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Effects of Low-Level Laser Therapy (LLLT) and Diclofenac (Topical and Intramuscular) as Single and Combined Therapy in Experimental Model of Controlled Muscle Strain in Rats

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ABSTRACT

Muscle injuries represent *ca* 30% of sports injuries and excessive stretching of muscle causes more than 90% of injuries. Currently the most used treatments are nonsteroidal anti-inflammatory drugs (NSAIDs), however, in last years, low-level laser therapy (LLLT) is becoming an interesting therapeutic modality. The aim of this study was to evaluate the effect of single and combined therapies (LLLT, topical application of diclofenac and intramuscular diclofenac) on functional and biochemical aspects in an experimental model of controlled muscle strain in rats. Muscle strain was induced by overloading tibialis anterior muscle of rats. Injured groups received either no treatment, or a single treatment with topical or intramuscular diclofenac (TD and ID), or LLLT (3 J, 810 nm, 100 mW) 1 h after injury. Walking track analysis was the functional outcome and biochemical analyses included mRNA expression of COX-1 and COX-2 and blood levels of prostaglandin E_2 (PGE_2). All treatments significantly decreased COX-1 and COX-2 gene expression compared with injury group ($P < 0.05$). However, LLLT showed better effects than TD and ID regarding PGE_2 levels and walking track analysis ($P < 0.05$). We can conclude that LLLT has more efficacy than topical and intramuscular diclofenac in treatment of muscle strain injury in acute stage.

INTRODUCTION

Muscle injuries represent *ca* 30% of sports injuries and excessive stretching of muscle causes more than 90% of injuries (1). Exercises involving eccentric contractions such as resistance exercises, jumping, plyometric and running with intermittent fast change of direction exercises are commonly responsible for this kind of injury (2).

Different kinds of pharmacological and nonpharmacological therapies have been used to prevent or at least mitigate the deleterious effects of this kind of injury. Among these therapies, we can highlight anti-inflammatory drugs, cryotherapy, massotherapy and electrotherapy.

Diclofenac is widely used in treatment of muscle injuries and this drug belongs to the category of nonsteroidal anti-inflammatory drugs (NSAIDs), and their mechanisms of action are not fully known. It is well-known that the main role of this drug is to reduce the activity of cyclooxygenase isoforms (COX-1 and COX-2), and consequently inhibits prostaglandin synthesis (3). However, it is important to highlight that when used orally, diclofenac may induce important adverse effects such as gastrointestinal bleeding, stomach ulcers, kidney and heart complications, among others (3, 4). However, in topical formulations these side effects seem to be decreased (3, 5–7).

An important meta-analysis published in the *British Medical Journal* (8) analyzed more than 13 000 patients with chronic inflammatory diseases taking NSAIDs, including coxibs. In this study, authors demonstrated that the effect of anti-inflammatory drugs were slightly superior to placebo and due to that, there is no evidence to support the use of such drugs in these diseases, especially taken into account the important and several side effects related to long-term use. This article has aroused considerable controversy in the scientific community concerning new therapies that could be used to treat chronic inflammatory diseases. In 2007, Bjordal *et al.* (9) have demonstrated in another meta-analysis of randomized, double-blinded, placebo-controlled trials that only topical application of diclofenac had a minimum clinically relevant efficacy in relieving pain in osteoarthritis of the knee.

In recent years, several randomized, placebo-control trials were performed, confirming that low-level laser therapy (LLLT) currently represents a consistent alternative therapy to treat different diseases (10–12). Particularly in the last 10 years, our research group has characterized the effects of the therapy in inflammatory paw edema (13), osteoarthritis (14), tendinitis (15) and skeletal muscle fatigue (16–18).

Recent studies in humans confirmed the fact that had already been hypothesized over the years. According to Samoilova *et al.* (19), LLLT is able to increase by 32% (2 min after irradiation),

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and 45% (20 min after irradiation) blood flow in cutaneous microcirculation. In addition, several other articles also reported similar increases in local microcirculation or vasodilatation in irradiated tissue (20). It is important to highlight that local vascularization is one of the most important factors that interfere in drug absorption.

Therefore, due to anti-inflammatory and vasodilator effects on the microcirculation of LLLT, we believe that laser irradiation can influence or even enhance the effect of topical application of diclofenac. In addition, pharmacological therapies and LLLT may act synergistically enhancing the anti-inflammatory effects of each therapy. With this line of thinking, the aim of this study was to evaluate the effect of single and combined therapies (LLLT, topical application of diclofenac and intramuscular diclofenac) on functional and biochemical aspects after an experimental model of controlled muscle strain in rats.

MATERIALS AND METHODS

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

Animal model of standardized muscle strain. The rats were anesthetized with ketamine/xylazine (100 and 20 mg kg^{-1}, respectively). The rat muscle strain model was the same used previously by our research group (21). The hind limb of each animal was positioned with the knee extended and ankle in 90° dorsiflexion (Fig. 1). A graduated weight corresponding to 150% of the animal body weight was attached to the right foot for 20 min, twice, with 3 min intervals. Animals were sacrificed with an overdose of halothane at the different outcome timepoints (3 and 6 h) for biochemical analyses. After the removal of skin and connective tissue, the tibialis anterior muscle was removed and processed for further analysis. For functional analysis, no invasive procedure or surgery was done until sacrifice at timepoints described below.

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

Figure 1. Experimental model of controlled muscle strain injury.

1 Control group (Control) — animals that did not undergo any type of procedure.
2 Injury group (Injury)—animals submitted to controlled muscle strain injury.
3 Topical diclofenac group (TD)—animals that underwent muscle injury and treated with topical application of diclofenac (11.6 mg g^{-1}).
4 Intramuscular diclofenac group (ID)—animals that underwent muscle injury and treated with intramuscular application of diclofenac (1 mg kg^{-1}).
5 LLLT group (LLLT)—animals that underwent muscle injury and treated with LLLT with a dose of 3 J.
6 LLLT + topical diclofenac group (LLLT + TD)—animals that underwent muscle injury and treated with LLLT with a dose of 3 J and topical application of diclofenac (11.6 mg g^{-1}).
7 LLLT + intramuscular diclofenac group (LLLT + ID)—animals that underwent muscle injury and treated with LLLT with a dose of 3 J and intramuscular application of diclofenac (1 mg kg^{-1}).

Treatments. All treatments were performed 1 h after muscle trauma.

Topical diclofenac: Animals of topical diclofenac group were treated with 5 mL of sodium diclofenac in gel solution (11.6 mg g^{-1}) been uniformly applied at right tibialis muscle belly 1 h after injury.

Intramuscular diclofenac: Animals of intramuscular diclofenac group were treated with sodium diclofenac (1 mg kg^{-1}; Voltaren injectable®, Novartis) injected in the gluteus muscle 1 h after injury.

Low-level laser therapy: A single LLLT session was performed 1 h after controlled muscle trauma with an infrared laser unit (DMC®; Sao Carlos, Brazil). The laser unit emitted a continuous optical output of 100 mW with a wavelength of 810 nm to a spot size area of 0.028 cm^2, giving a power density of 3.57 W cm^{-2}. The optical output of the laser unit was measured before, halfway through and after the experiment. Laser irradiation was performed in skin contact in the middle of anterior tibialis muscle on the belly with a dose of 3 J (107.14 J cm^{-2}), with a corresponding irradiation time of 30 s. The laser energy dose and parameters were chosen according to previous studies from our research group (17,18,21).

LLLT + TD and LLLT + ID groups were treated combining the above-mentioned treatments. It is important to highlight that in both groups, rats were irradiated 5 min before topical or intramuscular application of diclofenac.

Outcomes. Functional evaluation: Walking track index outcome—The walking track analysis was performed before induction of muscle strain and at 6 h after injury, this timepoint was chosen according to a previous study of our research group (21). Rats' paws were painted with black ink and then animals walked to the dark direction on a wooden track covered with blank squared paper. The walking index (sciatic index in the reference rabbit model) was then calculated according to a previously described mathematical formula (22).

Biochemical and molecular analysis. Analysis of gene expression by real-time reverse transcription polymerase chain reaction (RT-PCR) — First, muscles were thawed, and Trizol was immediately added (Gibco BRL, Life Technologies, Rockville, MD; 1 mL per 100 mg tissue). Then, 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 μg) was used for first-strand cDNA synthesis [reverse transcriptase (RT)] using SuperScript II. In addition, RNaseOUT 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 PCR amplification using Platinum Sybr QPCR Supermix-UDG and specific oligonucleotides. The primers used were: COX-1 (forward: CCGTGCGAGTACAGTCACAT; reverse: CCTCACCAGTCATTCCC TGT); COX-2 (forward: AGATCAGAAGCGAGGACCTG; reverse: CCATCCTGGAAAAGTCGAAG), 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. Delta-delta threshold cycle (ddCT) were calculated (the results were interpreted using the formula $2-\Delta\Delta Ct$ (Ct is the number of cycles required to reach the threshold value of fluorescence above background−Background)

relating the expression of the gene of interest compared with that of the housekeeping gene β-actin) and the results are expressed as fold increase. All oligonucleotides and reagents used in this protocol were purchased from Invitrogen Co.

Analysis of prostaglandin E_2 (PGE_2) levels in blood plasma by ELISA —For analysis of PGE_2, 3 mL of blood were 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 the analysis. The quantification of levels of PGE_2 was performed according instructions of manufacturer's using ELISA (R & D Systems, Minneapolis, MN).

Statistical analysis. Data used for functional evaluations were expressed in mean value and its standard error of the mean (SEM). The obtained data were first plotted for analysis of normal distribution, and statistical analysis was then performed with parametric tests if the data were normally distributed. After confirmation, obtained data were tested statistically by ANOVA with Student–Newman–Keuls posttest. The statistical level of significance was set at $P < 0.05$.

RESULTS

Figure 2 shows COX-1 gene expression of tibialis anterior muscle of rats in different experimental groups (control, injury, TD, ID, LLLT, LLLT + TD and LLLT + ID) at 3 and 6 h after controlled muscle strain injury. We can observe that in both experimental times, muscle strain injury significantly increased ($P < 0.05$) COX-1 gene expression in the injury group compared with the control group. On the other hand, all treatments significantly decreased COX-1 gene expression.

COX-2 gene expression was significantly increased at both experimental times in the injury group compared with the control group ($P < 0.05$). In addition, all treatments (TD, ID, LLLT, LLLT + TD and LLLT + ID) significantly decreased ($P < 0.05$) COX-2 gene expression compared with injury group. Results are summarized in Fig. 3.

Regarding PGE_2, we can observe that levels are increased in injury and TD groups compared with control group ($P < 0.05$) at 3 and 6 h. At 6 h, levels are also significantly increased in ID group compared with control group ($P < 0.05$). However, PGE_2 levels in LLLT, LLLT + TD and LLLT + ID groups are significantly decreased at both experimental times compared with injury and TD groups ($P < 0.05$), as well as at 6 h compared with ID group ($P < 0.05$). Figure 4 summarizes results regarding PGE_2 levels.

Regarding functional analysis, we observed that functional index showed a statistically significant increase in walking impairment in injury, TD and ID groups compared with control group ($P < 0.05$). However, we found that there was a statistically significant improvement ($P < 0.05$) in functional aspect in LLLT, LLLT + TD and LLLT + ID groups compared with injury, TD and ID groups. Results are summarized in Fig. 5.

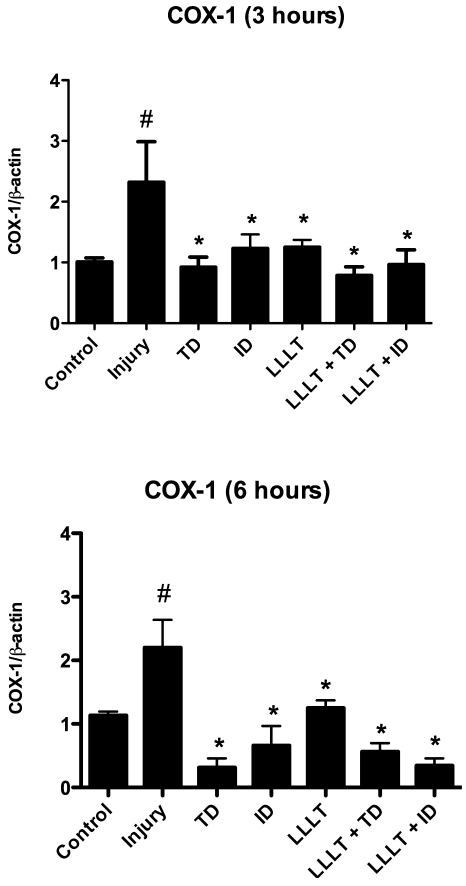

Figure 2. mRNA gene expression of COX-1. Values are represented by the mean values and error bars are SEM, $n = 6$ animals per group ($^{\#}P < 0.05$ *vs* control group; $^{*}P < 0.05$ *vs* injury group).

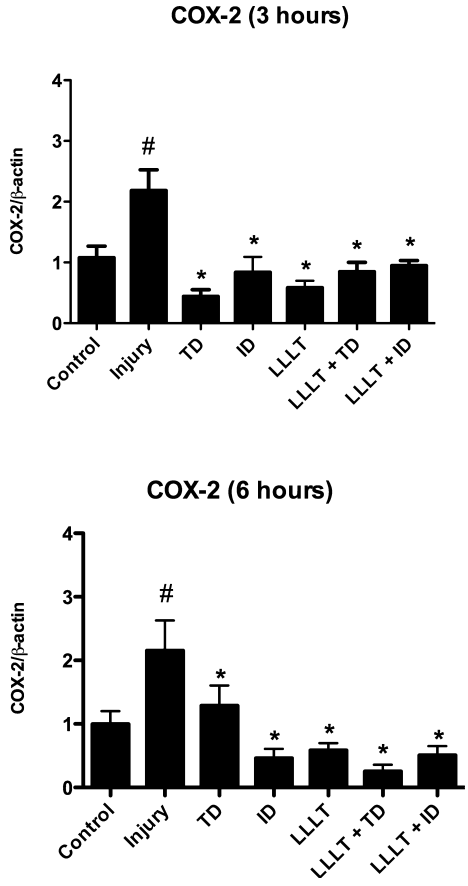

Figure 3. mRNA gene expression of COX-2. Values are represented by the mean values and error bars are SEM, $n = 6$ animals per group ($^{\#}P < 0.05$ *vs* control group; $^{*}P < 0.05$ *vs* injury group).

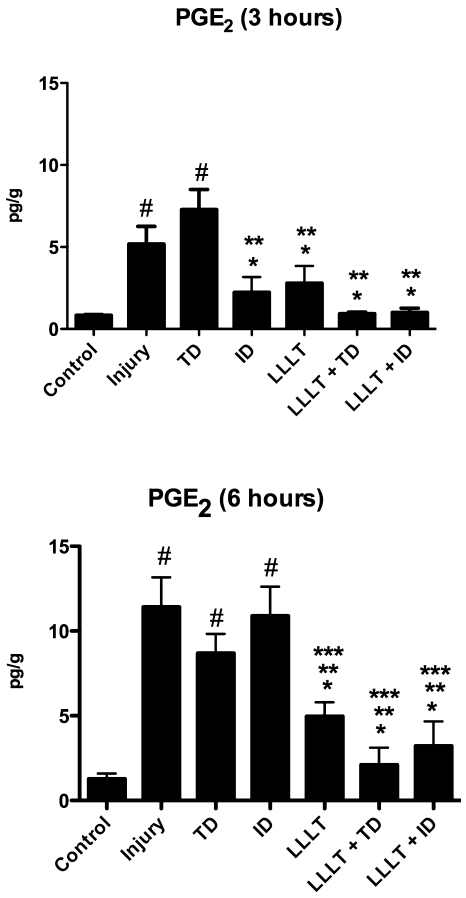

Figure 4. Levels of PGE_2 in blood plasma. Values are represented by the mean values and error bars are SEM, $n = 6$ animals per group ($^{\#}P < 0.05$ *vs* control group; $^{*}P < 0.05$ *vs* injury group; $^{**}P < 0.05$ *vs* TD group; $^{***}P < 0.05$ *vs* ID group).

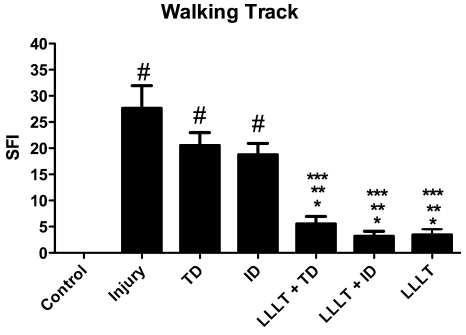

Figure 5. Walking Track analysis performed at 6 h after injury. Values are represented by the sciatic index mean values and error bars are SEM, $n = 6$ animals per group ($^{\#}P < 0.05$ *vs* control group; $^{*}P < 0.05$ *vs* injury group; $^{**}P < 0.05$ *vs* TD group; $^{***}P < 0.05$ *vs* ID group).

DISCUSSION

In the present study, we decided to employ an innovative model of muscle strain injury developed by our research group (21). This model was able to promote impairment of functional aspects and at the same time mimic a clinical condition where skeletal muscle injury occurs, excessive stretching. In the same study, we observed that LLLT with a dose of 3 J was the best dose in improvement of functional outcomes, and because of that we decided to use the same dose in the present study.

In the study of Ramos *et al.* (21), we also observed that the peak of impairment in Walking Track analysis occurs at 6 h after muscle strain injury. Due to this, we decided to perform this analysis at same timepoint.

We can observe that both topical diclofenac and intramuscular diclofenac used as a single therapy were unable to improve impairment caused by strain injury in Walking Track analysis, actually the results from both groups were very similar to the injury group (nontreated). On the other hand, LLLT as a single treatment significantly improved Walking Tracking analysis compared with injury, topical diclofenac, and intramuscular diclofenac groups. In addition, association of topical or intramuscular diclofenac with LLLT also significantly improved Walking Tracking analysis compared with injury, topical diclofenac, and intramuscular diclofenac groups. This lead us to believe that LLLT irradiation is the main responsible in improvement observed in three irradiated groups (LLLT, LLLT + TD and LLLT + ID), and do not necessarily the association of treatments.

Interestingly, our experimental model of muscle strain injury significantly increased gene expression of both COX isoforms in injury group at 3 and 6 h. All treatments tested in this study significantly decreased gene expression of COX-1 and COX-2 at both timepoints analyzed compared with injury group. Currently, the common sense is that the optimal anti-inflammatory therapy needs to act decreasing selectively COX-2 activity [for a review, see (23,24)]. As we can see in our results, all treatments do not show a significant decrease of COX-1 and COX-2 compared with the control group (uninjured). Which lead us to believe that all tested treatments are safe regarding this aspect. However, further investigation is needed about this issue.

On the other hand, regarding PGE_2 levels, we can observe that at 3 h topical diclofenac does not lead to positive effects. However, all other treatments significantly decreased PGE_2 levels compared with both injury and topical diclofenac group. At 6 h, both topical and intramuscular diclofenac do not decrease PGE_2 levels compared with the injury group. Only groups treated with LLLT as single or combined therapy showed a significant decrease in PGE_2 levels, both when compared with injury group as well as when compared with topical and intramuscular diclofenac groups. Furthermore, results of PGE_2 levels at 6 h are very similar with results of Walking Track, also performed at 6 h.

Therefore, merging outcomes observed in this study, we can observe that LLLT has better effects than topical and intramuscular diclofenac when used as a single treatment. Finally, LLLT as a single treatment has similar results of combined therapies (LLLT + TD and LLLT + ID), which lead us to believe that LLLT is the main responsible for positive results observed in this study.

CONCLUSION

We conclude that LLLT has more efficacy than topical and intramuscular diclofenac in the treatment of acute stage muscle strain injury. In addition, LLLT has the advantage to be a nonpharmacological therapy without reported side effects. Further studies are needed to confirm our results in clinical settings.

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