Iloprost and pentoxifylline attenuate ischemia-reperfusion injury in skeletal muscle in rabbit model

İloprost ve pentoksifilin tavşan modelinde iskelet kasındaki iskemi-reperfüzyon hasarını hafifletir

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BACKGROUND

We investigated the effects of iloprost and pentoxifylline on skeletal muscle ischemia-reperfusion injury in a rabbit model.

METHODS

Forty New Zealand white rabbits were grouped into four. In Group 1, iloprost was continuously infused starting half an hour before the reperfusion following a 2-hour ischemia formed by abdominal aortic occlusion, and it was continued during the 4-hour reperfusion period. Group 2 was treated with pentoxifylline, and Group 3 received saline solution. Group 4 was the sham group. Malondialdehyde levels and edema scores in gastrocnemius muscle were evaluated.

RESULTS

Edema score was significantly lower in Group 1 when compared with the control group (Group 1 vs Group 3, p=0.040; Group 2 vs Group 3, p=0.145; Group 1 vs Group 2, p=0.580). Malondialdehyde levels of the medicated groups were significantly lower when compared with the control group (Group 1: 60±11 nmol/g tissue, Group 2: 74±11 nmol/g tissue, Group 3: 95±10 nmol/g tissue; Group 1 vs Group 2, p=0.010; Group 1 vs Group 3, p<0.001; Group 2 vs Group 3, p<0.001; Group 1 vs Group 4, p<0.001; Group 2 vs Group 4, p<0.001; Group 3 vs Group 4: p<0.001).

CONCLUSION

Acute skeletal muscle ischemia is a common problem. We are of the opinion that in the early phase of skeletal muscle ischemia, iloprost and pentoxifylline medication may reduce ischemia-reperfusion injury.

Key Words: Cardiovascular agents; iloprost; ischemia-reperfusion injury/drug therapy; pentoxifylline; rabbit skeletal muscle.

AMAÇ

Bu çalışmada, iloprost ve pentoksifilinin tavşan iskelet kasında iskemi reperfüzyon (IR) hasarı üzerine etkileri araştırıldı.

GEREÇ VE YÖNTEM

Kırk adet Yeni Zelanda cinsi beyaz tavşan her grupta 10 adet olacak şekilde dört gruba ayrıldı. Grup 1'de iloprost iki saat abdominal aort ve iliyak dallarının klemplenmesiyle oluşturulan iskemiyi takiben dört saatlik reperfüzyon boyunca infüzyon olarak verildi. Grup 2'ye pentoksifilin, Grup 3'e ise serum fizyolojik verildi. Grup 4 sham grubu idi. Gastroknemyus kasından malondialdehit düzeyi ve mikroskopik ödem skoru incelendi.

BULGULAR

Grup 1'de kontrol grubuna göre mikroskoppik ödem skorlamasında anlamlı bir düşüklük saptandı (Grup 1 vs Grup 3, p=0,040; Grup 2 vs Grup 3, p=0,145; Grup 1 vs Grup 2, p=0,580). Malondialdehit düzeyleri bakımından Grup 1 ve Grup 2'de kontrol grubuna kıyasla anlamlı bir düşüklük saptandı (Grup 1: 60±11 nmol/gr, Grup 2: 74±11 nmol/gr, Grup 3: 95±10 nmol/gr, Grup 4: 29±7 nmol/gr) (Grup 1 vs Grup 2, p=0,010; Grup 1 vs Grup 3, p<0,001; Grup 2 vs Grup 3, p<0,001; Grup 1 vs Grup 4, p<0,001; Grup 2 vs Grup 4, p<0,001; Grup 3 vs Grup 4: p<0,001).

SONUÇ

İskelet kası akut iskemisi sık bir klinik sorundur, erken dönemde iloprost ve pentoksifilin tedavisinin IR hasarını azaltabileceği görüşündeyiz.

Anahtar Sözcükler: İloprost; iskemi reperfüzyon hasarı/ilaç tedavisi; kalp-damar ilaçları; pentoksifilin, tavşan iskelet kası.

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Restoration of blood flow to an acutely ischemic limb generates cellular and biochemical mediators that may generate oxygen-derived free radicals at the time of perfusion, which initiates reperfusion injury. Many studies have been performed concerning the effect of iloprost (IL), a stable prostacyclin analog, on reducing skeletal muscle ischemic injury.[1,2] IL was shown to decrease neutrophil activation and aggregation in addition to inhibition of oxygen-free radical production and release of lysosomal enzymes.[3,4]

Pentoxifylline (Ptx) was proven to attenuate reperfusion-associated membrane injury and tissue edema, to suppress leukocyte adhesion and to improve hindlimb blood flow during the reperfusion period.[5] It was also shown to improve ischemia-reperfusion (I/R) injury by attenuating neutrophil sequestration, production of reactive oxygen species, and platelet activation.[6]

In the current experimental study, we investigated the effects of IL and Ptx on skeletal muscle I/R injury in a rabbit model and compared the effects with the control groups. The primary hypothesis was that IL could provide better protection than Ptx on skeletal muscle I/R injury in a rabbit model.

MATERIALS AND METHODS

The experiment was done in accordance with the ethics of Medicine Faculty of Pamukkale University. All animals received humane care in compliance with Principles of Laboratory Animal Care, formulated by the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences (Institute of Laboratory Animal Resources, National Research Council. Guide for the Care and Use of Laboratory Animals. Washington DC: National Academy Press, 1996).

Forty New Zealand white rabbits of either sex weighing 2-2.5 kg were included into the study. The rabbits were randomized into four equal groups. Group 1 was treated with IL during the reperfusion after the ischemic period whereas Group 2 was treated with Ptx. Group 3 was the control group and did not receive any medication. Group 4 was the sham group from which normal muscle tissues were gathered. The study was funded by the authors.

The superficial veins on the ears of the rabbits were cannulated for venous line. The rabbits were sedated by intravenous ketamine injection with a dose of 30/kg, and anesthetized by intraperitoneal pentobarbital sodium (50 mg/kg), followed by intraperitoneal supplements (15 mg/kg) as required. The room temperature was kept between 28-30 °C. After midline laparotomy and heparin (400 U/kg) injection, the infrarenal abdominal aorta and the major branches were occluded by atraumatic bulldog clamps (Fig. 1). The laparotomy incisions were closed thereafter. The medications were started half an hour before the reperfusion so that they would be present in the blood at the start of the reperfusion period. This nearly simulates the normal human condition when a patient is admitted to an emergency clinic and undergoes a revascularization procedure. Continuous intravenous infusion of IL (2 ng/kg/min) was started in Group 1 animals half an hour before the reperfusion. The selected IL dose was the maximal dose for humans as indicated in the drug prospectus. The animals in Group 2 received intravenous bolus of Ptx (30 mg/kg) half an hour before the reperfusion, followed by continuous infusion at a rate of 0.1 mg/kg/min throughout the rest of ischemia.[5,7] Control animals received only normal saline solution. After 2-hour ischemia,[2] the aortas and major branches of the aorta of the animals in all groups were declamped and 4 hours of reperfusion was performed.[8] IL and Ptx infusions were maintained during the reperfusion period. After 4 hours of reperfusion, gastrocnemius muscle biopsies were taken from the left cruris of the animals. Normal muscle tissue materials without any

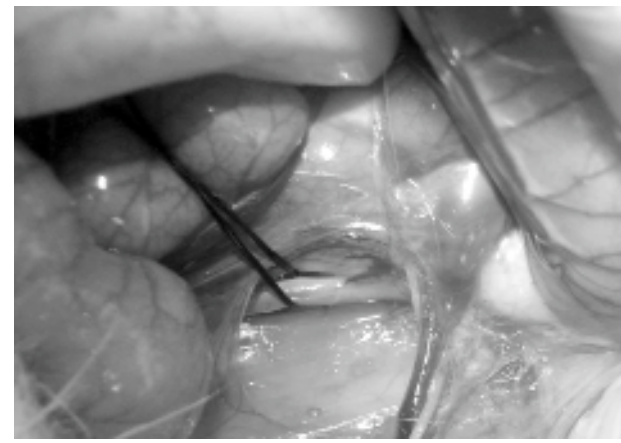

Fig. 1. Exploration of abdominal aorta and the major branches. The infrarenal abdominal aorta is seen proximally controlled by a silk suture.

medication or ischemia were taken from Group 4 for histologic and biochemical evaluation. All of the animals survived throughout the experiment. The animals were sacrificed after taking muscle biopsies.

Portions of the fixed muscles were examined microscopically (Nikon model Eclipse E600W). The specimens were fixed in 10% formalin. Paraffin blocks were cut at 5 µm and stained with hematoxylin-eosin. Histologically, reperfusion injury was considered in the presence of increased interstitial edema separating the muscle fibers.[2] This edema was scored by our pathologist according to the criteria hereunder. Muscle edema was evaluated and scored by the same pathologist, who was blinded to the study. Muscle edema was scored as:

Grade 0: No edema between the muscle fibers.

Grade 1: Mild-focal interstitial edema between the muscle fibers. The edema existed in few fields.

Grade 2: Moderate-intense focal interstitial edema between the muscle fibers. The edema existed in most of the microscopic fields.

Grade 3: Severe-intense diffuse interstitial edema between the muscle fibers. Edema existed in every microscopic field.

MDA is the end product of lipid peroxidation due to increased formation of free radicals in tissues. MDA levels were assessed according to the method described by Uchiama.[9] Muscle tissue samples were homogenized in ice cold 150 mmol KCl for the determination of MDA. MDA levels were used as an estimate of lipid peroxidation. Results were expressed as nmol MDA/g tissue.

Statistical analysis was done with SPSS 10.0 statistical software program (SPSS Inc, Chicago, IL). Tissue edema was classified as grade 0, 1, 2, or 3. Differences between the experimental and control groups were analyzed for their statistical significance using the chi-square test. Continuous variables were expressed as the mean ± 1 SD and evaluated with Mann-Whitney U-test. The p values less than 0.05 were considered as statistically significant.

RESULTS

Histologic evidence of reperfusion injury was the presence of increased interstitial edema separating the muscle fibers. Edema scores were significantly different between the groups ($p<0.001$). Edema scores were significantly lower in Group 1 when compared with the control group (Group 1 vs Group 3, $p=0.040$); however, edema scores in Group 2 were not statistically different (Group 2 vs Group 3, $p=0.145$). When the edema scores were compared between the IL and Ptx groups, there was no significant difference ($p=0.580$; NS) (Table 1).

Two animals were assessed as normal (grade 0: no edema) in Group 1, whereas no animal had grade 0 edema in the control group (Fig. 2). Three animals presented grade 3 edema in the control group, whereas there were no animals with grade 3 edema in the IL- and Ptx-treated groups (Fig. 3). The mean histologic score of Group 4 was significantly lower than in the other groups.

The MDA levels of the medicated groups were significantly lower when compared with the control group. Mean MDA levels were 60±11 nmol/g tissue in Group 1, 74±11 nmol/g tissue in Group 2, and 95±10 nmol/g tissue in Group 3 (Group 1 vs Group 2, $p=0.010$; Group 1 vs Group 3, $p<0.001$; Group 2 vs Group 3, $p<0.001$). The mean MDA level of Group 2 was higher than that of Group 1. Mean MDA level of the sham group was 29±7 nmol/g tissue and significantly lower than in the other three groups as well (Group 1 vs Group 4, $p<0.001$; Group 2 vs Group 4, $p<0.001$; Group 3 vs Group 4, $p<0.001$).

Table 1. Edema scores of the groups

	Grade 0	Grade 1	Grade 2	Grade 3
Group 1 (IL) (n=10)	2	6	2	
Group 2 (Ptx) (n=10)	1	5	4	
Group 3 (Control) (n=10)		2	5	3
Group 4 (Sham) (n=10)	8	2		

$p<0.001$ between the groups; Group 1 vs Group 3, $p=0.040$; Group 2 vs Group 3, $p=0.145$; Group 1 vs Group 2, $p=0.580$; Group 1 vs Group 4, $p=0.022$; Group 2 vs Group 4, $p=0.005$; Group 3 vs Group 4, $p=0.001$.

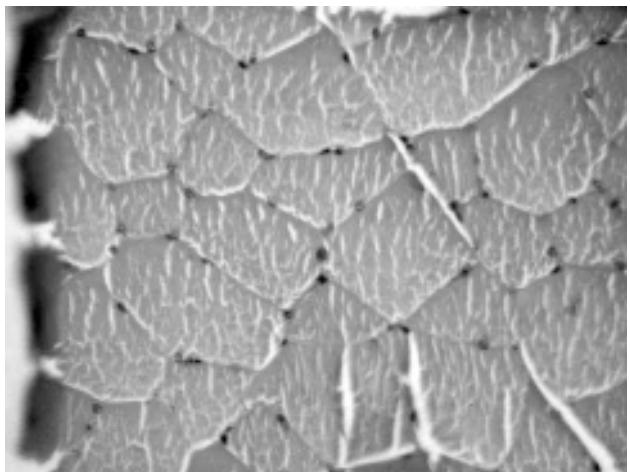

Fig. 2. Grade 0 edema: The nuclei of the cells are located around the muscle fibers. No edema between the muscle fibers (H-E x 200).

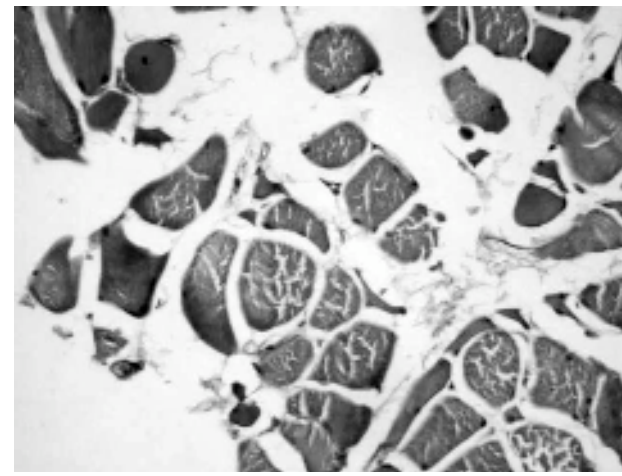

Fig. 3. Grade 3 edema: Severe-intense diffuse interstitial edema between the muscle fibers (H-E x 100).

DISCUSSION

The present study proved on a biochemical basis that Ptx and IL significantly reduced I/R injury. The primary postulation was not proven on comparison of the medicated groups but it was proven when the medicated groups were compared separately with the control group. On light microscopic basis, the Ptx group was statistically similar to the control group although the medians were different (median of histopathologic scores: Group 2=1, Group 3=2). On the other hand, the IL group showed significantly less edema when compared with the control group. However, IL did not show a significant difference when compared with Ptx on a histologic basis. Nevertheless, IL decreased MDA production to a greater extent than Ptx. There are few reports concerning the effects of IL and Ptx on skeletal muscle I/R injury. Some investigations have been made in efforts to decrease the I/R injury in skeletal muscle using free radical scavengers such as superoxide dismutase, catalase, and mannitol, and they have been reported to have therapeutic efficacy in experimental models of skeletal muscle ischemia.[10,11]

Reperfusion injury occurs during the initial period of reperfusion as highly active oxygen metabolites such as the superoxide and hydroxyl radicals accumulate and increase the cellular damage. This reaction increases membrane permeability and stimulates chemotaxis of leukocytes, which can release oxygen-derived free radicals and proteolytic enzymes when activated. Therefore, ischemic cell injury is worsened by reperfusion.[12] Reactive oxygen metabolites were found to contribute to the onset of microvascular perfusion deficits associated with the early reperfusion phase after 2 hours of normothermic no-flow ischemia of rat skeletal muscle.[13] This was simulated in our experiment by giving the medications in the early phase of reperfusion. Therefore, the experiment may be said to mimic the normal I/R process in acute ischemic limbs.

Prostacyclin has been shown to protect the myocardium against I/R injury in a canine model.[4] Possible potential properties of IL in the myocardium and skeletal muscle I/R models are membrane stabilization, decreased myocardial enzyme release and decreased release of membrane, and inhibition of neutrophil function, which are potential mediators of I/R injury in both cardiac and skeletal muscle. Neutrophils may cause local injury by the formation of oxygen-derived free radicals and the release of lysosomal enzymes. Neutrophil infiltration and activation, intracytosol calcium influx, complement activation, and generation of oxygen-free radicals are associated with reperfusion syndrome. Prostacyclin and IL were shown to be effective in reducing both experimental myocardial and skeletal infarct size.[14] IL has also been shown to decrease white blood cell aggregation and adhesion to vascular endothelium, to decrease superoxide radical production from stimulated canine and human neutrophils, and to decrease free radical for-

mation in the myocardium subjected to I/R injury. IL infusion begun before the ischemic injury was thought to reduce the effects of I/R injury.[1]

Skeletal muscle ischemia is associated with an increase in vascular permeability, which is characterized by the swollen and edematous muscle after unrelieved acute ischemia in the lower extremity. Microvascular permeability has been proven as a marker of skeletal muscle injury after I/R. Blebea and associates reported increased separation between muscle fiber bundles reflecting the presence of microscopic interstitial edema by light microscopy and concluded that increase in microvascular permeability was an indicator of I/R injury.[2] IL treatment was also shown to decrease plasma levels of MDA in I/R and this was thought to indicate a reduction in lipid peroxidation and cellular injury.[15] The present study demonstrated increased interstitial edema in I/R groups. The edema occurring in the IL group was less than in the control group, meaning that IL decreased reperfusion-induced edema and protected the muscle from I/R injury. On the other hand, this was not the same as in the Ptx group, which showed statistically similar edema with that of the control group. The analysis of MDA revealed a marked rise in lipid peroxidation in skeletal muscle in the control group. Furthermore, the MDA levels in the IL-treated group were significantly lower than in the Ptx group. It can be concluded that IL decreased the lipid peroxidation after I/R injury.

Belkin et al. reported that skeletal muscle ischemic injury was significantly reduced, from 57% to 16%, when pretreated with IL1. Blebea showed that IL might plateau by 2 hours of reperfusion due to an early beneficial effect on vascular permeability by closure of endothelial gap junctions. Furthermore, IL infusion was thought to counteract the aggregation of platelets and the vasoconstrictive effects of thromboxane that may lead to increased vascular permeability.[2] Pretreatment with IL before the onset of ischemia and continuation into the reperfusion phase was thought to decrease muscle infarct size and decrease the rise in vascular permeability.[1,2] However, IL given only during reperfusion did not significantly reduce the infarct size.[14] This was also simulated in our study by starting the medication 30 minutes before the initiation of reperfusion.

In the present study, we proved that IL and Ptx reduced the interstitial edema in I/R when compared with the untreated control group. Microscopically, neither Ptx nor IL was superior to the other. However, IL significantly decreased the lipid peroxidation when compared with Ptx. Another point is that IL showed better protection from I/R injury when compared with the control group.

These two agents have been studied in a renal I/R model,[16] but this is the only study that compares the effects of both IL and Ptx on skeletal muscle I/R injury. Ischemia in skeletal muscle, particularly in the lower extremities, is a frequent clinical problem and occurs as a consequence of trauma, hemorrhage, vascular stenosis, prolonged tourniquet application, traumatic amputations and thromboembolic events. We think that IL and Ptx treatment in the ischemic and early reperfusion periods can improve muscle viability.

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