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Effect of iloprost on contractile impairment and mitochondrial degeneration in ischemia-reperfusion of skeletal muscle

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Purpose: Acute lower extremity ischemia is still a main cause of mortality and morbidity in orthopedic traumatology and reconstructive surgery. In acute lower extremity ischemia, the skeletal muscles are the tissues that are the most vulnerable to ischemia. The aim of this study was to evaluate the effects of iloprost (IL) therapy on skeletal muscle contractile impairment and mitochondrial degeneration in an acute lower extremity ischemia-reperfusion rat model. *Main Methods:* Forty Wistar albino rats were randomly divided into a control group and four experimental groups. Experimental groups were either subjected to 2 h of lower extremity ischemia followed by a 4-h reperfusion period or to 4 h of ischemia followed by an 8-h reperfusion period. Except for the animals in the control group, all animals received IL (1 ng/kg/min) or saline (1 ml/kg) by intraperitoneal infusion for 10 min immediately before reperfusion. At the end of the recording of skeletal muscle electrical activity and contractility, all rats were sacrificed by decapitation and muscle samples of lower extremity were immediately harvested for histopathologic analyses. *Results:* After ischemia-reperfusion, a breakdown in the force–frequency curves of extensor digitorum longus muscle was observed, showing the diminished muscle contractility. However, IL significantly improved muscle contractility following injury induced by 2 h of ischemia followed by a 4-h reperfusion period. In addition, IL partially ameliorated mitochondrial degeneration in the muscle cells of ischemia groups. *Conclusion:* This study indicates that immediate IL therapy repairs muscle damage especially after 2 h of ischemia and 4 h of reperfusion and therefore that IL improves contractile function.

Keywords: ischemia-reperfusion injury, skeletal muscle, compound muscle action potential, contractility, iloprost, mitochondria damage

Introduction

Ischemia-reperfusion (I/R) injury is an occurrence that is observed very widespread in orthopedic traumatology and reconstructive surgery and can have detrimental effects on all tissues and organs involved. This event negatively influences the result of surgical procedures for the management of amputated or severely traumatized extremities in traumatology and patients who had a reconstructive surgery (10).

To date, acute lower extremity ischemia is still a main cause of mortality and morbidity. In acute lower extremity ischemia as in other ischemic diseases, the majority of injury

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to the soft tissues related to I/R happens during the reperfusion phase. In acute lower extremity ischemia, the skeletal muscles are tissues that are most vulnerable to ischemia. Long-term exposure to I/R is known to have harmful effects on skeletal muscle performance. Various methods have been used in experimental skeletal muscle ischemia models, such as temporarily blocking of the blood flow in the aorta and femoral artery and tourniquet application (19).

I/R injury triggers a chain of events in skeletal muscle. First, ischemia creates muscle necrosis due to the reduction of energy and ion instability. Then, reperfusion creates edema, inflammation, and excessive release of reactive oxygen and nitrogen species that causes further muscle injury, inducing muscle atrophy and weakness (2).

Physiological and anatomical studies indicate that irreversible muscle cell damage begins after 3 h of ischemia and is nearly complete at 6 h (2). It is important to know when to start therapy, that is, prior to, during, or immediately after ischemia.

Current therapies are intended to prevent I/R-induced damage to skeletal muscle tissue. Iloprost (IL) is used as a new pharmaceutical drug for the therapy of I/R.

The effects of IL (a long-acting prostacyclin analog) therapy on I/R injury in skeletal muscles have been investigated using many different methods, such as biochemical analyses and histopathological examination of muscle tissues, but there is no study that explores the effects of IL therapy on contractility and electrical activity of skeletal muscle.

The goal of this research was to demonstrate the influences of IL therapeutic interventions administered only during the initial period of reperfusion on I/R injury-induced contractile impairment in rat extensor digitorum longus (EDL) muscle. Therefore, this study was conducted to define the effects of IL according to ischemia time in terms of electrical and mechanical activities on EDL muscle following a reperfusion period in both femoral arteries of rats after tourniquet-induced occlusion.

Material and Methods

Animal preparation and I/R process

The study was conducted in the Biophysics Laboratory (Faculty of Medicine, Mersin University, Mersin, Turkey). In this study, the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (13) were applied. All experiments and protocols were confirmed by the Medical Faculty Experimentation Ethics Committee of Mersin University. Forty adult male Wistar albino rats (~10 weeks old; 200–250 g) were purchased from Clinical and Experimental Research Laboratory of Mersin University. They were housed under standard conditions (12-h light–dark cycle: 22 ± 2 °C, relative humidity: 50%–70%) and provided access to standard rat nutrients and purified drinking water ad libitum.

In this study, the process of ischemia (I/R) was induced using an atraumatic femoral artery rubber band tourniquet that was applied on both hind roots of the limb. Ischemia was followed by a reperfusion period (20). To determine the contractility features and electrical characteristics of the muscles left and right EDL muscles were used in all groups. EDL muscle was selected, because it predominantly contains fast-twitch fibers, which are more susceptible to IR injury than slow-twitch muscle fibers (e.g., fibers of the soleus) (4).

After 1 week of acclimatization, rats were randomly divided into a control group and four experimental groups, each containing eight rats:

Group C. This is the control group. Rats in Group C were allocated to sham operation. Group C was not subjected to I/R, but EDL muscles of the individuals were also used for recordings of electrical activity and contractility.

Experimental groups

Group I/R24SF. This group was subjected to 2 h of ischemia followed by a 4-h reperfusion period and received saline (SF) (dose: 1 ml/kg) by intraperitoneal (i.p.) infusion for 10 min immediately before reperfusion.

Group I/R24IL. This group was subjected to 2 h of ischemia followed by a 4-h reperfusion period and received IL (dose: 1 ng/kg/min) (Ilomedin®, Bayer Schering Pharma AG, Berlin, Germany) by i.p. infusion for 10 min immediately before reperfusion.

Group I/R48SF. This group was subjected to 4 h of ischemia followed by an 8-h reperfusion period and received saline (SF-dose: 1 ml/kg) by i.p. infusion for 10 min immediately before reperfusion.

Group I/R48IL. This group was subjected to 4 h of ischemia followed by an 8-h reperfusion period and received IL (dose: 1 ng/kg/min) (Ilomedin®) by i.p. infusion for 10 min immediately before reperfusion.

All dissection operations of animals and the records of the external electrical activity from a EDL muscle were carried out on a heating platform (DC-Heated Animal Operating Table–MAY QOT0801) to maintain the body temperature between 35 and 37 °C. Rectal temperature was measured throughout the process.

After recording the muscle electrical activity and contractility, all rats were sacrificed by decapitation after i.p. sodium-pentobarbital injection and skeletal muscle samples of lower extremity were immediately harvested for histopathologic analyses.

In vitro tissue preparation

Contractile properties of EDL muscles. The contractile properties of the EDL muscles were measured *in vitro*. Rats were anesthetized by i.p. injection of ketamin hydrochloride (Ketalar® flakon, Parke–Davis, USA) (50 mg/kg) and xylazin hydrochloride (Rompun flk, Bayer, Turkey) (2 mg/kg).

General dissection procedures in EDL muscles were performed as it reported by Gielen et al. (14). For this purpose, the whole length of the EDL muscle was dissected free from surrounding muscles. Only the blood supply and the neural connections of the muscle were left intact. The sciatic nerve was cut proximal to the common peroneal branch to prevent any reflex participation to the recorded signals. The EDL tendons of origin and insertion were dissected at the knee and proximal to the metatarsus, respectively. EDL muscles were immediately weighed after they were dissected. Prior to removal, 3/0 silk ligatures were tied to the tendons at both ends of the muscle. Each left muscle was mounted vertically in an isolated organ bath (Isolated Organ Bath Stand Set-IOB S99) and equilibrated for 30 min in a Krebs solution [118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 24 $NaHCO_3$, 1.2 KH_2PO_4, 11 glucose (mmol/L)] continuously perfused with 95% O_2–5% CO_2 gas. The distal tendon was fixed to a point at the bottom of the bath and the proximal tendon was connected to an isometric force transducer (FDT 05 Force Displacement Transducer, BIOPAC Systems Inc., Santa Barbara, CA, USA) and a holder attached to a micrometer that allowed adjustment of the preparation to optimal muscle length (Lo). The force transducers were calibrated using known weights. Bath solution temperature was thermostatically maintained at 37 °C using a digital heating circulator (Model MAY WBC 3044-PR). The isometric force transducer's

output was connected to a differential amplifier's input (MAY-GTA-200) through electrical connection. The signals were digitized with a 16-bit analog-to-digital converter at a sampling rate of 15,000 samples/s. EDL muscle contractile forces were recorded using the BIOPAC MP100 data acquisition and analysis system with AcqKnowledge software (Biopac MP100 system Inc., Santa Barbara, CA, USA). The muscles were directly stimulated by an electrical impulse traveling between two stainless steel wire electrodes, which were placed around it, using a 0.5 ms square wave pulse of supramaximal voltage. Stainless steel wire electrodes were connected to a stimulator module (STM100A, BIOPAC Systems Inc., Santa Barbara, CA, USA) with a stimulus-isolated power supply (MAY ISO150-A). Muscle length was adjusted so that the twitch force was maximal and at this optimal length, resting tension was measured to be about 2–2.5 g. Muscle length was maintained at this level throughout the study. Stimulus intensity was also increased until maximal twitch responses were obtained (40–50 V for direct stimulation). In this study, initially, a single muscle twitch was elicited (supramaximal voltage: 0.5 ms duration) with stimulation frequency of 0.1 Hz. From these records, twitch force, contraction time (time-to-peak force), and half-relaxation time (time for peak force to decay by 50%) were determined. The contraction time and half-relaxation time were measured as indices of isometric twitch kinetics. Next, force–frequency properties of the EDL muscles were found by sequentially stimulating the muscles every 5-min intervals with 500-ms trains of the following frequencies: 10, 20, 40, 80, 100, and 150 Hz.

To determine the relationship between force and frequency, the values were normalized for force obtained for each different stimulation frequencies, and expressed as a percentage of the maximum tetanic force advanced during each application.

The force of contraction was expressed in grams (g) and normalized for muscle cross-sectional area (CSA). Following the completion of the stimulation protocol, muscles were removed from the bath, blotted and weighed, and force per CSA were calculated according to the following equation: force/cm^2 = [force (g) × specific density of skeletal muscle (1.06 g/cm^3) × length of muscle (cm)]/[mass of muscle (g)] (18).

Electrical activity properties of EDL muscles

Prior to the electrical activity recordings, the rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar® flacon, Parke-Davis, ESA) and 2 mg/kg xylazin hydrochloride (Rompun flk, Bayer, Turkey), administered i.p. The right hind limb of the rats was placed in a standard position during the electrical activity recordings, and then the hind limb was shaved. To record electrical activities of EDL muscles, surface EMG electrodes (Medelec, number 017K006, Oxford, UK) were placed 3.0 mm separately. In this way, both electrodes were in contact with the muscle above the innervation region. The ground electrode (AgÏAgCl) was placed on the other thigh, to which the stimulation was not applied and so the compound motor action potentials (CMAPs) were not recorded. The diameter of the recording electrodes was approximately half the width of the muscle, allowing that a relatively large number of fibers could contribute to the CMAP (M waves) recordings. Stimulation was elicited through bipolar stimulating electrodes (Medelec small bipolar nerve electrodes, 6894T, Oxford, UK) placed at the end of the muscle distal to the M wave recording electrodes. Stimulation pulses of 10 V strength and 0.5 ms duration were used, which were found to be supramaximal for direct stimulation of muscle fibers. During electrical activity recording, the muscles and tendons were irrigated regularly with isotonic saline to prevent dehydration. BIOPAC Acqknowledge analysis software (AcqKnowledge 100 W) was used to determine amplitude, area, total time, depolarization duration, and half repolarization time of CMAP.

The amplitude of a given CMAP was defined as the height in millivolts (mV) from baseline to the peak of the negative phase. The area was measured under the curve from the first negative deflection to the first baseline crossing and the duration was measured from the first negative deflection to the first baseline crossing. Total time of CMAP was measured from the initial to the terminal deflection back to baseline. The half-repolarization time was defined as the time interval from the overshoot to 50% of the amplitude. Depolarization duration was measured from the initial to the overshoot point.

After the electrophysiological recording, all pups were euthanized by decapitation. The right EDL muscles were removed in all groups of animals for ultrastructural evaluation.

Sample preparation for electron microscopy

For transmission electron microscopic (TEM) evaluation, the muscle tissue samples were fixed with 2.5% gluteraldehyde, postfixed with 1% osmium tetroxide, dehydrated in graded alcohol series, cleared with propylene oxide, and embedded in epon. Thin sections (50–70 nm) were cut by a ultramicrotome (Leica UCT-125, Leica Microsystems GmbH, Wien, Austria) and contrasted with uranyl acetate and lead citrate. Sections were examined and photographed under a TEM (JEOL JEM-1011, Jeol Ltd., Tokyo, Japan).

Ultrastructural analysis of mitochondria

For ultrastructural analysis of mitochondria, the method used in our previous study was used (6). Briefly, electron micrographs were taken at 25,000 magnification randomly from muscles in all five groups. A total of 10 different fields for each biopsy and 10 mitochondria in every field were evaluated. Totally, 100 mitochondria per sample were graded.

Images were examined by one experienced investigator blinded for the order of sampling. Mitochondrial damage was evaluated by giving a numerical value of 0 through 3 to each mitochondrion. This mitochondrial classification was used by modification of the scores reported by Milei et al. (17).

Grading scale was: 0 – normal; 1 – initial swelling (separation of cristae, decreased matrix density); 2 – more marked swelling than in grade 1 and architectural disruption; 3 – findings as in grade 2 plus rupture of inner and outer mitochondrial membranes. The average obtained was expressed for each grade as a percentage of the total number of mitochondria counted per sample (17).

Statistical analyses

The data were processed and investigated using the Med Calc v.11.0 Statistical Package Program. Values were presented as mean ± standard deviation or median with interquartile range (25th to 75th percentiles) where appropriate (Table I). Kolmogorov–Smirnov test was used to determine whether all the parameters of electrical and mechanical activity of the EDL muscle, and ultrastructural data were normally distributed. All parameters were in normal distribution except half-repolarization time and area parameters of CMAP. If the groups are compatible with normal distribution, one-way analysis of variance (ANOVA) was used to examine the mean differences between the groups for all the parameters of electrical and mechanical activity of the EDL muscle. If the groups are not compatible with normal distribution, differences between groups were performed by Kruskal–Wallis test.

Levene's test was carried out to check the homogeneity of variance before comparing the groups with each other. While a homogenous group differences in variance were evaluated using ANOVA, Welch's *t*-test for the parameters of groups with unequal variances

was applied. Following these processes, Scheffe's *post-hoc* test was used to detect the significant pairwise differences between groups. Values of $p \leq 0.05$ were considered significant. All values in graphics have been expressed as mean ± standard error, together with their 95% confidence intervals.

Results

Following the induction of ischemia, motor deficits began to appear after the first hour of ischemia time in all rats. Cyanosis with coldness and edema was observed in the limb distal to the tourniquet in rats in which we had applied ischemia.

In the Group I/R48SF and Group I/R48IL, no rats died in the course of the study. However, one rat in the Group C died during the anesthesia procedure. In addition, one rat in the Group I/R24SF and one rat in the Group I/R24IL died during the ischemia process.

Electrical activity data of EDL muscles: CMAPs

The sample records of CMAPs in all five groups (control, I/R24SF, I/R24IL, I/R48SF, and I/R48IL) are shown in Fig. 1. The amplitudes in Group I/R24SF (Fig. 1B) and Group I/R48SF (Fig. 1C) were decreased when compared with Group C (Fig. 1A) ($p < 0.001$). IL (Fig. 1D and E) increased the amplitudes during I/R in comparison with I/R saline groups (Fig. 1B and C).

Descriptive statistics for CMAP parameters that were obtained from the EDL muscles are shown in Table I. The averages of amplitudes in Group I/R24SF and Group I/R48SF were decreased during ischemia and reperfusion when compared with Group C ($p < 0.001$). IL increased the amplitudes during I/R in comparison with I/R saline groups (Table I).

The IL treatment in Group I/R24IL decreased significantly ($p = 0.033$) the duration of depolarization of CMAPs when compared with Group I/R24SF.

The half-repolarization time was significantly prolonged in the Group I/R48SF compared with the Group C ($p < 0.001$). However, IL treatment to I/R animals almost completely reversed the prolongation in half-repolarization time (Table I). Therefore, it seems to improve the half-repolarization duration of IL treatment.

In addition, as seen in Table I, there were no statistically significant differences between control and experimental groups regarding area and total time of CMAP.

Mechanical activity data of EDL muscles

The force–frequency relationship. Force–frequency curves in all five groups are shown in Fig. 2. In this figure, data for 0.1 Hz represent twitch contraction parameters of EDL muscles.

As depicted in the figure, both I/R groups were characterized by reduced force production in EDL muscles at different proportions at all frequencies tested ($p < 0.001$). However, IL restored muscle contractility only at some low frequency values of the group subjected to the 4-h reperfusion period following 2 h of ischemia. On the other hand, IL has not improved muscle contractility in the group subjected to the 8-h reperfusion period following 4 h of ischemia.

The force production of Group I/R24IL significantly increased compared with Group I/R24SF at 0.1, 10, and 20 Hz frequencies (Fig. 2). However, there are no significant differences between Group I/R24SF and Group I/R24IL at frequency regions of 40–150 Hz, despite an increase in force production caused by IL therapy in this range.

The force–frequency graphics for the EDL muscles were similar in Group I/R48SF and Group I/R48IL (Fig. 2).

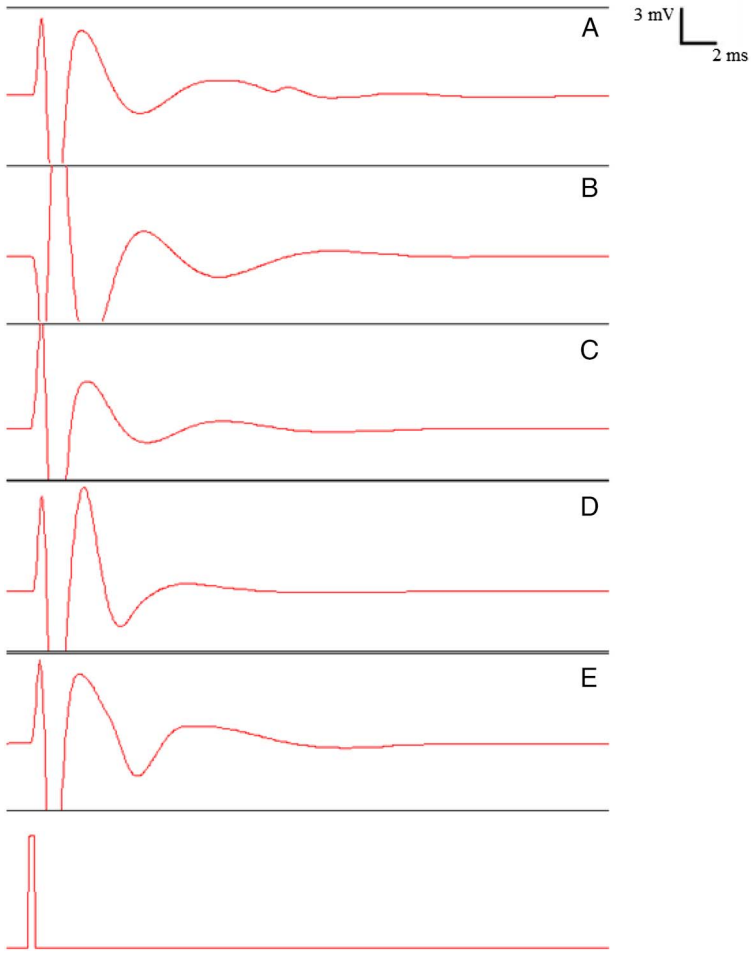

Fig. 1. Sample records of the compound motor action potential (CMAP) in control (C) A: saline-treated ischemia-reperfusion (B: I/R24SF and C: I/R48 SF) and iloprost-treated ischemia-reperfusion (D: I/R24IL and E: I/R48IL) groups. Groups I/R24SF and I/R24IL were subjected to 2 h of ischemia followed by a 4-h reperfusion period; Groups I/R48SF and I/R48IL were subjected to 4 h of ischemia followed by an 8-h reperfusion period. Calibrations for all traces are shown in the upper right; vertical bar = 3 mV; horizontal bar = 2 ms. The bottom curve is a sample of stimulus voltage of supramaximal strength (10 V) and 0.5 ms duration

The model of 4 h of ischemia followed by an 8-h reperfusion period caused significant decreases in the force output of the EDL muscles at all frequencies tested ($p < 0.001$); however, in this group, I/R-induced deficiency in force production in EDL muscles was not forestalled by IL pretreatment.

Muscle responses to twitch stimulation frequency (0.1 Hz). The effects of IL therapy and I/R-induced changes in twitch stimulation frequency (0.1 Hz) in EDL muscle are shown in Figs 2 and 3. As seen in Fig. 2, decreased force production could be observed in EDL muscles of both I/R groups at different proportions at twitch stimulation frequency of 0.1 Hz ($p < 0.001$). Almost complete inhibition of contraction force occurred in the EDL muscles of Group I/R48SF. The amount of inhibition of Group I/R48SF compared with Group C was

Table I. Descriptive statistics for compound motor action potential (CMAP) parameters that were recorded from EDL muscles

	Control ($n = 7$)	**I/R24SF ($n = 7$)**	**I/R24IL ($n = 7$)**	**I/R48SF ($n = 8$)**	**I/R48IL ($n = 8$)**	***p***
Amplitude (mV) (mean ± SD)	9.10 ± 3.98	4.79 ± 2.55[a]	10.37 ± 4.04[b]	5.71 ± 2.41[a]	8.39 ± 3.41[c]	0.016
Total time (ms) (mean ± SD)	8.41 ± 2.98	11.25 ± 1.92	9.09 ± 2.72	11.14 ± 1.65	13.18 ± 8.18	0.251
Area (mV.ms) (median-25th to 75th percentiles)	0.0118 (0.010–0.012)	0.0127 (0.012–0.014)	0.0109 (0.009–0.015)	0.0114 (0.009–0.014)	0.0126 (0.011–0.017)	0.482
Depolarization time (ms) (mean ± SD)	1.00 ± 0.20	1.81 ± 0.90	0.96 ± 0.24[b]	1.22 ± 0.32	1.42 ± 0.37	0.033
Half-repolarization time (ms) (median-25th to 75th percentiles)	1.30 (0.70–1.45)	0.85 (0.65–1.45)	1.10 (0.90–1.50)	1.47 (0.84–1.63)[a]	1.28 (0.96–1.44)	0.001

n: the number of rats in each group.
[a]Significantly different from control group.
[b]Significantly different from Group I/R24SF.
[c]Significantly different from Group I/R48SF

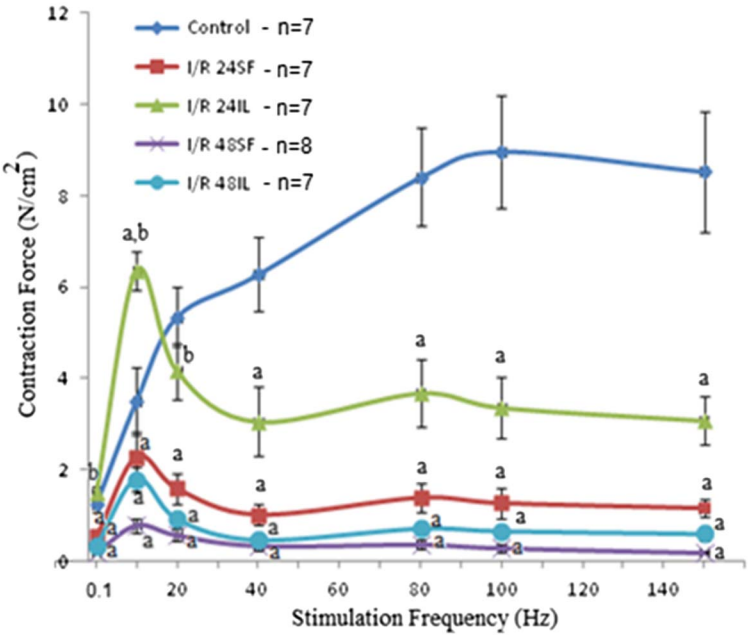

Fig. 2. Contraction forces of EDL muscle against stimulation frequencies. Values of forces are expressed with 95% confidence intervals of the mean for all groups in the study and as mean + standard error of the mean. [a]Significantly different from Group C. [a]$p < 0.001$. [b]Significantly different from Group I/R24SF. [b]$p < 0.001$. n = the number of rats in each group

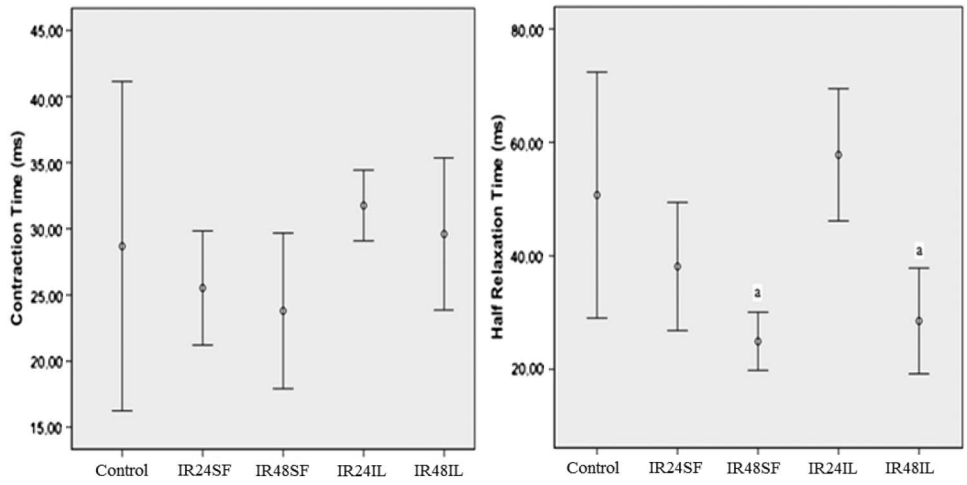

Fig. 3. The effects of ischemia-reperfusion injury and iloprost treatment on twitch configurations. Values are expressed with 95% confidence intervals of the mean obtained against the twitch stimulation frequency (0.1 Hz) in rat extensor digitorum longus for all groups. (A: contraction times and B: half-relaxation times). [a]Significantly different from Group C. [a]$p < 0.001$

83%. Whereas contraction force of Group I/R24SF muscles was decreased to 43% compared with Group C. However, it was determined that IL significantly improved the muscle contractility in only Group I/R24IL for 0.1 Hz stimulation frequency (Fig. 2).

The contraction times in twitch stimulation frequency (0.1 Hz) were slightly lower following I/R in EDL muscles treated with saline immediately before reperfusion compared with Group C (Fig. 3A). Following I/R, the contraction times of EDL muscles pretreated with IL have extended even more than in Group C.

Half-relaxation times of EDL muscles stimulated at twitch stimulation frequency were significantly shorter in only Group I/R48SF compared with the Group C ($p < 0.001$; Fig. 3B). Nevertheless, this reduction in the half-relaxation time caused by I/R was not improved by IL given immediately before reperfusion.

Electron microscopic findings

Qualitative evaluation. The muscle cells in Group C have normal morphological features. Myofibrils and all sarcoplasmic organelles were detected as normal (Fig. 4A and B). Although some muscle cells had normal morphological features, there were expansions in sarcoplasmic reticulum cisternae and mitochondrial degeneration in some muscle cells in the ischemia groups (Figs 5A, Band 6A, B). However, mitochondrial degeneration was decreased in IL-treated ischemia groups (Figs 5C and 6C).

Quantitative analysis. To estimate the mitochondrial damage of the EDL muscle cells, 10 fields were scored for each biopsy, as in our previous study (6). Table II indicates the data of quantitative grading of damage found after examination of 3,000 mitochondria in five groups. Biopsies of Group C indicated, as expected, that the large majority of mitochondria had minimal evidence of damage (grade 0: $98.67\% \pm 1.75\%$, grade 1: $1.33\% \pm 1.75\%$). In Group I/R24SF, mitochondrial injury improved and the proportion of mitochondria indicating normal morphology reduced (grade 0: $84.17\% \pm 24.02\%$), while the number of

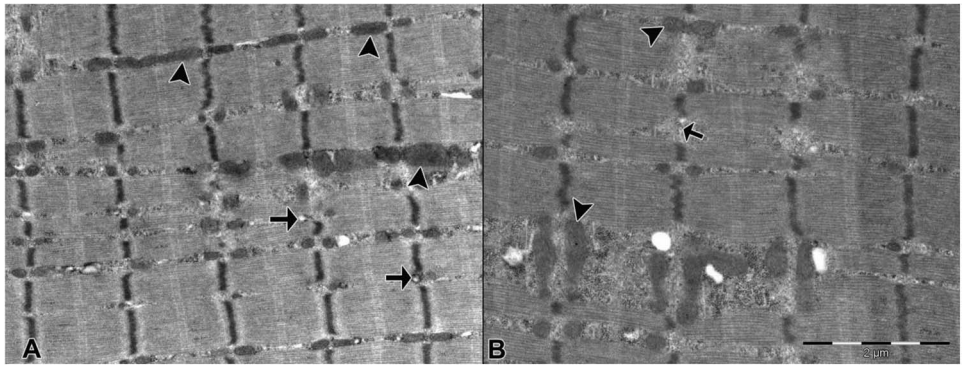

Fig. 4. (A and B) The electron microscopic findings of control group. The muscle cells have normal morphologic characteristics. Mitochondrion (arrow head), sarcoplasmic reticulum cisternaes (thin arrow). A: ×15,000, B: ×20,000

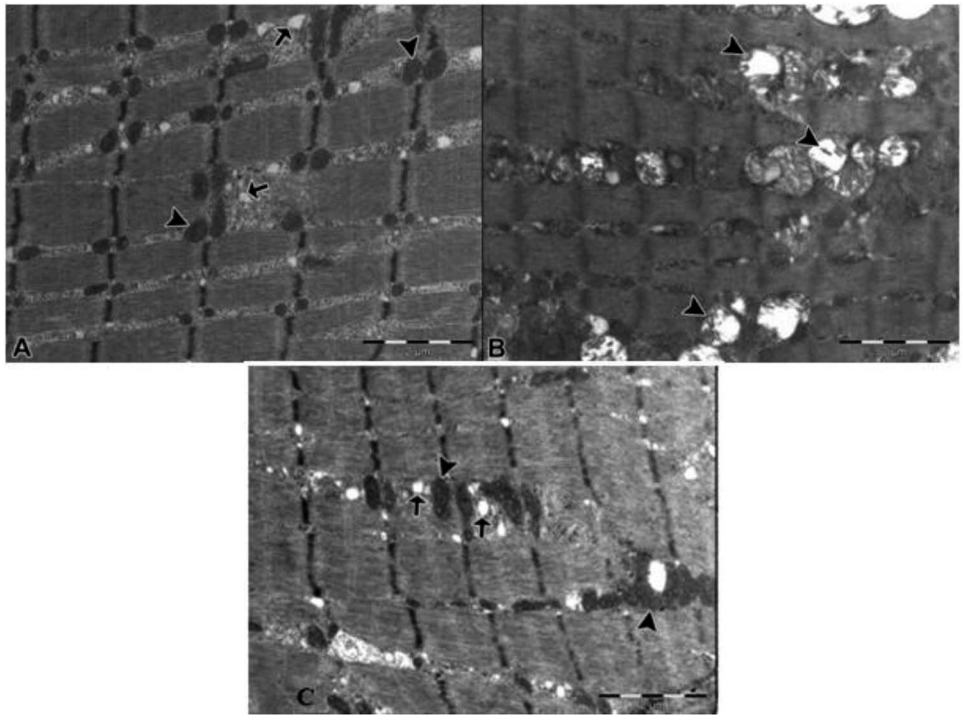

Fig. 5. (A, B, and C) The electron microscopic findings of ischemia-reperfusion and treatment groups. A: Group I/R24SF. Normal mitochondrion (arrow head), expansions in sarcoplasmic reticulum cisternae (thin arrow), ×15,000. B: Group I/R24SF. Mitochondrial degeneration (arrow head), ×15,000. C: Group I/R24IL. Normal mitochondrion (arrow head), expansions in sarcoplasmic reticulum cisternae (thin arrow), ×15,000

mitochondria indicating higher (i.e., worse) scores increased (grade 1: $8.33\% \pm 9.14\%$, grade 2: $7.17\% \pm 17.55\%$, grade 3: $0.33\% \pm 0.82\%$), but these results did not show statistically significant differences compared with Group C (Table II). Biopsies from Group

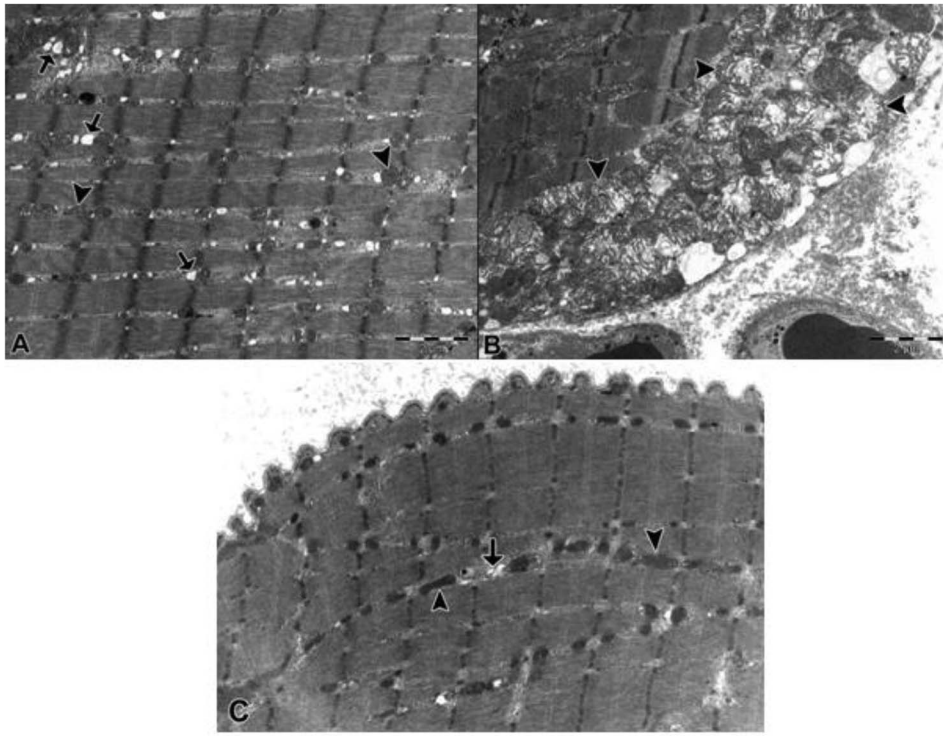

Fig. 6. (A, B, and C) The electron microscopic findings of ischemia-reperfusion and treatment groups. A: Group I/R48SF. Normal mitochondrion (arrow head), expansions in sarcoplasmic reticulum cisternae (thin arrow), ×10,000. B: Group I/R48SF. Mitochondrial degeneration (arrow head), ×10,000. C: Group I/R48IL. Normal mitochondrion (arrow head), sarcoplasmic reticulum cisternae (thin arrow), ×10,000

Table II. Ultrastructural grading scores for the experimental groups

Experimental groups	Grade 0	Grade 1	Grade 2	Grade 3
Control ($n = 600$)	98.67 ± 1.75	1.33 ± 1.75	0.0 ± 0.0	0.0 ± 0.0
I/R24SF ($n = 600$)	84.17 ± 24.02	8.33 ± 9.14	7.17 ± 17.55	0.33 ± 0.82
I/R24IL ($n = 600$)	98.17 ± 2.86	1.83 ± 2.86	0.0 ± 0.0	0.0 ± 0.0
I/R48SF ($n = 600$)	62.67 ± 31.21^{a}	$28.83 \pm 28.68^{a,b}$	8.50 ± 10.37	0.0 ± 0.0
I/R48IL ($n = 600$)	89.33 ± 19.07^{c}	10.17 ± 17.87^{c}	0.50 ± 1.23	0.0 ± 0.0

Data are mean ± SD of percentage mitochondria scored. Values in parentheses are the number of mitochondria scored for each group.
[a] $p < 0.05$ compared with the control group.
[b] $p < 0.05$ compared with Group I/R24SF.
[c] $p < 0.05$ compared with Group I/R48SF

I/R24IL showed similar proportions of normal morphology (grade 0: 98.17% ± 2.86%, grade 1: 1.83% ± 2.86%) versus those in Group C. In Group I/R48SF, mitochondrial injury developed and the proportion of mitochondria showing normal morphology decreased (grade 0: 62.67% ± 31.21%), while the number of mitochondria showing higher (i.e., worse) scores increased (grade 1: 28.83% ± 28.68%, grade 2: 8.50% ± 10.37%) compared with Group C ($p < 0.05$). Biopsies from Group I/R48IL did not demonstrate any significant difference compared with Group C. In addition, in Group I/R48IL, the proportion of mitochondria indicating normal morphology increased (grade 0: 89.33% ± 19.07%), while the number of mitochondria showing higher (i.e., worse) scores decreased (grade 1: 10.17% ± 17.87%, grade 2: 0.50% ± 1.23%) compared with Group I/R48SF ($p < 0.05$) (Table II).

Discussion

Ischemia is the absence or insufficiency of blood supply to tissues due to various reasons. Reperfusion, on the other hand, is the return of the blood supply to the tissue. During reperfusion, the release of free oxygen radicals – especially from the polymorphonuclear leukocytes that accumulate in the tissue and causing damage to biological molecules, such as nucleic acids, membrane lipids, enzymes, and receptors – accelerates tissue breakdown. This is referred to as reperfusion injury (21). Therefore, I/R reoxygenation causes cellular and subcellular changes characterized by weakened tissue oxygenation, reduced intracellular oxygen levels, interstitial edema, cellular swelling, variations in high-energy phosphate metabolism, and mitochondrial dysfunction (22).

Prostaglandins (PGs) are lipid compounds that arrange the variety of physiological and pathological functions in nearly all body tissues and organs. Prostacyclin [prostaglandin 12 (PGI2)], which is synthesized by the vascular endothelium, is a potent vasodilator, inhibits the aggregation of platelets *in vitro* and has cytoprotective effect on body tissues and organs (7). IL is a PGI2 analog, and has pharmacological features of its endogenous precursor. IL decreases neutrophil adhesion (12). It prevents the release of free radicals from neutrophils, thus decreasing the endothelial injury caused by neutrophils during reperfusion.

A number of studies have been performed to evaluate the effect of IL on skeletal muscle injury related to I/R using biochemical and/or histopathologic analyses, but there has not been studies, which explored the effects of IL therapy on contractility and electrical activity of skeletal muscle. Tiryakioglu et al. (21), Emrecan et al. (12), and Avci et al. (1) reported that IL has protective effects on I/R injury in skeletal muscle in a rodent model. Blebea et al. (3) showed that IL decreases the rise in vascular permeability in skeletal muscle after ischemia and reperfusion. Increased vascular permeability is an early and sensitive indicator of ischemic muscle injury, occurring before significant histologic or radionuclide changes are evident (3). Bozkurt (5) suggested that both vitamin E and IL are useful for attenuating oxidative muscle damage occurring after a period of skeletal muscle I/R.

In this study, it was demonstrated that the CMAP amplitude of EDL muscle decreased significantly in both I/R groups, suggesting a partial interruption of the signal through the muscle fibers. However, IL that was administered immediately prior to reperfusion showed a preventive effect on this partial interruption of the EDL muscle signal after I/R.

In this study, a statistically significant difference was found in Group I/R48SF compared with Group C for half-repolarization time in CMAP. An increase in half-repolarization time in Group I/R48SF compared with Group C suggested that the opening–closing kinetics of K^+ channels slowed down. As we could not find any difference between Group C and

Group I/R48IL regarding half-repolarization time, we suggest that IL treatment alters the opening and closing kinetics of voltage-gated potassium channels. Our results implicate the voltage-gated potassium channels as additional IL targets, opening up new perspectives for the pharmacological prevention of muscle signal conduction disturbances due to I/R injury.

In this study, there were no statistically significant differences between control and experimental groups regarding area of CMAP. Most likely, the fact that the changes observed in the amplitude do not occur in the area is due to the changes in the opposite direction in the time parameters (CMAP depolarization time and CMAP half-repolarization time). One of the time parameters has shortening, the other has elongation (please see Fig. 1 and Table I). The elongation or shortening in some of them will prevent any change in the final result of the "area." Amplitude is not affected by total changes in the time parameters, because the amplitude of a CMAP was defined as the height from baseline to the peak of the negative phase. However, area is affected by total changes in the time parameters. For this reason, while looking at the area parameter, one probably cannot see any effect of either the injury or the recovery.

In this study, it was observed that IL improved muscle contractility following injury at only low frequencies, and only when the intervention was 2 h of ischemia followed by a 4-h reperfusion period.

The effect of I/R derived oxygen-free radicals on skeletal muscle calcium metabolism has been examined by Cronenwett et al. (9). They have showed the involvement of oxygen-derived free radicals in abnormal Ca^{+2} transport observed in skeletal muscle after ischemia and reperfusion (9).

Lee et al. (16) reported that cytotoxic oxygen metabolites may contribute to skeletal muscle damage associated with ischemia and reperfusion by disturbing Ca^{+2} uptake by the sarcoplasmic reticulum of skeletal muscle in limbs subjected to periods of ischemia and reperfusion.

In this study, it was found that I/R reduced the force production in EDL muscles and that there were expansions in sarcoplasmic reticulum cisternae in muscle cells in the ischemia groups. Because terminal cisternae ensure rapid calcium delivery, they are well developed in muscles that contract quickly, such as fast-twitch skeletal muscle. Terminal cisterns then go on to release calcium. This event starts contraction process (15). Therefore, as seen in this study, expansions in sarcoplasmic reticulum cisternae of muscle cells in the I/R groups may reduce the force production by disrupting Ca^{+2} uptake by sarcoplasmic reticulum of skeletal muscle. Expansions in sarcoplasmic reticulum cisternaes of muscle cells may depend on oxygen-derived free radicals observed in skeletal muscles after ischemia and reperfusion.

In this study, mitochondrial degeneration in some muscle cells in the ischemia groups was observed by an electron microscope. Ultrastructural-morphometric analysis revealed that IL was able to diminish damages of the mitochondria after ischemia and reperfusion as well.

Some other studies have also reported a decrease in the muscle force production following acute I/R injury (8, 11, 23). The decrease in muscle force production can be due to (1) compromised excitation–contraction coupling, (2) disruption and/or loss of proteins involved in force generation and transmission, and (3) increase in non-contractile elements in muscle tissue. IL therapy in this study probably improved force production by influencing one or a combination of these factors.

The severity of I/R induced damage to EDL muscle was also evaluated by quantitative mitochondrial grading. The results confirmed only in Group I/R48SF that mitochondrial

injury had developed and the proportion of mitochondria showing normal morphology had decreased while the number of mitochondria showing higher (i.e., worse) scores had increased compared with the Group C. But the immediate treatment before reperfusion with a single-dose of IL prevented alterations and improved tissue oxygenation and mitochondrial oxidation in skeletal muscle I/R.

Although I/R reduces the muscle force production and creates expansions in sarcoplasmic reticulum cisternae of muscle cells in Group I/R24SF, mitochondrial injury developed only moderately according to the results obtained by quantitative mitochondrial grading and this result was not statistically significant compared with Group C. However, mitochondrial degeneration in some muscle cells in this ischemia group was observed by an electron microscope. This finding may be explained as quantitative mitochondrial changes in the ischemic skeletal muscle could occur after >2-h period of ischemia.

Using electrophysiological and histological methods, it has been found in this study that immediate IL therapy repairs muscle electrical and mechanical damage especially after a 2-h ischemia and 4-h reperfusion intervention and therefore that IL improves contractile function.

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