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ORIGINAL ARTICLE

Effects of photobiomodulation therapy and topical non-steroidal anti-inflammatory drug on skeletal muscle injury induced by contusion in rats—part 1: morphological and functional aspects

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Abstract Musculoskeletal injuries are very frequent and are responsible for causing pain and impairment of muscle function, as well as significant functional limitations. In the acute phase, the most prescribed treatment is with non-steroidal anti-inflammatory drugs (NSAIDs), despite their questionable effectiveness. However, the use of photobiomodulation therapy (PBMT) in musculoskeletal disorders has been increasing in the last few years, and this therapy appears to be an interesting alternative to the traditional drugs. The objective of the present study was to evaluate and compare the effects of PBMT, with different application doses, and topical NSAIDs, under morphological and functional parameters, during an acute inflammatory process triggered by a controlled model of musculoskeletal injury induced via contusion in rats. Muscle injury was induced by means of a single trauma to the animals' anterior tibialis muscle. After 1 h, the rats were treated with PBMT (830 nm; continuous mode, with a power output of 100 mW; 3.57 W/cm^2; 1 J–35.7 J/cm^2, 3 J–107.1 J/cm^2, and 9 J–321.4 J/cm^2; 10, 30, and 90 s) or diclofenac sodium for topical use (1 g). Morphological analysis (histology) and functional analysis (muscle work) were performed, 6, 12, and 24 h after induction of the injury. PBMT, with all doses tested, improved morphological changes caused by trauma; however, the 9 J (321.4 J/cm^2) dose was the most effective in organizing muscle fibers and cell nuclei. On the other hand, the use of diclofenac sodium produced only a slight improvement in morphological changes. Moreover, we observed a statistically significant increase of muscle work in the PBMT 3 J (107.1 J/cm^2) group in relation to the injury group and the diclofenac group ($p < 0.05$). The results of the present study indicate that PBMT, with a dose of 3 J (107.1 J/cm^2), is more effective than the other doses of PBMT tested and NSAIDs for topical use as a means to improve morphological and functional alterations due to muscle injury from contusion.

Keywords Photobiomodulation therapy · Low level laser therapy · Phototherapy · Skeletal muscle injury · Contusion · Topical non-steroidal anti-inflammatory drug

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Introduction

Musculoskeletal injuries are very frequent and represent a challenging traumatology problem, since they account for up to 30% of all injuries in the practice of professional sports [1].

Moreover, this type of injury causes pain and muscle function impairment, triggering important functional limitation [2, 3]. Different mechanisms, such as strains, ischemia, and neurological damage [2] may be involved in this type of injury; however, contusion is one of the most frequently observed mechanisms [4].

Musculoskeletal injuries resulting from contusion are characterized by compression of muscle cells, due to a large impact with a high load on the muscular surface. Thus, the severity of the injury depends upon the place of impact, the muscle involved, the age of the patient, and the presence or absence of muscular fatigue [4]. This type of injury can damage the contractile elements of the muscular structure, leading to a decrease in the properties of elasticity, extensibility, and contractility [3, 4].

The skeletal muscle is highly vascularized and, as a result of injury by contusion, compression and consequent rupture of blood vessels present at the site occurs, causing bruising [5, 6]. Moreover, mechanical destruction of muscle fibers, myofilaments, Z-line, and sarcomeres can be observed [1]. The injured fibers undergo a subsequent process of necrosis [7], and the activation of inflammatory cells induces a local inflammatory response [2].

In the acute phase of muscle injury, the most frequently prescribed therapy is pharmacological, specifically the use of NSAIDs, in which the use of topical application has been highlighted in recent years. The topical use of NSAIDs has become a potentially safer alternative, since the drug penetrates slowly and in small amounts into the circulation [8]. Thus, there is an increase in the concentration of the drug in the target tissue and a decrease in the total systemic exposure of the organism. This reduces the occurrence of systemic side effects [9], which are frequently observed with the prolonged use of NSAIDs and represent a major disadvantage for this therapy [10].

However, in the last few years, the application of photobiomodulation therapy (PBMT) has been shown to be an interesting strategy to accelerate the process of tissue regeneration [11] and to reduce the release of inflammatory mediators [12]. It has been observed that PBMT presents therapeutic properties in several musculoskeletal disorders [13–26] and does not present any side effects reported by the literature until the present moment. However, there are few studies in existence about the effects of PBMT on muscle injury by contusion, especially studies that investigate more than one application dose in an attempt to establish a therapeutic window. Also, there is a scarcity of studies comparing PBMT with the use of topical NSAIDs, the treatment of choice in this condition. Moreover, there are few studies that analyze the most important aspect when dealing with muscle injury (i.e., the recovery of muscle function).

With respect to these issues, the objective of the present study was to evaluate and compare the effects of PBMT, with different application doses, and the use of topical NSAIDs, according to morphological and functional parameters, during an acute inflammatory process triggered by a controlled model of musculoskeletal injury induced by contusion in rats.

Materials and methods

Animals

A total of 96 male Wistar rats from the central animal facility of the university, weighing around 250 g were used. The animals were kept under standard conditions of temperature (22–24 °C), relative humidity (40–60%), a 12-h light/dark cycle, and provided water and feed ad libitum. All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of our institution.

Experimental groups

The animals were randomized and divided into experimental groups of six animals per group, as described below:

- Control group: the animals were not subjected to any procedure or treatment.
- Injury group: the animals were submitted to muscle injury by contusion.
- Diclofenac group: the animals were submitted to muscle injury by contusion and, 1 h later, treated with diclofenac sodium for topical use.
- 1 J: the animals were submitted to muscle injury by contusion and, 1 h later, treated with PBMT with a dose of 1 J (35.7 J/cm^2).
- 3 J: the animals were submitted to muscle injury by contusion and, 1 h later, treated with PBMT with a dose of 3 J (107.1 J/cm^2).
- 9 J: the animals were submitted to muscle injury by contusion and, 1 h later, treated with PBMT with a dose of 9 J (321.4 J/cm^2).

Each experimental group was further divided into three experimental subgroups (comprised of six animals each) according to the time that they were slaughtered (i.e., 6, 12, or 24 h after injury) by overdose of ketamine and xilazin.

Procedures

Model of muscle injury by contusion Initially, the animals were anesthetized, intraperitoneally, with a mixture of Ketamine and Xylazine (90 and 10 mg/kg, respectively; König, Avellaneda, Argentina). Subsequently, the animals were submitted to the muscle contusion model, produced

by specific equipment (injury press), responsible for releasing a load of 186 g at a distance of 20 cm from the central region (most prominent) of the anterior tibial muscle, thus causing the muscle contusion (in the ventral region). The contusion was performed with the animal in the lateral decubitus position, and the right hind paw was manually immobilized in a stretching position, by means of the plantar flexion of the ankle.

Treatments

Application of PBMT A diode laser with a wavelength of 830 nm (infrared); continuous mode; 0.028 cm^2 spot area; 100 mW power; 3.57 W/cm^2 power density; 35.7 J/cm^2, 107.1 J/cm^2 and 321.4 J/cm^2 energy density; and either 1 J (10 s), 3 J (30 s) or 9 J (90 s) doses of energy was used. Only one single point on the ventral region of the animal's anterior tibialis muscle was irradiated. To irradiate the animals, the spot was kept in direct contact with the animal's skin, applying light pressure on the tissue. The application of PBMT was performed one hour after the induction of muscle injury by contusion. Table 1 shows parameters for PBMT.

Application of topical NSAIDs—diclofenac sodium A dose of 1 g of 10 mg/g diclofenac sodium generic gel was used (EMS®, Santo André, São Paulo, Brazil) and applied uniformly over the ventral region of the animals' anterior tibial muscle. The application of NSAIDs was performed 1 h after the muscle injury induced by contusion.

Collection of biological material

The biological material was collected with respect to the experimental times of 6, 12, and 24 h after the induction of muscle injury by contusion.

Collection of muscle tissue Initially, the animals were anesthetized with a mixture of Ketamine and Xylazine (90 and 10 mg/kg, respectively; König, Avellaneda, Argentina), administered intraperitoneally. Subsequently, the anterior tibial muscle was surgically removed and processed for future morphological analysis.

Analyses

Morphological analysis—histology The tissue samples were fixed in 10% formaldehyde for a period of 72 h. Subsequently, the samples were dehydrated and submitted to a gradual series of alcohol baths, starting with 50% and progressing to 100% absolute alcohol (SYNTH). The muscles were then diaphonized with Xylol for 4 h (SYNTH) for impregnation and inclusion of the samples in Paraplast®. Following this process, the samples were then placed in suitable aluminum containers with molten Paraplast® for 4 h. After impregnation, the samples were placed in a small container covered with molten paraffin wax and were left to cure, forming a block containing the tissue. For the microtomy, 5 μm microtome (LEICA RM 2125 RT) sections were washed and placed in a water bath. Once the samples were prepared, the sections were placed on slides to be stained with hematoxylin-eosin (H.E.) dye. After staining, the sections were mounted on permanent slides for further analysis under an optical microscope. The slides were photographed using a Dino-Lite Digital Microscope® microphotography system, the DinoEye AM423X model, connected to a microcomputer. Photos of all groups were obtained using the × 100 magnification. The images were presented with a similar photographic pattern.

Functional analysis The functional analysis was performed with respect to the experimental times of 6, 12, and 24 h after the induction of muscle injury by contusion. This protocol

Table 1 Parameters for PBMT

	Dose 1	Dose 2	Dose 3
Class	3B	3B	3B
Number of laser diodes	1	1	1
Beam profile	Top hat	Top hat	Top hat
Wavelength (nm)	830	830	830
Frequency (Hz)	Continuous	Continuous	Continuous
Optical output (mW), each	100	100	100
Spot size (cm^2), each	0.028	0.028	0.028
Power density (W/cm^2), each	3.57	3.57	3.57
Energy density (J/cm^2), each	35.7	107.1	321.4
Irradiation time per site (s)	10	30	90
Total energy delivered (J), each	1	3	9
Number of points irradiated	1	1	1
Application mode	Probe was kept in direct contact with the animal's skin, applying light pressure on the tissue.		

was previously used in other studies [13, 27, 28]. Initially, the animals were anesthetized, intraperitoneally, with a mixture of Ketamine and Xylazine (90 and 10 mg/kg, respectively; König, Avellaneda, Argentina), and then fixed on a surgical table. Subsequently, a small cross section was made on the skin of the animal, near the metatarsal plantar region, and with a pair of scissors, an avulsion was performed in order to separate the anterior tibial muscle from the subcutaneous tissue, together with the skin. Subsequently, with the aid of a scalpel, the tendon was separated from its insertion and tied to a thread followed by removal of the muscular fascia, for better isolation of the muscle. After sectioning, the muscle was drawn in the opposite direction to its insertion, through the thread, so as to be isolated from the tibia. Throughout the entire stimulation procedure, the anterior tibial muscle was maintained and hydrated with saline solution (0.09%). At the insertion region, near the metatarsal plantar region, the muscle, through its tendon, was connected to an isometric transducer (Ugo Basile®, Vareze, Italy) and the sciatic nerve to a bipolar electrode. The muscle was subjected to a constant tension of 0.1 N and was stimulated indirectly by pulses of 6–7 V, 0.2 Hz, with 2 ms duration, applied through a bipolar electrode in the sciatic nerve of the animals. In response to indirect stimuli, muscle contractions were recorded on a physiograph (GEMINI 7070 from UGO BASILE®) through an isometric transducer. To induce tetanic contractions, the frequency was raised to 60 Hz. Muscle fatigue was characterized by the inability of muscular contraction to be maintained, with the amplitude decaying by 50% of the maximum recorded, thus avoiding tissue death due to tetanus contraction. Tetanic contractions were performed every 10 min, in the 60-min period, making for a total of six contractions for each animal. The muscular work was analyzed from the records, defined through the area under the curve time vs. intensity.

Statistical analysis

Initially the data was tabulated and evaluated for normality using the Shapiro-Wilk test. As a normal distribution was determined, the one-way ANOVA test was used for the analysis of variance followed by the Bonferroni test for multiple comparisons. The level of statistical significance was set at $p < 0.05$. In the graphs, data are presented as mean and standard error of the mean (SEM).

Results

Histology by optical light microscopy

Figures 1, 2, and 3 demonstrate the morphological aspects of muscle tissue at 6, 12, and 24 h, respectively, after the induction of muscle injury by contusion. We observed that the experimental model adopted in the present study induces muscle damage and triggers signs of inflammation in the musculoskeletal tissue. Moreover, the application of PBMT in all experimental times decreases muscle damage and the morphological changes caused by muscle contusion, unlike the application of topical NSAIDs, which has not been shown to be effective in such a situation.

Analysis of muscle work

Figure 4 demonstrates the muscle work of the animals from all experimental groups, in all time points tested, after muscle injury by contusion. We observed a reduction of muscle work in the injury, diclofenac and PBMT 1 J (35.7 J/cm^2) and 9 J (321.4 J/cm^2) groups, when compared with the controls. Moreover, we verified that the PBMT 3 J (107.1 J/cm^2) group was the only group to increase muscle work, in relation to the injury and diclofenac groups.

Discussion

To the best of our knowledge, this is one of the first studies to investigate the effects of different doses of PBMT, as compared with the use of NSAIDs, currently, which is a classic treatment for muscle injury. In addition, the use of topical NSAIDs in the present study deserves to be highlighted, since it is a safer alternative to oral NSAIDs, for example. Finally, the functional analysis of the muscle, through the analysis of muscle work, also deserves attention, since this type of analysis is fundamental and difficult to find in the literature.

In the present study, we used an experimental model of controlled muscle trauma to reproduce one of the most frequently observed muscle injuries via muscle contusion. Moreover, we evaluated and compared the effects of using PBMT and NSAIDs, topically, in this condition.

According to the morphological and functional changes observed in the injury group in all experimental groups, it is important to note that our experimental model was effective in reproducing the typical aspects of a muscle injury, such as muscle fiber disorganization [1], presence of hemorrhage [5], and infiltration of inflammatory cells [2], in addition to the decrease in muscle work [29].

We observed that at 6 and 12 h after the injury, the three groups treated with PBMT showed an improvement of the morphological aspects when compared to the injury and diclofenac groups. In both experimental times, we found that the dose of 9 J (321.4 J/cm^2) was the most effective, among those tested, in organizing muscle fibers and cell nuclei. Moreover, we emphasize that 24 h after the injury, all groups treated with PBMT showed a reduction in the signs of muscle damage and inflammation. It should be noted that the diclofenac group showed only a slight improvement in the

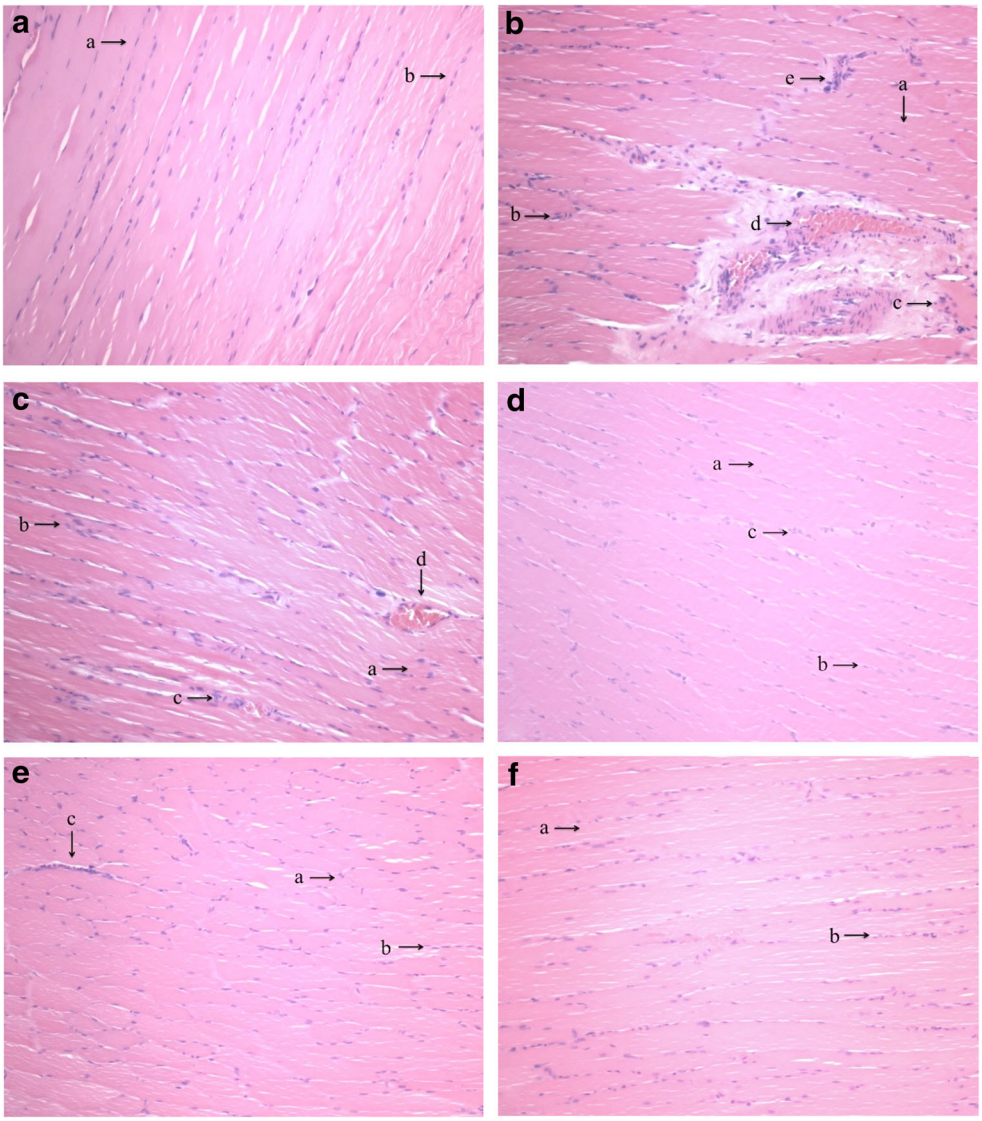

Fig. 1 Morphological changes observed in different experimental groups 6 h after injury of the anterior tibial muscle by contusion. **A** Control group: organization of muscle fibers (a); and cell nuclei located at the periphery of muscle fibers (b). **B** injury group: disorganization in muscle fibers (a); cell nuclei displaced to the center of the muscle fiber (b); clustering of cell nuclei (c); presence of hemorrhagic area (d); and presence of infiltrating inflammatory cells (e). **C** Diclofenac group: disorganization in muscle fibers (a); cell nuclei displaced to the center of muscle fibers (b); clustering of cell nuclei (c); and small hemorrhagic area (d). **D** PBMT group 1 J: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); and presence of small area of infiltrating inflammatory cells (c). **E** PBMT group 3 J: disorganization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); and small area with the presence of infiltrating inflammatory cells (c). **F** PBMT group 9 J: organization of muscle fibers (a); and cell nuclei located at the periphery of muscle fibers (b). Original magnification of × 100

morphological alterations triggered by muscle injury. Finally, the functional analysis showed that only the PBMT 3 J (107.1 J/cm^2) group showed significant improvement of muscle work when compared to the injury and diclofenac groups.

Similar to our study, De Almeida et al. [14], Rennó et al. [30], and Rodrigues et al. [31] observed that PBMT was effective in reducing inflammation, improving the organization of muscle fibers and reducing the area of cell necrosis and hemorrhage at the injury site. Rizzi et al. [32] demonstrated that PBMT was able to block the effects of reactive oxygen species (ROS) and reduce the trauma-induced inflammatory response. Liu et al. [33] observed that the PBMT dose of 8.4 J (43 J/cm^2) presented better results in relation to muscle damage and oxidative stress, 24 and 48 h, after the induction of muscle injury. It should be noted that the dose of 8.4 J (43 J/cm^2) used by Liu et al. [33] is very close to the best dose found in the present study (9 J–321.4 J/cm^2) to preserve the morphological characteristics of the muscle after injury by contusion.

It is interesting to note that the present study corroborates the aforementioned research and reinforces that high doses, 9 J (321.4 J/cm^2), are more effective in muscle regeneration after injury. However, it is important to note that the other doses used

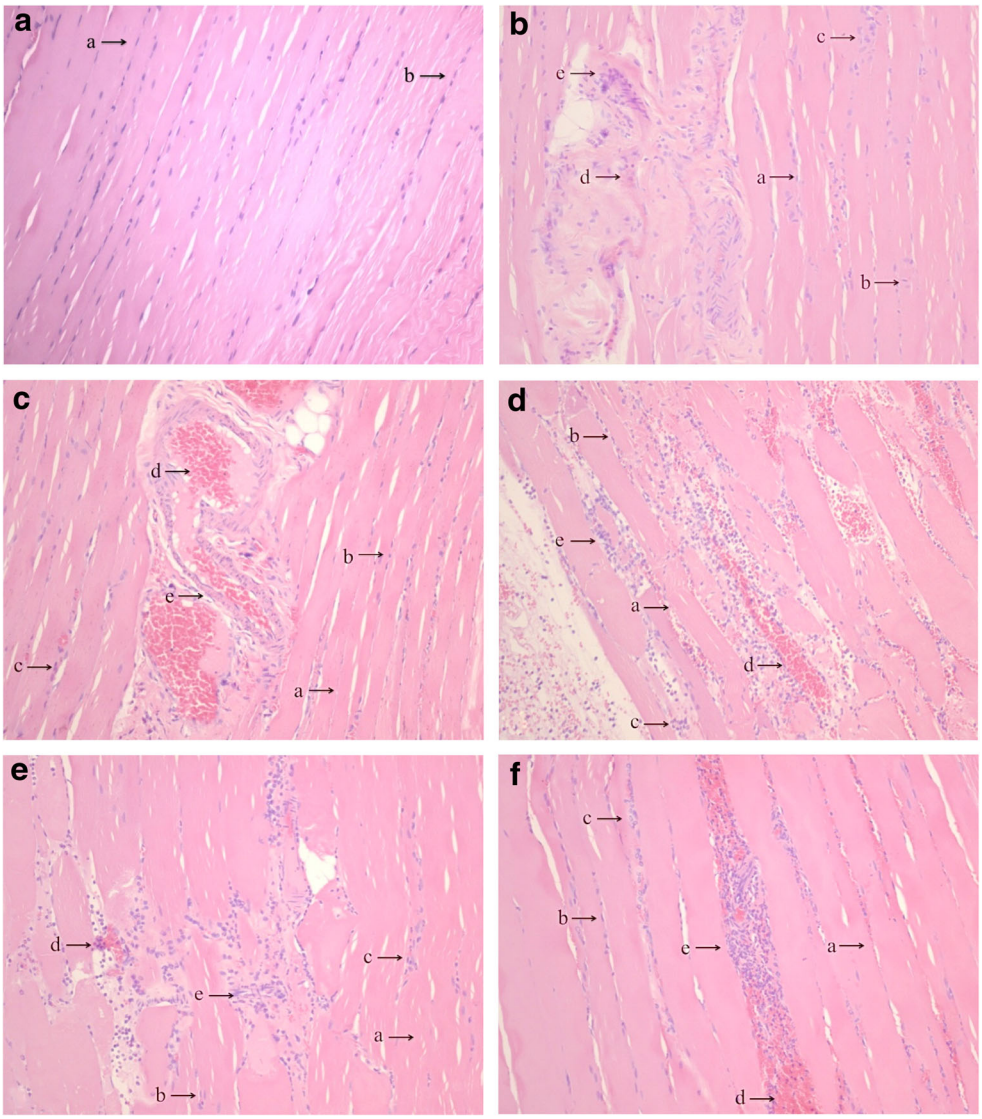

Fig. 2 Morphological changes observed in different experimental groups 12 h after injury of the anterior tibial muscle by contusion. **A** Control group: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b). **B** Injury group: disorganization of muscle fibers (a); regions with cell nuclei displaced to the center of muscle fibers (b); clustering of cell nuclei (c); hemorrhagic area (d); and extensive area of infiltrating cells (e). **C** Diclofenac group: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); clustering of cell nuclei (c); extensive area of hemorrhage (d); extensive areas of cellular infiltration (e). **D** PBMT group 1 J: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); areas of clustering of cell nuclei (c); hemorrhagic areas (d); and infiltrating inflammatory cells (e). **E** PBMT 3 J group: disorganization of muscle fibers (a); cell nuclei displaced to the center of muscle fibers (b); clustering of cell nuclei (c); small hemorrhagic area (d); and presence of infiltrating cells (e). **F** PBMT group 9 J: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); clustering of cell nuclei (c); hemorrhagic area (d); and area of infiltrating cells (e). Original magnification of × 100

in the present study, 1 J (35.7 J/cm^2) and 3 J (107.1 J/cm^2), were also able to improve the morphological characteristics of the muscle. Moreover, these different studies [14, 30, 31], using other experimental models, show that the PBMT is effective regardless of the muscle injury model and experimental time involved.

In addition to morphological changes, loss of performance is an important aspect observed in muscular injuries [29] and can be measured through the muscular work performed. De Almeida et al. [13] and Leal Junior et al. [28] verified that PBMT, with doses of 1 J (35.7 J/cm^2) and 3 J (107.1 J/cm^2), increased the muscular work when compared to the control group in the presence of muscular fatigue. Similarly, it is interesting to note in our results that there was an increase in muscle work in the group treated with 3 J (107.1 J/cm^2), in relation to the injury and diclofenac groups. This leads us to believe that PBMT, at said dose, may delay the onset of the expected decrease in muscle work in the presence of muscle injury.

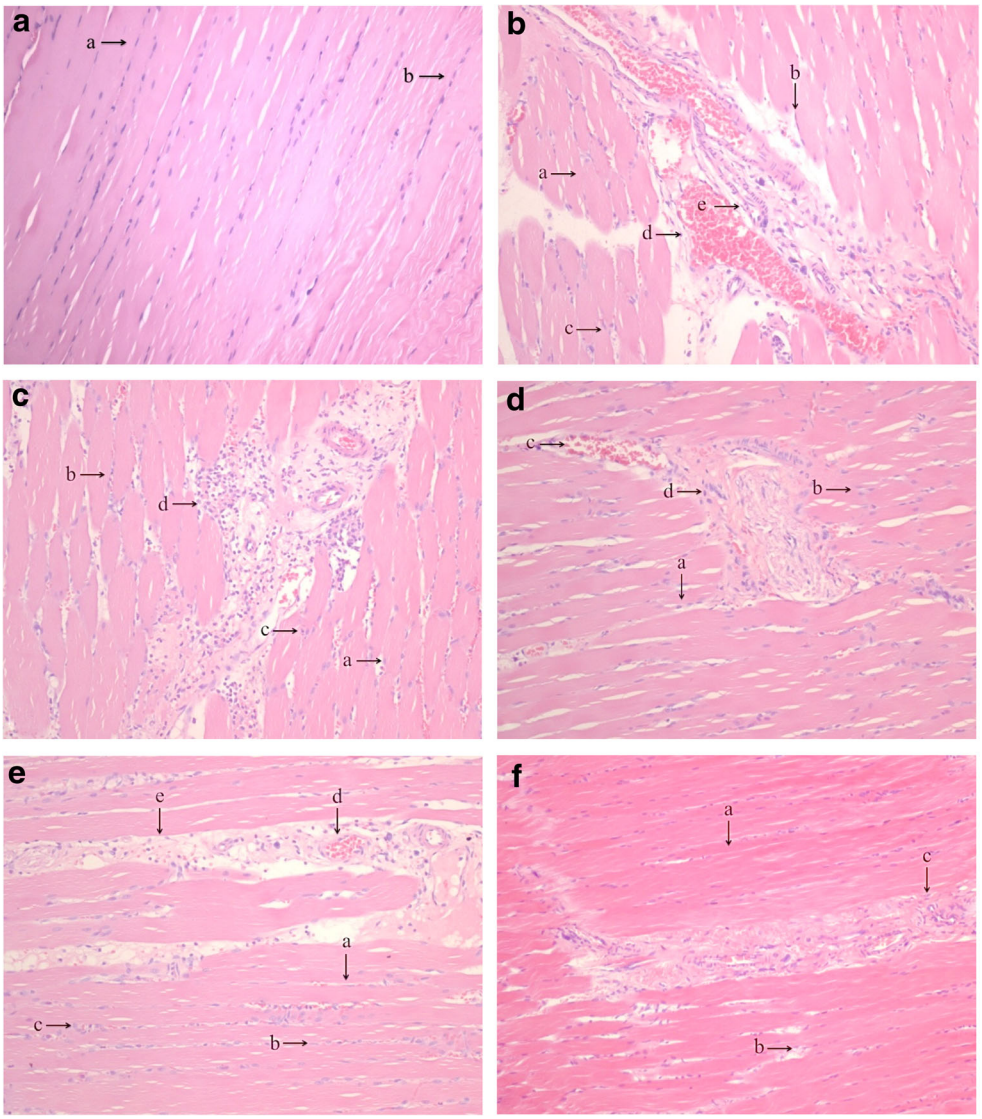

Fig. 3 Morphological changes observed in different experimental groups 24 h after injury of the anterior tibial muscle by contusion. **A** Control group: organization of muscle fibers (a); and cell nuclei located at the periphery of muscle fibers (b). **B** Injury group: disorganization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); clustering of cell nuclei (c); hemorrhagic area (d); and area of infiltrating cells (e). **C** Diclofenac group: disorganization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); area of clustering of cell nuclei (c); and area of infiltrating inflammatory cells (d). **D** PBMT 1 J: disorganization of muscle fibers (a); cell nuclei displaced to the center of muscle fibers (b); small hemorrhagic area (c); and small area with the presence of infiltrating inflammatory cells (d). **E** PBMT 3 J: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b), area of clustering of cell nuclei (c); small hemorrhagic area (d); and area with the presence of infiltrating cells (e). **F** PBMT 9 J group: organization of muscle fibers (a); cell nuclei located at the periphery of muscle fibers (b); and area presenting infiltrating inflammatory cells (c). Original magnification of × 100

The study by Ramos et al. [34] observed increased muscle work with the application of PBMT and also with the use of diclofenac sodium, differing somewhat from our results, since we did not observe statistically significant differences with the use of diclofenac sodium. However, the experimental model of lesion induction by Ramos et al. [34] (strain), and the route of use of the drug in question (intraperitoneally), were different from those used in the present study, which could justify the discrepancies found.

It is important to note that in the functional analysis, only the 3 J (107.1 J/cm^2) dose was able to improve muscle function. On the other hand, in the morphological analysis, we observed that the 9 J (321.4 J/cm^2) dose was the one that better preserved the characteristics of the muscle after muscle injury. However, the other doses (i.e., 1 J–35.7 J/cm^2 and 3 J–107.1 J/cm^2) also were demonstrated to improve the morphological aspects, when compared with the injury group. In view of these findings, we believe that the 3 J (107.1 J/cm^2) dose is the best choice to treat a muscle injury induced by contusion, since it improves the morphological characteristics of the muscle and is the only dose that led to a significant improvement in muscle function.

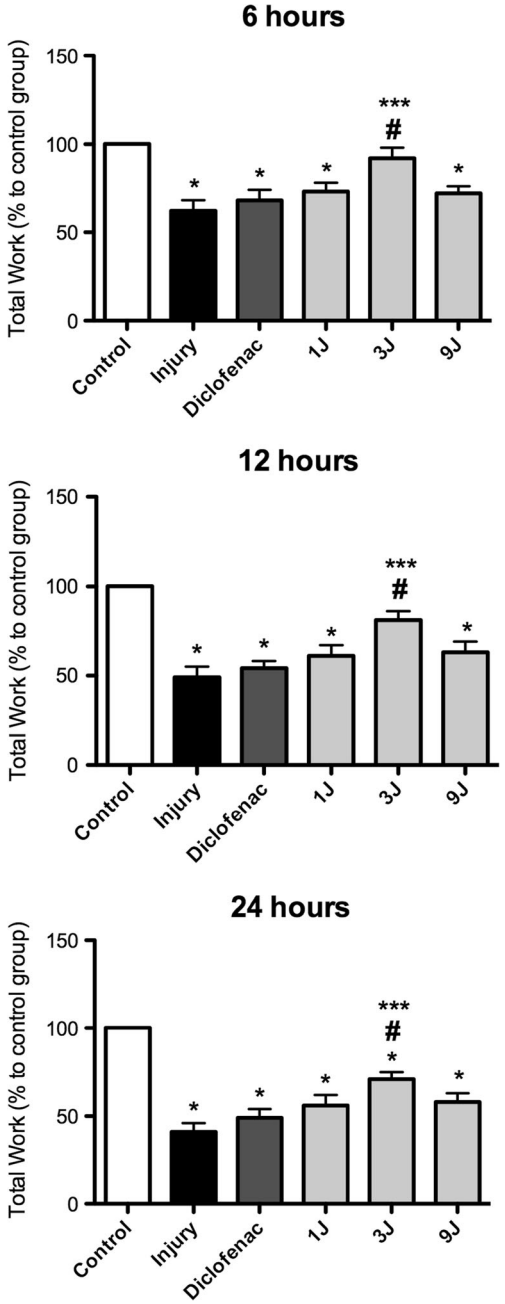

Fig. 4 Muscular work of the animals from all experimental groups 6, 12 and 24 h after the induction of muscle injury by contusion. *$p < 0.05$ vs. control group, #$p < 0.05$ vs. injury group, ***$p < 0.05$ vs. diclofenac group. Data represents the mean ± SEM

We believe that our results are interesting because they demonstrate that PBMT, with all doses tested, is effective in reducing morphological changes, and that a 3 J (107.1 J/cm^2) dose, specifically, is effective in improving the functional aspects of the muscle following induction of injury by contusion. However, high doses do appear to be more effective in assisting with the process of tissue regeneration, while intermediate doses suck, as 3 J (107.1 J/cm^2) appear to be more effective in improving functionality.

On the other hand, we verified that the application of diclofenac sodium for topical use does not seem to contribute in a significantly effective way to the reduction of damage nor tissue regeneration following muscle injury, with its use being questionable as a treatment of choice in the aforementioned condition. In contrast, these results lead us to believe that PBMT might be used as an alternate and safer therapy to the use of topical NSAIDs, since, so far, it does not appear to have contraindications or trigger adverse effects.

Conclusion

Our results indicate that PBMT, with a 3 J (107.1 J/cm^2) dose, is the best alternative therapy, when compared to other PBMT doses tested and topical diclofenac sodium, since it reduces morphological and functional changes resulting from the induction of muscle injury by contusion. However, further studies are required to analyze different markers of the inflammation. Moreover, the present study may open prospects for future clinical studies, since, to date, there is no clinical trial investigating the effects of PBMT on muscle injuries.

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Compliance with ethical standards

Conflict of interest Professor Ernesto Cesar Pinto Leal-Junior receives research support from Multi Radiance Medical (Solon, OH, USA), a laser device manufacturer. Multi Radiance Medical had no role in the planning of this study, and the laser device used was not theirs. They had no influence on study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining authors declare that they have no conflict of interests.

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Ethical approval All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of the University of Nove de Julho (UNINOVE) (Protocol AN0010/2011).

Informed consent Does not apply since it is an animal experiment.

Ethical aspects All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of the University of Nove de Julho (UNINOVE) (Protocol AN0010/2011).

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