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ORIGINAL ARTICLE

Effects of low-level laser therapy on ROS homeostasis and expression of IGF-1 and TGF-β1 in skeletal muscle during the repair process

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Abstract The aim of the present study was to determine the effects of low-level laser therapy (LLLT) on the homeostasis of reactive oxygen species (ROS) and expression of IGF-1 and TGF-β1 in the gastrocnemius muscles of rats following contusion. Muscle regeneration involves cell proliferation, migration, and differentiation and is regulated by growth factors. A growing body of evidence suggests that LLLT promotes skeletal muscle regeneration and accelerates tissue repair. Adult male Sprague-Dawley rats (n=96) were randomly divided into three groups: control group (no lesion, untreated, n=6), contusion group (n=48), and contusion-plus-LLLT group (n=42). Gallium aluminum arsenide (GaAlAs) laser irradiation (635 nm; beam spot, 0.4 cm^2; output power, 7 mW; power density, 17.5 mW/cm^2; 20 min) was administered to the gastrocnemius contusion for 20 min daily for 10 days. Muscle remodeling was evaluated at 0 h and 1, 2, 3, 7, 14, 21, and 28 days after injury. Hematoxylin and eosin and Van Gieson staining were used to evaluate regeneration and fibrosis; muscle superoxide dismutase (SOD) and malondialdehyde (MDA) were detected via biochemical methods; expression of transforming growth factor beta-1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) were investigated via immunohistochemistry. The results showed that LLLT markedly promoted the regeneration of

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muscle and reduced scar formation. LLLT also significantly enhanced muscle SOD activity and significantly decreased muscle MDA levels 1, 2, and 3 days after injury. LLLT increased the expression of IGF-1 2, 3, and 7 days after injury and decreased the expression of IGF-1 21 and 28 days after injury. LLLT decreased the expression of TGF-β1 3 and 28 days after injury but increased expression at 7 and 14 days after injury. Our study showed that LLLT could modulate the homeostasis of ROS and of the growth factors IGF-1 and TGF-β1, which are known to play important roles in the repair process. This may constitute a new preventive approach to muscular fibrosis.

Keywords IGF-1 · TGF-β1 · ROS · Low-level laser therapy · Skeletal muscle

Introduction

Fibrosis is one of the largest groups of diseases for which there is no effective therapy. Abnormal and exaggerated deposition of extracellular matrix is the hallmark of many fibrotic diseases, including systemic sclerosis and pulmonary, liver, muscle, and kidney fibrosis. The spectrum of affected organs, the usually progressive nature of the fibrotic process, the large number of affected persons, and the absence of effective treatment pose an enormous challenge to treatment.

A growing body of evidence suggests that low-level laser therapy (LLLT) can alleviate or prevent fibrosis. Fillipin et al. found LLLT at 904 nm reduces histological abnormalities such as fibrosis, collagen concentration, and oxidative stress in an experimental model of Achilles tendon injury induced by single-impact trauma [1]. Rizzi et al. found that LLLT at 904 nm markedly alleviated histological abnormalities such as fibrosis both 7 and 14 days after single-impact blunt trauma [2]. The effectiveness of LLLT on other fibrotic situations has also been reported recently. Nussbaum reported on LLLT therapy for benign fibrotic lumps in the breast following reduction mammoplasty [3]. Katz et al. reported that 595 nm pulsed dye lasers and 1,450 nm diode lasers in combination with intralesional triamcinolone/5-fluorouracil successfully treated hypertrophic scarring following a phenol peel [4]. Cassuto et al. reported similar results showing that the combined use of silicone gel sheeting and a 532-nm millisecond laser was an effective and safe treatment for hypertrophic scars and keloids [5].

The skeletal muscle repair process consists of several interdependent phases: degeneration, inflammation, regeneration, fibrosis/scar formation, and remodeling [6]. The fibrotic phase of muscle healing usually occurs around 14 days after injury, with a peak at 21 days. The fibrotic phase is characterized by the synthesis of collagen, which is the major component of the extracellular medium of muscles, especially types I and III [6].

During the inflammatory phase, factors such as phagocytic stimuli increase the regeneration of reactive oxygen species (ROS), leading to oxidative stress [7]. Evidence has suggested that increased ROS plays an important role in the formation of fibroses of the lung, liver, kidneys, heart, and skeletal muscle [8–12]. Neutrophil depletion prior to ischemia-reperfusion injury and blockage of the neutrophil respiratory burst have been shown to reduce muscle damage [13]. A recent study has revealed that antioxidant levels are a major determinant of the regenerative capacity of muscle stem cells [14]. It has been reported that LLLT promotes cellular redox activity [15]. However, other studies reported that LLLT can restrain oxidative stress by increasing antioxidant levels or activity [16, 17].

Growth factors play a variety of roles during muscle regeneration. IGF-1 has been implicated as a critical regulator of muscle regeneration [6, 18, 19]. In contrast, TGF-β1 was believed to play a key role in the development of fibrosis [6, 20, 21]. In this study, we determined whether LLLT at 635 nm could mitigate the dysfunctions in rat gastrocnemius contusions to prevent muscular fibrosis. We evaluated whether the LLLT could reduce the oxidative stress that occurs during the inflammation phases, up-regulate the expression of IGF-1, down-regulate the expression of TGF-β1, and finally prevent fibrosis in the contused muscle.

Materials and methods

Animals and experimental groups

This study was performed following the regulations of the local Animal Care Ethical Committee. Ninety-six 2-month-old male Sprague-Dawley rats weighing 180–220 g were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The rats were kept in individual cages with standard food and water ad libitum under proper environmental conditions. The rats were randomly divided into three groups: control (n=6), contusion (n=48), and contusion-plus-LLLT (n=42). The contusion and the contusion-plus-LLLT groups were further divided into subgroups according to the time of death as follows: immediately after contusion (0 h, 0-day group) and 1, 2, 3, 7, 14, 21, and 28 days after contusion (1-, 2-, 3-, 7-, 14-, 21-, and 28-day groups, respectively). Of these, subgroup 0 h is only for the contusion group. There were six rats in each subgroup.

Experimental skeletal muscle contusion

Many models have been created to replicate skeletal muscle contusions [22–25]. According to McBrier et al., when the

dropped weight was constant, height influenced the type of injury produced [26]. In order to replicate severe muscle contusions, we set up animal models using a load weighing 330 g dropping down from a height of 810 mm blown onto the left leg with the rat's limb extended and ankle in dorsiflexion. The impact spot was 0.785 cm^2 and 15 mm away from calcaneus. The impact kinetic energy delivered by the device was 2.62 J. During the procedure, rats were anesthetized with pentobarbital sodium (2.5 %, 0.2100 ml^{-1} g^{-1}). Control rats were also anesthetized to ensure standardization but without trauma.

Laser irradiation

A low-intensity GaAlAs laser (Erchonia EML (ML2), U.S.) with a 635-nm wavelength was applied at 17.5 mW/cm^2 (635 nm; beam spot, 0.4 cm^2; output power, 7 mW; power density, 17.5 mW/cm^2; 20 min). The rats in each contusion-plus-LLLT subgroup were anesthetized with pentobarbital sodium (2.5 %, 0.2100 ml^{-1} g^{-1}) and immobilized on a rodent fixation device with their legs stretched. GaAlAs laser irradiation (635 nm) was then applied directly to the injured muscle area. The laser beam was kept perpendicular to the irradiated surface. The rats in the contusion-plus-LLLT group received LLLT for 20 min continuously once per day for 10 days. The rats in the control group and the contusion group received no laser irradiation.

Tissue processing

The animals were anesthetized with pentobarbital sodium (2.5 %, 0.2100 ml^{-1} g^{-1}). After shaving and cleaning the skin, the gastrocnemius muscles of the injured areas were dissected and cleaned, half for histopathological and immunohistochemical examination, the other half for biochemical examination. The rats in the control group were anesthetized and sampled in the same way as those in contusion group and contusion-plus-LLLT group.

Half of the muscles used for histopathological and immunohistochemical analysis were immediately trimmed and fixed by immersion with 10 % buffered formalin for 24 h. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax.

The other half of muscles used for biochemical assays were immediately frozen in liquid nitrogen.

Histopathology and morphometric evaluation of muscle regeneration and fibrosis

Muscles were cut at 4 μm using a microtome (Leica-RM 2135, Germany). Serial sections were stained with hematoxylin and eosin (H&E). The sections were morphologically analyzed by light microscope.

Centronucleated cells were considered regenerating fibers [27, 28]. Nuclei with no discernible surrounding cytoplasm were discarded. The minor axis diameters (i.e., the smallest diameters) of the centronucleated myofibers in each of the images were measured using the image analysis software Image-Pro Plus under ×400 magnification.

Van Gieson (V.G.) staining was performed to determine the level of fibrin in the muscle tissue [29]. Slides were stained for 4 min following the manufacturer's protocol (V.G. staining kit, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Image-Pro Plus software was used to measure the area of fibrosis tissue under ×100 magnification [30].

Biochemical analysis

After rapid thawing and weighing, muscle samples were homogenized with an electrical homogenizer at 4 °C, in 9 volumes of ice-cold physiological saline. Homogenates were centrifuged for 15 min at 3,500 rpm (Eppendoff centrifuge 5417R centrifuge, Germany), and the resultant supernatants were collected for superoxide dismutase (SOD) and malondialdehyde (MDA) analysis.

Muscle SOD activity was detected by xanthine oxidase assay. SOD activity is expressed in units per milligram of protein. One unit here is defined as the amount of protein capable of inhibiting 50 % of the SOD of nitric ion production.

Muscle MDA content was determined by thiobarbituric acid assay. MDA content was expressed as nanomoles per milligram of protein.

All kits for SOD and MDA were provided by Nanjing Jiancheng Bioengineering Institute, Jiangsu, China.

Immunohistochemistry

Standard techniques were used to prepare serial 4 μm sections. For immunohistochemistry, the primary antibody, rabbit anti-rat IGF-1 and rabbit anti-rat TGF-β1 (1:50, Santa Cruz Biotechnology, CA) were used at the indicated dilutions. The next procedure was performed according to the instructions of PV-9000 kit (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China). Negative controls (staining without primary antibody) were performed concurrently with all immunohistochemical staining. IGF-1 and TGF-β1 were observed randomly in at least five portal areas under a light microscope and the areas of IGF-1 and TGF-β1 expression (brown after immunostaining) were measured with Image-Pro plus software under ×100 magnification.

Statistical analysis

Data were expressed as mean values±standard deviation. The groups were compared using one-way analysis of variance, and the Newman–Keuls test was used to determine

the significance of differences of among all experimental groups. Data were analyzed using Graphpad Prism 5.0 statistical software (Graphpad Software, San Diego, CA).

Results

Histopathological and morphometric evaluation of muscle regeneration and fibrosis

In the control group, muscle fibers showed visible cross striations and were juxtaposed into regularly arranged fascicles, without degeneration, hemorrhage, necrosis, inflammatory cell infiltration, or collagen proliferation.

In the contusion group, the gastrocnemius sections of rats showed degeneration and inflammation, regeneration, and fibrosis, in that order (Fig. 1). Immediately after contusion (0 h), the injured gastrocnemius showed hemorrhage, edema, degeneration, and the rupture of some muscle fibers. In the 1st-day subgroup, the injured gastrocnemius showed marked putrescence and inflammatory cell infiltration. In the 2nd-day subgroup, the inflammatory cells increased in number and mainly consisted of neutrophils and macrophages. In the 3rd day subgroup, a large number of nuclei could be seen in the injured muscle (Fig. 1a). In the 7th-day subgroup, some newborn muscle fibers were visible, with fibroblasts in the injured area (Fig. 1b). In the 14th-day subgroup, the injured muscle had not fully healed, with some lymphocytes clustered in the injured area (Fig. 1c). In the 21st-day subgroup, the injured muscle healed, showing some collagen fibers, which were not irregularly arranged. In the 28th-day subgroup, the scar in the injured area was more evident than in the 21st-day subgroup.

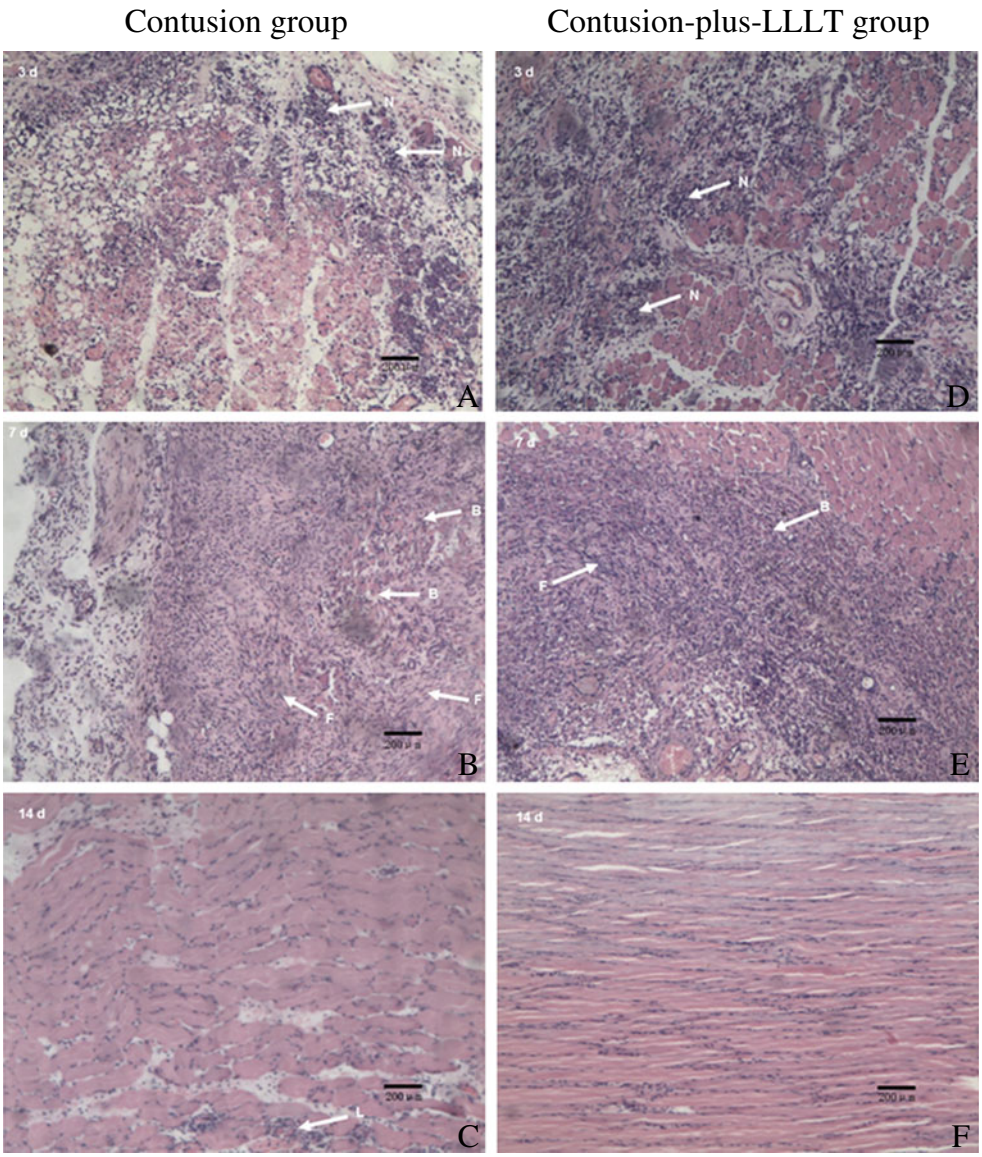

Fig. 1 Histological healing process of injured rat injured muscles in the contusion group and contusion-plus-LLLT group. **a**–**c** Injured muscles of group C rats 3, 7, and 14 days after injury, respectively. **d**–**f** Injured muscles of group L rats 3, 7, and 14 days after injury, respectively. H&E staining. *Scale bar*, 200 μm. The *letters in the graphs* represented: *N* nuclei, *B* newborn muscle fibers, *F* fibroblasts, *L* lymphocytes

In the contusion-plus-LLLT group, the histological abnormalities produced by the impact were markedly reduced, and the injured muscle exhibited better and quicker recovery than those of the contusion group. In the 1st-day subgroup, the injured gastrocnemius showed putrescence and centralized inflammatory cell infiltration. In the 3rd-day subgroup, the injured muscles showed more nuclei (Fig. 1d). In the 7th-day subgroup, more newborn muscle fibers and fewer fibroblasts were visible (Fig. 1e). From the 14th day on, the injured muscles healed, showing neogenetic muscles fibers arranged in an orderly fashion, and few collagen fibers could be seen (Fig. 1f).

The minor axis diameters (i.e., the smallest diameters) of the centronuleated myofibers in the contusion-plus-LLLT group were significantly larger than in the contusion group 14, 21, and 28 days after contusion (Fig. 2).

We used the V.G. staining to observe the formation of fibrosis tissue after injury in different groups. The results showed that the fibrotic area of the contusion-plus-LLLT group was significantly smaller that of contusion group 14, 21, and 28 days after injury ($P<0.01$) (Fig. 3).

Effects of LLLT on muscle superoxide dismutase activity and malondialdehyde content

Muscle SOD activity in the contusion group was significantly lower than in the control group at hour 0 and 1, 2, 3, and 7 days after contusion (Fig. 4a). SOD activity was not significantly different from the control group after the 14th day.

In the contusion-plus-LLLT group, muscle SOD activity was significantly higher than in the contusion group 1, 2, and 3 days after contusion, and the muscle SOD activity was not significantly different from that of the control group after the 7th day (Fig. 4a).

In the contusion group, MDA content was significantly higher than in the control group at hour 0 and 1, 2, 3, and 7 days after contusion (Fig. 4b). From the 14th day on, MDA content was not significantly different from the control group.

In the contusion-plus-LLLT group, MDA content was significantly lower than in the contusion group 1, 2, and 3 days after contusion, and the muscle MDA content was not significantly different from that of the control after the 7th day (Fig. 4b).

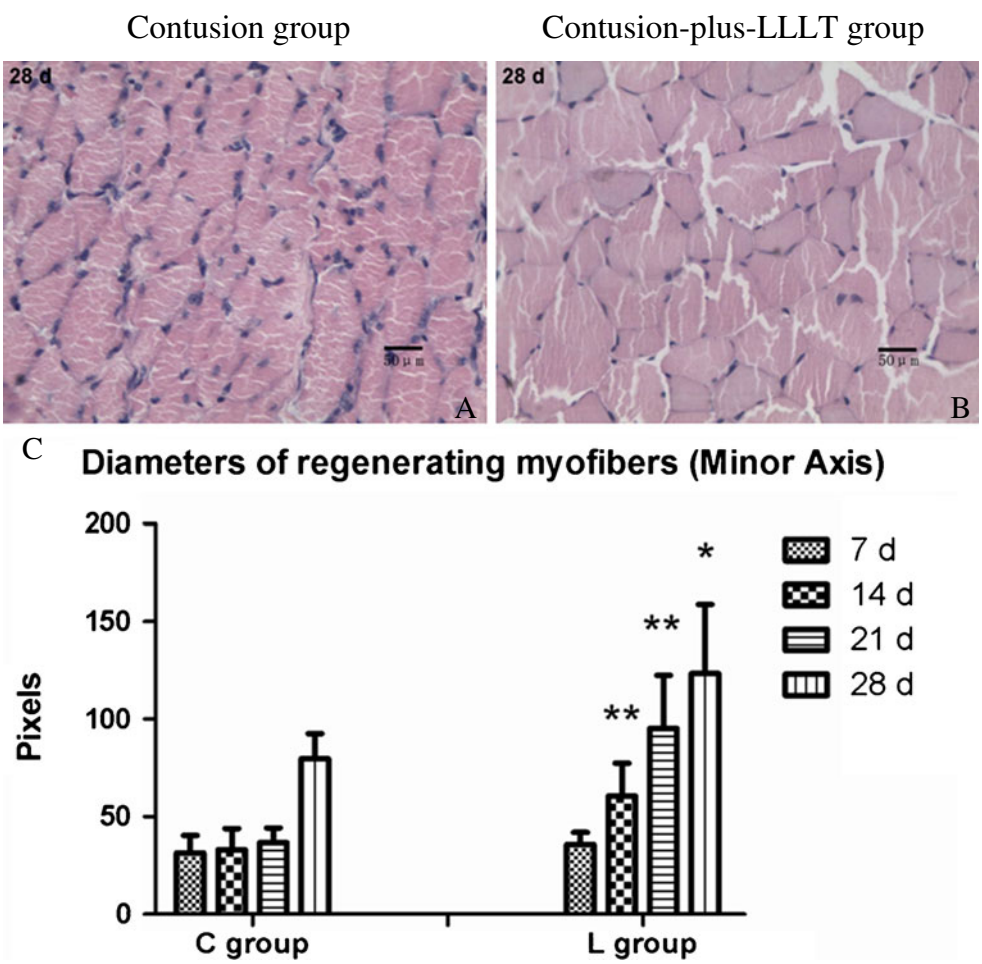

Fig. 2 Histological evaluation of the muscle regeneration at 28 days after injury of gastrocnemius muscle. **a** The contusion and **b** contusion-plus-LLLT groups. H&E staining. *Scale bar*, 50 μm. **c** Diameters of regenerating myofibers. *$P<0.05$; **$P<0.01$, significant differences between the two groups at a given time point

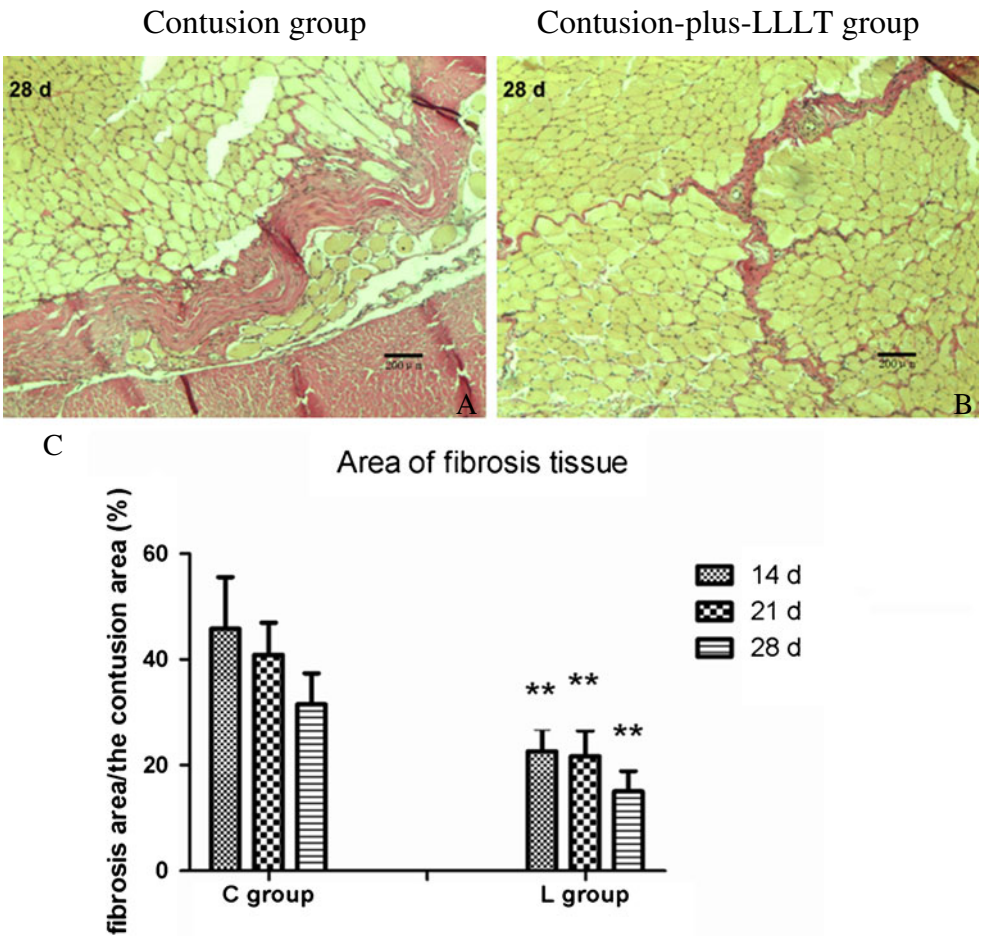

Fig. 3 Representative histological evaluation of fibrosis of gastrocnemius muscle 28 days after injury. **a** The contusion and **b** contusion-plus-LLLT groups. Fibrosis tissue was shown in *red* and muscles in *yellow*. Van Gieson staining. *Scale bar*, 200 μm. **c** Quantification of fibrotic area in the injured muscles. ** $P<0.01$, significant differences between the two groups at a given time point

Effects of LLLT on the expression of IGF-1 and TGF-β1 in injured muscle

The results of immunobiochemical assessment showed that the expression of IGF-1 in the injured region in the contusion group increased continuously. IGF-1 positive areas could be seen in the regenerative muscle fibers and in the connective tissue, especially 21 and 28 days after injury. The expression of IGF-1 in the contusion group showed a reverse V shape. It reached its highest point 21 days after injury and it dropped 28 days after injury (Fig. 5a).

The expression of IGF-1 in the contusion-plus-LLLT group showed a tendency similar to that observed in the contusion group, but the timing was different. The expression of IGF-1 reached its highest point 14 days after injury and then decreased continuously in the following days (Fig. 5b).

The expression of IGF-1 in contusion-plus-LLLT group was significantly higher than in the contusion group 2, 3, and 7 days after contusion ($P<0.01$). No significant differences were observed between the contusion and contusion-plus-LLLT group 14 days after injury. The expression of IGF-1 in the contusion-plus-LLLT group was significantly smaller than in the contusion group 21 and 28 days after injury ($P<0.01$) (Fig. 5c).

TGF-β1 expression was observed in both the newborn muscle cells and the intracellular substance. The expression of TGF-β1 in the contusion group increased significantly and showed a V-shape tendency. The lowest point of the TGF-β1 expression was 3 days after contusion. It increased continuously during the following days (Fig. 5d).

The expression of TGF-β1 in the contusion-plus-LLLT group reached its lowest point 3 days after injury and increased continuously from the 3rd through the 21st day. It dropped 28 days after injury (Fig. 5e).

The expression of TGF-β1 in the contusion-plus-LLLT group was significantly lower than in the contusion group 3 days after injury. It was significantly higher than in the contusion group 7 and 14 days after injury ($P<0.01$). No significant differences were observed between the contusion group and the contusion-plus-LLLT group 21 days after injury. The expression of TGF-β1 in the contusion-plus-LLLT group was significantly lower than in the contusion group 28 days after injury ($P<0.01$) (Fig. 5f).

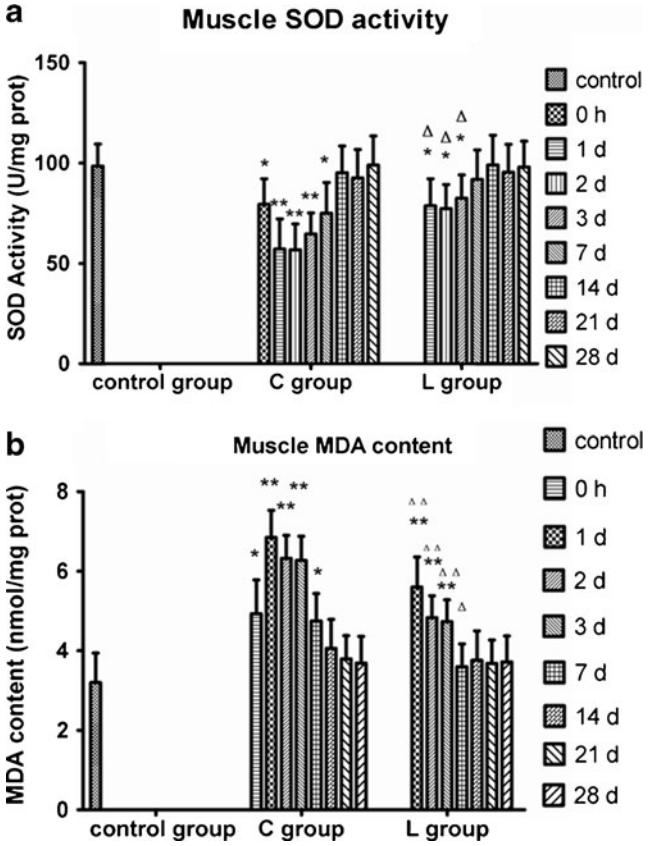

Fig. 4 The levels of muscle superoxide dismutase activity (**a**) and malondialdehyde content (**b**) in control, contusion, and contusion-plus-LLLT groups. *$P<0.05$; **$P<0.01$, significant differences from the control group. $^{\triangle}P<0.05$; $^{\triangle\triangle}P<0.01$, significant differences from the respective subgroup of group C at a given time point

Discussion

The present study demonstrated that LLLT could prevent fibrosis formation by promoting the regeneration of injured muscle. Muscles treated with LLLT showed more regenerating myofibers and less fibrotic scar formation than the untreated contusion group. The minor axis diameters of the regenerating myofibers in the contusion-plus-LLLT group were significantly larger than in the contusion group 14, 21, and 28 days after contusion.

First, we investigated the effects of LLLT on the homeostasis of ROS in injured muscle. MDA is an indicator of lipid peroxidation. LLLT decreased MDA level ($P<0.01$ 1, 2, and 3 days after injury and $P<0.05$ 7 days after injury), but it had no effect 14, 21, or 28 days after injury. SOD is an important antioxidant enzyme. LLLT increased SOD activity ($P<0.05$ 1, 2, and 3 days after injury), but it had no effect 7, 14, 21, or 28 days after injury. The results revealed that administration of LLLT dramatically decreased oxidative stress and increased the antioxidant enzyme activity during the inflammation phase, but it had no effect during the following phases, when the homeostasis of ROS had been restored.

Next, we investigated the effects of LLLT on the expression of IGF-1 and TGF-β1 in injured muscle. Many studies have identified a variety of different extracellular matrix proteins, growth factors, cytokines, and down-stream signaling pathways that orchestrate both muscle tissue homeostasis and the healing process [6, 18, 31, 32]. IGF-1 has been implicated as a critical regulator of muscle regeneration [6, 18, 19]. In contrast, TGF-β1 is believed to play a key role in the development of fibrosis [6, 20, 21]. Therefore, the promotion of signaling pathways activated by IGF-1 and inhibition of signaling pathways activated by TGF-β1 represent novel therapeutic approaches to fibrotic disorders [33]. However, in our study, LLLT was found to modulate the expression of IGF-1 and TGF-β1 in an extremely subtle manner that was entirely different from the drugs and therapies that solely promote the IGF-1 signaling pathway or solely inhibited the TGF-β1 signaling pathway.

Although IGF-1 has been implicated as a critical regulator of muscle regeneration, it is also potent mitogen for fibroblasts. IGF-1 can increase the production of matrix components such as collagen and decrease expression of matrix-degrading enzymes such as collagenase, potentially promoting fibrosis after muscle injury [6]. IGF-1 is also involved in the formation of lung fibrosis [34, 35]. In our study, the expression of IGF-1 in the contusion-plus-LLLT group was significantly higher than in the contusion group 2, 3, and 7 days after contusion. The first week was found to be the critical period for myoblast proliferation and differentiation [6]. These results indicate that LLLT may promote muscle regeneration by up-regulating the expression of IGF-1. The expression of IGF-1 in the contusion-plus-LLLT group was significantly lower than in the contusion group 21 and 28 days after injury, which suggested that LLLT might inhibit the expression of IGF-1 in the contusion group during the fibrosis and remodeling period.

TGF-β1 is a growth factor that plays different roles during different phases of the muscle healing process. There has been evidence indicating that TGF-β1 is not only a fibrotic inducer but also an inflammatory modulator in muscle injury [36]. A study by Shen et al. revealed that TGF-β1 might enhance the inflammatory response by enhancing the COX-2 pathway, especially the production of PGE2 [36]. Inflammation is an essential process of skeletal muscle healing [13, 37]. However, it has become evident that inflammation must be limited for effective healing to take place [13, 37]. Mesquita-Ferrari et al. found that LLLT modulated cytokine expression during short-term muscle remodeling, inducing a decrease in TGF-β [38]. In our study, the expression of TGF-β1 in the contusion-plus-LLLT group was significantly lower than in the contusion group 3 days after injury ($P<0.05$). This indicated that LLLT could regulate inflammation by inhibiting TGF-β1 signaling pathways. At 7 and 14 days after injury

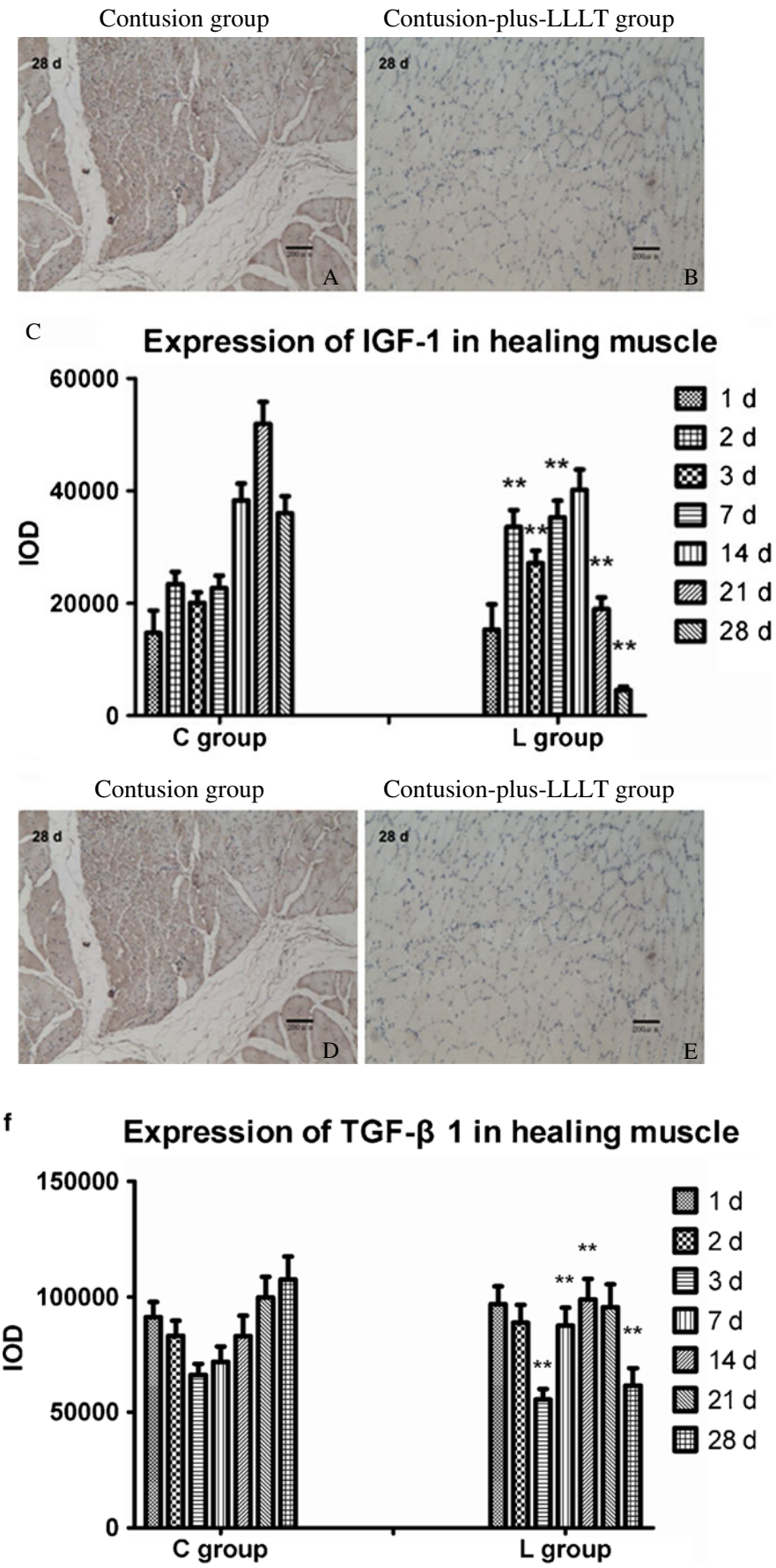

Fig. 5 **a**–**b** Immunostaining of IGF-1 expression in contusion and contusion-plus-LLLT groups 28 days after contusion. IGF-1-positive area was *brown* and cell nuclei was *blue*. **c** Expression of IGF-1 in healing muscles. ***P*<0.01, significant differences between the two groups at a given time point. *Scale bar*, 200 μm. **d**–**e** Immunostaining of TGF-β1 expression in contusion group and contusion-plus-LLLT group 28 days after contusion. TGF-β1-positive area was *brown* and cell nuclei were *blue*. **f** Expression of TGF-β1 in healing muscles. ***P*<0.01, significant differences between the two groups at a given time point. *Scale bar*, 200 μm

(regeneration was active during this period), the expression of TGF-β1 in the contusion-plus-LLLT group was significantly higher than in the contusion group ($P<0.01$). At 28 days after injury, the expression of TGF-β1 in the contusion-plus-LLLT group was significantly lower than in the contusion group ($P<0.01$), suggesting that LLLT can prevent fibrosis by inhibiting the expression of TGF-β1 during the fibrosis and remodeling period.

Conclusions

In summary, our study showed that LLLT can modulate the local homeostasis of ROS and of growth factors IGF-1 and TGF-β1 during the striated muscle repair. This may constitute a new preventive approach to muscular fibrosis.

Competing interests The authors have declared that no competing interests exist.

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