazma MDA, laktik dehidrogenaz (LDH) ve kreatin fosfokinaz (CPK) seviyeleri düşüktü ($p<0.05$). Vitamin C grubundaki iskelet kası MDA düzeyi iloprost grubuna göre düşük bulundu ($p<0.05$). İloprost ve C vitamini alan gruplar arasında reperfüzyon sonrasında pO_2, HCO_3, plazma MDA, LDH ve CPK düzeyleri açısından anlamlı farklılık bulunmadı ($p>0.05$).

Sonuç: Alt ekstremite İ/R sonucunda iskelet kasında belirgin hasar meydana gelir. Bu hasar C vitamini ve iloprostun reperfüzyon fazından önce verilmesiyle azaltılabilir.

Anahtar sözcükler: Askorbik asit/terapötik kullanım; hastalık modeli, hayvan; iloprost/terapötik kullanım; iskemi; kas, iskelet; periferal vasküler hastalık; sıçan; reperfüzyon hasarı.

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Skeletal muscle injury is a known and feared complication of acute arterial occlusion during abdominal and peripherial vascular surgery. Re-establishment of blood flow can cause more extensive muscular damage than does ischemia.[1] After the reintroduction of oxygenated blood to ischemic tissues, reactive oxygen species (ROS) are released and neutrophils activated. This phenomenon is called as ischemia/reperfusion (IR) injury.[2] Released ROS and cytokines cause both local and remote tissue damage in a number of organs.

In experimental and clinical studies, many pharmacologic agents including antioxidants and vasodilators have been used to attenuate I/R injury. Iloprost is a long-acting stable analog of prostacyclin (PgI_2).[3] In these studies, the results of exogenously administered prostaglandins are inconclusive.[3,4]

Vitamin C is an endogenous antioxidant in scavenging ROS by very rapid electron transfer.[5] It has been shown that patients with peripheral vascular disease have decreased levels of endogenous antioxidants such as vitamin C, vitamin E, and carotenes.[6]

The pathophysiological processes of I/R injury are complex and unclear. It is related to the development of ROS-mediated lipid peroxidation, which can be measured through by-products like malondialdehyde (MDA).[7]

The objective of this study was to investigate the effects of iloprost and vitamin C on skeletal muscle after I/R of lower extremities through assessment of biochemical parameters including arterial blood pH, pO_2, pCO_2, HCO_3, plasma MDA, lactate dehydrogenase (LDH), creatine phosphokinase (CPK), and soleus muscle MDA.

MATERIALS AND METHODS

Thirty-four healthy Wistar albino rats, weighing 180 to 200 g, were used in this study. All the animals received humane care in compliance with the European Convention on Animal Care. The study was approved by the institutional ethic committee.

During surgical procedures, anesthesia was induced and then maintained with intramuscular (IM) injection of 30 mg/kg ketamine hydrochloride (Ketalar, Pfizer) and 2 mg/kg xylazine hydrochloride (Rompun, Bayer). Body temperature was maintained using a heating pad filled with water. Rectal temperature was monitored and maintained close to 38 °C under a warming light. A jugular venous line was established for intravenous infusion of fluids and drugs through a neck incision. The animals were then given heparin (1000 units/kg) via the right jugular vein.

The abdominal aorta was exposed through a midline abdominal incision just below the renal arteries. A microvascular bulldog clamp was used for aortic occlusion. Reperfusion was confirmed visually and by Doppler assessment in the femoral region.

The rats were divided into five groups. In the I/R group (n=6), the aorta was cross-clamped for about three hours followed by an hour of reperfusion. In vitamin C group (n=8), the animals were pretreated with 100 mg/kg ascorbic acid via the left jugular vein before aortic cross-clamping. In the iloprost group (n=8), the animals were pretreated with 20/ng/kg/min iloprost by constant intravenous infusion via the left jugular venous cannula. In the sham group (n=6), the abdomen was left open throughout the same period. In the control group (n=6), the kidneys were removed and blood samples were taken immediately after sternotomy without any treatment given. At the end of four hours, arterial blood pH, pO_2 (mmHg), pCO_2 (mmHg), HCO_3 (mmol/L), and plasma MDA (nmol/ml) values were determined. Biopsies were taken from the right soleus muscle to determine tissue MDA levels (nmol/g wet tissue). Blood Ph, pO_2, pCO_2, and HCO_3 values were determined on Medica Easystat blood gas analyzer. As an index of lipid peroxidation, plasma and tissue MDA levels were measured spectrophotometrically using thiobarbituric acid reaction as described by Yagi.[8] The principle of the method depends on the measurement of the pink color produced by interaction of the barbituric acid with MDA elaborated as a result of lipid peroxidation.

The results were presented as mean ± standard deviation. Statistical analyses were performed using the analysis of variance (ANOVA) test. A p value of less than 0.05 was considered statistically significant.

RESULTS

The mean values of pH, pO_2, pCO_2, and HCO_3, are presented in Table 1. Plasma and soleus muscle MDA levels are shown in Table 2, and plasma CPK and LDH levels are shown in Table 3.

At the end of the reperfusion period, arterial blood pO_2 and HCO_3 levels were significantly high ($p<0.05$); skeletal muscle MDA, plasma MDA, LDH and CPK levels were significantly low ($p<0.05$) in both the iloprost and vitamin C groups when compared to the IR group. In the vitamin C group, skeletal muscle MDA levels were significantly lower than the iloprost group ($p<0.05$). After the reperfusion period, no significant differences were found between the rats pretreated with iloprost and vitamin C with regard to pO_2, HCO_3, plasma MDA, LDH, and CPK ($p>0.05$).

DISCUSSION

Cross-clamping in the abdominal part of the aorta make the skeletal muscle vulnerable to ischemia. Chronic and acute ischemia in the lower extremities followed by

Table 1. The mean values of pH, pO_2, pCO_2, and HCO_3 in five groups

	pH	pO_2	pCO_2	HCO_3
Control	7.37±0.10*	98.33±3.44*	40.33±1.96	19.68±4.58*
Sham	7.32±0.08	97.50±4.23*	41.00±2.19	18.85±4.34*
I/R	7.18±5.49	71.00±11.15	42.83±1.72	8.15±1.90
Ilomedin	7.28±0.09	94.37±5.65*	41.50±1.60	15.78±3.45*
Vitamin C	7.25±0.09	90.75±5.72*	42.12±1.55	14.16±3.11*

*Significantly higher than the I/R group by ANOVA ($p<0.05$).

reperfusion is an important and common clinical event. Both clinical observations and animal experiments indicate that re-establishment of blood flow can save the lower extremities, but results in local and distant organ damage, even death.[9] Ischemia alone, even for prolonged periods of time, does not result in systemic injury in the absence of subsequent reperfusion.[10] Release of the aortic clamp is also important because sudden re-establishment of the circulation to the lower torso results in the generation of ROS and systemic vasoconstrictors, and neutrophil activation. These mediators cause both local and distant tissue damage in a number of organs including the kidneys and lungs.[2] Such I/R-induced injury is characterized by edema, impaired blood flow, muscle injury, and loss of muscle function.[11]

It is generally accepted that polymorphonuclear leukocytes (particularly neutrophils) and reactive oxygen metabolites are key mediators of reperfusion-induced injury in organs such as the skeletal muscle by modifying and/or distributing the structure and function of any cellular or noncellular component.[12]

Table 2. The mean levels of plasma and lung tissue malondialdehyde (MDA)

	Plasma MDA	Muscle MDA
Control	2.95±1.10*	36.20±8.43*
Sham	5.56±1.55*	41.76±4.45*
I/R	14.41±2.43	96.26±14.49
Ilomedin	11.35±2.10*	68.33±12.39*
Vitamin C	10.33±1.61*	56.08±7.37*

*Significantly lower than the I/R group by ANOVA ($p<0.05$).

Table 3. The mean levels of CPK and LDH

	CPK	LDH
Control	523.70±122.23*	410.49±134.17*
Sham	640.54±108.62*	585.26±39.08*
I/R	1349.63±219.69	1231.47±205.84
Ilomedin	860.96±104.89*	821.62±113.76*
Vitamin C	817.95±134.03*	738.11±185.81*

*Significantly lower than the I/R group by ANOVA ($p<0.05$). CPK: Creatine phosphokinase; LDH: Lactate dehydrogenase

Vitamin C is a highly effective scavenger of free oxygen radicals by very rapid electron transfer and has been used in clinic and experimental models to attenuate oxidant stress and I/R-mediated injury.[5,13] Patients with peripheral vascular disease have insufficient amounts of endogenous antioxidants such as vitamin C, vitamin E, and carotenes.[6] There is evidence for continuous oxidant injury in smokers with increased lipid peroxidation.[6] After supplementation with vitamin C, *in vivo* protection has been observed against this oxidant-mediated injury.[5] Lehr et al.[5] showed that vitamin C played a protective role for microvascular endothelium against neutrophil adhesions and, therefore, prevented injury to the microvascular circulation. Furthermore, Herbaczynska-Cedro et al.[14] demonstrated that vitamin C reduced the production of oxygen free-radicals in human circulating neutrophils.

PgI_2 is biosynthesized from arachidonic acid in endothelial cells, and is a powerful vasodilator as well as the most potent inhibitor of platelet aggregation known.[15] PgI_2 may be protective through different pharmacologic properties, including inhibition of platelet and leukocyte aggregation,[16,17] scavenging of free radicals,[18] and direct cytoprotection.[19] Iloprost is a synthetic prostacyclin analog (Schering AG, Berlin, Germany) which is ten times more potent with regard to thrombus prevention than natural prostacyclin.[20] Natural prostacyclin has a life of up to 30 minutes in the human body. After release from endothelial cells, it attempts to locate a receptor on either platelets, vascular muscle, or other cells. When attached to platelets, it inactivates them by elevating cyclic adenosine monophosphate (cAMP) levels.[21] The beneficial effect of prostacyclin on ischemia/reperfusion or otherwise inflammatorily activated tissues has been shown in several studies.[17,22] There is experimental evidence that iloprost attenuates tissue injury induced by ischemia.[23] Simpson et al.[24] demonstrated iloprost-induced inhibition of neutrophil function in a canine model.

We evaluated the effects of vitamin C and iloprost on skeletal muscle injury caused by IR injury to the lower extremities. Ischemia caused by nontraumatic vascular clamping was maintained for three hours, fol-

lowed by an hour of reperfusion. At the end of the reperfusion period, skeletal muscle injury was assessed by biochemical parameters. We found that the plasma and soleus muscle MDA levels in the control group were similar to those in the sham group, and were significantly lower than those in the IR group. Ascorbic acid or iloprost administration improved lipid peroxidation, which was shown to be associated with the lack of MDA overgeneration in plasma and soleus muscle. The exogenous administration of ascorbic acid and iloprost during ischemic period significantly protected the skeletal muscle from I/R damage in this experiment. This finding was consistent with clinical observations reported by Akar et al.[1] Lower MDA levels found in the iloprost group compared to the I/R group can be explained by such properties of iloprost including attenuation of neutrophil-mediated ROS formation, vasodilatation, and platelet disaggregation.[25]

It was also found that vitamin C and iloprost administrations were associated with higher plasma LDH and CPK levels compared to the I/R group. Additionally, a massive increase in tissue injury occurred in the I/R group, which was correlated with high plasma and skeletal muscle MDA levels.

Higher arterial pH, pO_2, HCO_3 levels in the vitamin C and iloprost groups also suggested the preservation of pulmonary functions. Similarly, Bozkurt also showed that pO_2 levels were lower in the IR groups when compared to the iloprost groups, which was related to the lung damage caused by IR injury to the skeletal muscle.[26]

In conclusion, our findings demonstrate that vitamin C pretreatment before, or iloprost infusion during, the clamping of the abdominal aorta decrease I/R-related skeletal muscle injury in the lower extremities. Moreover, we believe that, administration of these agents before abdominal aortic or peripheral vascular surgery may play a preventive role in postoperative pulmonary dysfunctions.

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