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Low-Level Laser Therapy (808 nm) Reduces Inflammatory Response and Oxidative Stress in Rat Tibialis Anterior Muscle After Cryolesion

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Abstract

Background and Objective—Muscle regeneration is a complex phenomenon, involving coordinated activation of several cellular responses. During this process, oxidative stress and consequent tissue damage occur with a severity that may depend on the intensity and duration of the inflammatory response. Among the therapeutic approaches to attenuate inflammation and increase tissue repair, low-level laser therapy (LLLT) may be a safe and effective clinical procedure. The aim of this study was to evaluate the effects of LLLT on oxidative/nitrative stress and inflammatory mediators produced during a cryolesion of the tibialis anterior (TA) muscle in rats.

Material and Methods—Sixty Wistar rats were randomly divided into three groups ($n = 20$): control (BC), injured TA muscle without LLLT (IC), injured TA muscle submitted to LLLT (IRI). The injured region was irradiated daily for 4 consecutive days, starting immediately after the lesion using a AlGaAs laser (continuous wave, 808 nm, tip area of 0.00785 cm², power 30 mW, application time 47 seconds, fluence 180 J/cm²; 3.8 mW/cm²; and total energy 1.4 J). The animals were sacrificed on the fourth day after injury.

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Results—LLLT reduced oxidative and nitrative stress in injured muscle, decreased lipid peroxidation, nitrotyrosine formation and NO production, probably due to reduction in iNOS protein expression. Moreover, LLLT increased SOD gene expression, and decreased the inflammatory response as measured by gene expression of NF- κ B and COX-2 and by TNF- α and IL-1 β concentration.

Conclusion—These results suggest that LLLT could be an effective therapeutic approach to modulate oxidative and nitrative stress and to reduce inflammation in injured muscle.

Keywords

low-level laser therapy; photobiomodulation; muscle cryolesion; inflammatory mediators; nitrative stress; oxidative stress

INTRODUCTION

Skeletal muscle injuries are common consequences of sport and labor activities. Depending on the severity of the injury, they can affect muscle function, leading to atrophy, contracture, pain, and increased likelihood of re-injury [1–3].

Muscle repair is very complex and involves several highly organized molecular and cellular processes. Immediately following the disruption of the myofibers, neutrophils, and macrophages infiltrate to the lesion area, producing pro-inflammatory cytokines and proteases responsible for necrotic tissue removal and further propagation of the inflammatory response [4–6]. These processes is reflected clinically by edema formation, local hematoma and significant increase in the serum levels of creatine kinase (CK), IL-1 β , and IL-6 cytokines, and lactate dehydrogenase [7].

The inflammatory phase of the muscle injury process is accompanied by an increase in the production of reactive oxygen and nitrogen species (ROS and RNS) and a reduction in the activity of antioxidant defense enzymes [8]. This imbalance between pro-oxidants and antioxidants, in favor of pro-oxidants, can generate oxidative and nitrative stress in the tissue that contributes to activate NF- κ B, a pleiotropic transcription factor responsible for multiple changes in gene expression in the inflammatory process [9,10]. These gene products include pro-inflammatory cytokines, growth factors, chemokines, and adhesion molecules. Excessive production of these mediators can be harmful for muscle repair, as it intensifies inflammation and can inhibit differentiation and fusion of myoblasts, impairing the regeneration of muscle tissue [11,12].

It is known that in muscle lesions, IL-1 β and TNF- α act synergistically increasing the expression of inducible nitric oxide synthase (iNOS) and consequently increasing NO levels in the tissue [13,14]. NO from the inflammatory site can react with superoxide anion (O₂⁻) produced by inflammatory cells, forming peroxynitrite (ONOO⁻). Peroxynitrite is a potent oxidant and may cause protein modifications by oxidizing thiol groups of proteins, by nitrating tyrosine residues, and by increasing lipid peroxidation, nucleic acid damage, and mitochondrial respiratory chain inhibition [15]. The severity of this process depends on the intensity and duration of the inflammatory response [8]. Thus, during rehabilitation of muscle injury, therapeutic approaches that focus on avoiding oxidative damage and lessening the inflammatory process can be helpful for promoting repair and reestablishing homeostasis.

Low-level laser therapy (LLLT) has been considered a safe and efficient technique for the clinical treatment of a variety of diseases and injuries as it possesses anti-inflammatory, analgesic and reparative properties. One area that is attracting growing interest is the use of

LLLT to treat skeletal muscle disorders [16–18]. The fact that LLLT can penetrate into the skeletal muscle could allow non-invasive treatment to be carried out with a low likelihood of treatment-related adverse events [19]. LLLT promotes the regeneration of skeletal muscle in animal and human, where it is able to induce the activation and proliferation of quiescent satellite cells [20,21], stimulate the formation of myotubes, increase the number of muscle fibers and mitochondrial density, as well as stimulate angiogenesis [22–25]. Recent studies have showed that LLLT can reduce muscle fatigue induced by electrical stimulation in rats [26] and reduce pain in patients with delayed onset muscle soreness (DOMS) [27].

LLLT may have beneficial effects in the acute treatment of skeletal muscle injuries due its ability to reduce the inflammatory cells and enzymes responsible for release of chemotactic factors in the early phase of inflammation [28,29], inhibit prostaglandin and inflammatory cytokine synthesis [2,17], and increase antioxidant enzyme levels in several models of inflammation [16]. Although knowledge is steadily increasing concerning the biological mechanisms of this process more studies are needed to determine which signaling pathways are triggered by LLLT. This study therefore focused on exploring the effect of LLLT on inflammation in injured muscle, evaluating its effect on oxidative and nitrative stress and on local inflammatory mediators produced during a cryogenic muscle lesion in rats.

MATERIALS AND METHODS

Experimental Groups and Freezing Muscle Injury (Cryolesion)

Adult male Wistar rats (*Rattus norvegicus*) weighing 300 g were used in this study. Good laboratory animal practice was observed according to the international standards for animal experimentation and following approval by our institution's Animal Care and Ethics Committee.

The animals ($n = 60$) were randomly divided into three groups ($n = 20$ per group): control group—animals with no interventions (BC); injured TA muscle without treatment (IC); and injured TA muscle submitted to laser irradiation treatment (IRI).

Surgical procedures (cryolesion) were performed based on those described by Miyabara et al. [30], under anesthesia with 40 mg/kg ketamine (Dopalen; Vetbrands, São Paulo, Brazil) and 20 mg/kg xylazine (Anasedan; Vetbrands, São Paulo, Brazil). After anesthesia the skin around the right TA muscle was shaved and cleaned. Then, a transversal cut (about 1 cm) of the skin over the middle of the muscle was carried out, exposing the muscle. A rectangular iron bar ($6 \times 30 \text{ mm}^2$), frozen in liquid nitrogen, was then kept for 10 seconds on the center of the muscle. The procedure was repeated twice consecutively, with a time interval of 30 seconds.

The cryolesion is a well-known and highly reproducible model, which reproduces the natural response of muscle to damaging lesions, as well as its regeneration capacity [30]. Therefore, it was employed in the present study for evaluation of the effectiveness of LLLT. The model is characterized by the development of a homogeneous and well-defined lesion area, which can be macroscopically observed by the white color that develops once it is established (Fig. 1). This lesion produces myonecrosis, tissue disruption, edema, hypercontracted fibers, inflammatory cell infiltration (especially neutrophils and macrophages). Inflammatory cytokines TNF- α and TGF- β are increased in this model. During the regeneration phase (after 10 days) the tissue shows less inflammatory cell infiltration, basophilic regenerating cells, centrally nucleated cells, and fibers with enlarged diameter. The final stage of regeneration occurs after 21 days when most cells have a mature appearance [17,30,31].

Finally, the skin was sutured. The right TA muscle was chosen because it is a superficial muscle, making the surgery easy. After surgery, the animals were housed in single plastic cages in a room with controlled environmental conditions and fed rat chow and water *ad libitum*.

LLLT Protocol

Photobiostimulation was performed using a gallium–aluminum–arsenide (GaAlAs) diode laser (Photon Laser II, DMC® Equipment Ltd., SP, São Carlos, Brazil), with the following parameters: continuous radiation mode, 808 nm wavelength, 30 mW power output, 47 seconds irradiation time, 0.00785 cm² spot area, dose 180 J/cm², irradiance 3.8 W/cm², and 1.4 J total energy per point. The equipment was previously calibrated in a laser power energy monitor (Masterfield, Filter Coherent, CA) supplied by Physics Institute of São Carlos (IFSC; São Carlos, Brazil).

The skin having been shaved at the surgery site, the laser was applied in a single point at in the middle of right TA muscle (lesion area). LLLT was performed daily and at the same time for 4 consecutive days, with the first application immediately after skin suturing. Laser was applied by contact technique, with the optical fiber kept perpendicular to the skin.

Muscle Evaluation

Animals were weighed and euthanized with an anesthetic overdose (twofold the anesthetic dose) and the right TA muscles removed and weighed. Subsequently, ten animals from each group were used for morphological analysis and the others ten animals for real-time polymerase chain reaction (qPCR) analysis, thiobarbituric acid-reactive substances (TBARS), NO production, immunoblotting, cytokine measurements and dot blot (Fig. 2). For histological evaluation, the muscle fragment was immediately frozen in isopentane pre-cooled in liquid nitrogen, and then stored in a freezer at –80°C (Forma Scientific, Marietta, OH). For further analysis, the muscle fragments from the injured site were each divided transversally into two equal parts and pulverized in liquid nitrogen with a mortar and pestle. The proximal fragment was used for RNA extraction protocol. The distal samples were used for the other analyses and were homogenized in RIPA buffer containing 10 mM Tris–HCl (pH 7.5), 1% Tergitol, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, and proteolytic enzyme inhibitors (Protease Inhibitor Cocktail; Sigma, St. Louis, MO). After debris separation by centrifugation for 45 minutes at 14,000g the supernatants were collected and the protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL). All samples were stored at –80°C until analysis.

Muscle Morphological Analysis

Histological serial muscle cross-sections were obtained (one section of 10 µm in each 100 µm) in a cryostat microtome (Microm HE 505, Jena, Germany), across the middle of the TA muscle.

For morphological evaluation by light microscopy (Axiolab, Carl Zeiss, Germany) tissue sections were stained using toluidine blue and/or submitted to acid phosphatase staining. Toluidine blue staining was used to evaluate the morphological pattern of the muscle fibers and the presence of muscle fiber injury, because it permits the identification of the myonuclei, areas of myonecrosis, and the basophilic regions of the muscle fibers. Acid phosphatase staining was used to identify areas of necrosis. Normal muscles fibers do not show a positive acid phosphatase reaction that indicates a high concentration of lysosomes, which is considered evidence of tissue necrosis and consequent phagocytosis.

Lipid Peroxidation

The level of lipid peroxides was measured using the TBARS method in the muscle samples. The tissue homogenates (200 μ l) were then added to a reaction mixture consisting of 1.5 μ l of 0.8% thiobarbituric acid, 200 μ l of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5), and 600 μ l distilled water and heated at 90°C for 1 hour. After cooling to room temperature, samples were cleared by centrifugation (10,000g, 10 minutes) and their absorbance measured at 532 nm in a Spectramax Plate Reader (Molecular Devices, Sunnyvale, CA). 1,1,3,3-Tetramethoxypropane was used as an external standard. Results were expressed as μ M malondialdehyde (TBARS)/mg protein. Tissue protein concentration was quantified by the Bradford protein assay (Bio-Rad, Hercules, CA).

NO Production

NO production was assessed using the Griess reaction. Tissue NO concentration was assayed in the supernatant by mixing 50 μ l of the sample with 50 μ l of solution A (1% sulfanilamide in 5% phosphoric acid) and adding 50 μ l of solution B (0.1% naphthylethylenediamine in distilled water), and incubating for 10 minutes. The optical density was read at 595 nm in a Spectramax Plate Reader (Molecular Devices, Sunnyvale, CA).

Immunoblotting

Protein expression was performed using SDS–polyacrylamide gel electrophoresis under reducing conditions. Tissue extracts (25 μ g) were boiled in equal volumes of loading buffer (150 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 15% β -mercaptoethanol, and 0.01% bromophenol blue) and were subjected to electrophoresis in 10% polyacrylamide gel. Following electrophoretic separation, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and 0.5% Tween 20 (TBST) for 1 hour. Primary antibody (Ab) against the following was employed: iNOS (rabbit polyclonal, 1:1,000, Santa Cruz). Ab was diluted in TBST with 0.5% bovine serum albumin (BSA) and incubated in 4°C overnight. After washing twice with TBST, secondary Ab horseradish peroxidase conjugate (Sigma–Aldrich, St. Louis, MO) was applied at dilution 1:1,000 for 1 hour. Blot was washed in TBST for 30 minutes, incubated in enhanced chemiluminescence reagents (Super signal detection kit; Pierce) and exposed and photographed using GBox Gel Document System (Syngene, Frederick, MD). The band intensity was quantified using Gene Tools software (Syngene).

Dot Blot

For detection of nitrotyrosine formation using dot blot, 5 μ g of protein was blotted onto nitrocellulose membranes using a vacuum dot blotter (Bio-Rad). Membranes were blocked with 5% BSA in PBS with Tween 20 (PBST) and incubated with primary antibody against nitrotyrosine residues (rabbit monoclonal Abcam; 1:10,000) overnight. After, membrane were washed 3 times in PBST, followed by incubation with secondary antibody (anti-rabbit IgG, 1:2,500, Sigma–Aldrich) for 2 hours. Then blot was washed in PBST for 30 minutes, incubated in enhanced chemiluminescence reagent and quantified as described above.

Cytokine Measurements

Tissue was prepared as described above and cytokine measurements was performed in the homogenates by enzyme-linked immunosorbent assay (ELISA). Cytokine measurement kits were used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) using a Spectramax Plate Reader (Molecular Devices, Sunnyvale, CA). The results are expressed in pg per μ g of protein.

Total RNA Isolation and Real Time Polymerase Chain Reaction

Tissues fragments were homogenized in 1 ml TRIzol[®] reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA integrity was assessed by 260/268 nm ratio and on a 1% agarose gel electrophoresis stained with ethidium bromide. Two nanograms mRNA was used to real-time PCR. The amplification was performed in a thermal cycler (Applied Biosystems StepOne[™]) at 50°C for 10 minutes, 95°C for 5 minutes, and then 95°C for 15 seconds followed by 60°C for 30 seconds, and 72°C for 30 seconds for 40 cycles. Real-time PCR was performed in a 15 µl reaction mixture containing 7.5 µl 2× SYBR Green Reaction Mix (Invitrogen), 0.3 µl each primer, 0.3 µl Super Script III RT/Platinum Taq Mix (10 pmol/µl), 0.15 µl ROX Reference Dye, and 5 µl sample in water. Quantification was performed by $2^{-\Delta\Delta C_t}$ method, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. This gene was chosen after genome analysis (<http://medgen.ugent.be/~jvdesomp/genorm/>) of 5 housekeeping genes. The following primers were used: SOD forward: GGCAAGCGGTGAACCAGTTG, reverse: TGCCCAGGT-CTCCAACATGC; COX-2 forward: TGTATGCTACCATCTGGCTTCGG, reverse: GTTTGGAACAGTCGCTCGTC-ATC; NF-κβ forward: TCCGAGATAATGACAGCGTGTG, reverse: GGTCCATCCTGCCATAATTG; and GAPDH forward: ATGATTCTACCCACGGCAAG, reverse: CTG-GAAGATGGTGATGGGTT.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Shapiro–Wilk's and Levene's test were applied to evaluate the normality and homogeneity of the results, respectively. Comparisons between experimental groups were performed by analysis of variance (one-way ANOVA), and the Tukey post-test used to compare individual groups. A *P*-value <0.05 was considered significant. All analyzes were performed using Sigma Stat Statistical Software (v.3.1).

RESULTS

Morphological Analysis

By histochemical methods (Fig. 3), it was possible to qualitatively analyze the injured muscle alone or after LLLT. The morphological aspects were similar in both injured experimental groups (IC and IR). The region affected by the cryolesion was restricted to the surface of the TA muscle and could be visualized in the TB-stained cross-sections. The interface between the intact area and the lesion site is clearly defined. The fibers from the intact region are typical of healthy skeletal muscle tissue with polygonal shape, peripheral nucleus, fascicular organization, and negative reaction to acid phosphatase staining. While in the lesion site next to the interface, recent formed fibers are characterized by centralized nuclei and a smaller transverse section. In the superficial region of the lesion, it was possible to identify a high concentration of mononuclear cells (inflammatory and/or myogenic), absence of newly formed muscle fibers, abundant extracellular matrix and numerous lysosomes, indicating tissue necrosis and phagocytosis (positive acid phosphatase reaction). Moreover, the non-injured groups (BC) presented no alteration in fiber morphology and had negative reaction for acid phosphatase activity.

Lipid Peroxidation

Lipid peroxidation, evaluated by TBARS, increased in the injured muscle groups (IC and IRI), compared to control group (BC; *P* < 0.01, Fig. 4). TBARS levels in the injured muscle significantly decreased after LLLT (IRI, *P* < 0.05 vs. IC).

Nitrotyrosine Formation

Nitration of tyrosine residues was found in the injured muscle groups but not control (IC and IRI; $P < 0.01$ vs. BC), whereas LLLT significantly reduced nitrotyrosine formation in the injured muscle (IRI; $P < 0.01$ vs. IC; Fig. 5).

iNOS Protein Expression and NO Production

The cryolesion elevated the expression of iNOS protein and increased NO levels in the muscle homogenate from the injured muscle groups IC and IRI ($P < 0.01$ vs. BC). LLLT significantly reduced iNOS protein expression in injured muscle (IRI; $P < 0.05$ vs. IC, Fig. 6A) and also reduced NO levels (IRI; $P < 0.01$ vs. IC, Fig. 6B).

Muscle Cytokines Levels

TNF- α and IL-1 β cytokines were quantified in the muscle homogenates by ELISA. Both cytokines were increased in injured muscle groups (IC and IRI; $P < 0.01$ vs. BC; Fig. 7A and B). LLLT decreased levels of both TNF- α and IL-1 β in the injured muscle groups (IRI; $P < 0.05$ vs. IC).

Gene Expression of NF- κ B, COX-2 e SOD

NF- κ B gene expression levels as measured by mRNA was increased after muscle lesion (IC and IRI; $P < 0.01$ vs. BC; Fig. 8A). LLLT diminished the gene expression level of NF- κ B in the injured muscle ($P < 0.01$ vs. IC).

COX-2 gene expression levels showed the same pattern observed in the NF- κ B mRNA quantification (Fig. 8B). COX-2 levels were increased in injured muscle groups (IC and IRI) compared to control group (BC; $P < 0.01$). LLLT reduced COX-2 mRNA levels in the injured muscle ($P < 0.01$ vs. IC).

SOD mRNA, an important antioxidant enzyme, was increased in cryolesioned muscle groups (IC and IRI, $P < 0.01$ vs. BC; Fig. 8C). LLLT further increased SOD mRNA levels in the injured muscle group (IRI; $P < 0.01$ vs. IC).

DISCUSSION

LLLT has been demonstrated to be effective in reducing the inflammatory response, to be able to promote tissue repair, to attenuate pain and to reduce muscle fatigue in several studies in both animal models and in clinical trials [27,32,33]. Although most of the studies demonstrated beneficial effects with this therapy, little is known about how exactly LLLT is able to affect cellular systems and what are the molecular mechanisms involved in these processes. In particular, the question of whether LLLT increases or decreases reactive oxygen species and oxidative stress remains unanswered, as there are reports supporting both sides of the question [33,34]. We used a model of muscle cryolesion in rats in order to evaluate the influence of LLLT mediated by an 808 nm laser on oxidative stress and inflammatory mediators in the injured area.

Several experimental models have been utilized to evaluate the influence of LLLT in skeletal muscle post-injury [16,22]. To some extent contusion and exercise-induced muscle injury models are able to reproduce the lesions described in clinical reports, however, these models generate diffuse and complex lesions, and are usually difficult to analyze. In this study, the cryolesion procedure was chosen to be a homogeneous and highly reproducible lesion model, causing damage to satellite cells, basal lamina, blood vessels, and nerve fibers, and inducing a rapid and extended necrosis of myofibrils, followed by a relatively slow regeneration process. Even though this model does not reproduce the lesions from clinical

reports, it has been utilized as a tool for mechanistic studies of laser action on the wounded tissue [2,18].

Morphological analysis demonstrated that induction of cryolesion in the tibialis anterior muscle generated an inflammatory response characterized by modifications in the muscle structure, increased intercellular edema, and an inflammatory infiltrate with a high concentration of lysosomes considered to be a marker of phagocytosis and myonecrosis [17,30,31]. We did not observe morphological differences between irradiated and non-irradiated groups. As light microscopy is a limited morphological technique, further studies using transmission electron microscopy may be necessary for a more thorough analysis. In spite of this limitation, we were able to confirm a smaller lesion area in the irradiated group [35].

We also investigated the effect of LLLT on oxidative and nitrative stress. During inflammation, the presence of lipid peroxidation, iNOS expression, NO, and nitrotyrosine generation suggests that local oxidative stress could amplify the severity of the lesion and modify, both structural and functionally, proteins and lipids from cell membranes, promoting changes in the signaling pathways that serve to increase the overall inflammatory response [1,36]. Lipid peroxidation can occur enzymatically, that is, activating cyclooxygenases and lipoxygenases and/or non-enzymatically through generation of exogenous ROS, RNS, liberation of transition metals, and production of reactive species [8,37]. Our results show that LLLT reduced lipid peroxidation, keeping it close to basal levels. Even though the mechanism of this reduction is unknown, it is a relevant observation that there was less COX-2 mRNA and more SOD mRNA after LLLT irradiation.

In addition, NO may also be involved with local changes downstream of muscle lesion. Here, LLLT was shown to exert an important effect in reducing iNOS protein expression. We also observed a lower level of NO that may be a consequence of lower levels of iNOS. It is clear from several published studies and reviews that the overall effects of NO and its by products are complex and ambiguous. It is known that NO is an important mediator of inflammation, contributing to the activation and inactivation of several molecules.

NO react with superoxide anion, generating peroxynitrite, which causes nitrative stress [38]. Peroxynitrite is a potent oxidizing and nitrating agent, that intensifies inflammatory response and causes nitration of tyrosine residues in proteins, contributing to cellular necrosis and/ or apoptosis [37]. Enhanced NO production correlates with the increase in lipid peroxidation and nitrotyrosine formation in the injured muscle group, suggesting that considerable oxidative damage may be occurring.

In contrast, in the LLLT group, reduced formation of nitrotyrosine is indicative of reduced iNOS expression and lower NO generation and decreased peroxynitrite production. In this case, LLLT was able to reduce oxidative and nitrative stresses and, consequently, decrease deleterious effects to the injured tissue. These findings suggest that LLLT could be used to lessen the damaging effects of ROS and RNS generated in the muscle lesion.

Regarding the antioxidant effects of irradiation, SOD mRNA increased after LLLT, and this increase may contribute to preventing the additional formation of superoxide that leads to peroxynitrite and other reactive species production [16,29]. Therefore, results shown here suggest that LLLT had a positive biological effect in modulating the redox balance, reducing nitrative and oxidative stress and lessening oxidative damage that would delay the recovery of the injured tissue. Similar results were obtained by others: Rizzi et al. [39], described that LLLT inhibited oxidative stress, iNOS expression and the activation of NF- κ B in a model of muscle trauma. Studies utilizing different experimental models have suggested that LLLT is

able to induce SOD expression, decreasing the available superoxide anion and, as a result, reduce peroxynitrite production [16,40].

The inflammatory response involves multiple mediators and LLLT could influence overall NF- κ B activation during this process. NF- κ B is a transcription factor rapidly activated in response to oxidative stress and which participates in the induction of many inflammation-related genes such as COX-2, iNOS, and pro-inflammatory cytokines [9,10]. We found trauma was able to elicit NF- κ B mRNA activation, leading to production of inflammatory mediators such as COX-2, iNOS, TNF- α , and IL-1 β . LLLT, however, decreased NF- κ B gene expression, suggesting that this effect could be responsible for the reduced amount of pro-inflammatory mediators in this model. As discussed above, LLLT mediated anti-inflammatory effects could be a consequence of NF- κ B reduction or inactivation [41–43].

In summary, our results indicate that near-infrared laser (808 nm) at the dose selected was able to attenuate oxidative and nitrative stress in the skeletal muscle, decrease NO production, lipid peroxidation, nitrotyrosine formation, and iNOS and could increase SOD expression in an experimental animal model. Additionally, TNF- α , IL-1 β , and COX-2 were reduced in the lesion area, leading to an anti-inflammatory effect. Accordingly, these experimental data may contribute to further understanding beneficial effects of LLLT on muscle inflammation.

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References

1. Filippin LI, Cuevas MJ, Lima E, Marroni NP, Gonzalez-Gallego J, Xavier RM. The role of nitric oxide during healing of trauma to the skeletal muscle. *Inflamm Res*. 2011; 60(4):347–356. [PubMed: 21076852]
2. Renno AC, Toma RL, Feitosa SM, Fernandes K, Bossini PS, de Oliveira P, Parizotto N, Ribeiro DA. Comparative effects of low-intensity pulsed ultrasound and low-level laser therapy on injured skeletal muscle. *Photomed Laser Surg*. 2011; 29(1):5–10. [PubMed: 21166589]
3. Li Y, Cummins J, Huard J. Muscle injury and repair. *Curr Opin Orthop*. 2001; 12:6.
4. Tidball JG. Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol*. 2005; 288(2):R345–R353. [PubMed: 15637171]
5. Huard J, Li Y, Fu FH. Muscle injuries and repair: Current trends in research. *J Bone Joint Surg Am*. 2002; 84-A(5):822–832. [PubMed: 12004029]
6. Jarvinen TA, Jarvinen TL, Kaariainen M, Kalimo H, Jarvinen M. Muscle injuries: Biology and treatment. *Am J Sports Med*. 2005; 33(5):745–764. [PubMed: 15851777]
7. Buford TW, Cooke MB, Sheldahl BD, Hudson GM, Redd L, Willoughby DS. Effects of eccentric treadmill exercise on inflammatory gene expression in human skeletal muscle. *Appl Physiol Nutr Metab*. 2009; 34(4):745–753. [PubMed: 19767811]
8. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev*. 2002; 82(1):47–95. [PubMed: 11773609]

9. Adams V, Nehrhoff B, Spate U, Linke A, Schulze PC, Baur A, Gielen S, Hambrecht R, Schuler G. Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: An in vitro and in vivo study. *Cardiovasc Res.* 2002; 54(1):95–104. [PubMed: 12062366]
10. van den Berg R, Haenen GR, van den Berg H, Bast A. Transcription factor NF-kappaB as a potential biomarker for oxidative stress. *Br J Nutr.* 2001; 86(Suppl 1):S121–S127. [PubMed: 11520430]
11. Barnes PJ, Karin M. Nuclear factor-kappaB: A pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.* 1997; 336(15):1066–1071. [PubMed: 9091804]
12. Langen RC, Van Der Velden JL, Schols AM, Kelders MC, Wouters EF, Janssen-Heininger YM. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. *FASEB J.* 2004; 18(2):227–237. [PubMed: 14769817]
13. Kaminski HJ, Andrade FH. Nitric oxide: Biologic effects on muscle and role in muscle diseases. *Neuromuscul Disord.* 2001; 11(6–7):517–524. [PubMed: 11525879]
14. Filippin LI, Moreira AJ, Marroni NP, Xavier RM. Nitric oxide and repair of skeletal muscle injury. *Nitric Oxide.* 2009; 21(3–4):157–163. [PubMed: 19682596]
15. El-Remessy AB, Al-Shabrawey M, Platt DH, Bartoli M, Behzadian MA, Ghaly N, Tsai N, Motamed K, Caldwell RB. Peroxynitrite mediates VEGF's angiogenic signal and function via a nitration-independent mechanism in endothelial cells. *FASEB J.* 2007; 21(10):2528–2539. [PubMed: 17384142]
16. Liu XG, Zhou YJ, Liu TC, Yuan JQ. Effects of low-level laser irradiation on rat skeletal muscle injury after eccentric exercise. *Photomed Laser Surg.* 2009; 27(6):863–869. [PubMed: 19697999]
17. Mesquita-Ferrari RA, Martins MD, Silva JA Jr, da Silva TD, Piovesan RF, Pavesi VC, Bussadori SK, Fernandes KP. Effects of low-level laser therapy on expression of TNF-alpha and TGF-beta in skeletal muscle during the repair process. *Lasers Med Sci.* 2011; 26(3):335–340. [PubMed: 21053039]
18. Ramos L, Leal Junior EC, Pallotta RC, Frigo L, Marcos RL, de Carvalho MH, Bjordal JM, Lopes-Martins RA. Infrared (810 nm) low-level laser therapy in experimental model of strain-induced skeletal muscle injury in rats: Effects on functional outcomes. *Photochem Photobiol.* 2012; 88(1): 154–160. [PubMed: 22053933]
19. Hamblin MR. Introduction to experimental and clinical studies using low-level laser (light) therapy (LLLT). *Lasers Surg Med.* 2010; 42(6):447–449. [PubMed: 20662020]
20. Ben-Dov N, Shefer G, Irintchev A, Wernig A, Oron U, Halevy O. Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. *Biochim Biophys Acta.* 1999; 1448(3):372–380. [PubMed: 9990289]
21. Shefer G, Oron U, Irintchev A, Wernig A, Halevy O. Skeletal muscle cell activation by low-energy laser irradiation: A role for the MAPK/ERK pathway. *J Cell Physiol.* 2001; 187(1):73–80. [PubMed: 11241351]
22. Iyomasa DM, Garavelo I, Iyomasa MM, Watanabe IS, Issa JP. Ultrastructural analysis of the low level laser therapy effects on the lesioned anterior tibial muscle in the gerbil. *Micron.* 2009; 40(4): 413–418. [PubMed: 19269186]
23. Amaral AC, Parizotto NA, Salvini TF. Dose-dependency of low-energy HeNe laser effect in regeneration of skeletal muscle in mice. *Lasers Med Sci.* 2001; 16(1):44–51. [PubMed: 11486338]
24. Nakano J, Kataoka H, Sakamoto J, Origuchi T, Okita M, Yoshimura T. Low-level laser irradiation promotes the recovery of atrophied gastrocnemius skeletal muscle in rats. *Exp Physiol.* 2009; 94(9):1005–1015. [PubMed: 19525315]
25. Silveira PC, Silva LA, Fraga DB, Freitas TP, Streck EL, Pinho R. Evaluation of mitochondrial respiratory chain activity in muscle healing by low-level laser therapy. *J Photochem Photobiol B.* 2009; 95(2):89–92. [PubMed: 19232497]
26. Lopes-Martins RA, Marcos RL, Leonardo PS, Prianti AC Jr, Muscara MN, Aimbire F, Frigo L, Iversen VV, Bjordal JM. Effect of low-level laser (Ga–Al–As 655 nm) on skeletal muscle fatigue induced by electrical stimulation in rats. *J Appl Physiol.* 2006; 101(1):283–288. [PubMed: 16627677]

27. Douris P, Southard V, Ferrigi R, Grauer J, Katz D, Nascimento C, Podbielski P. Effect of phototherapy on delayed onset muscle soreness. *Photomed Laser Surg.* 2006; 24(3):377–382. [PubMed: 16875447]
28. Cressoni MD, Dib Giusti HH, Casarotto RA, Anaruma CA. The effects of a 785-nm AlGaInP laser on the regeneration of rat anterior tibialis muscle after surgically-induced injury. *Photomed Laser Surg.* 2008; 26(5):461–466.
29. Dourado DM, Favero S, Baranauskas V, da Cruz-Hofling MA. Effects of the Ga–As laser irradiation on myonecrosis caused by *Bothrops moojeni* snake venom. *Lasers Surg Med.* 2003; 33(5):352–357. [PubMed: 14677163]
30. Miyabara EH, Martin JL, Griffin TM, Moriscot AS, Mestril R. Overexpression of inducible 70-kDa heat shock protein in mouse attenuates skeletal muscle damage induced by cryolesioning. *Am J Physiol Cell Physiol.* 2006; 290(4):C1128–C1138. [PubMed: 16291818]
31. Baptista J, Martins MD, Pavesi VC, Bussadori SK, Fernandes KP, Pinto Junior Ddos S, Ferrari RA. Influence of laser photobiomodulation on collagen IV during skeletal muscle tissue remodeling after injury in rats. *Photomed Laser Surg.* 2011; 29(1):11–17. [PubMed: 20701543]
32. Leal Junior EC, Lopes-Martins RA, Frigo L, De Marchi T, Rossi RP, de Godoi V, Tomazoni SS, Silva DP, Basso M, Filho PL, de Valls Corsetti F, Iversen VV, Bjordal JM. Effects of low-level laser therapy (LLLT) in the development of exercise-induced skeletal muscle fatigue and changes in biochemical markers related to postexercise recovery. *J Orthop Sports Phys Ther.* 2010; 40(8):524–532. [PubMed: 20436237]
33. Servetto N, Cremonozzi D, Simes JC, Moya M, Soriano F, Palma JA, Campana VR. Evaluation of inflammatory bio-markers associated with oxidative stress and histological assessment of low-level laser therapy in experimental myopathy. *Lasers Surg Med.* 2010; 42(6):577–583. [PubMed: 20662035]
34. Chen AC, Arany PR, Huang YY, Tomkinson EM, Sharma SK, Kharkwal GB, Saleem T, Mooney D, Yull FE, Blackwell TS, Hamblin MR. Low-level laser therapy activates NF- κ B via generation of reactive oxygen species in mouse embryonic fibroblasts. *PloS ONE.* 2011; 6(7):e22453. [PubMed: 21814580]
35. Assis L, Moretti AI, Abrahao TB, de Souza HP, Hamblin MR, Parizotto NA. Low-level laser therapy (808 nm) contributes to muscle regeneration and prevents fibrosis in rat tibialis anterior muscle after cryolesion. *Lasers Med Sci.* 2012
36. Shi X, Garry DJ. Muscle stem cells in development, regeneration, and disease. *Genes Dev.* 2006; 20(13):1692–1708. [PubMed: 16818602]
37. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol.* 1996; 271(5 Pt 1):C1424–C1437. [PubMed: 8944624]
38. Kroncke KD, Fehsel K, Suschek C, Kolb-Bachofen V. Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol.* 2001; 1(8):1407–1420. [PubMed: 11515808]
39. Rizzi CF, Mauriz JL, Freitas Correa DS, Moreira AJ, Zettler CG, Filippin LI, Marroni NP, Gonzalez-Gallego J. Effects of low-level laser therapy (LLLT) on the nuclear factor (NF)- κ B signaling pathway in traumatized muscle. *Lasers Surg Med.* 2006; 38(7):704–713. [PubMed: 16799998]
40. Kim YG, Pak SC, Lee SR. Hairless mouse epidermal antioxidants and lipid peroxidation assessed by He–Ne laser. *Lasers Surg Med.* 2000; 27(5):420–426. [PubMed: 11126436]
41. Pallotta RC, Bjordal JM, Frigo L, Leal Junior EC, Teixeira S, Marcos RL, Ramos L, de Moura Messias F, Lopes-Martins RA. Infrared (810-nm) low-level laser therapy on rat experimental knee inflammation. *Lasers Med Sci.* 2012; 27(1):71–78. [PubMed: 21484455]
42. Bjordal JM, Lopes-Martins RAB, Joensen J, Iversen VV. The anti-inflammatory mechanism of low level laser therapy and its relevance for clinical use in physiotherapy. *Phys Ther Rev.* 2010; 15(4):7.
43. Filippin LI, Mauriz JL, Vedovelli K, Moreira AJ, Zettler CG, Lech O, Marroni NP, Gonzalez-Gallego J. Low-level laser therapy (LLLT) prevents oxidative stress and reduces fibrosis in rat traumatized *Achilles tendon*. *Lasers Surg Med.* 2005; 37(4):293–300. [PubMed: 16196040]

Fig. 1. Freezing right tibialis anterior muscle injury (cryolesion) model. **A:** Dissection and muscle exposition; **(B and C)** Cryolesion procedure; and **(D)** Suture after surgical procedure. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]

Fig. 2.

Diagram for the collection and storage of the right tibialis anterior muscle. **A:** Muscles from 10 animals for morphological analysis; **(B)** 10 muscles were cut transversely into two equal parts: the proximal fragment was used for RNA extraction protocol; the distal fragments were homogenate in RIPA buffer and used for de TBARs, Griess, ELISA, Western blot, and dot blot analyses. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]

Fig. 3.

Serial transversal cross-sections in the medial region of right TA muscle. **A:** Toluidine blue staining; **(B)** acid phosphatase staining of: normal TA muscle—control (BC); injured TA muscle without LLLT (IC); injured TA muscle submitted to infrared laser irradiation (IRI); Bar: 100 μ m (magnification: 200 $\times$). ●Normal tissue, *injured area; ◆regenerating fiber; (–) negative reaction to acid phosphatase; and (+) positive reaction to acid phosphatase showing high concentration of lysosomes as a tissue necrosis marker. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]

Fig. 4. Lipid peroxidation. Normal TA muscle—control (BC); injured TA muscle without LLLT (IC); injured TA muscle submitted to infra-red laser irradiation (IRI); LLLT decreased lipid peroxidation levels ($^{\dagger}P < 0.05$ vs. IC).

Fig. 5. Nitrotyrosine formation. Normal TA muscle—control (BC); injured TA muscle without LLLT (IC); injured TA muscle submitted to infrared laser irradiation (IRI); LLLT decreased nitrotyrosine formation ($^{\dagger}P < 0.01$ vs. IC).

Fig. 6. Protein expression of iNOS and NO generation. **A:** iNOS expression and **(B)** NO production. Normal TA muscle—control (BC); Injured TA muscle without LLLT (IC); injured TA muscle submitted to infrared laser irradiation (IRI). LLLT decreased iNOS expression ($^{\dagger}P < 0.05$ vs. IC) and NO production ($^{\dagger}P < 0.01$ vs. IC).

Fig. 7.

Cytokine levels. **A:** TNF- α and **(B)** IL-1 β . Normal TA muscle—control (BC); injured TA muscle without LLLT (IC); injured TA muscle submitted to infrared laser irradiation (IRI). LLLT has decreased the concentration of inflammatory cytokines ($^{\dagger}P < 0.05$ vs. IC).

Fig. 8. NF-κB (A), COX-2 (B), and SOD (C) gene expression. Normal TA muscle—control (BC); Injured TA muscle without LLLT (IC); injured TA muscle submitted to infrared laser irradiation (IRI). LLLT has decreased gene expression of NF-κB and COX-2, and enhanced SOD mRNA ($^{\dagger}P < 0.01$ vs. IC).