

The effects of iloprost and alprostadil on ischemia-reperfusion injury in preventing inflammation, tissue degeneration, and apoptosis in rat skeletal muscle

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Background/aim: The protective effects of prostaglandin (PG) analogs on ischemia-reperfusion (I/R) have been well documented; however, comparative studies are lacking. The aim of the present study was to determine whether iloprost or alprostadil is more effective in preventing muscle I/R injury.

Materials and methods: Thirty-two rats were divided into four groups (n = 8): sham, control, IL (I/R + iloprost), and AL (I/R + alprostadil). I/R was induced by a tourniquet in the hindlimb for 3 h/3 h. The IL and AL groups received iloprost (0.5 ng kg⁻¹ min⁻¹) and alprostadil (0.05 µg kg⁻¹ min⁻¹) during reperfusion, respectively. After 6 h, blood and muscles were collected for analyses.

Results: Serum TNF-α and IL-1β levels were decreased in the IL and AL groups compared with the control group (P < 0.05), whereas IL-6 levels did not change significantly. Tissue malondialdehyde levels were significantly lower in the IL and AL groups (P < 0.05). Tissue catalase levels showed no difference. The histological damage scores and apoptosis scores were both significantly decreased in the IL and AL groups compared with the control group (P < 0.05).

Conclusion: The present study indicated that iloprost and alprostadil attenuated I/R injury in skeletal muscle. However, no comparable difference was evident regarding the efficacies of either PG analog.

Key words: Ischemia, reperfusion injury, iloprost, alprostadil, skeletal, muscle

1. Introduction

While reestablishing circulation to tissues in an ischemic state may contribute to their survival, it may also cause simultaneous damage during reperfusion. This phenomenon is defined as ischemia-reperfusion (I/R) injury. I/R injury in skeletal muscle occurs during revascularization involving the extremities as a result of both local and systemic inflammatory responses. Endothelial cells and leukocytes produce chemotactic cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) following reperfusion (1). Hypoxic insult and subsequent reperfusion activate a complex cascade of events involving radical oxygen species (ROS) production, leukocyte chemotaxis and adhesion, platelet-leukocyte aggregation, and increased permeability of microvessels (2). Reestablishing reperfusion following a long period of ischemia may pose

clinical problems following the revascularization of an ischemic limb and has a negative impact on both morbidity and mortality as it can necessitate limb amputation and cause distant organ injury-related damage (3).

Protective responses are also activated during the pathophysiological processes associated with I/R injury. The release of antioxidant enzymes (catalase, superoxide dismutase, and nitric oxide synthase) and local hormones such as prostaglandins (PGs) serves as a natural protective mechanism against I/R injury (4,5). PGI₂ and PGE₁ are end-products of arachidonic acid metabolism and are synthesized primarily by endothelial cells. They inhibit aggregation of platelets, chemotaxis of inflammatory cells, and production of ROS (4). Moreover, PGs are potent vasodilators and regulate microvascular permeability (5). They are also useful in maintaining tissue microcirculation by reducing the levels of inflammatory cytokines (TNF-α,

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IL-1 β , and IL-6) (1). Although the effects of the two are similar, it is thought that PGE₁ is more potent than PGI₂ (6). This difference in modulation of the inflammatory response depends on different prostanoid receptors of individual PG mediators (5).

Iloprost and alprostadil are synthetic analogs of PGI₂ and PGE₁, respectively. Several studies have been reported concerning the effect of iloprost in the setting of skeletal muscle ischemic injury (4,7–12). However, less is known regarding the protective effects of alprostadil on skeletal muscle following I/R injury (13–15). Comparative studies regarding the effects of PGs are more scarce in the literature (6,16). Only one recent study compared the effects of iloprost and alprostadil on I/R injury (17).

The aim of the present study is to compare the potential effects of iloprost and alprostadil on the serum levels of inflammatory cytokines and to explain the logic of PG use in the prevention of tissue damage and apoptosis in the setting of acute I/R injury in skeletal muscle.

2. Materials and methods

All research procedures were approved by the animal care committee of Eskişehir Osmangazi University (Protocol #325-2/2014) and complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All experiments were conducted at the Center for Medical and Surgical Research (TICAM), Eskişehir, Turkey. Until the day of the experiments, the animals had unlimited access to standard food pellets and tap water.

Iloprost (Ilomedin, 20 μ g/mL) and alprostadil (Alprostadil, 20 μ g/mL) were purchased from Bayer Inc. (Berlin, Germany) and VEM İlaç Sanayi Ltd. (İstanbul, Turkey), respectively. The ELISA kits for TNF- α (Invitrogen, Catalog #KRC3011) and IL-6 (Invitrogen, Catalog #KRC0061) were purchased from Invitrogen Corp. (Camarillo, CA, USA), and that for IL-1 β (Platinum ELISA, Catalog #BMS630) was purchased from Bender MedSystems GmbH (Vienna, Austria). Malondialdehyde (MDA) and catalase assays were performed in the Department of Biochemistry. Hematoxylin and eosin and caspase-3 stainings were performed by Merter Medical Laboratory Services LTD (Eskişehir, Turkey).

Thirty-two adult female Sprague Dawley rats weighing 250–350 g were used in the study. The animals were divided randomly into the following four groups (n = 8 each): sham, control, I/R + iloprost (IL), and I/R + alprostadil (AL) groups. The sham group was not exposed to I/R injury and received no treatment. I/R injury was induced in the control group without treatment. Both the IL and AL groups were subjected to I/R injury and received the appropriate drugs. The dosages of iloprost and alprostadil were based on those used in previous studies (7,8).

2.1. Experimental procedures

The rats were anesthetized with sodium thiopental (40–50 mg/kg Pental Sodium, İE Ulagay, İstanbul, Turkey) via intraperitoneal injection. Before beginning the experimental procedures, 26-gauge polyethylene catheters were inserted into the right jugular veins for the administration of a drug or vehicle (0.9% NaCl). Complete ischemia of the right hindlimb was induced by placing a tourniquet around the proximal femur in the control, IL, and AL groups. Following 3 h of ischemia, the tourniquets were released, and 3 h was allotted for the reperfusion of the affected limbs. In the IL and AL groups, iloprost (0.5 ng kg⁻¹ min⁻¹) and alprostadil (0.05 μ g kg⁻¹ min⁻¹) were administered via jugular catheters beginning 20 min before tourniquet release and maintained during the reperfusion period. The sham and control groups received equal amounts of vehicle solution. At the end of the reperfusion period, the gastrocnemius muscles were excised and cut into three pieces for tissue MDA and catalase analyses, as well as histopathologic examinations. The animals were subsequently euthanized via high-volume exsanguination from the heart. The obtained blood samples were reserved for IL-1 β , IL-6, and TNF- α assays after the serum was separated via centrifugation at 4000 rpm for 10 min. The serum and two pieces of muscle were stored at -80 °C until used for measurements. The last muscle piece was preserved in a 10% formaldehyde solution.

2.2. Biochemical analysis

Concentrations of immunoreactive TNF- α , IL-1 β , and IL-6 were determined using ELISA kits according to the manufacturer's instructions. Briefly, the serum was reacted with the assay reagents in the TNF- α , IL-1 β , and IL-6 kits and then analyzed spectrophotometrically using a VICTOR X3 Multilabel Plate Reader (PerkinElmer Inc., Waltham, MA, USA) at an absorbance of 450 nm. The levels of cytokines were calculated using the kit standards and expressed as pg/mL.

MDA was assayed via a thiobarbituric acid reaction according to the method devised by Ohkawa et al. (18). The tissues were homogenized in ice-cold 0.15 mM KCl buffer using a homogenizer (Ultra Turrax 125-Janke Kunkol; IKA, Staufen, Germany) and centrifuged at 1000 rpm for 15 min. Equal amounts of supernatant and 0.5% thiobarbituric acid (w/v) were mixed in 20% trichloroacetic acid and allowed to react. The absorbance of the pink-colored thiobarbituric acid reactive substances was measured using a spectrophotometer (UV-1601; Shimadzu, Tokyo, Japan) at 532 nm. The levels of tissue MDA were expressed as nmol/mg.

Catalase was assayed using the Beutler method (19). After the tissues were homogenized in ice-cold 1 M phosphate buffer (pH 7) and centrifuged at 600 rpm for 10 min, enzymatic activity based on H₂O₂ degradation was

measured using a spectrophotometer at an absorbance of 230 nM. The level of tissue catalase was expressed as U/mg.

2.3. Histopathological examinations

The muscle specimens placed in 10% formalin were routinely processed following paraffin embedding. The sections were sliced at a thickness of 5 μ m, stained with hematoxylin and eosin (H&E), and examined under a light microscope with a computer connection (Olympus BX-50F4, Tokyo, Japan) by a blinded histologist. The histologic damage in each muscle sample was evaluated quantitatively in ten visual fields using the following parameters: muscle fiber degeneration, nuclear centralization, infiltration of the inflammatory cells, vasocongestion, and disorganization. The damage was estimated by summing of the parameters (0 as normal; 1 as mild; 2 as moderate; and 3 as severe). The lowest score was 0 and the highest score was 15.

The paraffin blocks underwent additional processing for immunohistochemical evaluation of caspase-3 expression. Histologic sections of 6 μ m in thickness were stained using the primary cleaved anticaspase-3 (RB-1197-P1) antibody and antirabbit antibody (Thermo Scientific, Fremont, CA, USA) according to the manufacturer's guidelines. The caspase-3 expression was quantified in a blinded fashion. Brown-stained myocyte nuclei indicated apoptosis. The numbers of caspase-positive nuclei per field were counted to determine the score across ten fields of each muscle section among all study groups.

2.4. Statistical analysis

The data were analyzed using IBM SPSS 19.0 (IBM Corp., Armonk, NY, USA). The normal distribution of continuous variables was assessed by the Shapiro-Wilk test of normality for compliance within each group. The normally distributed variables were evaluated by one-way analysis of variance (ANOVA). The nonparametric Kruskal-Wallis test with the Tukey multiple comparison

test was used for comparisons between the groups due to the lack of a normal data distribution. The values were expressed as means \pm standard deviations. The significance level was set at either $P < 0.01$ or $P < 0.05$.

3. Results

3.1. Biochemical results

The serum levels of TNF- α and IL-1 β were significantly higher in the control group compared with the sham, IL, and AL groups ($P < 0.05$). The differences in the TNF- α and IL-1 β levels between the IL and AL groups were not significant. The serum IL-6 level was significantly higher in the control group compared with the sham group ($P < 0.05$). However, there was no significant difference in the IL-6 levels between the IL and AL groups compared with the control group (Table 1).

The tissue MDA levels were significantly lower in the sham (5.33 ± 1.33 nmol/mg), IL (7.21 ± 1.66 nmol/mg), and AL (6.16 ± 1.31 nmol/mg) groups compared with the control group (10.63 ± 3.66 nmol/mg) ($P < 0.05$). There was also a significant difference in MDA levels when the sham group was compared with the IL and AL groups ($P < 0.05$). However, no significant difference was found in the levels of MDA between the IL and AL groups (Figure 1a). No significant difference in tissue catalase activities was evident in the sham (66.48 ± 28.64 U/mg), control (120.32 ± 51.94 U/mg), IL (104.75 ± 35.39 U/mg), and AL (109.43 ± 47.41 U/mg) groups (Figure 1b).

3.2. Histological and immunohistological results

The sham group exhibited almost no damage (Figure 2a). However, severe tissue injury characterized by inflammatory cell infiltration, vasocongestion, and disorganization was evident in the control group (Figure 2b). The morphological features of tissue injury were less evident in the IL and AL groups (Figures 2c and 2d). The total damage score was significantly elevated in the

Table 1. The serum levels of TNF- α , IL-1 β , and IL-6 according to the study groups.

Groups (pg/mL)	Sham (n = 8)	Control (n = 8)	IL (n = 8)	AL (n = 8)	P < 0.05
TNF- α *	8.34 \pm 1.07	10.86 \pm 2.16	8.90 \pm 1.50	7.84 \pm 2.36	Sham vs. control; control vs. IL and AL
IL- 1 β **	32.02 \pm 15.60	61.26 \pm 25.63	38.13 \pm 10.36	40.99 \pm 23.67	Sham vs. control; control vs. IL and AL
IL -6**	22.83 \pm 7.11	35.39 \pm 11.08	28.25 \pm 7.44	32.77 \pm 11.27	Sham vs. control

*: $P < 0.05$, one-way analysis of variance (ANOVA) for serum TNF- α levels in sham vs. control and control vs. IL and AL. **: $P < 0.05$, Kruskal-Wallis one-way ANOVA for IL-1 β levels in sham vs. control and control vs. IL and AL. **: $P < 0.05$ Kruskal-Wallis one-way ANOVA for IL-6 levels in sham vs. control. IL: Iloprost group, AL: alprostadil group.

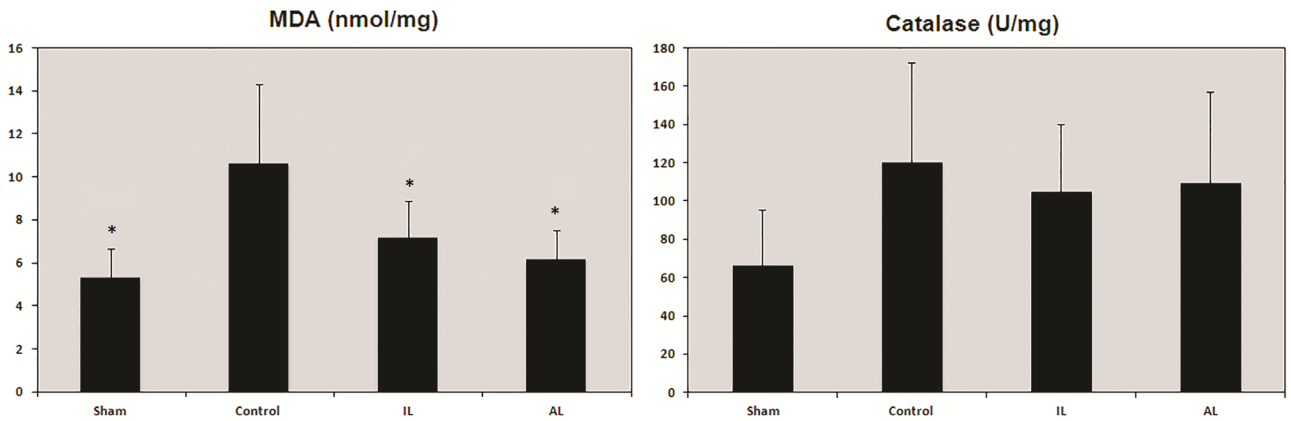


Figure 1. (a) MDA and (b) catalase levels in the skeletal muscle tissues of the rats. *: $P < 0.05$, Kruskal–Wallis test for tissue MDA levels in control vs. sham, IL, and AL; sham vs. IL and AL. No significant differences for tissue catalase levels in all groups. IL: Iloprost group, AL: alprostadil group.

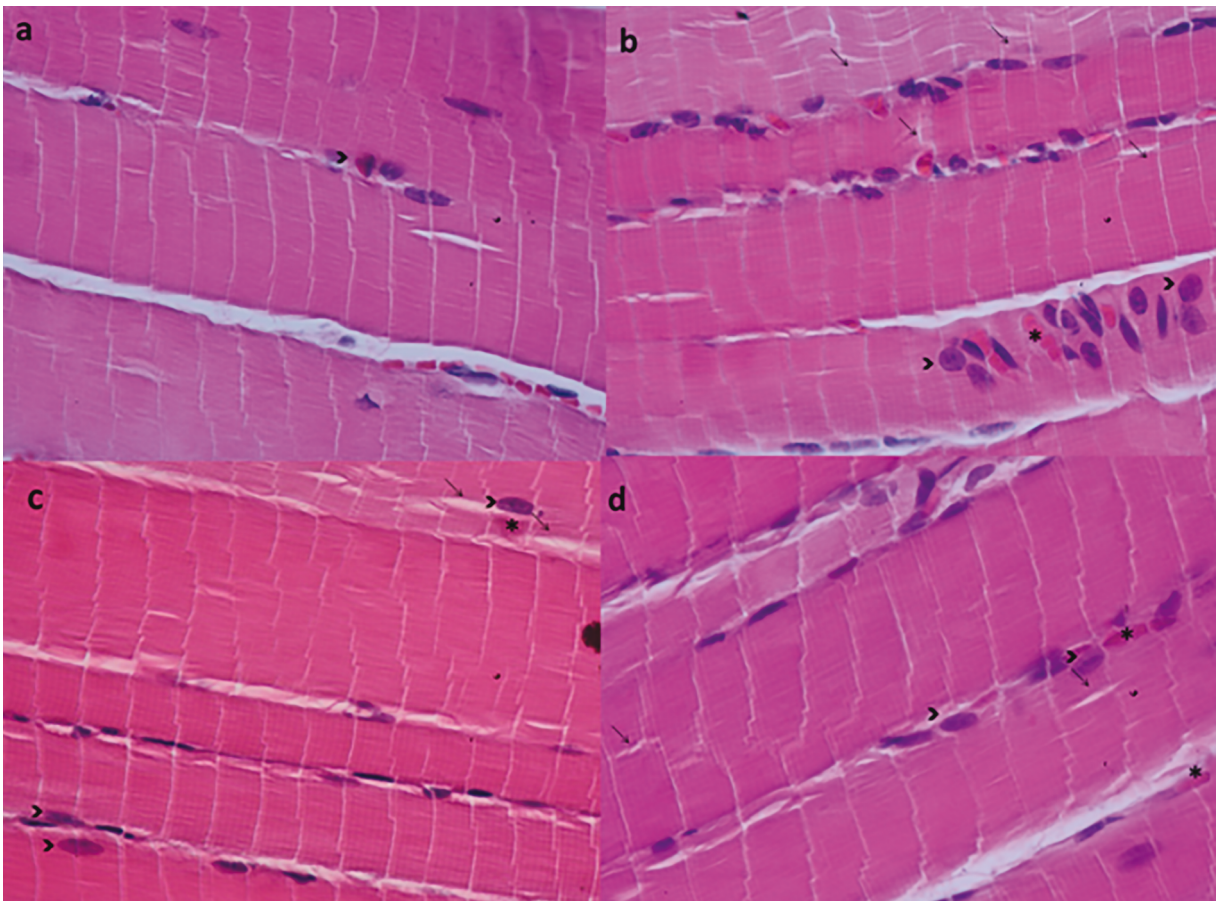


Figure 2. Histologic changes in skeletal muscle sections according to the study groups: (a) normal muscle histology with minimal changes was demonstrated in the sham group; (b) extensive damage was demonstrated in the control group (I/R); (c, d) the damage was attenuated in both the IL and AL groups. The arrow indicates muscle fiber degeneration; the arrowhead indicates inflammatory cell infiltration; vasocongestion is marked with an asterisk. H&E, original magnification 40 \times . IL: Iloprost group, AL: alprostadil group.

control group compared with the other groups ($P < 0.05$). The difference in total damage scores was not significant

between the IL and AL groups. The scores for each of the study groups are given in Table 2.

Table 2. The damage scores according to the study groups. *: $P < 0.05$, Kruskal–Wallis test, control vs. sham, IL, and AL. IL: Iloprost group, AL: alprostadil group.

Groups	Sham (n = 8)	Control (n = 8)	IL (n = 8)	AL (n = 8)
Degeneration	0.25 ± 0.08	1.64 ± 0.07	1.33 ± 0.05	1.64 ± 0.07
Centralization	0.16 ± 0.05	1.05 ± 0.71	0.84 ± 0.13	0.66 ± 0.32
Inflammatory cells	0.26 ± 0.07	1.96 ± 0.12	1.71 ± 0.06	1.27 ± 0.64
Vasocongestion	0.43 ± 0.09	2.64 ± 0.20	1.53 ± 0.07	1.27 ± 0.48
Disorganization	0.15 ± 0.05	2.50 ± 0.12	1.31 ± 0.06	1.31 ± 0.06
Total score	1.25 ± 0.14*	9.79 ± 0.58	6.71 ± 0.54*	5.88 ± 1.47*

The patterns of positive caspase-3 staining among all of the groups were different (Figures 3a–3d). The apoptosis scores were significantly elevated in the control group (3.16 ± 0.07) compared with the sham (1.9 ± 0.06), IL (1.59 ± 0.08), and AL (1.62 ± 0.09) groups ($P < 0.01$). Statistical significance was also evident when the sham group was compared with the IL and AL groups ($P < 0.01$). The difference between the IL and AL groups regarding apoptosis was not statistically significant (Figure 4).

4. Discussion

Iloprost and alprostadil are commonly used as adjuvant treatments following revascularization procedures involving ischemic limbs. Both drugs were retested for their efficacy in the setting of I/R injury in many experimental studies (Table 3). However, comparative studies of iloprost and alprostadil are infrequent in the literature (6,16,17). The present study compared them and showed that PG analogs iloprost and alprostadil

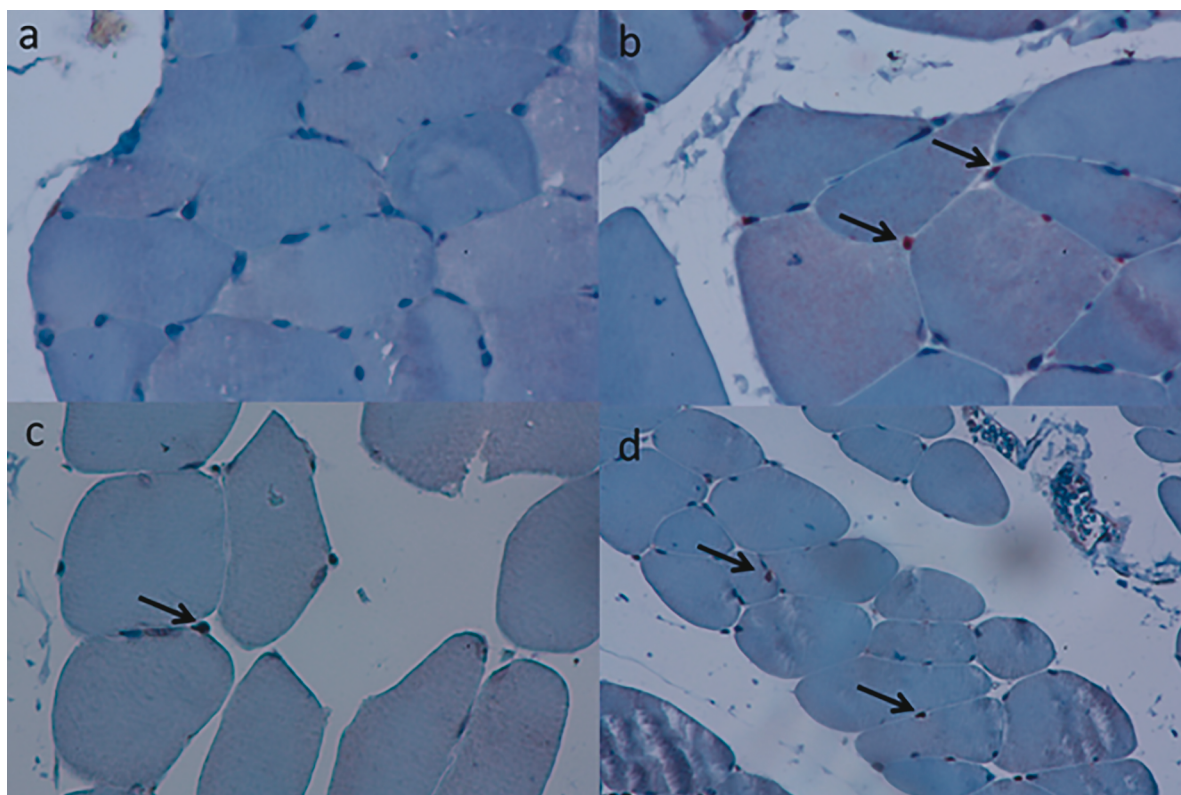


Figure 3. Caspase-3 immunostaining study demonstrating apoptosis: (a) no staining was noted in the sham group; (b) extensive caspase-3 positive staining of myocyte nuclei was noted in the control group; (c, d) caspase-3 staining was decreased in both the IL and AL groups. The arrow indicates positively stained nuclei (brown) Caspase-3 immunostaining, original magnification 40×. IL: Iloprost group, AL: alprostadil group.

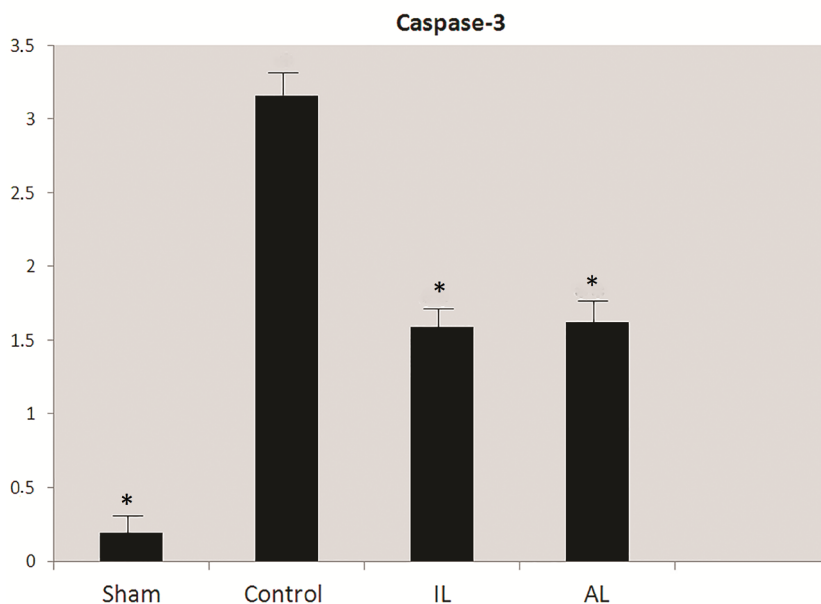


Figure 4. Apoptosis scores according to the study groups. *: $P < 0.01$, Kruskal-Wallis test, control vs. sham, IL, and AL; sham vs. IL and AL. IL: Iloprost group, AL: alprostadil group.

were almost equally effective in preventing I/R injury in skeletal muscle. Iloprost is a stable long-acting analog of PGI_2 and exerts cytoprotective effects mediated by several possible mechanisms, including vasodilation, membrane stabilization (7), reduced ROS production, antiplatelet activity (3,5), increased red cell deformability (20), and inhibition of leukocyte-endothelial adhesion (1,21). It also regulates microcirculation by decreasing the levels of proinflammatory cytokines (1). The effects of iloprost on injury caused by I/R in skeletal muscle (6–12,17) have been well documented, but they are still controversial in the literature. Earlier studies have focused on circulation research. In an intravital microscopy study, Thomson et al. demonstrated that iloprost reduced leukocyte-induced reperfusion injury in extensor digitorum muscle venules by simulating a femorodistal bypass surgery model (21). The possible mechanism of action was postulated as downregulation in the expressions of adhesion molecules such as ICAM-1 and ELAM-1 in response to iloprost (1). Blebea et al. demonstrated that iloprost attenuated increased microvascular permeability in the setting of I/R injury in rat cremaster muscles (8). However, the ischemia period utilized in their study was brief and was characterized by only increased interstitial edema, an early sign of I/R injury. In 1992, Mohan et al. reported that the protective effect of iloprost was not related to the inhibition of neutrophil activation (4). Therefore, the potential therapeutic role of iloprost remained undefined until recently. Most recent studies based on biochemical and histologic findings demonstrated that

iloprost administration attenuated the effects of I/R injury in skeletal muscle (10–12,17). In clinical practice, iloprost has been used as an adjuvant to surgery. In a study involving 192 patients over 70 years of age, iloprost significantly decreased the combined incidences of death and amputation; however, it did not delay amputations (3). In another report, iloprost was found to exert beneficial effects following the microsurgical repair of arterial injuries in four infants (22).

Although alprostadil exerts effects similar to those of iloprost (a PGI_2 analog), it (as a PGE_1 analog) has been used less often in the setting of acute skeletal muscle I/R injury (14,17). The reason for this may be the rapid metabolism of alprostadil by the lungs, which decreases its plasma concentrations (23). This may cause less effectivity in target organs. Antonio et al. (14) and recently Erer et al. (17) reported that alprostadil did not significantly reduce inflammatory changes and subsequent muscular damage. However, alprostadil has been found to be protective in other organs such as the lungs (17), kidneys (17), and neural tissue (15). On the contrary, we have found that alprostadil protected muscle against I/R injury, similar to iloprost. This finding is supported by other studies. PGE_1 increased the survival of musculocutaneous flaps, as demonstrated by Hong et al. (13). They chose an intraflap injection to minimize the adverse effects of PG. They hypothesized that PGE_1 exerts protective effects against I/R injury by decreasing leukocyte-endothelial cell adhesion via the decreased expression of ICAM-1. Huk et al. investigated the role of PGE_1 in NO production in

Table 3. Summary of PG studies on I/R muscle injury.

Authors/reference	Agent(s) or drug(s)	Time of I/R	Animal, site of injury	Mechanism of action	Conclusion
Belkin et al., 1990 (7)	Iloprost	6 h/1 h	Dog, gracilis muscle	Not indicated in the article	Preventing muscle I/R injury but no effect on platelet sequestration
Blebea et al., 1990 (8)	Iloprost	2 h/2 h	Rat, cremaster muscle	Decreasing the rise in vascular permeability	Preventing muscle I/R injury
Mohan et al., 1991 (9)	Iloprost	6 h/48 h	Dog, gracilis muscle	Decreasing leukocyte activity with continuous infusion	Protective effect on muscle I/R injury if administered continuously during reperfusion
Mohan et al., 1992 (4)	Iloprost	6 h/48 h	Dog, gracilis muscle	Leukocyte accumulation or increased microvascular permeability cannot be inhibited	No protective effect on I/R injury and muscle necrosis
Rowlands et al., 1999 (16)	PGE ₁ , PGE ₂ , and iloprost	6 h/4 h	Rat, hindlimb muscles	Inducing vasodilation	Improving blood flow in I/R muscle injury
Huk et al., 2000 (24)	PGE ₁	2.5 h/2 h	Rabbit, adductor magnus muscle	Indirect reduction of superoxide and peroxynitrite production to physiological levels	Protective effect on muscle I/R injury
Hong et al., 2001 (13)	PGE ₁	4 h/5 days	Rat, transverse rectus abdominis muscle-skin flap	Decreasing leukocyte-endothelial cell adhesion through decreased expression of ICAM-1	Preventing muscle I/R injury
Bozkurt, 2002 (10)	Iloprost	4 h/1 h	Rat, hindlimb muscles (gastrocnemius)	Cytoprotection	Preventing muscle I/R injury
Tauber et al., 2004 (6)	PGE ₁ and PGI ₂	4 h/24 h	Hamster, dorsal skin striated muscles	No-reflow and reflow paradox	Attenuation of leukocyte adhesion, but no effect on microvascular dysfunction
Fräßdorf et al., 2006 (26)	PGE ₁	3 h/3 h	Rabbit, hindlimb muscles	Positive effect on local hemodynamics (increased intravascular volume)	Attenuation of I/R injury in terms of edema formation, but not neuromuscular function
Emreçan et al., 2008 (11)	Iloprost	2 h/4 h	Rabbit, hindlimb muscles (gastrocnemius)	Cytoprotection	Preventing muscle I/R injury
Abdel-Rahman et al., 2009 (25)	PGE ₁	6 h/90 min	Pig, hindlimb muscles (gastrocnemius)	Protecting regional blood flow Increasing serum O ₂ and glucose consumption Decreasing muscle ATP consumption	Reducing local hemodynamic effects of I/R injury in addition to controlled limb perfusion
Antonio et al., 2009 (14)	Alprostadil	5 h/1 h	Rat, hindlimb muscles (gastrocnemius)	Not indicated in the article	No protective effect on muscle I/R injury
Avci et al., 2014 (12)	Iloprost	2 h/2 h	Rat, hindlimb muscles	Cytoprotection	Preventing muscle I/R injury
Erer et al., 2016 (17)	Iloprost and alprostadil	2 h/2 h	Rat, hindlimb muscles	Not indicated in the article	Preventing muscle I/R injury

the setting of skeletal muscle I/R injury and hypothesized that there exists an inverse relationship between PGE₁ and nitric oxide levels (24). Abdel-Rahman et al. reported that PGE₁ reduced the local hemodynamic effects of I/R injury in addition to controlling reperfusion, primarily via the attenuation of the “reflow paradox” (25). However, the exact effect of PGE₁ on I/R injury was not clear, as PGE₁ was not used in the setting of uncontrolled reperfusion in the study in question. Fräßdorf et al. reported that I/R injury was attenuated by PGE₁ treatment, as they observed decreased edema and creatine kinase release; however, PGE₁ had no influence on the recovery of neuromuscular function (26). In clinical reports, PGE₁ appears to have yielded more encouraging results regarding its ability to protect against I/R injury in skeletal muscle (27).

The present study also compared the protective effects exerted by two synthetic PG analogs, iloprost and alprostadil, against I/R injury. To evaluate the systemic response to I/R injury, the levels of three primary proinflammatory cytokines, TNF- α , IL-1 β , and IL-6, were

assayed in the serum. Iloprost and alprostadil decreased the levels of both TNF- α and IL-1 β ; however, they had no significant effect on the level of IL-6. The local tissue injury caused by I/R has been assessed by MDA and catalase assays. MDA is a stable end-product of lipid peroxidation, which indicates the damage of the cellular membrane (28). MDA levels, which were increased in the setting of I/R, were reduced via the administration of both iloprost and alprostadil. The levels of catalase, a protective enzyme against ROS, were increased in both the IL and the AL groups, as well as in the control group. Therefore, we concluded that the administration of the PG analogs did not affect tissue protective mechanisms such as catalase production, but did decrease the extent of tissue injury. Our histologic examinations demonstrated that tissue injury was also decreased in both the IL and AL groups. Inflammatory cell counts, which contributed primarily to the damage scores, decreased following the infusion of both PG types. Caspase-3 immunostaining demonstrated that the apoptosis scores were lower in both

the IL and AL groups compared with the control group. Apoptosis is a final determinant of tissue injury, as said injury often results in programmed cell death. To the best of our knowledge, this study is the first to demonstrate that iloprost and alprostadil prevent apoptosis. The possible role of prostaglandins in decreasing apoptosis can be investigated in future studies.

Different dosages of iloprost and alprostadil have been proposed in previous studies to exert maximal protective effects against I/R injury and exert only minimal adverse effects (12,14,15,17,25). In light of the currently available literature, we used $0.5 \text{ ng kg}^{-1} \text{ min}^{-1}$ iloprost (12) and $0.05 \text{ } \mu\text{g kg}^{-1} \text{ min}^{-1}$ alprostadil (15). The efficacy of both drugs may change if administered at either higher or lower doses. Iloprost has a half-life of 30 min and is effective when administered intravenously (8). It is metabolized in the kidneys. However, in determining the systemic dose of alprostadil, it should be taken into consideration that the plasma concentration of this drug decreases rapidly following intravenous administration, as the drug may be metabolized by as much as 80% following a single pass through the lungs (23). Therefore, alprostadil was administered at higher doses than iloprost in the present study. The dosages of the drugs should be higher when administered systemically as opposed to locally, as is the case in both femorodistal bypass surgery (21) and flap surgeries (13). Although hypotension has been reported following systemic administration (13), we did not observe deterioration in the health status of the animals during the experiments.

The present study has a few limitations. First of all, it showed only early protective effects of the given drugs

on I/R injury. The late effects on I/R injury have not been demonstrated. The tourniquet use to establish ischemia in the experiments does not exactly represent ischemia due to intravascular thrombosis in most clinical scenarios. The iloprost and alprostadil were not administered in a combined manner. Therefore, their additive effects are unknown. Muscle function has not been evaluated with tests such as electromyography in the current study.

The results of these investigations regarding the effects of PG analogs against I/R injury in skeletal muscle remain inconclusive. The protective effects exerted by PGs and their analogs have been demonstrated in many studies. However, there have also been studies that found PGs and their analogs to be less effective in the setting of I/R injury. In the present study iloprost and alprostadil were found effective in attenuating I/R injury and reducing apoptosis in skeletal muscle. However, no significant difference was found between their efficacies. This result may guide clinicians in considering adverse effects and cost-effectiveness in decisions on drug administration.

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References

- Della Bella S, Molteni M, Mocellin C, Fumagalli S, Bonara P, Scorza R. Novel mode of action of iloprost: in vitro down-regulation of endothelial cell adhesion molecules. *Prostaglandins Other Lipid Mediat* 2001; 65: 73-83.
- Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. *Br Med Bull* 2004; 70: 71-86.
- De Donato G, Gussoni G, Cao P, Setacci C, Pratesi C, Mazzone A, Ferrari M, Veglia F, Bonizzoni E, Settembrini P et al. Acute limb ischemia in elderly patients: can iloprost be useful as an adjuvant to surgery? Results from the ILAILL study. *Eur J Vasc Endovasc Surg* 2007; 34: 194-198.
- Mohan C, Marini C, Gennaro M, Ascer E. The value and limitation of iloprost infusion in decreasing skeletal muscle necrosis. *J Vasc Surg* 1992; 16: 268-273.
- Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 2011; 31: 986-1000.
- Tauber S, Menger MD, Lehr HA. Microvascular in vivo assessment of reperfusion injury: significance of prostaglandin E1 and I2 in postischemic "no-reflow" and "reflow-paradox". *J Surg Res* 2004; 120: 1-11.
- Belkin M, Wright JG, Hobson RW. Iloprost infusion decreases skeletal muscle ischemia-reperfusion injury. *J Vasc Surg* 1990; 11: 77-83.
- Blebea J, Cambria RA, DeFouw D, Feinberg RN, Hobson RW, Duran WN. Iloprost attenuates the increased permeability in skeletal muscle after ischemia and reperfusion. *J Vasc Surg* 1990; 12: 657-666.
- Mohan C, Ascer E, Marini C, Maughan R, Gennaro M. Does iloprost mediate thromboxane activity and polymorphonuclear leukocyte sequestration in ischemic skeletal muscle? *J Cardiovasc Surg (Torino)* 1991; 33: 613-618.
- Bozkurt A. Alpha-tocopherol (vitamin E) and iloprost attenuate reperfusion injury in skeletal muscle ischemia/reperfusion injury. *J Cardiovasc Surg (Torino)* 2002; 43: 693-696.

11. Emreçan B, Tulukoğlu E, Bozok Ş, Aksun M, Yağdı S, Özcan AV, Saçar M, Gürbüz A. Iloprost and pentoxifylline attenuate ischemia-reperfusion injury in skeletal muscle in rabbit model. *Ulus Travma Acil Cer* 2008; 14: 182-187.
12. Avci T, Erer D, Kucuk A, Oztürk Y, Tosun M, Oktar GL, Arslan M, Iriz E, Kavutcu M, Tatar T. The effects of iloprost on ischemia-reperfusion injury in skeletal muscles in a rodent model. *J Surg Res* 2014; 187: 162-168.
13. Hong JP, Chung YK, Chung SH. The effect of prostaglandin E1 versus ischemia-reperfusion injury of musculocutaneous flaps. *Ann Plast Surg*. 2001; 47: 316-321.
14. Antonio L, Evora P, Piccinato C. Use of alprostadil, a stable prostaglandin E1 analogue, for the attenuation of rat skeletal muscle ischemia and reperfusion injury. *Minerva Chir* 2009; 64: 559-564.
15. Milcan A, Arslan E, Bagdatoglu OT, Bagdatoglu C, Polat G, Kanik A, Talas DU, Kuyurtar F. The effect of alprostadil on ischemia-reperfusion injury of peripheral nerve in rats. *Pharmacol Res* 2004; 49 : 67-72.
16. Rowlands T, Gough M, Homer-Vanniasinkam S. Do prostaglandins have a salutary role in skeletal muscle ischaemia-reperfusion injury? *Eur J Vasc Endovasc Surg* 1999; 18: 439-444.
17. Erer D, Özer A, Demirtaş H, Gönül İI, Kara H, Arpacı H, Çomu FM, Oktar GL, Arslan M, Küçük A. Effects of alprostadil and iloprost on renal, lung, and skeletal muscle injury following hindlimb ischemia-reperfusion injury in rats. *Drug Des Devel Ther* 2016; 10: 2651-2658.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358.
19. Beutler E. *Red Cell Metabolism: A Manual of Biochemical Methods*. 2nd ed. New York, NY, USA: Grune & Stratton; 1975.
20. Arslan M, Donmez T, Erer D, Tatar T, Comu F, Alkan M. Effect of iloprost on erythrocyte deformability in rat's lower extremity undergoing an ischemia reperfusion injury. *Bratisl Lek Listy* 2012; 114: 189-191.
21. Thomson I, Egginton S, Hudlicka O, Sims M. Iloprost reduces leukocyte adhesion in skeletal muscle venules following ischaemia in a rat model of femorodistal bypass. *Eur J Vasc Surg* 1994; 8: 335-341.
22. Iriz E, Erer D, Kula S, Olgunturk R, Halit V. Use of microsurgery and iloprost in the infantile arterial injuries. *Circ J* 2007; 71: 554-558.
23. Lin X, Zhang Y, Cui Y, Wang L, Wang J, Tang X. Determination of alprostadil in rat plasma by ultra performance liquid chromatography-electrospray ionization-tandem mass spectrometry after intravenous administration. *J Pharm Biomed Anal* 2009; 49: 983-988.
24. Huk I, Brovkovych V, Nanobashvili J, Neumayer C, Polterauer P, Prager M, Patton S, Malinski T. Prostaglandin E1 reduces ischemia/reperfusion injury by normalizing nitric oxide and superoxide release. *Shock* 2000; 14: 234-242.
25. Abdel-Rahman U, Risteski P, Klaeffling C, Mitrev Z, Ackermann H, Matheis G, Moritz A, Beyersdorf F. The influence of controlled limb reperfusion with PGE1 on reperfusion injury after prolonged ischemia. *J Surg Res* 2009; 155: 293-300.
26. Fräßdorf J, Luther B, Müllenheim J, Otto F, Preckel B, Schlack W, Thämer V. Influence of groin incision, duration of ischemia, and prostaglandin E1 on ischemia-reperfusion injury of the lower limb. *J Cardiothorac Vasc Anesth* 2006; 20: 187-195.
27. Sako H, Hadama T, Miyamoto S, Anai H, Wada T, Iwata E, Hamamoto H, Tanaka H, Urushino K, Shuto T. Effect of prostaglandin E1 on ischemia-reperfusion injury during abdominal aortic aneurysm surgery. *Surg Today* 2006; 36: 140-146.
28. Valenzuela A. The biological significance of malondialdehyde determination in the assessment of tissue oxidative stress. *Life Sci* 1991; 48: 301-309.