

# Effects of light-emitting diode (LED) therapy on skeletal muscle ischemia reperfusion in rats

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**Abstract** Low-level laser therapy has been shown to decrease ischemia–reperfusion injuries in the skeletal muscle by induction of synthesis of antioxidants and other cytoprotective proteins. Recently, the light-emitting diode (LED) has been used instead of laser for the treatment of various diseases because of its low operational cost compared to the use of a laser. The objective of this work was to analyze the effects of LED therapy at 904 nm on skeletal muscle ischemia–reperfusion injury in rats. Thirty healthy male Wistar rats were allocated into three groups of ten rats each as follows: normal (N), ischemia–reperfusion (IR), and ischemia–reperfusion+LED (IR+LED) therapy. Ischemia was induced by right femoral artery clipping for 2 h followed by 2 h of reperfusion. The IR+LED group received LED irradiation on the right gastrocnemius muscle ( $4 \text{ J/cm}^2$ ) immediately and 1 h following blood supply occlusion for 10 min. At the end of trial, the animals were euthanized and the right gastrocnemius muscles were submitted to histological and histochemical analysis. The

extent of muscle damage in the IR+LED group was significantly lower than that in the IR group ( $P<0.05$ ). In comparison with other groups, tissue malondialdehyde (MDA) levels in the IR group were significantly increased ( $P<0.05$ ). The muscle tissue glutathione (GSH), superoxide dismutases (SOD), and catalase (CAT) levels in the IR group were significantly lower than those in the subjects in other groups. From the histological and histochemical perspective, the LED therapy has alleviated the metabolic injuries in the skeletal muscle ischemia reperfusion in this experimental model.

**Keywords** Light-emitting diode · Skeletal muscle · Ischemia reperfusion

## Introduction

Prolonged ischemia and reperfusion frequently occur in a variety of conditions such as reconstructive plastic surgery, orthopedic surgery, thrombolytic therapy, transplantation, and limb trauma such as crush injury and fractures. The events during ischemia reperfusion are basically similar in different organs and tissues but the time scale of the event varies [1–8]. Ischemia leads to muscle cell energy failure, inflammatory reaction, and biochemical alterations. These are worsened by reperfusion, which triggers high free radical production and neutrophil activation, making local and systemic lesions more severe [1–9]. The effect of ischemia reperfusion on skeletal muscle has been well described in several studies [10–15]. Therefore, it is important to find preservation methods for the skeletal muscle that decrease injuries caused by ischemia reperfusion.

Recently, phototherapy with light-emitting diode (LED) has attracted attention for inducing tissue repair through several mechanisms [16–18]. LED radiation has many biological effects similar to those of lasers, such as increases the

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adenosine triphosphate (ATP) synthesis, angiogenesis, collagen and protein synthesis, fibroblast proliferation; inhibits pain and oxidative stress; and decreases inflammatory reactions [16–25]. Phototherapy in wavelengths ranging from <600 to 1000 nm leads to activation of cytochrome c oxidase and improvement of ATP synthesis in cell cultures, which favors the expression of genes related to tissue repair [19–21, 26, 27]. No information is available about the applicability of LED therapy on muscle reperfusion injury. Since LED therapy can counteract many of the deleterious effects induced by high intensity exercise on the skeletal muscle [28, 29], this treatment may be a suitable method for preventing symptoms of skeletal muscle damage after ischemia reperfusion. This approach may be useful for decreasing injuries during ischemia reperfusion but this possibility has yet to be tested. The aim of this study, therefore, was to investigate the effects of 940-nm wavelength LED therapy on a skeletal muscle injury in a rat model following acute ischemia reperfusion. The study hypothesis was that LED therapy could decrease muscle damage induced by ischemia reperfusion.

## Material and methods

Thirty healthy male Wistar rats, aged between 3 and 4 months with body weight between 300 and 320 g, were purchased from the Pasteur Institute of Iran. This study was conducted according to the guidelines of the animal care review board of the Islamic Azad University Faculty of Specialized Veterinary Sciences and adhering to the guide for care and use of laboratory animals, and the study is approved by the ethics committee. They were kept in a controlled temperature and humidity, 12 h/12 h light/dark cycle, with ad libitum access to commercial food and filtered tap water.

### Experimental groups

The rats were divided randomly into three groups of ten rats each (of these ten, five were used for histochemical assays and five for histological analysis), normal (N), ischemia–reperfusion (IR), and ischemia–reperfusion+LED (IR+LED) therapy.

### Surgery

Surgery was performed at the Laboratory of Experimental Surgery, Science and Research Faculty of Specialized Veterinary Sciences. The rats were weighed and anesthetized using an intramuscular injection of ketamine hydrochloride 10 % and xylazine hydrochloride 2 % (50 mg/kg and 10 mg/kg, respectively). The animals were placed on a table, in dorsal recumbency, with their fore- and rear limbs immobilized with adhesive tape. After clipping, disinfecting

with antiseptic povidone-iodine solution, and draping, a skin incision was made on the medial surface of the right hind limb. After the isolation of the femoral artery and vein from the surrounding tissues, the femoral artery was exposed. Ischemia was induced by 2 h of femoral artery occlusion with a mini vascular clamp and followed by 2 h of reperfusion. Rats were maintained in dorsal recumbency and kept anesthetized (additional doses were given in case of necessity) throughout the ischemic period. Body temperature was maintained with a heating pad and monitored using a rectal thermometer. Following the ischemic period, the vascular forceps were removed and the surgical site was routinely closed with 3/0 nylon sutures.

### LED therapy

LED equipment was developed for this study according to parameters that were suggested by Costa Santos et al. [28]. The optical output power of the light source was verified prior to experimental procedures. The animals in IR+LED group were submitted to irradiation at a wavelength of 940 nm, delivering an energy intensity of 4 J/cm<sup>2</sup> and a power density of 9.5 mW/cm<sup>2</sup> to 1 cm<sup>2</sup> of area immediately and 1 h following blood supply occlusion for 10 min. The radiation source was attached to a support and kept perpendicular 1 cm above the skin surface, on the middle region of the right gastrocnemius muscle belly.

### Specimen collection

At the end of the trial, euthanasia was performed by using an overdose of pentobarbital (300 mg/kg) intraperitoneally. The right gastrocnemius muscle was harvested immediately, accomplished by incision in the posterior face of the leg then it was submitted for histological and biochemical assays.

### Histological analysis

The muscle samples were paraffin-embedded. Using standard techniques, paraffin sections were obtained at 5 μm, stained with hematoxylin and eosin, and studied under optical microscopy by two pathologists who were blinded to the experiment and data. Histological changes were scored on a scale from 0 to 3 where 0=absence (<5 % of myocytes injury), 1=mild (<10 %), 2=moderate (15–20 %), and 3=severe (20–25 %) [30]. A total of ten fields of view from each muscle sample were randomly screened, and the mean was accepted as the representative value of the sample.

### Biochemical assays

Glutathione (GSH), superoxide dismutases (SOD), catalase (CAT), and malondialdehyde (MDA) were detected in skeletal

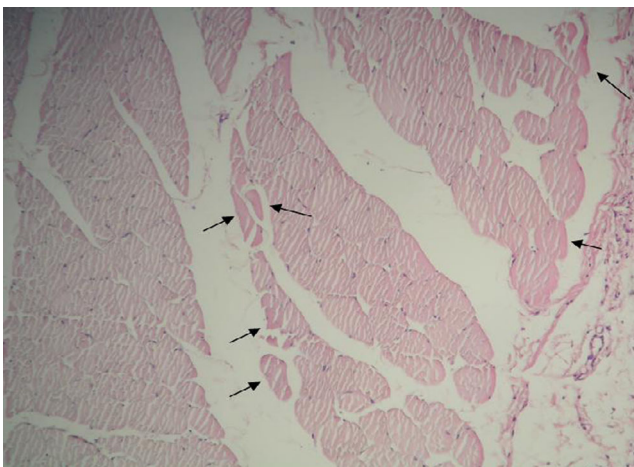
muscle tissue cuts. Each tissue was stocked in a separate bowl at  $-80^{\circ}\text{C}$  until analysis. Then, tissues were homogenized with cold Tris–HCl buffer (pH 7.4) to make a 10 % homogenate. GSH measurements were taken using a modification of the Ellman procedure [31]. The results were expressed as nanomoles/100 mg protein in tissue samples. The total SOD activity was measured kinetically by a method described by Sun et al. [32]. SOD activity was expressed as units/milligram of tissue protein. Muscle tissue CAT activity was measured according to Aebi's method [33]. Results were expressed as  $k$  (rate constant per gram protein;  $k/\text{g}$  protein). The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid-reactive substance formation as described previously [34]. The results are expressed as nanomole MDA/gram tissue.

### Statistical analysis

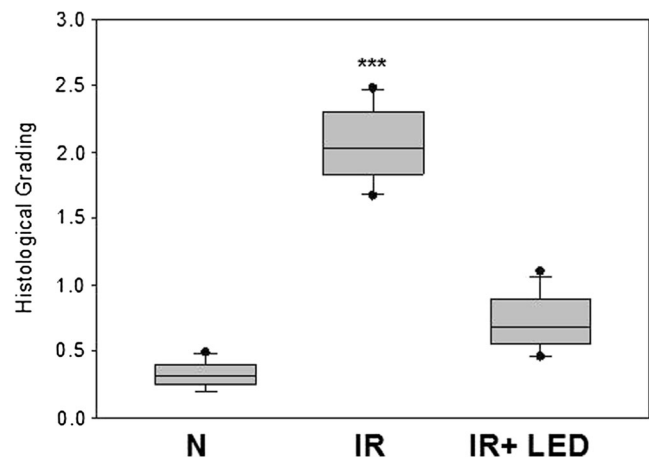
Data were analyzed by using SPSS statistical software package (version 18). Distribution of the groups was analyzed with one sample Kolmogorov–Smirnov test. The results were analyzed by using analysis of variance (ANOVA) for the comparison of multiple means with post hoc test analysis. Results of the study were expressed as the mean  $\pm$  standard deviation. Values of  $P < 0.05$  were considered as statistically significant.

### Results

The experimental procedure was well tolerated, and no animal died during the experiment. The muscles in the IR group exhibited destruction of muscle fibers with edema and inflammatory cell infiltration 2 h after reperfusion. Shrinkage was



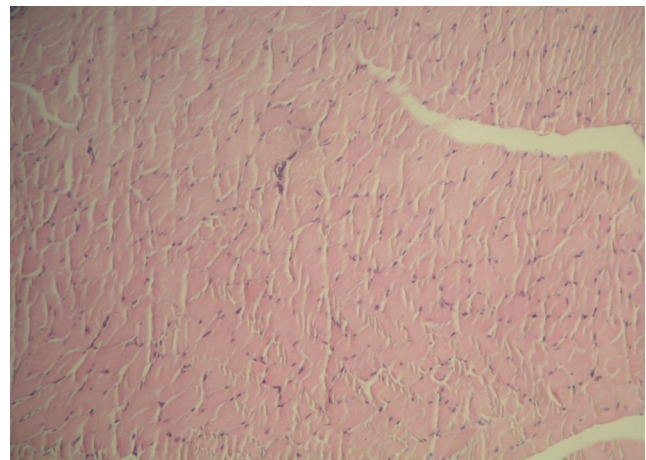
**Fig. 1** Histological section of rat skeletal muscle stained with hematoxylin and eosin. The skeletal muscle of a rat in the ischemia–reperfusion group showing irregular muscle fiber morphology with inconsistent color and necrosis (arrows) (original magnification  $\times 40$ )



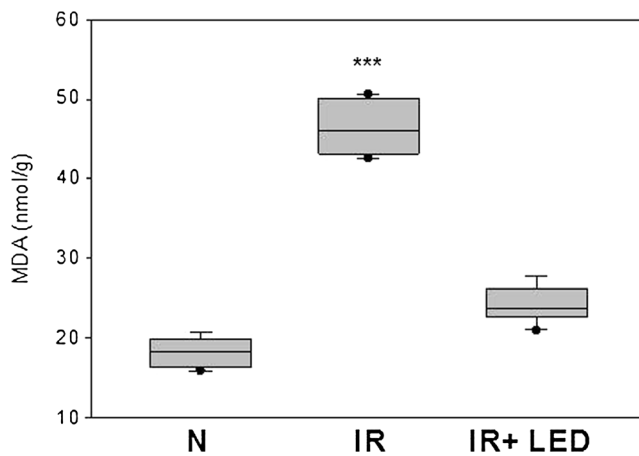
**Fig. 2** Histogram of histological score measurements in normal (N), ischemia–reperfusion (IR), and ischemia–reperfusion+LED (IR+LED) therapy gastrocnemius muscles 24 h after ischemia reperfusion injury. \*\*\* $P < 0.001$  compared with the normal and ischemia–reperfusion+LED group

observed in muscle structure, and cell gaps were obviously widened. The degree of injuries was largely uniform in most subjects, and all subjects had a high histologic damage score after ischemia–reperfusion injury (Fig. 1). In contrast to the muscle tissues observed after reperfusion injury in the IR group, damage to muscle fibers was rarely observed in the IR+LED therapy group (Fig. 2). Degeneration was not obvious and cell gap, slightly wide; however, there were some cases of inflammatory cell infiltration and edema between muscle fibers (Fig. 3). Histological findings indicate that the scavenging of free radicals by LED therapy may inhibit inflammatory cascades.

In IR+LED therapy group, treatment of phototherapy significantly decreased ( $P < 0.05$ ) skeletal muscle MDA level compared to the IR group (Fig. 4). SOD, CAT, and GSH levels were measured in the skeletal muscle. Results of



**Fig. 3** In ischemia–reperfusion+LED group, muscle fiber morphology was more regular and staining was uniform, vacuolar degeneration was not obvious, and cell gap was slightly wide (H & E staining, original magnification  $\times 100$ )

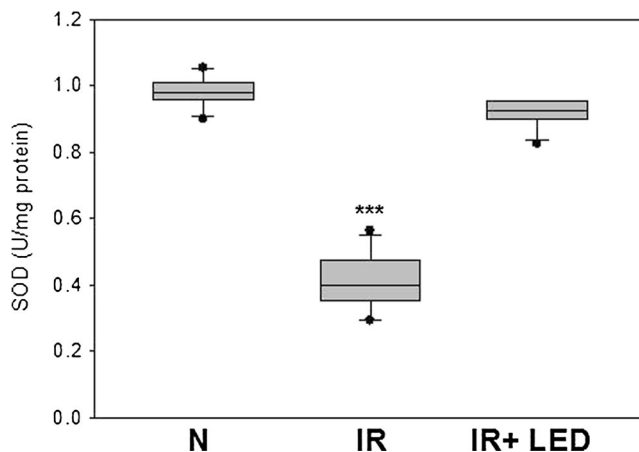


**Fig. 4** Histogram of malondialdehyde (*MDA*) levels in normal (*N*), ischemia–reperfusion (*IR*), and ischemia–reperfusion+LED (*IR+LED*) therapy gastrocnemius muscles 24 h after ischemia–reperfusion injury. \*\*\* $P<0.001$  compared with the normal and ischemia–reperfusion+LED group

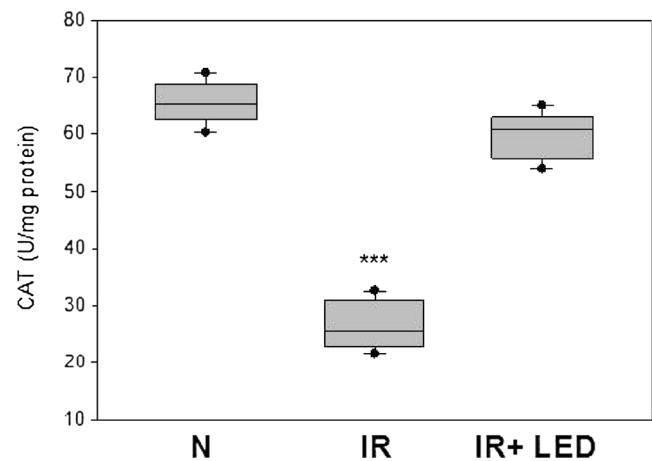
oxidative stress markers in each group were shown in Figs. 5, 6, and 7. The muscle tissue GSH levels in the IR group were significantly lower ( $P<0.05$ ) than those in the LED treatment group. Ischemia–reperfusion injury to the skeletal muscle significantly decreased ( $P<0.05$ ) SOD activity muscle tissues. CAT activity in muscle tissue decreased ( $P<0.05$ ) markedly after reperfusion. A significant decrease in the activity of CAT was observed in the IR group compared with the IR+LED group.

## Discussion

At the onset of ischemia, the biochemical alterations in the basic cellular functions can be reversed; however, when the ischemia time is longer, another sequence of reactions is



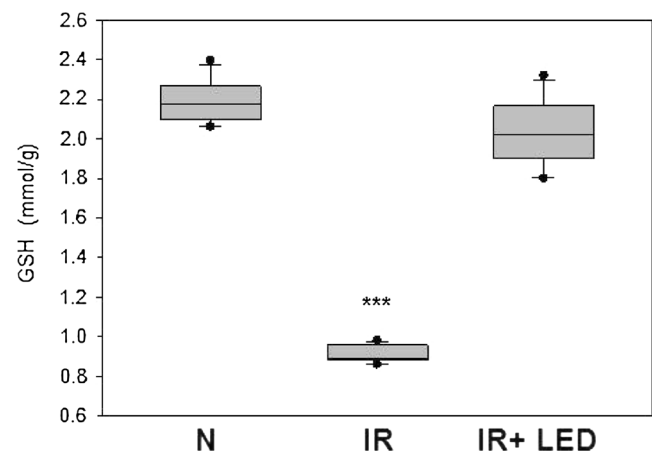
**Fig. 5** Histogram of superoxide dismutase (*SOD*) activity in normal (*N*), ischemia–reperfusion (*IR*), and ischemia–reperfusion+LED (*IR+LED*) therapy gastrocnemius muscles 24 h after ischemia–reperfusion injury. \*\*\* $P<0.001$  compared with the normal and ischemia–reperfusion+LED group



**Fig. 6** Histogram of catalase (*CAT*) activity in normal (*N*), ischemia–reperfusion (*IR*), and ischemia–reperfusion+LED (*IR+LED*) therapy gastrocnemius muscles 24 h after ischemia–reperfusion injury. \*\*\* $P<0.001$  compared with the normal and ischemia–reperfusion+LED group

triggered as a consequence of cellular energetic failure [35, 36]. The anaerobic metabolism prevails up to 2 h of ischemia, increasing the amount of lactate and inorganic phosphate and reducing pH, ATP, and creatine [37–39].

The ATP synthesized by the anaerobic metabolism maintains the ion pumps, the membrane potential, and the contractile function, although the production of lactic acid infuses into the interstitial space, causing edema and acidemia [40]. When this energetic source is over, the pumps fail and the ionic gradient of the cells is altered with potassium and magnesium release and sodium and calcium input into the intracellular medium, causing edema in the cell, matrix, or mitochondrial crests [37, 40, 41]. Increased intracellular calcium concentration activates the cytoplasmic proteases. In combination with the high concentration of hypoxanthine resultant from the uninterrupted degradation of ATP, the proteases



**Fig. 7** Histogram of glutathione (*GSH*) levels in normal (*N*), ischemia–reperfusion (*IR*), and ischemia–reperfusion+LED (*IR+LED*) therapy gastrocnemius muscles 24 h after ischemia–reperfusion injury. \*\*\* $P<0.001$  compared with the normal and ischemia–reperfusion+LED group

convert the enzyme xanthine dehydrogenase into the oxidase enzyme. This enzyme has an important role in reperfusion injuries. Calcium also activates the lysosomal enzymes that impair organelles and the phospholipase enzyme  $A_2$ , which degrades the arachidonic acid, originating inflammation mediators like leukotrienes, prostaglandins, prostacyclins, and thromboxanes [37, 39, 40]. Neutrophils are activated by the leukotriene B4 [37].

Paradoxically, although reperfusion is critical for reverting ischemia, it worsens injuries that occurred during the ischemia period. The production of exceeding free radicals and the intense participation of neutrophils increase the inflammatory reaction thereby promoting muscular edema formation, tissue necrosis, impairment of systemic clinical conditions and lead to limb loss and even death.

The photobiomodulation has been ascribed to activation of the mitochondrial respiratory chain, which leads to activation of several intracellular signaling cascades and cell proliferation [16, 18, 21]. Cytochrome c oxidase is the photon acceptor of LED irradiation, and increased ATP synthesis may be one of the beneficial effects of phototherapy. Other benefits include increased tissue repair and the maintenance of tissue homeostasis [19, 20, 24]. LED irradiation also acts as a radical scavenger, reducing reactive oxygen species production and inhibiting activation of phospholipase  $A_2$ . This mechanism avoids oxidative damage to muscle and reduces inflammation caused by the release of prostaglandins [24]. Another mechanism involved in muscle protection that is affected by LED therapy is the expression of several genes related to angiogenesis, synthesis of basement membrane components, and muscle differentiation [21]. Other biological effects of phototherapy include the alleviation of pain and nociception [24, 42, 43]. Effects on tissue repair and inflammation can be reached in wavelengths ranging from 600 to 1000 nm [18, 21, 23, 44].

Some studies have suggested that 940-nm LED irradiation could decrease serum markers of muscle injury and inflammation after exercise [28, 29]. On the basis of these studies, the LED equipment was developed for this study according to parameters that were suggested [28, 29]. In the present study, we observed that LED irradiation at 940 nm has the potential to prevent skeletal muscle injury from ischemia reperfusion and local inflammatory cell infiltration. LED irradiation decreased the damaged muscle fibers, decreased areas of necrosis, and reduced leukocyte infiltration.

## Conclusion

The results of this study indicate that 940-nm LED phototherapy is effective for reducing muscle inflammation and muscle fiber injury induced by ischemia reperfusion. Its effect may be related to anti-inflammatory action and the preservation of muscle fiber cell membrane integrity.

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