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ORIGINAL ARTICLE

What is the best treatment to decrease pro-inflammatory cytokine release in acute skeletal muscle injury induced by trauma in rats: low-level laser therapy, diclofenac, or cryotherapy?

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Abstract Currently, treatment of muscle injuries represents a challenge in clinical practice. In acute phase, the most employed therapies are cryotherapy and nonsteroidal anti-inflammatory drugs. In the last years, low-level laser therapy (LLLT) has becoming a promising therapeutic agent; however, its effects are not fully known. The aim of this study was to analyze the effects of sodium diclofenac (topical application), cryotherapy, and LLLT on pro-inflammatory cytokine levels after a controlled model of muscle injury. For such, we performed a single trauma in tibialis anterior muscle of rats. After 1 h, animals were treated with sodium diclofenac (11.6 mg/g of solution), cryotherapy (20 min), or LLLT (904 nm; superpulsed; 700 Hz; 60 mW mean output power; 1.67 W/cm^2; 1, 3, 6 or 9 J; 17, 50, 100 or 150 s). Assessment of interleukin-1β and interleukin-6 (IL-1β and IL-6) and tumor necrosis factor-alpha (TNF-α) levels was performed at 6 h after trauma employing enzyme-linked immunosorbent assay method. LLLT with 1 J dose significantly decreased ($p<0.05$) IL-1β, IL-6, and TNF-α levels compared to non-treated injured group as well as diclofenac and cryotherapy groups. On the other hand, treatment with diclofenac and cryotherapy does not decrease pro-inflammatory cytokine levels compared to the non-treated injured group. Therefore, we can conclude that 904 nm LLLT with 1 J dose has better effects than topical application of diclofenac or cryotherapy in acute inflammatory phase after muscle trauma.

Keywords Phototherapy · Anti-inflammatory drugs · Cryotherapy · Inflammation · Skeletal muscle injury

Patrícia de Almeida and Shaiane Silva Tomazoni have equal participation in study development.

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Introduction

Currently, treatment of muscle injuries represents a challenge in clinical practice. Strains, contusions, ischemia, and

neurological damage are some of mechanism that can lead to skeletal muscle injury [1].

In muscle injuries caused by contusion, tissue is strongly compressed, and it is usually due a single trauma. Injured muscle suffers degeneration and regeneration processes, which lead to biochemical and morphological changes at injured site [1–3].

Inflammatory response begins immediately after injury, and it starts with migration of leukocytes to the site of injury. There is migration of neutrophils after few hours until 24 h from injury, and macrophages arrive to injury site after 24 h until 14 days from injury [4]. Neutrophils and macrophages contribute to tissue degradation through the release of reactive oxygen species [5] and production of pro-inflammatory cytokines such as interleukin 1-beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-alpha (TNF-α) [6]. Consequently, inflammatory response in acute phase after muscle injury is predominantly a pro-inflammatory response.

In acute phase, the most employed therapies in the treatment of acute skeletal muscle injuries are cryotherapy and nonsteroidal anti-inflammatory drugs (NSAIDs). In the last years, low-level laser therapy (LLLT) has becoming a promising therapeutic agent in treatment of several musculoskeletal conditions such as arthritis [7], tendinitis [8, 9], neck pain [10–12], low back pain [13], and skeletal muscle fatigue [14–21]. On the other hand, there are few studies (animal trials) investigating the effects of LLLT in skeletal muscle injuries [22–24]. Additionally, there is a lack of studies that compare LLLT effects with other treatments like cryotherapy and NSAIDs for instance, and we strongly believe that this kind of studies could start to introduce LLLT as the first choice for treatment of musculoskeletal conditions mentioned before, which certainly would represent an interesting turn in clinical practice and could increase the popularity of LLLT.

With this perspective in mind, we aimed to test and compare the effects of sodium diclofenac with topical application, cryotherapy, and LLLT in pro-inflammatory cytokine levels during acute inflammation after a controlled model of skeletal muscle injury induced by trauma in rats.

Materials and methods

Animal model of standardized muscle trauma

The experiments were carried out with male Wistar rats weighing 200 g, with food and water ad libitum. The Central Animal House of Nove de Julho University provided the animals. All rats were randomly divided into groups of six animals. The policies and procedures of the animal laboratory are in accordance to Brazilian laws and with those detailed by the US Department of Health and Human Services. The experimental protocol was submitted and approved by the Animal Research and Care Committee of the Nove de Julho University.

Rats were anesthetized with ketamine/xylazine (100 and 20 mg/kg, respectively). Each animal's right hind limb was positioned with the knee extended and ankle in 90° dorsiflexion. Then, a single trauma was performed employing a mini guillotine comprised a block weight of 200 g with a blunt edge 2 mm wide that was dropped from 20 cm guided by supports [24]. Animals were sacrificed with an overdose of halothane at 6 h for biochemical analysis. After the removal of skin and connective tissue, tibialis anterior muscle was removed and processed for further analysis.

Experimental groups

Each group was composed of six animals randomly divided into eight experimental groups as follows:

- Control group—animals that did not undergo any type of procedure
- Injury group—animals submitted to muscle injury
- Diclofenac group—animals that underwent muscle injury and treated with topical application of diclofenac (11.6 mg/g of solution)
- Cryotherapy group—animals that underwent muscle injury and treated with cryotherapy for 20 min
- LLLT 1 J group—animals that underwent muscle injury and treated with LLLT with dose of 1 J/point
- LLLT 3 J group—animals that underwent muscle injury and treated with LLLT with dose of 3 J/point
- LLLT 6 J group—animals that underwent muscle injury and treated with LLLT with dose of 6 J/point
- LLLT 9 J group—animals that underwent muscle injury and treated with LLLT with a dose of 9 J/point

Treatments

All treatments were performed 1 h after muscle trauma.

Cryotherapy In this group, we tried to mimic RICE protocol. For such, animals were treated for 20 min with small rubber bags containing crushed ice. The rubber bags were fixed at the region of right tibialis muscle belly with rubber bands, and the right hind limb of rats was kept elevated.

Diclofenac Rats of diclofenac group were treated with sodium diclofenac with topical application 1 h after injury. For such, 5 ml of diclofenac (Cataflam® Emulgel) in gel solution (11.6 mg/g of solution) was applied uniformly at right tibialis muscle belly. This amount and dose of diclofenac were applied to sufficiently cover the injured muscle belly and also

trying to mimic clinical conditions. Additionally, manufacturer's instruction about the use of medication was followed.

Low-level laser therapy A single LLLT treatment was performed 1 h after controlled muscle trauma with an infrared laser unit (Irradia®, Stockholm, Sweden). The laser unit operated in pulsed mode with a peak power of 20 W, pulse width of 200 ns (10^{-9} s), frequency of 700 Hz, mean output power of 60 mW, spot size of 0.0364 cm^2, and power density of 1.67 W/cm^2. The optical power was calibrated using a Newport multifunction optical meter model 1835C. The stability of laser during the laser irradiation was measured collecting light with a partial reflect (4 %). The optical power output of the laser unit was measured before, halfway through, and after the experiment. All measurements to state parameters were performed at laser aperture, and manufacturer gave laser beam information. Laser irradiation was performed in skin contact at the middle of anterior tibialis muscle belly with doses (energy) of 1, 3, 6, and 9 J/point and corresponding irradiation times of 17, 50, 100 and 150 s, respectively. Energy densities were 27.47, 82.42, 164.84 and 247.25 J/cm^2 respectively. The laser energy doses were chosen according to previous studies from our research group [23, 24].

Analyses

For all analyses, we used a sample of six animals. A blinded observer performed all analyses. Initial analysis was performed at Nove de Julho University, Brazil. To ensure consistency in analyses and reproducibility of results, one other laboratory at the University of São Paulo (Brazil) duplicated the analyses.

Evaluation of inflammatory mediators (IL-1β, IL-6, and TNF-α) The levels of IL-1β, IL-6, and TNF-α in the muscle samples were determined by enzyme-linked immunosorbent assays, using a commercial kit and following the manufacturer's instructions (R&D System). For this purpose, 96-well plates were coated with 100 μL of monoclonal antibody for each cytokine (anti-IL-1β and IL-6) and diluted in sodium carbonate buffer (0.1 M, pH 9.6), whereas anti TNF-α was diluted in sodium phosphate buffer (0.2 M, pH 6.5). The plates were incubated (4 °C) for 18 h. For blocking, the plates were washed with PBS containing 0.05 % Tween 20 (PBST) four times and then filled with 300 L/well of blocking solution (3 % gelatin in PBST; Sigma) at 37 °C for 3 h and subjected to a new cycle of washes. Next, 100 μL of properly diluted samples or standards of recombinant cytokines were added to the plate and left for 18 h at 4 °C. After washing, 100 μL of the respective biotinylated antibodies for the specific detection of each cytokine was added and left for 1 h at room temperature. After washing the plates, 100 μL of streptavidin-peroxidase was added and left for 1 h at room temperature (22 °C), followed by further washing. The reaction was revealed by adding 100 μL/well solution of 3,3′,5,5-tetramethylbenzidine and stopped by adding 50 μL/well of sulfuric acid (2 M). Readings were performed in a Spectrum Max Plus 384 spectrophotometer (Sunnyvale, CA) at a wavelength of 450 nm, with correction at 570 nm. Sample concentrations were calculated from standard curves obtained from recombinant cytokines. The limit of detection was 1.95 pg mL^{-1} for IL-1β and TNF-α and 3.13–300 pg mL^{-1} for IL-6.

Statistical analysis

A blinded observer performed the statistical analysis. Data are expressed as mean and standard error (±) of the mean (SEM). All data were statistically evaluated by analysis of variance, followed by the Tukey–Kramer post hoc. Values with $p<0.05$ were considered statistically significant.

Results

Our experimental model significantly increased ($p<0.05$) IL-1β levels in injured non-treated group. LLLT with 1 J was the only treatment that significantly decreased IL-1β levels compared to injury group ($p<0.05$). Additionally, treatment with 1 J LLLT was significantly better ($p<0.05$) than diclofenac and cryotherapy. Figure 1 summarizes results regarding IL-1β.

Similarly, the experimental model of skeletal muscle trauma employed in this study also significantly increased ($p<0.05$) IL-6 levels in the injured non-treated group. Again, treatment with 1 J LLLT was the only one to significantly

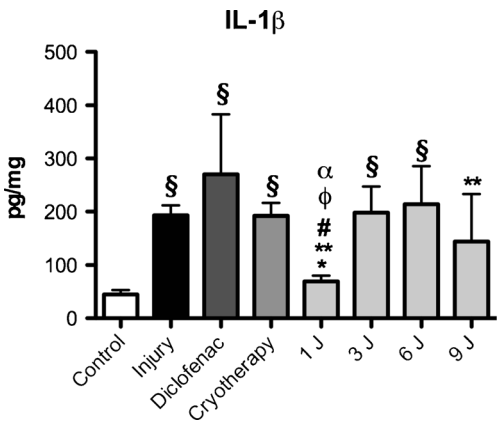

Fig. 1 IL-1β levels. The samples were collected at 6 h after muscle trauma. The values are the mean and *error bars* are SD; $n=6$ animals per group ($^{§}p<0.05$ vs control group; $^{*}p<0.05$ vs injury group; $^{**}p<0.05$ vs diclofenac group; $^{\#}p<0.05$ vs cryotherapy group; $^{\Phi}p<0.05$ vs 3 J group; $^{\alpha}p<0.05$ vs 6 J group)

decrease IL-6 levels compared to injury group ($p<0.05$). LLLT with 1 J showed significantly better results than diclofenac and cryotherapy. Results regarding IL-6 are summarized in Fig. 2.

The same pattern was observed regarding TNF-α levels. Experimental model of muscle trauma significantly increased ($p<0.05$) TNF-α levels in injured non-treated group. One more time treatment with 1 J LLLT was the only therapy to significantly decrease TNF-α levels compared to injury group ($p<0.05$). LLLT with 1 J again showed significantly better results than diclofenac and cryotherapy. Figure 3 summarizes results regarding TNF-α levels.

Discussion

In this study, we employed an experimental model of controlled muscle trauma trying to mimic contusion that is one of the most common mechanisms of skeletal muscle injury. Firstly, it is important to highlight that our experimental model was able to promote the typical increase of pro-inflammatory cytokine release that occurs in acute phase according as observed in non-treated injury group.

We observed that only treatment with LLLT at 1 and 9 J doses was able to significantly decrease IL-1β levels compared to the injury group ($p<0.05$). Additionally, LLLT with 1 J dose was significantly better ($p<0.05$) than cryotherapy and diclofenac, as well as 3 and 6 J LLLT doses tested.

On the other hand, regarding IL-6 and TNF-α levels, only LLLT at 1 J dose significantly decreased the levels of these cytokines compared to injury group ($p<0.05$). Similarly to the results of IL-1β levels, LLLT with 1 J dose was significantly better ($p<0.05$) than cryotherapy, diclofenac, and 3 and 6 J LLLT.

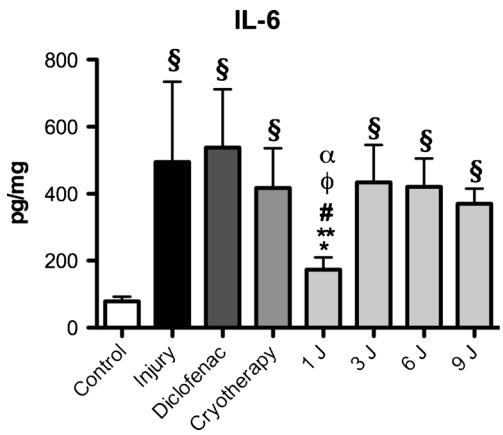

Fig. 2 IL-6 levels. The samples were collected at 6 h after muscle trauma. The values are the mean and *error bars* are SD; $n=6$ animals per group ($^{§}p<0.05$ vs control group; $^{*}p<0.05$ vs injury group; $^{**}p<0.05$ vs diclofenac group; $^{\#}p<0.05$ vs cryotherapy group; $^{\phi}p<0.05$ vs 3 J group; $^{\alpha}p<0.05$ vs 6 J group)

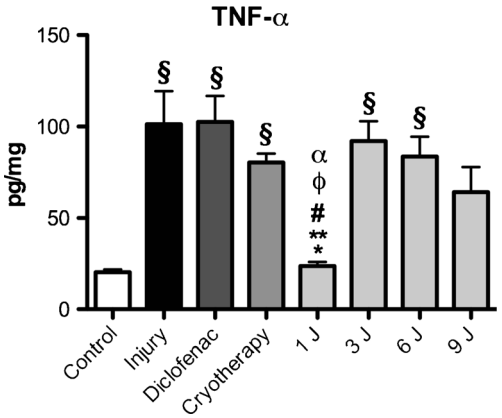

Fig. 3 TNF-α levels. The samples were collected at 6 h after muscle trauma. The values are the mean and *error bars* are SD; $n=6$ animals per group ($^{§}p<0.05$ vs control group; $^{*}p<0.05$ vs injury group; $^{**}p<0.05$ vs diclofenac group; $^{\#}p<0.05$ vs cryotherapy group; $^{\phi}p<0.05$ vs 3 J group; $^{\alpha}p<0.05$ vs 6 J group)

In a recent study performed by our research group, we employed the same experimental model of muscle trauma; however, we used 830 nm wavelength and we analyzed other aspects such as muscle morphology and gene expression of inflammatory markers [24]. Differently of the results observed in the present study, all LLLT doses tested (1, 3, and 9 J) significantly improved both morphological and biochemical aspects of acute inflammation compared to injury and diclofenac groups. However, 9 J dose showed slightly better results than other doses. Despite similarity of experimental model and doses tested, as well as difference of aspects evaluated between studies, this illustrates how difficult it is to establish an optimal dose and better parameters for LLLT, and also that different wavelengths have different patterns regarding optimal dose.

In other study [25], we analyzed the effects of LLLT (3 J), topical and intramuscular diclofenac used as single or combined therapy on functional aspect (walking index), and also inflammatory markers (cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2), respectively) after an experimental model of controlled muscle strain. Despite that all treatments (single or combined) have improved COX-1 and COX-2 gene expression compared to the injury group ($p<0.05$), only groups where LLLT was used (as single or combined treatment) showed a significant improvement regarding PGE_2 levels and walking index analysis.

Still regarding inflammatory markers, we recently observed that a single treatment of LLLT (904 nm, 15 mW, dose of 1 J) before tetanic contraction of tibialis anterior in rats significantly increases skeletal muscle performance and decreases skeletal muscle damage and COX-2 gene expression. These findings mean that LLLT seems to protect muscles against damage and inflammation induced by exercise [18]. It is important to highlight that 1 J energy dose that showed these protective effects in muscle tissue is the same to what was used in the present study.

Surprisingly, in the present study, two of treatments widely used in acute phase after muscle trauma do not show significant effects compared to the non-treated injured group in none of outcomes tested, which demonstrates that LLLT is an interesting alternative to classic treatments like NSAIDs and cryotherapy, and this increases scientific evidence regarding the use of LLLT in the treatment of musculoskeletal disorders.

Conclusion

We conclude that 904 nm LLLT with dose of 1 J decreases pro-inflammatory cytokine release in acute inflammatory phase after muscle contusion. Furthermore, LLLT is better than topical application of diclofenac and local application of cryotherapy. Further studies are needed to translate these findings to clinical settings.

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