Research Article

Pharmacokinetic and Pharmacodynamics of Sodium Diclofenac (Topical and IM) Associated with Laser Photobiomodulation on Skeletal Muscle Strain in Rats

Rodrigo Leal de Paiva Carvalho,¹ P. S. L. M. Leonardo,² Gustavo Duarte Mendes,^{3,4} Fernanda Púpio Silva Lima,⁵ Mário Oliveira Lima,⁵ Rodrigo Labat Marcos,⁶ and Rodrigo Alvaro Brandão Lopes-Martins²

¹Department of Physical Education, Universidade Estadual Paulista - UNESP, Bauru, SP, Brazil

²Laboratory of Biophotonics and Experimental Therapeutics, Instituto de Pesquisa & Desenvolvimento - IP&D, Universidade do Vale do Paraíba - UNIVAP, São José dos Campos, SP, Brazil

³Universidade Metropolitana de Santos-UNIMES, Programa de Pós-Graduação em Saúde e Meio Ambiente, Brazil

⁴Faculdade de Medicina São Leopoldo Mandic, Campinas, SP, Brazil

⁵Laboratory of Sensory Motor Rehabilitation, Instituto de Pesquisa & Desenvolvimento-IP&D, Universidade do Vale do Paraíba-UNIVAP, São José dos Campos, SP, Brazil

⁶Postgraduate Program in Biophotonics Applied to Health Sciences, Universidade Nove de Julho, São Paulo, Brazil

Correspondence should be addressed to Rodrigo Alvaro Brandão Lopes-Martins; ralopesmartins@gmail.com

Received 8 August 2018; Accepted 10 December 2018; Published 3 March 2019

Academic Editor: Kei Ohkubo

Copyright © 2019 Rodrigo Leal de Paiva Carvalho et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Purpose. The practice of physical activities is considered a primary factor for the maintenance of good health status. However, exhaustive or unusual physical activities can lead to muscle injuries. Several treatments are used to recover muscle injuries; however, systemic NSAIDs often result in serious adverse events. In this study, we aimed to investigate the association of laser therapy (LLLT) and topical diclofenac, evaluating the kinetics of the drug and its pharmacodynamic effect in stretching-induced muscle injury in rats. **Methods.** Male Wistar rats weighing 200 g were randomized and divided into groups of 6 animals. Plasma concentrations of diclofenac were quantified by mass spectrometry at different times (15 min to 24 hours) in all animals. The laser energy used was 3 Joules (830 nm; 100 mW, 30s). Treated groups received diclofenac at the dose of 1 mg/kg IM or topically applied with or without laser therapy. The electric stimulation was used to study the functional status of the muscles. **Results.** After topical administration of diclofenac, the peak plasma concentration (t_{max}) occurred for 30 minutes in the irradiated group and 4 hours in the nonirradiated group. The AUC (0-24 hs) was 442 (ng/h/mL⁻¹) in the nonirradiated group and 712 (ng/h/mL⁻¹) in the irradiated group. **Conclusion.** LLLT was effective to provide a significant improvement in functional patterns. Taken together, our results demonstrate the synergistic effect between LLLT and topical diclofenac in muscle injury induced by stretching in rats.

1. Introduction

Unusual and exhaustive physical exercise may lead to structural, ultrastructural, and biochemical changes of the skeletal muscle [1]. Such changes have characteristics of an acute disease called Exercise Myopathy (ME). There is evidence that the great abundance of signs and symptoms

that are associated with ME is dependent on the type of contraction (eccentric vs. concentric), intensity, duration [2, 3], and individual factors, such as age and their level of physical conditioning.

Muscle injuries account for about 30% of sports injuries, of which more than 90% are caused by force or excessive stretching of the muscle [4]. The most common exercises that

can induce injuries are those with high eccentric components, such as resisted exercises, jumping exercises, plyometrics, and intermittent races with rapid change of direction [5].

There are several evidences already described in the literature regarding indirect methods of measuring muscle injury, especially in humans, including decreases of maximum voluntary isometric force [6–8], increases in muscle discomfort [9–11], significant increases in cytokines concentrations (IL-1 β and IL-6), and lactate dehydrogenase and glutamic oxalacetic transaminase [12]. Levels of these mediators are usually elevated when acute muscle injury occurs.

Different types of pharmacological and nonpharmacological therapies have been used with the aim of preventing or at least mitigating the deleterious effects of exercise-related injuries. Among these, we can cite (a) anti-inflammatory drugs, (b) nutritional supplements, (c) cryotherapy, (d) massage therapy, (e) electrotherapy, and (f) low-level laser therapy.

Diclofenac is part of the nonsteroidal anti-inflammatory category and is a weak organic acid (pKa = 4.00). It is known that its main action is the ability to decrease the activity of the isoforms of the cyclooxygenase enzyme and consequent inhibition of prostaglandin synthesis [13, 14].

When orally used, diclofenac may present important adverse effects such as gastrointestinal bleeding, gastric ulcer, renal and cardiac complications, among others [13, 15]. However, in topical formulations, these adverse effects are practically not found, and their efficacy is possibly as good as oral or IM for skeletal muscle lesions [13, 16].

According to Miyatake et al. [16], the efficacy of oral nonsteroidal anti-inflammatory drugs (NSAIDs) in muscle and synovial tissues is reasonably established. In their study, they compared the plasma concentration between diclofenac given orally and topically applied, using recommended dosages in clinical prescriptions. The authors concluded that topical application is also an effective method.

The efficacy of diclofenac absorption applied topically depends on the ease of transposing the different layers of the skin [17], the low molecular weight (<500 Daltons), and both hydrophilic and lipophilic characteristics to cross the *stratum corneum*.

1.1. Laser Photobiomodulation. Previous clinical trials have confirmed the fact that has been reported countless times. According to Samoilova et al. [18], the low-level laser therapy (LLLT) was able to increase in 32% after 2 minutes of irradiation, and in 45% after 20 minutes, the blood flow in the cutaneous microcirculation. This effect was blocked by the local application of nitric oxide synthesis inhibitor (LNMA). Several other articles also report this increase in local microcirculation due to vasodilation in the irradiated tissue [19–23]. It seems that laser light produces vascular relaxation with elevation of cGMP [24].

In vivo experiments demonstrated a significant induction of iNOS after laser irradiation in the iliac artery of rabbits [25], and according to the results found, if laser irradiation elevates cGMP levels, a significant local vasodilation would probably occur, thus facilitating the transport of nutrients

to the irradiated region as well as the removal of metabolic substances that would impair muscle contraction.

In view of the anti-inflammatory effects of low-level laser associated with its vasodilatory effects on microcirculation, we believe that laser radiation can influence or even potentiate the absorption of diclofenac applied topically. In addition, AINE's and laser therapy may act synergistically, potentiating the anti-inflammatory effects of each one with reduced adverse effects when compared to systemic NSAIDs. In this context, the purposes of the present study were to investigate the effect of laser photobiomodulation on the pharmacokinetic of sodium diclofenac topically and intramuscularly applied and to compare the effects of both treatments on stretch-induced muscle lesion in rats.

2. Material and Methods

2.1. Animals. A total of 252 female Wistar rats from the Biotery of the University of São Paulo (USP) weighing around 200 g were used. The animals were kept under standard conditions of temperature (22–24°C), relative humidity (40–60%), light-dark cycle for 12 hours with water, and ad libitum feed. Rats were randomized and divided into groups of 6 animals. Our experimental protocols were approved by the Ethics Committee on Animal Experimentation of the Institute of Biomedical Sciences of the University of São Paulo (ICB_USP) n° 30 fls. page 87. For a better understanding of the experimental procedures, please see the graphical flow chart in Figures 1 and 2.

2.2. Pharmacokinetics Analysis

2.2.1. Experimental Groups for Determination of Concentrations of Diclofenac in Rat Plasma. The following experimental groups were used for the determination of plasma concentrations of diclofenac in noninjured animals: (a) healthy - healthy animals, (b) diclofenac IM, (c) laser + diclofenac IM, and (d) topical diclofenac laser + topical diclofenac;

Plasma concentrations of diclofenac were quantified by mass spectrometry at different times (0, 15, 30 min, 1, 2, 4, 6, 12, and 24 hours) in all treated and untreated animals. The laser energy used was 3 Joules. Treated groups received diclofenac at the dose of 1 mg/kg (milligram per kilogram) IM and topically applied.

2.2.2. Determination of Diclofenac Concentrations in Plasma. The determinations of the concentrations of diclofenac in rat plasma were carried out in the Cartesius Analytical Unit, Laboratory of Prof. Dr. Gilberto De Nucci in the Department of Pharmacology of the ICB/USP, according to the Analytical Protocol for the Validation of METGRU Method 08-07 - Determination of Diclofenac in human plasma by LC-MS/MS.

2.2.3. Protocol for Collection of Plasma Concentration Samples. The blood samples from the animals were collected via retro orbital at different times (mentioned above). At each predetermined time of collection, about 500 μ L of blood was withdrawn with capillary and placed in a clean

FIGURE 1: Shows the protocol timeline for determination of blood diclofenac concentrations.

FIGURE 2: Shows the experimental protocol of muscle stretching used to induce muscle damage in rats.

heparinized tube. Samples were centrifuged for 10 minutes at 4,000 RPM (Beckman GPR Centrifuge, São Paulo, Brazil). Plasma was separated into plastic tubes with a screw cap and sealing ring identified and stored in a freezer at -20°C for further analysis.

Samples from each animal were analyzed on the same day, thus avoiding interassay variations. Plasma concentrations of diclofenac were measured by reverse phase high-performance liquid chromatography (HPLC), and the peaks were monitored by mass spectrometry (MS/MS) detectors.

2.2.4. Extraction of Plasma Samples. The procedures described were applied not only to unknown samples but also to standard curve extraction and quality controls. During the run of extraction of the unknown samples, these occurred under yellow light, the following experimental protocol was followed. An appropriate number of 12×120 mm disposable glass test tubes were placed in a grid. The tubes were numbered according to the assay protocol; the plasma ($100\ \mu\text{L}$) of the animal samples were added to each tube; in each tube, $50\ \mu\text{L}$ of internal standard ($2\ \mu\text{g}/\text{mL}$ Naproxen solution) was added using a calibrated autopipette, and the sample was homogenized (vortexed) for approximately 10 s; $25\ \mu\text{L}$ of formic acid was added; the sample was homogenized (vortex) for 10 seconds; 4 ml of ethyl ether/hexane (80/20; v/v) was added to all tubes, and extraction was carried out by homogenization (vortex) for 40 s; the upper organic phase was then transferred to another set of clean glass tubes and evaporated under N_2 flow at 45°C ; the dried residues were dissolved with 1 mL acetonitrile/water (50/50, v/v) with 1 mM acetic acid and homogenized for 20 seconds (vortex), and then

the solutions were transferred to PCR plates using automatic pipettes with disposable plastic tips; the PCR plates were capped, and the plates were placed in the self-injector racks. Diclofenac and the internal standard Naproxen were extracted from plasma and analyzed by combining LC-MS/MS with turbospray negative and MRM detection mode. In order to conduct the tests, the quality of the reference standards used in the preparation of the solutions was evaluated. As authenticity of the reference standards, the origin and batch number of the analytical standards were recorded.

2.3. Pharmacodynamics Analysis. The pharmacodynamics analysis consists in the study of the biochemical and physiologic effects of drugs, i.e., how a drug affects an organism. Here, we investigate the effects of topical and IM diclofenac alone or associated with laser irradiation.

2.3.1. Passive Stretching Muscle Injury Model. For the tibialis muscle elongation protocol in this work, the stretching protocol was performed, as briefly described below [26].

The animals were anesthetized with ketamine: xylazine (80:16 mg/kg, König, Avellaneda, Argentina) intraperitoneally (i.p) before being submitted to the passive stretching protocol of the anterior tibial muscle. After weighing the animal, it was placed in dorsal decubitus on cork attached to the stretching system. The right hind limb was firmly fastened with a line, which passed through a pulley and attached itself to a pissette with a volume of water corresponding to 150% of the animal's body. This line was fixed on the back of the animal's paw, performing a plantar flexion, stretching the anterior tibial muscle of the right hind paw of the animal. The

protocol was performed only once and the animal received the traction for 20 minutes, resting for 3 minutes, and a second traction for 20 minutes.

2.3.2. Electromyographic Study. The animal was anesthetized with ketamine and xylazine (100 mg/kg and 20 mg/kg, respectively, IM) and fixed on a surgical table. The animal was then subjected to a cut in the skin near the metatarsal plantar region. Subsequently, a vulsion was performed, separating the tibial muscle from the subcutaneous tissue together with the skin; for this procedure, we enter the small cut already done with scissors closed on the muscle, returning in the caudal direction with the open scissors, exposing all the muscle of the animal. Then, with the aid of a scalpel, the tendon was separated from its insertion and tied to a thread. The muscle fascia was removed, making it easier to isolate the muscle. After the section, the muscle was pulled in the opposite direction to its insertion through the wire, so as to be isolated from the tibia. Throughout the procedure of stimulation of the anterior tibial muscle, it was kept hydrated with saline solution (0.09%). In the insertion region, near the metatarsal plantar region, the muscle through the tendon was connected to an isometric transducer (Ugo Basile®; Varese, Italy) and the sciatic nerve to a bipolar electrode.

The muscle was subjected to a constant tension of 10 g. The preparation was indirectly stimulated by pulses of 6–7 V, 0.2 Hz, and 2 ms. of duration. Muscle contractions in response to indirect stimuli were recorded on the UGO BASILE® GEMINI 7070 physiographer via the isometric transducer. In order to induce tetanic contraction, the frequency was raised to 60 Hz. Muscle fatigue was characterized by the inability to maintain muscle contraction, with the amplitude decaying by 50% of the maximum recorded, to avoid tissue death, resulting from tetanic contraction. For each group, tetanic contractions were performed every 10 minutes in the period of 30 minutes, making a total of 3 contractions for each animal in each group.

From the records, we analyzed (a) the intensity of the contraction force (amplitude) in grams, (b) the time required for the contraction to drop to one-half of maximal (50% of muscle fatigue) in seconds, and (c) the area under the time curve X intensity.

2.3.3. Laser Irradiation. A Thera-lase type laser (DMC®) was used, operating at the wavelength of 830 nm in a continuous mode, at the total energy dose of 3 Joules, time of irradiation of 30 seconds, spot size of 0.028 cm², and 100 mW of power. Laser irradiation was performed in the contact mode, at only one point in the middle region of the anterior tibial muscle of the rats, after induction of muscle injury.

2.3.4. Statistical Analysis. The results were expressed as means $\pm$ SEM and submitted to the unpaired Student *t*-test or analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Pharmacokinetics Analysis

3.1.1. Quantification of the Time Concentration Curve of Diclofenac, Applied Topically with and without Low-Power Laser Irradiation. Figure 3(a) shows the time concentration curve of diclofenac applied topically with and without low-intensity laser irradiation and quantification of plasma concentrations at different times (0, 15, 30 min, 1, 2, 4, 6, and 12 hours).

After topical administration of diclofenac, the peak plasma concentration (*t* max) occurred for 30 minutes in the irradiated group and 4 hours in the nonirradiated group, thus presenting a statistically significant difference for $*p < 0.05$. The AUC (0–24hs) was 442 (ng h mL⁻¹) in the nonirradiated group and 712 (ng h mL⁻¹) in the irradiated group, where it also presented a statistically significant difference for $*p < 0.05$.

Figure 3(b) shows the time concentration curve of diclofenac applied intramuscularly with and without low-intensity laser irradiation and the quantification of plasma concentrations at different times (0, 15, 30 min, 1, 2, 4, 6, 12, and 24 hours).

Following intramuscular administration of diclofenac, the peak plasma concentration (*t* max) occurred for 15 minutes in both groups. The mean plasma concentration (*w/w*) was 755 (ng h mL⁻¹) in the nonirradiated group and 899 (ng h mL⁻¹) in the irradiated group, showing a statistically significant difference for $*p < 0.05$. The mean AUC (0–24hs) was 757 (ng h mL⁻¹) in the nonirradiated group and 1082 (ng h mL⁻¹) in the irradiated group, where it also presented a statistically significant difference for $*p < 0.05$.

3.2. Pharmacodynamics Analysis

3.2.1. Effect of Tetanic Contraction on Indirect Stimulation in Rat Tibial Muscle, Postinjury, and Treatment with Low-Power Laser Therapy and Diclofenac Topic (DT) 3 and 6 Hours after the Lesion Protocol. Figure 4(a) shows the intensity of the muscle contraction force by electrical stimulation during three tetanus in a 3-hour protocol: DT, laser + DT, and laser. A muscle contraction force gain was observed in all 3 tetanus in the group treated with laser + DT (86.04 $\pm$ 3.57, 84.98 $\pm$ 3.58, and 84.78 $\pm$ 3.07) when compared to the lesion group (69.28 $\pm$ 5.75, 68.50 $\pm$ 4.43, and 67.02 $\pm$ 4.81) with $*p < 0.05$ and the DT group (70.83 $\pm$ 4.71, 69.40 $\pm$ 5.45, and 68.30 $\pm$ 5.68) with $*p < 0.05$.

Figure 4(b) shows the intensity of the muscle contraction force by electrical stimulation during three tetanus in a 6-hour protocol: DT, laser + DT, and laser. A muscle contraction force gain was observed in all 3 tetanus in the group treated with laser + DT (92.90 $\pm$ 3.46, 91.58 $\pm$ 3.34, and 89.88 $\pm$ 2.51) when compared to the lesion group (70.48 $\pm$ 4.14, 70.25 $\pm$ 4.82, and 70.08 $\pm$ 4.28) with $*p < 0.05$ and the DT group (76.59 $\pm$ 3.97, 74.14 $\pm$ 6.96, and 72.67 $\pm$ 6.91) with $*p < 0.05$. In the group treated with laser (85.78 $\pm$ 5.01, 82.98 $\pm$ 4.48, and 81.79 $\pm$ 4.02), a strength gain of muscle contraction was observed in all 3 tetanus when compared to the DT group.

FIGURE 3: Panel (a) shows the time concentration curve of diclofenac applied topically with and without low-intensity laser irradiation and quantification of plasma concentrations at different times (0, 15, 30 min, 1, 2, 4, and 6 hours; $n = 6/\text{group}$). Panel (b) shows the time concentration curve of diclofenac applied intramuscularly with and without low-intensity laser irradiation and quantification of plasma concentrations at different times (0, 15, 30 min, 1, 2, 4, 6, 12, and 24 hours; $n = 6/\text{group}$).

Figure 5(a) shows the intensity of the muscle contraction force by electrical stimulation during three tetanus in a 3-hour protocol: D.IM, laser + D.IM, and laser. It was observed in all 3 tetanus a muscle contraction force gain in the group treated with laser + D.IM (85.07 ± 3.30 , 84.31 ± 3.95 , and 84.02 ± 3.75) compared to the lesion group (69.28 ± 5.75 , 68.50 ± 4.43 , and 67.02 ± 4.81) with $*p < 0.05$ and the D.IM group. (72.55 ± 4.08 , 71.59 ± 5.97 , and 69.97 ± 5.68) with $^+p < 0.05$.

Figure 5(b) shows the intensity of the muscle contraction force by electrical stimulation during three tetanus in a 6-hour protocol: D.IM, laser + D.IM, and laser. It was observed in all 3 tetanus a muscle contraction force gain in the group treated with laser + D.IM (93.71 ± 3.45 , 91.89 ± 3.27 , and 90.69 ± 2.49) when compared to the lesion group (70.48 ± 4.14 , 70.25 ± 4.82 , and 70.08 ± 4.28) with $*p < 0.05$ and to the D.IM group. (77.98 ± 3.98 , 71.07 ± 6.95 , and 70.76 ± 6.92) with $^+p < 0.05$. In the group treated with laser (85.78 ± 5.01 , 82.98 ± 4.48 , and 81.79 ± 4.02), there was a

gain of muscle contraction force in all 3 tetanus when compared to the lesion group and only in the second tetany when compared to the D.IM group.

4. Area under the Fatigue Curve

Figure 6(a) shows a significant increase in all 3 contractions of the area under the muscle fatigue curve, after tetanic contraction and the ratio of muscle contraction intensity with time, so that this tension decreased by 50% of the maximum amplitude in a protocol of 3 hours when compared to the laser + DT groups (71.98 ± 5.33 , 73.67 ± 5.28 , and 72.89 ± 5.46) and to the lesion group (53.74 ± 5.39 , 54.95 ± 5.94 , and 53.12 ± 5.63) with $*p < 0.05$.

Figure 6(b) demonstrates a significant increase in all 3 tetanus of the area under the muscle fatigue curve, after tetanic contraction and the ratio of muscle contraction intensity over time, so that this voltage decays 50% of the maximum amplitude in a protocol of 6 hours when

FIGURE 4: Panel (a) shows the intensity of muscle contraction force induced by indirect electrical stimulation every 10 minutes, in a total of 3 tetanic contractions in healthy, lesion, and topical diclofenac-treated animals, 03 hours after stretching. Panel (b) shows the intensity of muscle contraction force induced by indirect electrical stimulation every 10 minutes, in a total of 3 tetanic contractions in healthy animals, lesion, and treated, 06 hours after stretching. The data represent the mean $\pm$ SEM, $n = 6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany $*p < 0.05$ vs. lesion and 1st, 2nd, and 3rd tetany $^{+}p < 0.05$ vs. DT).

compared to the laser + DT groups (88.27 ± 5.89 , 89.97 ± 5.27 , and 88.63 ± 5.46) and to the lesion group (62.19 ± 6.12 , 60.49 ± 5.28 , and 61.96 ± 5.72) with $p = 0.05$ and the DT group (68.97 ± 5.65 , 67.43 ± 5.44 , and 66.09 ± 5.62) with $^{+}p < 0.05$.

Figure 7(a) shows a significant increase in all 3 tetanus of the area under the muscle fatigue curve, after tetanic contraction and the ratio of the muscular contraction intensity with time, so that this tension decreased by 50% of the maximum amplitude in a protocol of 3 hours when compared to laser + D.IM groups (72.49 ± 5.32 , 74.79 ± 5.07 , and 74.11 ± 5.39) and to the injury group (53.74 ± 5.39 , 54.95 ± 5.94 , and 53.12 ± 5.63) with $*p < 0.05$.

Figure 7(b) demonstrates a significant increase in all 3 tetanus of the area under the muscle fatigue curve, after tetanic contraction and the ratio of the muscular contraction intensity with time, so that this tension decays 50% of the maximum amplitude in a protocol of 6 hours when

compared to laser + D.IM groups (90.79 ± 5.65 , 92.14 ± 5.73 , and 90.20 ± 5.48) and the laser group (88.27 ± 5.89 , 89.97 ± 5.27 , and 88.63 ± 5.46) to the injury group (62.19 ± 6.12 , 60.49 ± 5.28 , and 61.96 ± 5.72) with 0.05 and the D.IM group (69.83 ± 5.38 , 65.15 ± 5.72 , and 65.98 ± 5.90) with $^{+}p < 0.05$.

5. Discussion

In this work, the effects of low-level laser therapy operating in the infrared spectral region on the absorption and pharmacokinetics of NSAIDs (diclofenac topical and IM) and their performances separately on skeletal muscle injury were studied, using the experimental model of controlled muscle strain in the tibial muscle of rats.

The present experimental model of injury was characterized by Ramos et al. [26]. In his work, the dose of 3 J proved to be the most effective for this protocol. It was also

FIGURE 5: Panel (a) shows the intensity of muscle contraction force induced by indirect electrical stimulation every 10 minutes, in a total of 3 tetanic contractions in healthy, lesion, and intramuscular- (IM-) treated animals, 03 hours after stretching. Panel (b) shows the intensity of muscle contraction force induced by indirect electrical stimulation every 10 minutes, in a total of 3 tetanic contractions in healthy animals, lesion, and treated, 06 hours after stretching. The data represent the mean $\pm$ SEM, $n=6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany * $p < 0.05$ vs. lesion and 1st, 2nd, and 3rd tetany + $p < 0.05$ vs. DT).

demonstrated that the peak of the lesion in most of the analyses was reached 6 hours after the stretching, such as Evans blue plasma extravasation, COX-2 gene expression, TNF- α , C-reactive protein, and the walking track test.

Muscle injuries often lead to a long time of immobilization and wide use of anti-inflammatory drugs, which can lead, beside the side effects, to an increase in the numbers of collagen fibers as well as a decrease in the amount of muscle fibers. Such effects contribute to the changes in the biomechanical properties of the muscle [27].

Low-level laser therapy (LLLT) represents an alternative for the treatment of musculoskeletal injuries and may have inhibitory or stimulatory effects, depending on the parameters used in the treatment. In this sense, it is common to use the term photobiomodulation to cite its effects on biological tissues. The clinical objectives of the use of low-level laser therapy in muscle injury situations are aimed at reducing the adverse effects of anti-inflammatory drug use, reducing

immobilization time, and inhibiting or even reducing inflammatory process and enhance tissue repair, restoring the functional characteristics of the tissue.

As previously mentioned, the use of NSAIDs after muscle injury can lead to important adverse effects such as gastrointestinal bleeding, gastric ulcer, renal and cardiac complications, among others. Therefore, the use of topical NSAIDs has been shown also to be an interesting alternative, which, through several studies, demonstrated a good efficacy when compared to NSAIDs used by IM and oral routes, with the advantage of having few adverse effects [13, 28].

Currently, the dermatological area has been studying ways to improve the transport or transdermal absorption of some substances or drugs. To do this, a variety of media such as chemical agents, immersion, microneedles, and ultrasound is being used. However for some drugs, none of them can successfully overcome the *stratum corneum* (SC) layer [29, 30].

FIGURE 6: (a) Relation of muscle contraction intensity over time so that this force decays 50% of maximum amplitude, in a total of 3 tetanic contractions in healthy, lesion, and topical diclofenac-treated animals, 03 hours after stretching. The data represent the mean $\pm$ SEM, $n = 6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany $*p < 0.05$ vs. INJURY). (b) Relation of muscle contraction intensity over time so that this force decays 50% of maximum amplitude, in a total of 3 tetanic contractions in healthy animals, lesion, and topical diclofenac-treated animals, 06 hours after stretching. The data represent the mean $\pm$ SEM, $n = 6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany $*p < 0.05$ vs. LESION and $+p < 0.05$ DT).

Several types of laser have been tested with the objective of studying the facilitation of absorption and penetration of drugs through SC in a more effective way and without causing tissue damages such as CO₂, Nd:YAG, IPL (intense pulsed light), and diodes [29, 30]. However, such studies do not assess the plasma bioavailability of the drugs used.

In few studies, high-power laser application demonstrated a great capacity of improvement in the penetration of drugs and substances through the SC, without causing damage to the skin, demonstrated through analyzes such as optical coherence tomography, VIS-NIR fiber spectrometer, spectrophotometer, and spectral scanner [29–32]. This improvement in the permeability attributed to the laser is still not fully understood.

The low-intensity laser has as its characteristics the increase of the local microcirculation and the vasodilatation and recruitment of the collateral vascularization. These characteristics are widely accepted and demonstrated in studies with muscle tissues, healing, skin flaps, etc. [19–23, 33], which could further aid in the absorption function of drugs or other substances.

The bioavailability results of the present study corroborate the studies cited above. Thus, a statistically significant difference was observed in the group irradiated with laser 5 minutes before receiving diclofenac applied topically. This group had on average a t_{max} of 30 minutes and as mean AUC (0–12 hs) the value of 712 (ng h mL⁻¹), whereas the groups that received only topical diclofenac were evidenced

FIGURE 7: (a) Relation of muscle contraction intensity over time so that this force decays 50% of maximum amplitude, in a total of 3 tetanic contractions in healthy, lesion, and intramuscular (IM) diclofenac-treated animals, 03 hours after stretching. The data represent the mean $\pm$ SEM, $n = 6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany $*p < 0.05$ vs. INJURY). (b) Relation of muscle contraction intensity over time so that this force decays 50% of maximum amplitude, in a total of 3 tetanic contractions in healthy, lesion, and intramuscular- (IM-) treated animals, 06 hours after stretching. The data represent the mean $\pm$ SEM, $n = 6$ (ANOVA, followed by the Student-Newman-Keuls test, 1st, 2nd, and 3rd tetany $*p < 0.05$ vs. LESION and $^+p < 0.05$ DT).

averages of 3 hours and 442 ng h mL^{-1} , respectively. In the group where diclofenac IM was applied, statistically significant results were found for AUC (0-24 hs) (ng h mL^{-1}) and C (max) (ng h mL^{-1}), 1082 and 899, respectively, with $p < 0.05$, for the groups irradiated with laser 5 minutes before the application of the drug.

This response of the anti-inflammatory drug preceded by laser irradiation could be acting to a more rapid resolution of the inflammatory process, triggered after the occurrence of the stretch lesion, as well as better repair of the injured tissue.

In the present study, we evaluated the reduction of muscle fatigue in tibial muscle of rats after tetanic contraction by indirect electrical stimuli after stretching muscle injury, in groups treated with low-level laser therapy and with diclofenac, both applied topically and intramuscularly.

There are some definitions for muscle fatigue, and one of them says that fatigue is the inability to maintain muscle contraction force over a period of time. This disability was found in our study. The data found in our study corroborate previous results, such as those demonstrated in a study conducted by Ramos et al. [26] in our laboratory.

As we can observe in Figures 2 and 3, the association of laser therapy and diclofenac (both topical and IM) significantly preserved the peak force of tibial muscle after 03 but especially 06 hours of the lesion. Possibly, the application of two distinguished therapies produced a synergistic effect, inhibiting the inflammatory amplification phase after muscle damage. Interestingly, topical anti-inflammatory diclofenac plus laser irradiation presented a significant enhancement of absorption and the best effects on muscle force preservation.

Concerning on muscle fatigue, we previously demonstrated that the normal behavior of nontreated muscles is a decrease of peak force and AUC in a sequence of contractions. Here, we observed that laser therapy is still the best treatment to delay the time to fatigue. Rats in the lesion group showed a decrease in fatigue resistance in all protocols, while in the laser-treated group prior to the application of topical and intramuscular diclofenac demonstrated an increase in this parameter.

According to the theory of blood flow regulation, vasoactive metabolites are released from the muscle fiber in proportion to the diffusion activities, and this metabolic vasodilatation increases the supply of oxygen and nutrients in response to the tissue demand [34, 35].

Kipshidze et al. [24] have demonstrated in their studies that visible ultraviolet light produces vascular relaxation with elevation of cGMP. The absorption of light by vascular tissue, through induction of NO, is speculated to elevate cGMP. Vasodilation would be triggered by the hypotensive effect of both phosphodiesterase inhibitors and circulating cGMP nitrovasodilators.

In vivo experiments in laboratories demonstrated a significant induction of iNOS after laser irradiation in the iliac artery of rabbits [25], and according to our results, if laser irradiation elevates cGMP levels, a significant local vasodilation would probably occur, thus facilitating the transport of nutrients to the irradiated region as well as the removal of metabolic substances that would impair muscle contraction. Therefore, we can assume that a vasodilation or an increase in the local microcirculation caused by laser irradiation could be acting on the muscle cells, increasing the time for the tetanus muscle to decrease its maximum contraction intensity until reaching its half (50%).

When analyzing the area on the fatigue curve recorded in the electrophysiograph, it was possible to observe a very similar picture to that described above. Muscle fatigue presents as characteristics mainly a decrease of muscle strength and impaired motor control, with subsequent muscular pain. The presence of muscle injury also causes a decrease in performance and a reduction in the capacity to generate force.

The lesion of the skeletal muscle has as a characteristic the immediate loss of force production. One of the causes of this loss of force would be the rupture or loss of the force-generating structures, such as actin and myosin [36].

One of the mechanisms that triggers muscle injury is the excessive influx of calcium from the interstitium into the muscle fiber, resulting in elevated levels of intracellular calcium, which in turn is generated by damage to the sarcoplasm or the sarcoplasmic reticulum of the fiber. The process leads to the loss of homeostasis and stimulation to the calcium-dependent proteolysis, thus provoking a tissue degeneration [37]. In the intracellular medium, excess calcium causes the mitochondria to accumulate this ion, which inhibits cellular respiration and energy production, compromising the cell's ability to actively remove calcium from within. Calcium overload then precipitates an autogenic phase where an increase in the action of proteases and phospholipases results in the degradation of myofibrils

and the cell membrane, resulting in a compromise in glycogen synthesis when the muscle is injured [37].

The irradiated animals increased the fatigue resistance in the three tetanus in the groups treated with laser + DT, and also in the group treated with laser alone (6 hours), demonstrating an increase in fatigue resistance superior to the groups treated with diclofenac only.

Recent studies by our group have demonstrated that the preirradiation of low-power laser athletes, both in the red and infrared wavelength range, was able to increase resistance to muscle fatigue and to accelerate the fall in the concentrations of plasma lactic acid and CK [39–41].

Skeletal muscle has a considerable ability to regenerate muscle fiber, which is limited by the satellite cell population, revascularization, and local reinnervation. All this process is slow and usually incomplete, with a great scar formation, fibrous consistency, and little elastic and may alter muscle strength and extensibility [26]. Both the extent of the lesion and the treatment are important, because as mentioned above, muscle regeneration ends up being a complex process, causing morphological changes of the regenerated fibers, which can affect postinjury muscle performance [26].

According to Amaral et al. [42], direct laser radiation, used in the first days after the partial excision of the rat gastrocnemius muscle, promoted the regenerative process and the muscular maturation of the injured region. In his research, he used different doses, demonstrating that only the dose of 2.6 J of the He-Ne laser promoted significant changes, such as increased muscle fiber area and increased mitochondrial density, thus suggesting that low doses of laser irradiation would be more effective in promoting biostimulatory effects.

Concerning on laser and drug absorption, it is well known that the circulatory load of a given area is one of the main factors affecting local absorption from the pharmacological point of view. Recently, Oishi et al. [43] demonstrated that the abdominal acute application of PBM at 660 nm is able to induce a long-lasting hypotensive effect in hypertensive rats and vasodilation by a NO-dependent mechanism. In the same way, Kazemikhoo et al. [44] demonstrated that it may present a beneficial effect for diabetic patients via decreasing arginase expression and activation of the NOS/NO pathway which increases NO production and vasodilation, and before that, Plass et al. [45] demonstrated that low-level laser irradiation induced photorelaxation in coronary arteries and overcomes vasospasm of internal thoracic arteries. Taken together, these studies can definitely suggest that laser irradiation is able to produce a local vasodilatation and, in this case, enhance local absorption of topically applied drugs.

From our results, we can conclude that low-level laser therapy (830 nm) was effective in reducing/accelerating the inflammatory process and muscle damage induced by muscle stretching in rats, in addition to providing a significant improvement in functional patterns. Taken together, our results demonstrate the synergistic effect between low-power laser therapy and diclofenac applied by both the topical and IM routes in the experimental model of muscle injury induced by stretching in rats.

Data Availability

All data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was funded by FAPESP/CEPOF 2013/07276-1 and 2015/06502-3; Patrícia Sardinha Leonardo Lopes Martins was supported by PhD Scholarship, CAPES, Brazil.

References

- [1] J. M. Roberts and K. Wilson, "Effect of stretching duration on active and passive range of motion in the lower extremity," *British Journal of Sports Medicine*, vol. 33, no. 4, pp. 259–263, 2007.
- [2] P. Magalhães, "Alterações Hematológicas Agudas Induzidas Por Diferentes Protocolos De Exercício Físico Exaustivo E Inabitual. 148 F. Dissertação (Mestrado Em Ciências Do Desporto)," Faculdade De Ciências Do Desporto E De Educação Física Da Universidade Do Porto, Porto, 2000.
- [3] J. M. C. Soares and J. A. Duarte, "Miopatia do exercício. Etiologia, fisiopatologia e factores de prevenção," *Rev. Port. Med. Desp.*, vol. 7, pp. 187–200, 1989.
- [4] T. Wright-Carpenter, P. Klein, P. Schäferhoff, H. J. Appell, L. M. Mir, and P. Wehling, "Treatment of muscle injuries by local administration of autologous conditioned serum: a pilot study on sportsmen with muscle strains," *International Journal of Sports Medicine*, vol. 25, no. 8, pp. 588–593, 2004.
- [5] C. Twist and R. Eston, "The effects of exercise-induced muscle damage on maximal intensity intermittent exercise performance," *European Journal of Applied Physiology*, vol. 94, no. 5-6, pp. 652–658, 2005.
- [6] D. L. MacIntyre, W. D. Reid, D. M. Lyster, I. J. Szasz, and D. C. McKenzie, "Presence of Wbc, decreased strength, and delayed soreness in muscle after eccentric exercise," *Journal of Applied Physiology*, vol. 80, no. 3, pp. 1006–1013, 1996.
- [7] D. J. Newham, G. McPhail, K. R. Mills, and R. H. T. Edwards, "Ultrastructural changes after concentric and eccentric contractions of human muscle," *Journal of the Neurological Sciences*, vol. 61, no. 1, pp. 109–122, 1983.
- [8] K. Nosaka and P. M. Clarkson, "Influence of previous concentric exercise on eccentric exercise-induced muscle damage," *Journal of Sports Sciences*, vol. 15, no. 5, pp. 477–483, 1997.
- [9] H.-J. Appell, J. M. C. Soares, and J. A. R. Duarte, "Exercise, muscle damage and fatigue," *Sports Medicine*, vol. 13, no. 2, pp. 108–115, 1992.
- [10] P. Clarkson, W. Byrnes, K. McCormick, L. Turcotte, and J. White, "Muscle soreness and serum creatine kinase activity following isometric, eccentric and concentric exercise," *International Journal of Sports Medicine*, vol. 7, no. 3, pp. 152–155, 1986.
- [11] J. Friden, P. N. Sfikianos, and A. R. Hargens, "Muscle soreness and intramuscular fluid pressure: comparison between eccentric and concentric load," *Journal of Applied Physiology*, vol. 61, no. 6, pp. 2175–2179, 1986.
- [12] D. B. Chen, Z. M. Yang, R. Hilsenrath, S. P. Lê, and M. J. Harper, "Stimulation of prostaglandin (PG) $F_{2\alpha}$ and PGE_2 release, tumor necrosis factor- α and Interleukin-1 α in culture human luteal phase endometrial cells," *Human Reproduction*, vol. 10, no. 10, pp. 2773–2780, 1995.
- [13] M. Banning, "Topical diclofenac: clinical effectiveness and current uses in osteoarthritis of the knee and soft tissue injuries," *Expert Opinion on Pharmacotherapy*, vol. 9, no. 16, pp. 2921–2929, 2008.
- [14] L. A. Rodrigues, J. F. Fracasso, and C. E. Siqueira, "Diclofenaco," *Rev. Cienc Farm Basica Aplic*, vol. 28, pp. 45–49, 2007.
- [15] P. L. McCormack and L. J. Scott, "Diclofenac sodium injection (Dyloject): in postoperative pain," *Drugs*, vol. 68, no. 1, pp. 123–130, 2008.
- [16] S. Miyatake, H. Ichiyama, E. Kondo, and K. Yasuda, "Randomized clinical comparisons of diclofenac concentration in the soft tissues and blood plasma between topical and oral applications," *British Journal of Clinical Pharmacology*, vol. 67, no. 1, pp. 125–129, 2009.
- [17] S. P. Stanos, "Topical agents for the management of musculoskeletal pain," *Journal of Pain and Symptom Management*, vol. 33, no. 3, pp. 342–355, 2007.
- [18] K. A. Samoilova, N. A. Zhevago, N. N. Petrishchev, and A. A. Zimin, "Role of nitric oxide in the visible light-induced rapid increase of human skin microcirculation at the local and systemic levels: II. Healthy volunteers," *Photomedicine and Laser Surgery*, vol. 26, no. 5, pp. 443–449, 2008.
- [19] T. Kami, Y. Yoshimura, T. Nakajima, T. Ohshiro, and T. Fujino, "Effects of low-power diode lasers on flap survival," *Annals of Plastic Surgery*, vol. 14, no. 3, pp. 278–283, 1985.
- [20] Y. Maegawa, T. Itoh, T. Hosokawa, K. Yaegashi, and M. Nishi, "Effects of near-infrared low-level laser irradiation on microcirculation," *Lasers in Surgery and Medicine*, vol. 27, no. 5, pp. 427–437, 2000.
- [21] A. M. Miro, U. Shivaram, and P. J. P. Finch, "Noncardiogenic pulmonary edema following laser therapy of a tracheal neoplasm," *Chest*, vol. 96, no. 6, pp. 1430–1431, 1989.
- [22] A. Schindl, G. Heinze, M. Schindl, H. Pernerstorfer-Schön, and L. Schindl, "Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy," *Microvascular Research*, vol. 64, no. 2, pp. 240–246, 2002.
- [23] F. R. M. Ihsan, "Low-level laser therapy accelerates collateral circulation and enhances microcirculation," *Photomedicine and Laser Surgery*, vol. 23, no. 3, pp. 289–294, 2005.
- [24] N. Kipshidze, M. H. Keelan, J. R. Petersen et al., "Photoactivation of vascular iNOS and elevation of cGMP in vivo: possible mechanism for photovasoelaxation and inhibition of restenosis in an atherosclerotic rabbit model," *Photochemistry and Photobiology*, vol. 72, no. 4, pp. 579–582, 2000.
- [25] J. Moitra, S. Sammani, and J. G. N. Garcia, "Re-evaluation of Evans blue dye as a marker of albumin clearance in murine models of acute lung injury," *Translational Research*, vol. 150, no. 4, pp. 253–265, 2007.
- [26] L. Ramos, E. C. P. Leal Junior, R. C. Pallotta et al., "Infrared (810 nm) low-level laser therapy in experimental model of strain-induced skeletal muscle injury in rats: effects on functional outcomes," *Photochemistry and Photobiology*, vol. 88, no. 1, pp. 154–160, 2012.
- [27] T. A. H. Järvinen, L. Józsa, P. Kannus, T. L. N. Järvinen, and M. Järvinen, "Organization and distribution of intramuscular

- connective tissue in normal and immobilized skeletal muscles. An immunohistochemical, polarization and scanning electron microscopic study," *Journal of Muscle Research & Cell Motility*, vol. 23, no. 3, pp. 245–254, 2002.
- [28] C. Gómez, Á. Costela, I. García-Moreno, F. Llanes, J. M. Teijón, and M. D. Blanco, "Skin laser treatments enhancing transdermal delivery of ALA," *Journal of Pharmaceutical Sciences*, vol. 100, no. 1, pp. 223–231, 2011.
- [29] C. Liu, Z. Zhi, V. V. Tuchin, Q. Luo, and D. Zhu, "Enhancement of skin optical clearing efficacy using photo-irradiation," *Lasers in Surgery and Medicine*, vol. 42, no. 2, pp. 132–140, 2010.
- [30] A. P. Raphael, O. R. Wright, H. A. Benson, and T. W. Prow, "Recent advances in physical delivery enhancement of topical drugs," *Current Pharmaceutical Design*, vol. 21, no. 20, pp. 2830–2847, 2015.
- [31] D. J. McAuliffe, S. Lee, T. J. Flotte, and A. G. Doukas, "Stress-wave-assisted transport through the plasma membrane in vitro," *Lasers in Surgery and Medicine*, vol. 20, no. 2, pp. 216–222, 1997.
- [32] S. Lee, D. J. McAuliffe, N. Kollias, T. J. Flotte, and A. G. Doukas, "Photomechanical delivery of 100-nm microspheres through the stratum corneum: implications for transdermal drug delivery," *Lasers in Surgery and Medicine*, vol. 31, no. 3, pp. 207–210, 2002.
- [33] B. T. Ivandic and T. Ivandic, "Low-level laser therapy improves vision in patients with age-related macular degeneration," *Photomedicine and Laser Surgery*, vol. 26, no. 3, pp. 241–245, 2008.
- [34] T. L. Jacobs and S. S. Segal, "Attenuation of vasodilatation with skeletal muscle fatigue in hamster retractor," *The Journal of Physiology*, vol. 524, no. 3, pp. 929–941, 2000.
- [35] R. S. Santos, M. T. T. Pacheco, R. A. B. L. Martins et al., "Study of the effect of oral administration of L-arginine on muscular performance in healthy volunteers: an isokinetic study," *Isokinetics and Exercise Science*, vol. 10, no. 3, pp. 153–158, 2002.
- [36] J. L. Thompson, E. M. Balog, R. H. Fitts, and D. A. Riley, "Five myofibrillar lesion types in eccentrically challenged, unloaded rat adductor longus muscle—a test model," *The Anatomical Record*, vol. 254, no. 1, pp. 39–52, 1999.
- [37] P. du Souich, G. Roederer, and R. Dufour, "Myotoxicity of statins: mechanism of action," *Pharmacology & Therapeutics*, vol. 175, pp. 1–16, 2017.
- [38] T. I. Karu, N. Afanas'eva, S. Kol'iakov, and L. V. Piatibrat, "Change in the absorption spectrum of a monolayer of live cells under low-intensity laser irradiation," *Doklady Akademii Nauk*, vol. 360, no. 2, pp. 267–270, 1998.
- [39] E. C. P. Leal Junior, R. Á. B. Lopes-Martins, B. M. Baroni et al., "Comparison between single-diode low-level laser therapy (LLLT) and LED multi-diode (cluster) therapy (LEDT) applications before high-intensity exercise," *Photomedicine and Laser Surgery*, vol. 27, no. 4, pp. 617–623, 2009.
- [40] R. Á. B. Lopes-Martins, R. L. Marcos, P. S. Leonardo et al., "Effect of low-level laser (Ga-Al-As 655 nm) on skeletal muscle fatigue induced by electrical stimulation in rats," *Journal of Applied Physiology*, vol. 101, no. 1, pp. 283–288, 2006.
- [41] R. A. B. Lopes-Martins, F. Pazzelo Mafra, and G. De Nucci, "Laser therapy and muscle fatigue: a promising research area," *Photomedicine and Laser Surgery*, vol. 34, no. 7, pp. 273–275, 2016.
- [42] A. C. Amaral, N. A. Parizotto, and T. F. Salvini, "Dose-dependency of low-energy Hene laser effect in regeneration of skeletal muscle in mice," *Lasers in Medical Science*, vol. 16, no. 1, pp. 44–51, 2001.
- [43] J. C. Oishi, T. F. De Moraes, T. C. Buzinari, E. C. Cárnio, N. A. Parizotto, and G. J. Rodrigues, "Hypotensive acute effect of photobiomodulation therapy on hypertensive rats," *Life Sciences*, vol. 178, pp. 56–60, 2017.
- [44] N. Kazemikhoo, A. F. Sarafnejad, F. Ansari, and P. Mehdipour, "Modifying effect of intravenous laser therapy on the protein expression of arginase and epidermal growth factor receptor in type 2 diabetic patients," *Lasers in Medical Science*, vol. 31, no. 8, pp. 1537–1545, 2016.
- [45] C. A. Plass, G. M. Wieselthaler, B. K. Podesser, and A. M. Prusa, "Low-level-laser irradiation induces photorelaxation in coronary arteries and overcomes vasospasm of internal thoracic arteries," *Lasers in Surgery and Medicine*, vol. 44, no. 9, pp. 705–711, 2012.