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ORIGINAL ARTICLE

PBMT and topical diclofenac as single and combined treatment on skeletal muscle injury in diabetic rats: effects on biochemical and functional aspects

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Abstract

Physical exercise generates several benefits in a short time in patients with diabetes mellitus. However, it can increase the chances of muscle damage, a serious problem for diabetic patients. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat these injuries, despite the serious adverse effects. In this way, photobiomodulation therapy (PBMT) with low-level laser therapy (LLLT) and/or light emitting diode therapy (LEDT) can be used as an alternative in this case. However, its efficacy in tissue repair of trauma injuries in diabetes mellitus until now is unknown, as well as the combination between PBMT and NSAIDs. The objective of the present study was to evaluate the effects of NSAIDs and PBMT applied alone or combined on functional and biochemical aspects, in an experimental model of muscle injury through controlled trauma in diabetic 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; 3 J; 107.1 J/cm^2, 30 s), diclofenac sodium for topical use (1 g), or combination of them. Our results demonstrated that PBMT + diclofenac, and PBMT alone reduced the gene expression of cyclooxygenase-2 (COX-2) at all assessed times as compared to the injury and diclofenac groups ($p < 0.05$ and $p < 0.01$ respectively). The diclofenac alone showed reduced levels of COX-2 only in relation to the injury group ($p < 0.05$). Prostaglandin E_2 levels in blood plasma demonstrated similar results to COX2. In addition, we observed that PBMT + diclofenac and PBMT alone showed significant improvement compared with injury and diclofenac groups in functional analysis at all time points. The results indicate that PBMT alone or in combination with diclofenac reduces levels of inflammatory markers and improves gait of diabetic rats in the acute phase of muscle injury.

Keywords Photobiomodulation therapy · Low-level laser therapy · Skeletal muscle injury · Topical nonsteroidal anti-inflammatory drug · Diabetes mellitus · Rats

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Introduction

According to the International Diabetes Federation, a DM (diabetes mellitus) epidemic is developing [1]. It is now estimated that the world population with diabetes is in the order of 397 million individuals and will reach 700 million in 2045 [1].

Diabetes mellitus is characterized by a heterogeneous group of metabolic disorders, hyperglycemia, and angiopathies, among others [2]. Hyperglycemia causes imbalance in growth factors such as keratinocytes, fibroblasts, and endothelial cells in tissue repair; increases the expression of proinflammatory cytokines; and impairs immune cell functions, causing DNA damage. This deregulation provides an increase in ROS, making it difficult to resolve the inflammatory process, and increases cellular apoptosis, culminating in delayed tissue repair [2, 3].

Physical exercise is undoubtedly of great importance for the DM patient. It is known that in a short time, it generates several benefits, among them the increase in glucose consumption [4, 5]. It is also known that weight control and increased physical activity decrease insulin resistance [4, 5].

Physical activity as a therapy in diabetes can reduce oxidative stress and β cell damage, also acting by increasing insulin content and basal insulin secretion. The same authors report in their study that physical exercise increases the viability of pancreatic β cells through the signaling of interleukin-6 (IL) [5]. However, exercise can increase the chances of muscle damage during running, jumping, and eccentric contractions, among others, which is a serious problem for diabetic patients, who suffer from delayed tissue repair [6, 7].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat these injuries, with short-term beneficial effects, inhibiting the action of cyclooxygenases (COX-1 and COX-2) and consequently the synthesis of prostaglandins [8]. However, these drugs cause serious adverse effects such as kidney and heart complications, stomach ulcers, and gastrointestinal problems, among others [9, 10].

Photobiomodulation therapy (PBMT) is a therapeutic resource used in the treatment of short-term musculoskeletal injuries. Although its biological mechanisms of action have not been fully elucidated, PBMT has been widely used in experimental and clinical studies, demonstrating the positive action on muscle regeneration and absence of adverse effects [10–13]. PBMT seems to work very well in traumatic muscle injuries. Recently, in two studies, Tomazoni et al. [14, 15] demonstrated the efficacy of PBMT in the repair of trauma injuries, regarding morphological, biochemical, and functional aspects, using an 830-nm laser and a dose of 3 J. Furthermore, works can be found in the literature with positive results of treatment with photobiomodulation therapy in wound healing and cryolesions [16, 17]. However, the efficacy of photobiomodulation therapy in the repair of trauma injuries in diabetic rats, as well as the synergistic effects of using of NSAIDs and photobiomodulation therapy combined, is unknown. In addition, these therapies may act synergistically to ameliorate acute anti-inflammatory effects.

With this line of thought, the objective of the present study was to evaluate the effects of therapies applied alone or combined, on functional and biochemical aspects, in an experimental model of muscle injury through controlled trauma in diabetic rats.

Material and methods

Animals

The experiments were carried out using male Wistar rats weighing 300 g, with access to food and water ad libitum. The rats were provided by animal facility of the university. All rats were randomly divided into groups of six. A total of 108 rats were used and divided into 36 rats at each time analyzed (12, 24, and 48 h) (Fig. 1). The policies and procedures of the animal laboratory are in accordance with Brazilian laws and those detailed by the US Department of Health and Human Services. In addition, the experimental protocol was submitted to and approved by the ethics committee for the use of animals (CEUA number: 3294021216.

Experimental groups

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

- Control group (control)—animals that did not undergo any type of procedure.
- Diabetes group (DM)—animals with induced diabetes, which did not undergo any type of procedure.
- Diabetes injury group (not treated = NT)—animals submitted to controlled muscle trauma injury that did not receive treatment.
- Diabetes topical diclofenac group (TD)—animals that underwent muscle injury and were treated with topical application of diclofenac.
- Diabetes PBMT + topical diclofenac group (PBMT + TD)—animals that underwent muscle injury and were treated with PBMT and topical application of diclofenac.
- Diabetes PBMT group (PBMT)—animals that underwent muscle injury and were treated with PBMT at a dose of 3 J.

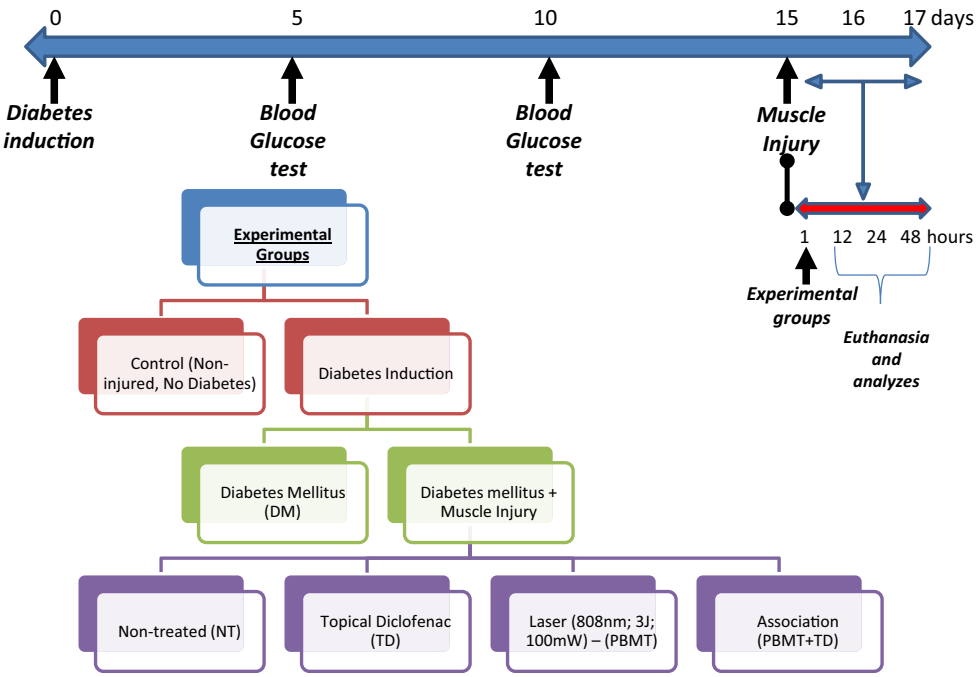

Fig. 1 Experimental design

Procedures

Chemical induction of diabetes

The animals were fasted with access to water for 12 h prior to the induction of diabetes. Diabetes was induced, 15 days prior to the trauma protocol, with a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 50 mg/kg of body mass, freshly dissolved in 0.05 M citrate buffer [18, 19]. Blood glucose levels were monitored each 5 days following STZ injection. Animals with fasting blood glucose greater than 220 mg/dL were selected for the experiment. All animals that received STZ developed experimental diabetes.

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 using 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 (Fig. 2). The contusion was performed with the animal in the lateral decubitus position, and the right hind paw was manually immobilized in a stretched position, by means of the plantar flexion of the ankle [11].

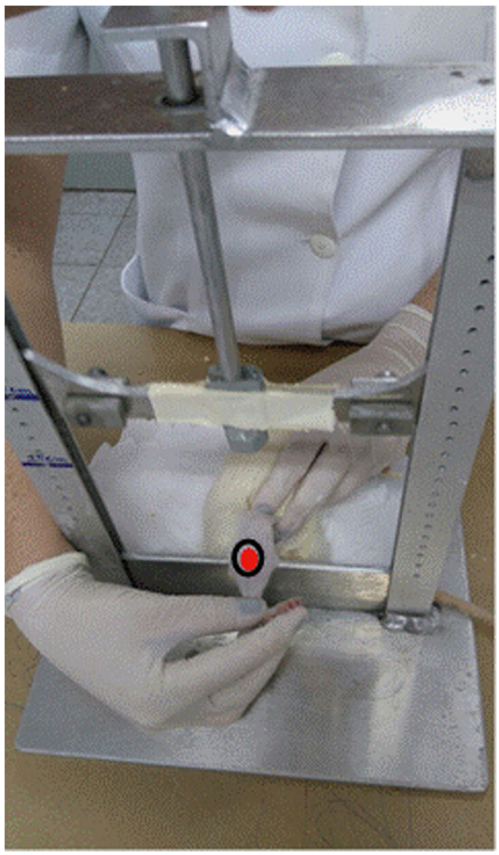

Fig. 2 Site of injury (0) and treatments ( )

Treatments

All treatments were performed 1 h after the muscle trauma [10, 11].

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; 107.51 J/cm^2 energy density; and 3 J (30 s) dose of energy per point, was used. Only one single point on the ventral region of the animal's anterior tibial muscle was irradiated. The optical output of the laser unit was measured before, halfway through, and after the experiment. During irradiation, the spot was kept in direct contact with the animal's skin, applying light pressure on the tissue. The parameters of PBMT were chosen according to previous studies from our research group [10, 11]. The PBMT + TD group was treated with a combination of the abovementioned treatments. It is important to highlight that in this group, the rats received irradiation 5 min before topical application of diclofenac [10].

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 animal's anterior tibial muscle.

Collection of biological material

The biological material was collected 12, 24, and 48 h after 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 analysis. For functional analysis, no invasive procedure or surgery was performed until euthanasia at the time points described below.

Outcomes

Biochemical and molecular analysis

Analysis of gene expression by real-time reverse transcription polymerase chain reaction First, muscles were thawed, and Trizol was immediately added (Gibco BRL, Life Technologies, Rockville, MD; 1 mL per 100 mg tissue). Next, muscles were homogenized for the recovery of total RNA, according to the manufacturer's instructions. To obtain a pure RNA sample, DNase I was added and the integrity of RNA was verified using agarose gel electrophoresis. Total RNA (2 lg) was used for first-strand cDNA synthesis [reverse transcriptase (RT)] using SuperScript II. In addition, RNase OUT was also added to protect the RNA during this process. Three pooled RNA aliquots were routinely sham reverse transcribed (i.e., RT omitted) to ensure the absence of DNA contaminants. Diluted RT samples (1:10) were submitted to real-time polymerase chain reaction (PCR) amplification using Platinum Sybr QPCR Supermix-UDG and specific oligonucleotides. The primers used were as follows: COX-1 (forward: CCGTGCGAGTACAGTCACAT; reverse: CCTCACCA GTCATTCCCTGT) and COX-2 (forward: AGATCAGA AGCGAGGACCTG; reverse: CCATCCTGGAAAAG TCGAAG), and beta-actin was used as an internal control (forward: AAGATTTGGCACCACACTTTCTACA; reverse: CGGTGAGCAGCACAGGGT). The conditions for PCR were as follows: 50 °C–2 min; 95 °C–2 min, followed by 30 cycles of 95 °C–15 s; 60 °C–1 min, and 72 °C–15 s. Ct values were recorded for each gene, and the results of genes of interest were normalized to results obtained with the internal control gene. The delta threshold cycles (ddCT) were calculated (the results were interpreted using the formula 2_DDCt (Ct is the number of cycles required to reach the threshold value of fluorescence above background) relating the expression of the gene of interest compared with that of the housekeeping gene beta-actin) and the results are expressed as fold increase. All oligonucleotides and reagents used in this protocol were purchased from Invitrogen Co.

Analysis of PGE_2 levels in blood plasma by ELISA For analysis of PGE_2, 3 mL of blood was collected from animals by cardiac puncture immediately prior to euthanasia. For blood collection, we used a syringe containing heparin. After collection, samples were centrifuged and only the content of the supernatant resulting from centrifugation was stored in a freezer at − 80 °C in the form of plasma until analysis. The quantification of levels of PGE was performed according to the manufacturer's instructions using ELISA (R & D Systems, Minneapolis, MN).

Functional evaluation

Walking track index outcome The walking track analysis was performed prior to induction of muscle strain and 12, 24, and 48 h after injury. The functional evaluation was performed using the walking track analysis, described by De Medinacelli et al. [20]. In this analysis, the animal was placed in a transparent acrylic corridor with access to a dark environment. A ruler for measurement calibration (cm) was placed under the corridor. The footsteps on the floor were recorded by a camcorder, attached to a tripod, always maintaining the same distance from the equipment to the acrylic corridor. The images were used for the functional analysis, through a computer software. This procedure was repeated twice with each animal. All the rats were allowed adaptation and exploration of the site, as well as recording of the normal behavior of each animal, prior to the injury procedure. The following measures

were collected and analyzed through the software: (1) the space between the second and fourth distal phalanx (ITS intermediate toe spread), (2) the space between the first and fifth distal phalanx (TS toe spread), and (3) the space between the proximal edge of the foot and the third distal phalange (PL print length).

Statistical analysis

The obtained data were first plotted for analysis of normal distribution, after which statistical analysis was performed with parametric tests if the data were normally distributed. After confirmation, the obtained data were tested statistically by ANOVA with the Student–Newman–Keuls post-test. The statistical level of significance was set at $p < 0.05$. Data are expressed in mean value and standard error of the mean (SEM).

Results

Gene expression analysis by reverse transcription polymerase chain reaction

Figure 3a shows COX-1 gene expression of the tibialis anterior muscle of rats in different experimental groups (control, DM, NT, TD, PBMT + TD, PBMT) 12, 24, and 48 h after muscle trauma injury. It can be observed that at all experimental moments, muscle trauma injury significantly increased ($p < 0.05$) COX-1 gene expression in the injury group compared with the control and diabetes groups. On the other hand, all treatments significantly decreased COX-1 gene expression. Figure 3b shows COX-2 gene expression was significantly increased at all experimental moments in the injury group compared with the control and diabetes groups ($p < 0.05$). In addition, all treatments (TD, PBMNT + TD, and PBMT) significantly decreased ($p < 0.05$) COX-2 gene expression compared with the injury group.

Analysis of PGE_2 levels by ELISA

Regarding PGE_2, it can be seen that at all experimental moments, levels are increased in the injury and TD groups compared with control and DM groups ($p < 0.05$). However, PGE_2 levels in the PBMT + TD and PBMT groups are significantly decreased at both experimental moments compared with the injury and TD groups ($p < 0.05$). Figure 4 summarizes the results regarding PGE levels.

Functional analysis

We observed that the functional index showed a statistically significant increase in walking impairment in the injury and TD groups compared with control and diabetes groups ($p < 0.05$). However, we found that there was a statistically significant improvement ($p < 0.05$) in functional aspect, at all time points, in the PBMT + TD and PBMT groups compared with injury and TD groups Fig. 5.

Discussion

To our knowledge, this is the first study to investigate the effects of PBMT used in combination with NSAIDs in diabetic rats, compared with NSAID treatment alone, which is a first-line drug for treatment of muscle injury [14].

In addition, the use of NSAIDs in a topical manner deserves to be highlighted, since it presents lower adverse effects than oral NSAIDs (for example, [10, 14]). Finally, the functional analysis of the rats, through the walking track analysis, also deserves attention, since the main objective at the end of a treatment for muscular injury is the return of normal function of the treated segment.

It is known that hyperglycemia can generate an imbalance in the production of growth factors, an increase in the inflammatory process, and impair cellular functions, thus causing a delay in the process of tissue repair [2, 3]. According to Tomazoni et al. [14, 15], NSAID therapy is the most commonly used treatment for muscular lesions and photobiomodulation therapy (PBMT) is effective in reducing inflammatory markers, as well as improving repair of injured musculoskeletal tissue, at the energy density of 107.1 J/cm^2 and total energy of 3 J.

In the present study, we observed that the gene expression of COX-1 and COX-2 increased at all moments (12, 24, and 48 h) in the diabetic injured and untreated (NT) groups. All treatments tested in the present study significantly decreased the gene expression of COX-1 and COX-2 at all moments compared to the NT group. However, groups treated with topical diclofenac alone (TD) presented significantly decreased gene expression of COX-1 at all moments. The groups treated with PBMT alone or in combination with diclofenac presented a statistically significant reduction in the gene expression of COX-2 when compared to the NT and TD groups, and the LLLT + TD group demonstrated the greatest decrease. According to de Paiva Carvalho [10], who also observed similar results in muscle lesions, but in non-diabetic rats, the most interesting result in an anti-inflammatory therapy is the decrease in COX-2. Tomazoni et al. [15], using the same injury protocol and the same parameters of PBMT, demonstrated a decrease in the gene expression of COX-2 in non-diabetic animals. Our findings are in agreement with several studies that demonstrate a decrease in inflammatory markers in different experimental models of diseases [15, 21–24]. Ramos et al. [25] found that PBMT (3 J) decreased gene expression of inflammatory markers after

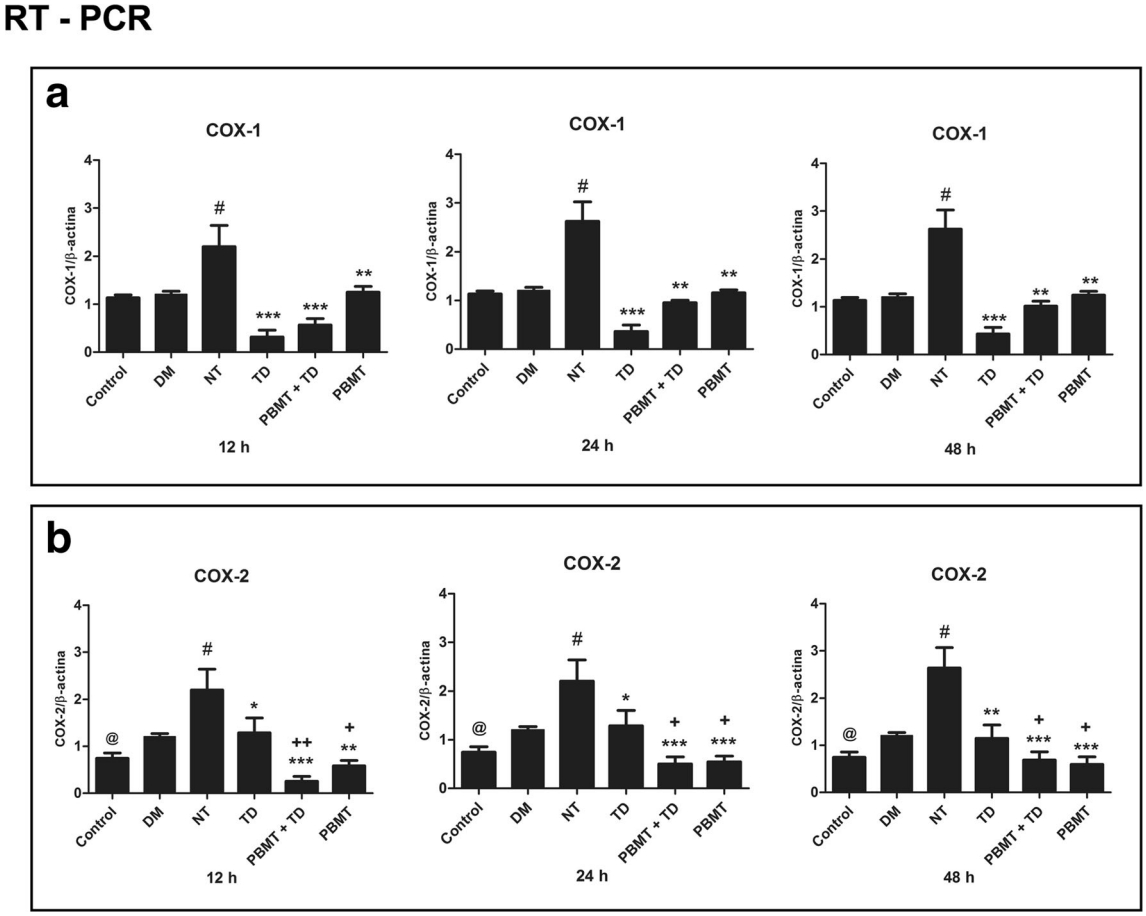

Fig. 3 **a** Gene expression levels of COX-1 in the anterior tibial muscle of all experimental groups at all assessed times. $^{**}p < 0.05$ vs. NT group, $^{***}p < 0.05$ vs. NT group, $^{\#}p < 0.05$ vs. control and DM group, $^{*}p < 0.05$ vs. NT group. Data represents the mean ± SEM; there were six animals per group. **b** Gene expression levels of COX-2 in the anterior tibial muscle of all experimental groups at all assessed times. $^{**}p < 0.05$ vs. NT group, $^{***}p < 0.05$ vs. NT group, $^{\#}p < 0.05$ vs. control and DM group, $^{*}p < 0.05$ vs. NT group, $^{+}p < 0.05$ vs. TD group, $^{++}p < 0.05$ vs. TD group, $^{@}p < 0.05$ vs. DM group. Data represents the mean ± SEM; there were six animals per group

stretch muscle injury. Alves et al. [12] also demonstrated that PBMT modulated the gene expression of inflammatory markers (3.2 J) during muscle tissue repair.

Thus, in a previous study by our group [10], we observed that the groups treated only with PBMT and with PBMT together with diclofenac demonstrated a significant decrease in

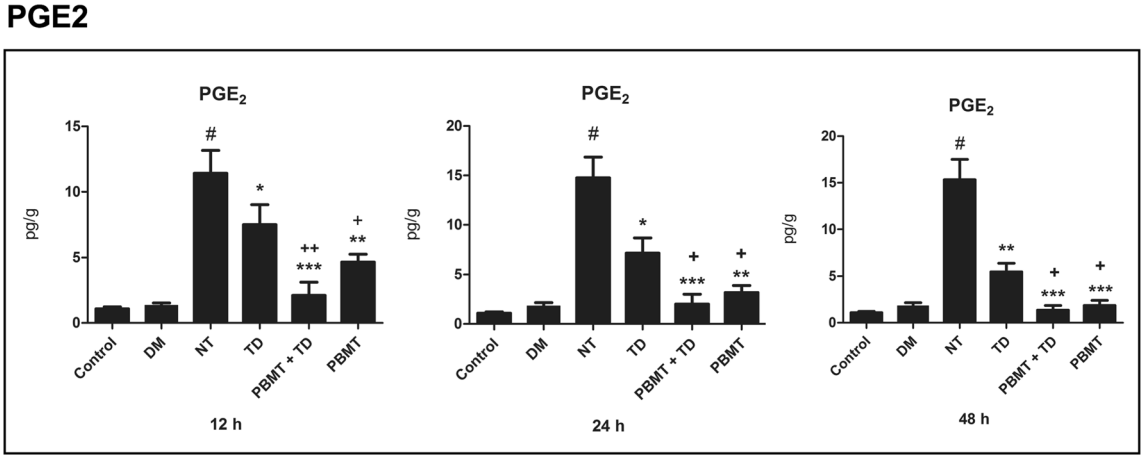

Fig. 4 Levels of PGE_2 in blood samples of all experimental groups at all assessed times. $^{*}p < 0.05$ vs. NT group, $^{**}p < 0.05$ vs. NT group, $^{***}p < 0.05$ vs. NT group, $^{\#}p < 0.05$ vs. control and DM group, $^{+}p < 0.05$ vs. TD group, $^{++}p < 0.05$ vs. TD group. Data represents the mean ± SEM; there were six animals per group

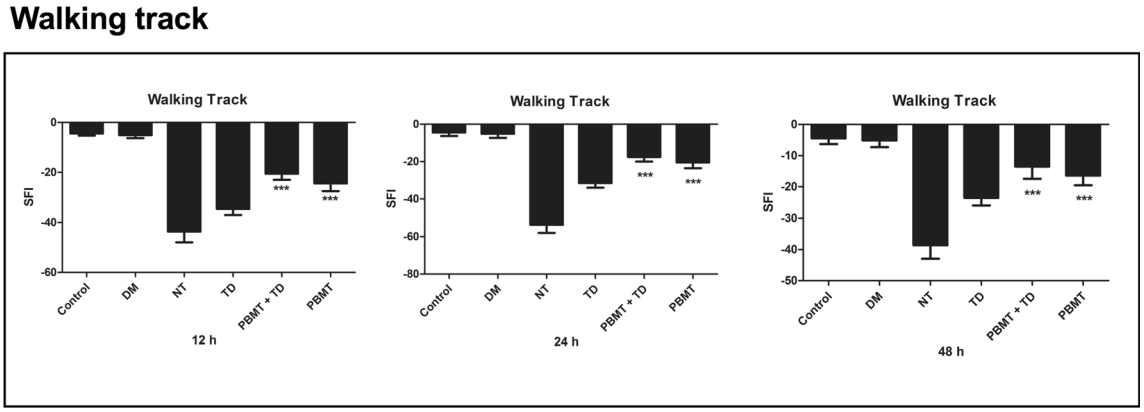

Fig. 5 Functional index of all experimental groups at all assessed times. ***$p < 0.05$ vs. NT group. Data represents the mean ± SEM; there were six animals per group

PGE_2 levels when compared to the group treated with diclofenac alone and the NT group, at all moments analyzed. Moreover, we observed that the group treated with PBMT together with diclofenac presented a statistically significant decrease, when compared to the group treated only with PBMT 12 and 24 h after the injury protocol. However, 48 h after the injury protocol, we observed that there was no statistical difference between these two groups, with both reaching baseline levels of PGE_2, compared with the control group. On the other hand, the group treated with TD in isolation showed a decrease in PGE_2 levels only compared with the NT group. These results demonstrate the maintenance of the inflammatory process, consistent with the diabetic state of the rats [3].

It is important to emphasize that functional tests are very important in the analysis of muscle tissue, to better understand its evolution [14]. In our study, we used the functional analysis called walking track analysis to evaluate the gait of the rats. We observed that the rats in the group treated with TD alone did not present improved walking at any moment analyzed. On the other hand, the rats in the groups treated with PBMT alone or in combination demonstrated improved gait at all the three moments analyzed. Ramos et al. [25] and de Paiva Carvalho et al. [10] found similar results; however in both studies, the authors used shorter analysis times (3 and 6 h) and non-diabetic rats. Our results (PBMT group) appear to demonstrate a decrease in the inflammatory process (through the decrease in inflammatory markers) and a significant improvement in function 48 h after the injury induction protocol. However, we also observed that in less time, the groups irradiated with PBMT in combination with TD demonstrated statistically significant results, although after 48 h, the groups treated with only PBMT presented similar results to the LLLT + TD group. This leads us to believe that PBMT can present better results in the medium and long terms. In summary, our findings demonstrate that PBMT may contribute to future clinical practices as a therapeutic, non-pharmaceutical, noninvasive intervention that has no reported side effects, and contributes positively to the prognosis even in complicated conditions [26] such as diabetes [16].

Conclusion

We conclude that PBMT therapy, alone or in combination with diclofenac, reduces levels of inflammatory markers and improves gait of diabetic rats in the acute phase of muscle injury. However, further experimental studies are needed before PBMT can be fully implemented in the clinical treatment of patients. Since it is a noninvasive, nonpharmacological intervention, PBMT arises as a promising therapy for the treatment of muscular impairments in diabetes.

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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.

Ethical aspects All experimental protocols were submitted and approved by the Animal Experimentation Ethics Committee of the Universidade Sagrado Coração (Protocol 3294021216).

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