

Low-level laser therapy in IL-1 β , COX-2, and PGE2 modulation in partially injured Achilles tendon

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Abstract This study evaluated IL-1 β , COX-2, and PGE2 modulation in partially injured Achilles tendons treated with low-level laser therapy (LLLT). Sixty-five male Wistar rats were used. Sixty were submitted to a direct injury on Achilles tendon and then distributed into six groups: LASER 1 (a single LLLT application), LASER 3 (three LLLT applications), and LASER 7 (seven LLLT applications) and Sham 1, 3, and 7 (the same injury but LLLT applications were simulated). The five remaining animals were allocated at control group (no procedure performed). LLLT (780 nm) was applied with 70 mW of mean power and 17.5 J/cm² of fluency for 10 s, once a day. The tendons were surgically removed and assessed immunohistochemically for IL-1 β , COX-2, and PGE2. In comparisons with control (IL-1 β : 100.5 \pm 92.5 / COX-2: 180.1 \pm 97.1 / PGE2: 187.8 \pm 128.8) IL-1 β exhibited (mean \pm SD) near-normal level ($p>0.05$) at LASER 3 (142.0 \pm 162.4). COX-2 and PGE2 exhibited near-normal levels ($p>0.05$) at LASER 3 (COX-2: 176.9 \pm 75.4 /

PGE2: 297.2 \pm 259.6) and LASER 7 (COX-2: 259.2 \pm 190.4 / PGE2: 587.1 \pm 409.7). LLLT decreased Achilles tendon's inflammatory process.

Keywords Achilles tendon · Collagen · Inflammation · Low-level laser therapy · Tendinopathy

Introduction

Achilles tendon is the largest and strongest tendon of the human body. However, it is frequently injured because it is placed under high overload and functional demands during physical and sports activities [1]. Acute and subacute Achilles tendon injuries involve tissue inflammation and the control of this process is one of the main treatment objectives [2, 3].

Furthermore, the presence of specific pro-inflammatory agents such as interleukin-1 β (IL-1 β), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) are associated with pain [4], collagen fibers degradation [4, 5], inhibition of collagen type I synthesis, and tendon rigidity reduction [6], thereby impairing the repair process and making the tendon more susceptible to complete ruptures [1, 7]. Therefore, the modulation of these inflammatory factors in injured Achilles tendons has become extremely important.

Low-level laser therapy (LLLT) has shown excellent results in inflammation control [8–15] because it does not cause significant increases in tissue temperature, it modulates the inflammatory process, and achieves similar results to other non-steroidal anti-inflammatory drugs (NSAID), without undesirable side effects (irritation of the gastrointestinal tract and collagen reduction) [3, 8–10, 12–16].

Although some reports show pain control and collagen fibers reorganization at injured Achilles tendons treated with LLLT. There is still a need for further studies to investigate LLLT effectiveness on inflammatory process modulation and

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degradation control of injured tendons. Thus, the aim of this study was to assess LLLT influence on IL-1 β , COX-2, and PGE2 modulation in injured Achilles tendons.

Methods

The present study was approved by the Ethics Committee of the *Universidade Federal de São Paulo*—protocol number 0074/2011.

Sixty-five male 12-week-old Wistar rats (*Rattus norvegicus*: var. *albinus*, *Rodentia*, *Mammalia*), weighing between 270–300 g, were used. The animals were kept in cages, in a light/dark cycle of 12 h, with temperatures of approximately 20 °C and humidity of 65 %. They received water and rat chow ad libitum.

Injury procedure

Sixty animals were randomly anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (50 mg/kg). The five remaining animals were not submitted to anesthesia.

After anesthesia, manual trichotomy of the right hind paw was performed (Achilles tendon area). The animal was positioned at injury device base—a mini-guillotine dispositive designed to drop weight and promote injury by direct trauma. The right hind paw was placed on the equipment base, and mild traction was exerted through ankle dorsiflexion until the dorsal region of the paw rested against the injury device base. Finally, a weight of 186 g was released from a height of 20 cm onto the Achilles tendon of each animal, corresponding to a potential energy of 364.9 mJ at the time of trauma [1, 17].

Distribution of groups

After the Achilles tendon of the 60 animals had been partially injured, they were randomly distributed into six groups:

- Sham 1: 10 tendons submitted to simulated LLLT for 1 day.
- LASER 1: 10 tendons submitted to LLLT application for 1 day.
- Sham 3: 10 tendons submitted to simulated LLLT for 3 days.
- LASER 3: 10 tendons submitted to LLLT application for 3 days.
- Sham 7: 10 tendons submitted to simulated LLLT for 7 days.
- LASER 7: 10 tendons submitted to LLLT application for 7 days.

The other five animals (10 tendons) were placed at control group and were not submitted to any procedures.

LLLT treatment

The AsGaAl LLLT equipment was used with a wavelength (λ) of 780 nm (near infrared), a beam area of 0.04 cm², and a mean power of 70 mW.

Laser was applied manually once a day at central portion of injured Achilles tendons, with the animal gently caged and immobilized during treatment and/or simulation period. Treatment started 1 min after induced injury and was applied $\Delta E = 17.5$ J/cm² for 10 s using an irradiance dose of 0.7 J. The contact technique and continuous emission were used in all irradiations. The animals were treated for one day (LASER 1), three consecutive days (LASER 3), and seven consecutive days (LASER 7) [1, 7, 17].

The animals in sham groups (1, 3, and 7) only received simulated LLLT application, with the device turned off. However, contact was maintained between the equipment and the injured area for the same time as the effective treatment.

Euthanasia

The animals in LASER 1/Sham 1, LASER 3/Sham 3, and LASER 7/Sham 7 groups were euthanized (anesthetic overdose) 24 h, 4 days and 8 days after the induced injury, respectively. The animals in control group were euthanized on the last day of the experiment (8th day).

Samples processing

After euthanasia, tendons of the two hind paws were surgically removed from animals in control group, as well as the 60 tendons (right hind paw) of animals belonging to the other groups.

The tendons were immediately washed in saline solution (0.9 %) and then fixed for 12 h in paraformaldehyde 4 % in Millionig 0.1 M buffer (pH 7.2–7.4).

After fixation, the tendons were washed in water and dehydrated in increasing solutions of ethyl alcohol (from 70 to 100 %) for 45 min in each one. Next, they were diaphanized in xylene (two baths of 45 min each) and included in paraffin, positioned in such a way as to obtain longitudinal slices. The slices were performed in microtome with a thickness of 5 μ m for immunohistochemical technique (IL-1 β , COX-2, and PGE2). The slides were silanized for slices adhesion.

Each slide was prepared with two slices of each tendon for staining and quantification (immunohistochemistry) of IL-1 β , COX-2, and PGE2. This process was repeated three times. Therefore, three nonconsecutive slides were obtained for each analysis from each animal studied.

All of the slices were dewaxed, hydrated, and submitted to the method listed below.

Immunohistochemistry analysis (inflammatory process)

Specific antibodies kits for IL-1 β (IL-1 β polyclonal; dilution: 1/300; source/brand: Bioss bs-0812R), COX-2 (COX-2 polyclonal; dilution 1/300; source/brand: Spring Bioscience E3030) and PGE2 (PGE2 polyclonal; dilution 1/200; source/brand: Bioss bs-2639R) were used in this study.

The dewaxed slices were washed in distilled water and Tris-phosphate buffer pH 7.4 (TBS). Antigen retrieval was performed in humid heat (95 °C) with citric acid solution (10 mM—pH 6.0) for 45 min. The blocking of endogenous peroxidase was conducted with oxygenated water 10v (3 %), and the slices were then washed in distilled water and TBS.

The slides were incubated with the primary antibodies diluted in specific diluent for 24 h at 4 °C and then incubated with the secondary antibody Histofine for 45 min at 37 °C. After this stage, the slides were washed in water.

The slices were revealed in diaminobenzidine (DAB) for 10 min at room temperature. Finally, the slices were counterstained with Harris hematoxylin and prepared with Entellan.

The effect of the Achilles tendon inflammatory modulation (IL1- β , COX-2, and PGE2) was analyzed at myotendinous junction by identifying and quantifying the types of positive cells in each reaction. Ten images were randomly captured at $\times 200$ (objective $\times 20$) using a CoolSNAP-Pro ef camera coupled with a Nikon Eclipse-E800 microscope. After the images obtainment, the cells that exhibited positive markings for each type of protein were identified and quantified through the computerized system of Image-ProPlus, version 4.5. The different types of cells (neutrophils, fibroblasts, lymphocytes, and macrophages) were then individually summed by slide.

Statistical analysis

The Statistical Package for Social Sciences (SPSS version 15.0) was used, with the level of significance set at 5 % ($p \leq 0.05$). The Shapiro-Wilk and Levene tests were used to assess the distribution and homogeneity of data, respectively. The parametric data were reported in mean and standard deviation values and tested by analysis of variance (ANOVA) of one factor and post-hoc tests were performed when there was no homogeneity of the variances (Tukey's or Games-Howell).

Results

Table 1 presents the total positive inflammatory cells for IL-1 β , COX-2, and PGE2. The experimental groups (LASER 1 and Sham 1) exhibited higher levels of inflammatory agents studied than the control group ($p < 0.05$), showing injury and the inflammatory process presences in these Achilles tendons.

The intergroup analysis (Sham 1, 3, and 7 versus LASER 1, 3, and 7) in relation to number of applications (1, 3, and 7) did not reveal significant differences ($p > 0.05$).

No significant differences ($p > 0.05$) were found in comparisons for IL-1 β , COX-2, and PGE2 levels between groups LASER 3 and control, and also in groups LASER 7 and control for COX-2 and PGE2. LASER 3 and 7 presented inflammatory levels close to normal (Fig. 1). The LLLT was able to modulate and reduce IL-1 β presence with three applications (LASER 3), as well as COX-2 and PGE2 with three (LASER 3) and seven applications (LASER 7).

Discussion

The aim of this controlled laboratory study was to assess the LLLT effect on rats' Achilles tendons inflammatory process modulation. The main findings of the present study were reductions in IL-1 β , COX-2, and PGE2 to near-normal levels after three and seven applications of LLLT.

Injured Achilles tendons exhibit a slow repair process, and this characteristic is directly associated with the presence of IL-1 β , COX-2, and PGE2 [4, 7, 18, 21]. LLLT has been used in these clinical situations to modulate inflammatory process and consequently, potentiate and advance tissue repair [17, 19, 20].

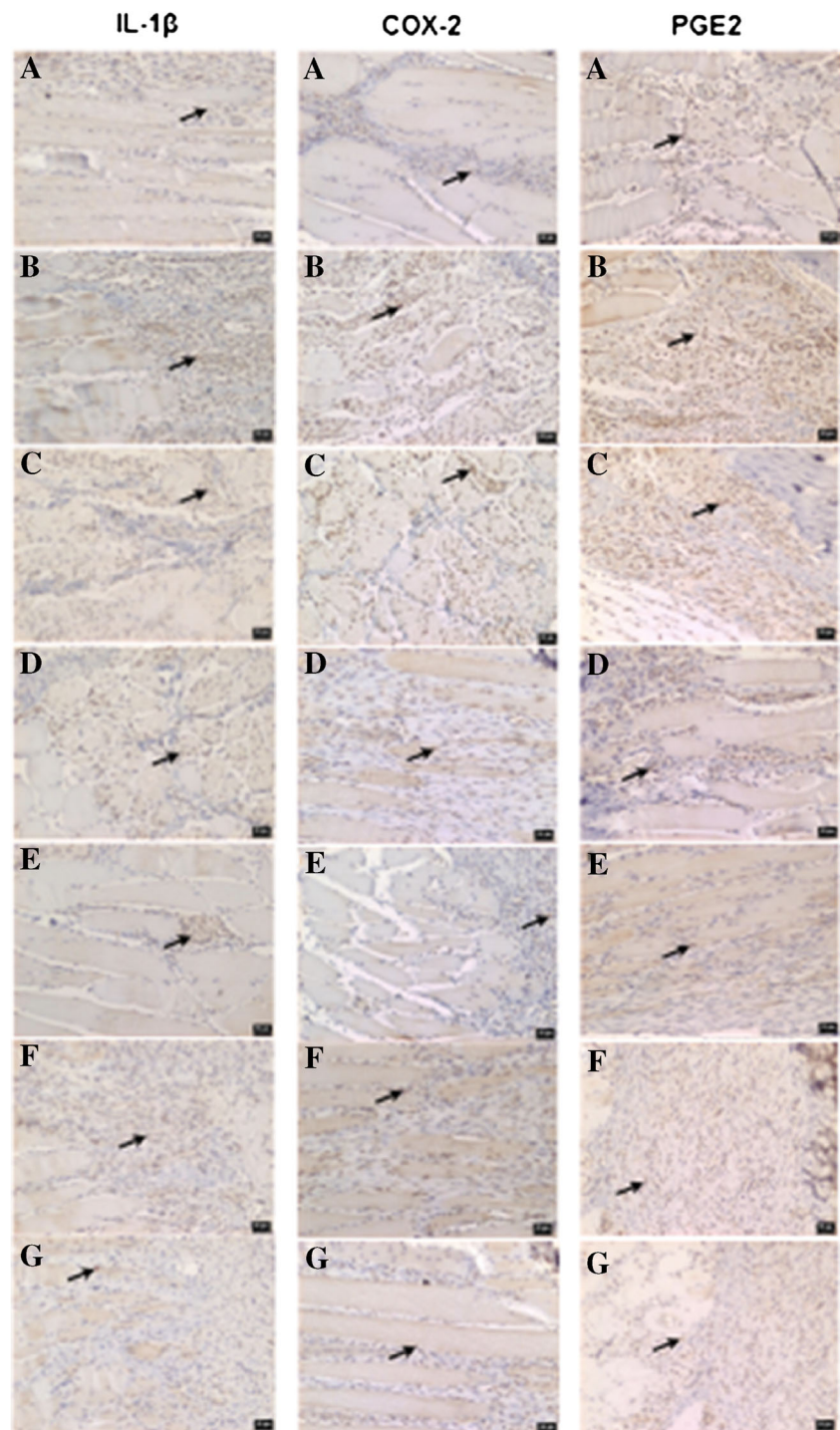
There was a decrease in all inflammatory agents studied at groups Sham 3 and LASER 3 in comparison with groups Sham 1 and LASER 1; probably, the inflammatory process triggered by traumatic injury has been partially modulated by the animal's own metabolism (Sham 3) and also by the LLLT interaction on the injured tendons (LASER 3) [14, 22–28].

In addition, it was noted an increase in inflammatory levels at groups Sham 7 and LASER 7 in comparison with Sham 3 and LASER 3. The tendon repair process is a complex phenomenon depending upon modulatory and proliferative responses; perhaps, the inflammatory modulation previously occurred has reduced the pain (PGE2 reduction) and

Table 1 Total positive inflammatory cells (mean \pm SD) for IL-1 β , COX-2, and PGE2

	Control	Sham 1	LASER 1	Sham 3	LASER 3	Sham 7	LASER 7
IL-1 β	100.5 \pm 92.5	493.1 \pm 288.0	487.8 \pm 223.7	285.9 \pm 201.9	142.0 \pm 162.4	393.6 \pm 320.8	264.1 \pm 330.0
COX-2	180.1 \pm 97.1	859.6 \pm 481.0	800.1 \pm 388.2	445.2 \pm 379.2	176.9 \pm 75.4	500.1 \pm 314.3	259.2 \pm 190.4
PGE2	187.8 \pm 128.8	901.3 \pm 388.3	689.4 \pm 363.8	684.5 \pm 423.2	297.2 \pm 259.6	644.5 \pm 403.8	587.1 \pm 409.7

Fig. 1 Photomicrography of the positive cells (✓) for IL-1 β , COX-2, and PGE2 (immunohistochemistry) at $\times 10$: **a** Control. **b** Sham 1. **c** LASER 1. **d** Sham 3. **e** LASER 3. **f** Sham 7. **g** LASER 7



stimulated early load on the tendon still in repairing process (in general, proliferation and collagen alignment are obtained with 5-day LLLT) [1, 17, 19].

This phenomenon is described as the iceberg theory, where a false sense of tendinopathy resolution occurs only because there is no pain; however, the entire degenerative process is still present—and this early overload could have triggered again an increase in inflammatory levels [29].

The LLLT anti-inflammatory mechanisms have not been completely clarified. However, in this case, LLLT probable modulated the pro-inflammatory agents by reducing the IL-1 β and COX-2 mRNA expression and consequently reduced PGE2 levels, and also by reducing cell migration and the quantity of neutrophils, mast cells, and macrophages in the injured tissue [22, 23]. Macrophages and mast cells secrete IL-1 β , which in turn recruit

COX-2, enzyme that convert arachidonic acid in PGE2 [24, 25].

Furthermore, near infrared LLLT (780 nm) can modulate inflammatory processes [14, 26], including in Achilles tendons injuries [27]. Perhaps, the early modulation of pro-inflammatory mediators can prevent excess tissue degradation and promote pain control. This modulation can also stimulate the tendon repair, passing ahead the inflammatory phase to the proliferative phase of this process [28].

There are several reports highlighting some other factors that can also influence the LLLT inflammatory modulation. Laraia et al. [30] and Pires et al. [27] presented IL-1 β and COX-2 modulations. However, these modulations were reached quicker (a single application of LLLT) than in this study (three and seven applications). This difference may be associated with the injury methods used. The abovementioned authors used more aggressive methods, which could significantly elevate the inflammatory levels and facilitate the detection of LLLT modulation.

The elevated dose and wavelength (near infrared) of LLLT can also directly affect inflammatory modulation. There are some studies showing a reduction of IL-1 β , COX-2, and PGE2 associated with these factors, since they present high rates of penetration and energy absorption through the tissue, advancing and potentiating the Achilles tendon repair process [3, 27, 30, 31].

The frequency of LLLT treatment can also promote the inflammation control. Shimizu et al. [32] reported a reduction of PGE2 levels with three and five LLLT applications (once a day). These results are similar to this study. Thus, the wavelength, dose, frequency of treatment, and injury method can all affect the injured Achilles tendons inflammatory modulation [27, 31].

Although the present study demonstrates a satisfactory interaction between LLLT and the injured Achilles tendon, modulating the inflammatory agents studied, there is still a need for further studies about this interaction, using different injury methods and exploring more inflammatory agents, such as matrix metalloproteinases (MMP). There is also a need to investigate other LLLT dosimetry parameters used in treatment.

Conclusion

Low-level laser therapy decreased the injured Achilles tendons inflammatory levels and is an adequate resource to avoid excessive degradation and stimulate repair process.

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