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Effect of Low-Level Laser Therapy (660 nm) on Acute Inflammation Induced by Tenotomy of Achilles Tendon in Rats

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ABSTRACT

In this study, we aimed to analyze the effects of low-level laser therapy (LLLT; 660 nm) on levels of protein expression of inflammatory mediators after cutting Achilles tendon of rats. Thirty Wistar male rats underwent partial incisions of the left Achilles tendon, and were divided into three groups of 10 animals according to the time of euthanasia after injury: 6, 24 and 72 h. Each group was then divided into control group and LLLT group (treated with 100 mW, 3.57 W cm^{-2}, 0.028 cm^2, 214 J cm^{-2}, 6 J, 60 s, single point). In LLLT group, animals were treated once time per day until the time of euthanasia established for each group. The group treated with LLLT showed a significant reduction of IL-1β compared with control groups at three time points (6 h: $P = 0.0401$; 24 h: $P = 0.0015$; 72 h: $P = 0.0463$). The analysis of IL-6 showed significant reduction only in the LLLT group at 72 h compared with control group ($P = 0.0179$), whereas IL-10 showed a significant increase in the treated group compared with control group at three experimental times (6 h: $P = 0.0007$; 24 h: $P = 0.0256$; 72 h: $P < 0.0001$). We conclude that LLLT is an important modulator of inflammatory cytokines release after injury in Achilles tendon.

INTRODUCTION

The sudden rupture of a tendon is a relatively common occurrence in sports, affecting both professional and amateur athletes alike (1). This assertion is also true when it comes to the Achilles tendon, which is the strongest, thickest tendon in the human body and results from the fusion of the tendons of the gastrocnemius and soleus muscles.

The incidence of acute tendon rupture is highest in men in the third and fourth decades of life. The main injury mechanisms are sudden, forced plantar flexion and dorsiflexion or unexpected sudden dorsiflexion starting from plantar flexion. There are a number of theories on the etiology to torn tendons, such as the use of topical corticosteroids, constant use of antibiotics, hyperthermia induced by physical exercise and biomechanical changes in the ankle (2). The rupture of the Achilles tendon usually occurs 3–6 cm above its insertion into the calcaneus. This may be explained by repetitive strain and poor blood supply of this region of tendon. Tendon healing usually requires long-term rehabilitation, and the use of immobilization cast may predispose the tendon to several complications (3).

The total healing of a calcaneal tendon can take weeks or even months, making adherence to the treatment regimen difficult. Because of the high incidence of these injuries, there is a need for studies focusing in the improvement of tendon repair, reducing recovery time and time required to return to normal activities (4). According to Khan *et al.* (2), the treatment of tendon ruptures may be surgical (in the case of young, lean athletes) or conservative (for the elderly and individuals unable to undergo a surgical procedure).

The healing of soft tissue consists of an integrated process involving cell activity and vascular responses. The regeneration of tendons involves an inflammatory phase, proliferative or reparative phase and a final phase of matrix remodeling. The maturation of the scar tissue is preceded by the chemotaxis of neutrophils and macrophages, angiogenesis and collagen deposition (1,5,6).

The role of inflammation process after injury is to remove the offending agent and help restore the damaged tissue to return to homeostasis. Wojciak and Crossan (7) reported the presence of inflammatory infiltrate with lymphocytes and macrophages in the synovial sheath and epitenon of the tendon during healing. The increased number of inflammatory cells is due to the interaction between the inflamed tissue and circulating leukocytes in blood. Following the inflammatory stimulus, the endothelium initiates signaling for the expression of adhesion molecules, which facilitate the migration of inflammatory cells to the inflamed tissue. Released by resident cells, inflammatory mediators, such as prostaglandins (PGE_2), thromboxane (TXA2), leukotrienes (LTD4), nitric oxide and tumor necrosis factor-alpha and interleukins (IL-1β and IL-6), modify vascular tonus through vasodilation, thereby contributing to increased vascular permeability and, consequently, increasing the number of inflammatory cells (monocytes and

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neutrophils) at the site of injury (8). According to Schulze-Tanzil *et al*. (9), a tendon rupture induces a local inflammatory response characterized by the induction of pro-inflammatory cytokines. Cytokines are regulatory proteins that bind to specific high-affinity receptors on the cell surface and may influence either an increase or reduction in the production of other cytokines.

Cytokines are small proteins with the properties of locally acting hormones. These substances are essential to the healing of connective tissues following injury. The function of cytokines is to regulate the immune and inflammatory responses, counteracting the effect of pro-inflammatory and anti-inflammatory mediators (10,11). According to Pires *et al*. (12), inflammatory symptoms in tendons are caused by pro-inflammatory cytokines, such as tumor necrosis factor-alpha, IL-1 β and IL-6.

According to Casalechi *et al*. (10), researchers are still looking for the best method for accelerating the tissue repair process and restoring function. For a specific therapy to be considered effective, it should reduce the acute inflammatory phase and accelerate tissue regeneration. Recent studies report that the application of ultrasound and low-level laser can improve the quality of the scar tissue and offers beneficial effects regarding tendon healing. In addition, low-intensity pulsed ultrasound promoted restoration of mechanical strength and collagen alignment in healing tendons only when applied at early healing stages (3,13).

According to Fillipin *et al*. (14), The laser reduces histological abnormalities, collagen concentration and oxidative stress in an experimental model of Achilles tendon injury. Reduction of fibrosis could be mediated by the beneficial effects on the oxidant/antioxidant balance.

Photobiostimulation through low-level laser therapy (LLLT) is widely used in the fields of medicine and dentistry. Attention has been focused on the effects of this therapy on a number of pathological conditions, such as injuries, musculoskeletal complications and pain. Clinical studies have demonstrated the efficacy of laser irradiation regarding the acceleration of the tissue healing process, but further investigation is need to clarify the mechanisms behind this acceleration.

According to Joensen *et al*. (15), in most studies addressing this issue, tendons were treated with LLLT daily for three to 21 days. Some studies have investigated the effect of LLLT in the first 24 h after acute inflammation or the treatment of inflammation with three or four sessions of LLLT. In these studies, tissues treated with LLLT exhibited reduced concentrations of inflammatory markers and cells in comparison to untreated controls.

The aim of the present study was to investigate the effects of low-level laser on the acute inflammatory phase following Achilles tendon injury with regard to the expression of pro-inflammatory and anti-inflammatory proteins.

MATERIALS AND METHODS

Experimental animals. Thirty male Wistar rats (*Rattus norvegicus*) aged *ca* 90 days and weighing 250–300 g were maintained under controlled light and temperature, with access to water and chow (Nuvilab CR1; Nuvital Nutrients, Colombo, Brazil) *ad libitum*. All experimental procedures received approval from the Ethics Committee of the Anhanguera Educational Center (Brazil) under process number 2-046/10 and were carried out in compliance with the standards of the Brazilian College of Animal Experimentation and the International Council for Laboratory Animal Science.

Experimental groups. The sample size calculus was based on studies by Joensen *et al*. (15), with the application of ANOVA, a test power of 80% and alpha error of 0.05, resulting in a minimum sample of 30 animals. The animals were randomly distributed into six groups (G1, G2, G3, G4, G5 and G6), each containing five animals (Table 1).

Production of tendon injuries. The animals were anesthetized with an intraperitoneal injection of ketamine (10%) and xylazine (2%) combined in one syringe (1 mL of ketamine [100 mg] and 1 mL of xylazine [20 mg]), administered as 0.1 mL of solution per 100 g of body weight. After removing the hair from the left leg on the back of the tibiotarsal joint, asepsis was performed caudally to the common access to the calcaneal (Achilles) tendon. Partial tenotomy was made in region between the osteotendinous and myotendinous calcaneal junction, using a scalpel blade. The tissues were then united with the Bunnell suture technique, using 4–0 polyamine nonabsorbable thread.

Irradiation with indium-gallium-aluminum-phosphide laser. An indium-gallium-aluminum-phosphide laser was used (Photon Lase III; DMC Equipment, Sao Carlos, Brazil), with power output of 100 mW (power density of 3.57 W cm^{-2}), beam area of 0.028 cm^2 (according manufacturer specification) and wavelength (λ) of 660 nm. Application was performed in a single point, with direct contact of probe on skin at posterior region of the calcaneal tendon, with energy density of 214 J cm^{-2}, 6 J of energy and 60 s exposure time. Before and after experiments, the laser device was checked with a power checker (13PEM001/J, Melles Griot, The Netherlands). The application was initiated immediately after surgery and extended in accordance with the experimental group (*i.e*. 6, 24 and 72 h).

Treatment protocols. Treatment in the three groups undergoing LLLT (G2, G4 and G6) began immediately after tendon injury. Three periods of euthanasia were established: 6, 24 and 72 h after injury (Table 2). The animals were treated daily until the time of euthanasia established for each group.

Euthanasia and histopathological analysis. For the histological analysis, the animals were euthanized in a CO_2 chamber. For such, the gas cylinder valve was opened for 30 s. The animals in a given group ($n = 5$) were then placed inside, avoiding excessive gas exhaust. When animals exhibited drowsiness, the cylinder valve was opened again for *ca* 60 s. After 3–5 min, the animals had dilated pupils and fixed eyes and had stopped moving and breathing. The removal of the calcaneal tendon was performed with dissection from calcaneal insertion into the myotendinous junction. The tendons were placed separately in duly identified containers containing 4% formaldehyde for fixation and sent for histopathological analysis. After fixation, the tendons were dehydrated, cleared and embedded in paraffin and the blocks were cut on a microtome in semiserial sections measuring 5 μm in thickness. Five sections per sample were stained with hematoxylin and eosin.

Table 1. Composition of experimental groups.

Group	No. animals	Euthanasia time (h)	Procedure
G1	5	6	Untreated
G2	5	6	Treated with laser
G3	5	24	Untreated
G4	5	24	Treated with laser
G5	5	72	Untreated
G6	5	72	Treated with laser

Table 2. Application protocol in groups treated with laser irradiation.

Group	First application	No. applications	Euthanasia (h)
G2	Immediately after induction	1	6
G4	Immediately after induction	2	24
G6	Immediately after induction	4	72

Evaluation of inflammatory mediators (IL-1β, IL-6 and IL-10). The levels of IL-1β, IL-6 and IL-10 in the tendon samples were determined by enzyme-linked immunosorbent assays (ELISA), using a commercial kit and following the manufacturer's instructions (R & D System). For this purpose, 96-well plates were coated with 100 μL of monoclonal antibody for each cytokine (anti-IL1β and IL-6) diluted in sodium carbonate buffer (0.1 M, pH 9.6), whereas anti IL-10 was diluted in sodium phosphate buffer (0.2 M, pH 6.5). The plates were incubated (4°C) for 18 h. For blocking, the plates were washed with PBST (PBS containing 0.05% Tween 20) four times and then filled with 300 L per well of blocking solution (3% gelatin in PBST; Sigma) at 37°C for 3 h and subjected to a new cycle of washes. Next, 100 μL of properly diluted samples or standards of recombinant cytokines were added to the plate and left for 18 h at 4°C. After washing, 100 μL of the respective biotinylated antibodies for the specific detection of each cytokine were added and left for 1 h at room temperature. After washing the plates, 100 μL of streptavidin-peroxidase were added and left for 1 h at room temperature (22°C), followed by further washing. The reaction was revealed by adding 100 μL per well solution of 3,3′, 5,5′-tetramethylbenzidine (TMB) and stopped by adding 50 μL per well of sulfuric acid (2 M). Readings were performed in a Spectrum Max Plus 384 spectrophotometer (Sunnyvale, CA) at a wavelength of 450 nm, with correction at 570 nm. Sample concentrations were calculated from standard curves obtained from recombinant cytokines. The limit of detection was 1.95 pg mL^{-1} for IL-1β and IL-10 and 3.13–300 pg mL^{-1} for IL-6.

Statistical analysis. The data were tabulated using the MICROSOFT EXCEL 2007 software and initially assessed for normality using the Shapiro–Wilk test. As normal distribution was determined, the Student's *t*-test was used for comparisons between the control and LLLT groups and ANOVA with Tukey's *post hoc* test was used for comparisons between periods. All data are expressed as mean and standard deviation values. The Prism 5 (GraphPad, La Jolla, CA, USA) software program was used, with the null hypothesis considered $P < 0.05$.

RESULTS

Table 1 displays the composition of the experimental groups. Groups 1, 3 and 5 were the untreated controls sacrificed at 6, 24 and 72 h, respectively. Groups 2, 4 and 6 were treated with laser irradiation and sacrificed at 6, 24 and 72 h, respectively. Table 2 shows the application protocol in groups treated with laser irradiation. Laser treated was initiated immediately following the induction of injury in all three groups. G2 received a single application, G4 received two applications and G6 received four applications prior to euthanasia.

A statistically significant reduction in IL-1β was found in the laser group in comparison to the control group at all three evaluation times ($P < 0.05$; Fig. 1). A statistically significant reduction in IL-6 was found in the laser group in comparison to the control group only at 72 h following tendon injury ($P < 0.05$; Fig. 2). A statistically significant increase in IL-10 was found in the laser group in comparison to the control group at all three evaluation times ($P < 0.05$; Fig. 3).

Histological analysis

In histological analysis, we can observe the myotendinous junction with intense presence of inflammatory cells, hemorrhagic areas and neovascularization. The analysis was performed at 72 h after induction of injury.

DISCUSSION

In the present study, the expression of pro-inflammatory and anti-inflammatory mediators (IL-1β, IL-6 and IL-10) was measured to determine the effect of LLLT on the inflammatory phase following Achilles tendon injury. IL-1β causes the degradation of the extracellular matrix, the suppression of Type-I collagen, which leads to a reduction in tendon stiffness, and the induction of elastin, which leads to increased elasticity of the tissue (16). Other reported effects of the presence of

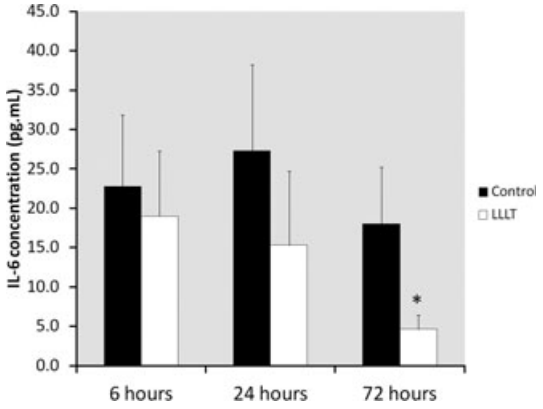

Figure 2. Comparison of mean and standard deviation values for IL-6 concentration determined by ELISA at 6, 24 and 72 h after induction of tendon injury; *$P < 0.05$, compared with control group in same period (Student's *t*-test).

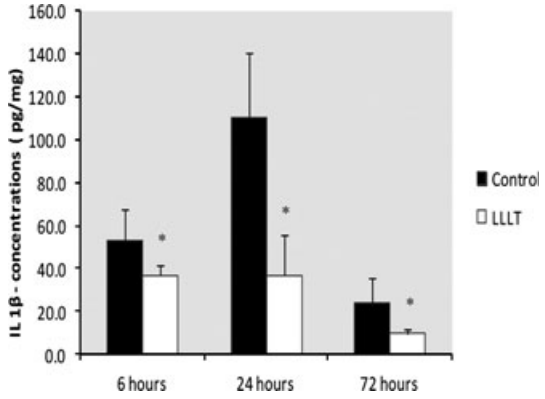

Figure 1. Comparison of mean and standard deviation values for IL-1β concentration determined by ELISA at 6, 24 and 72 h after induction of tendon injury; *$P < 0.05$, compared with control group in same period (Student's *t*-test).

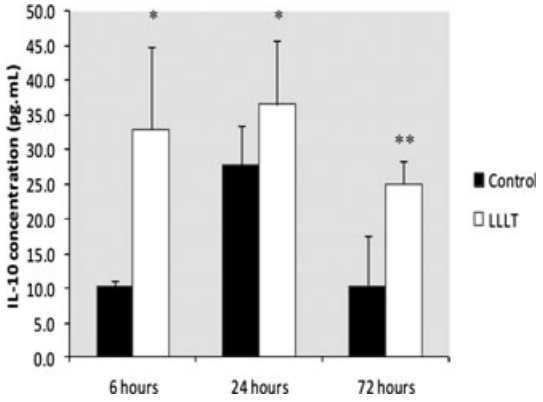

Figure 3. Comparison of mean and standard deviation values for IL-10 concentration determined by ELISA at 6,24 and 72 h after induction of tendon injury; *$P < 0.05$, **$P < 0.001$ compared with control group in same period (Student's *t*-test).

interleukins in tendon tissue include the induction of inflammatory and catabolic mediators, such as COX-2, PGE2 and matrix metalloproteinases (MMPs), which accelerate the degradation of the extracellular matrix in the tendon and cause the loss of biomechanical strength and durability in this tissue (17–19).

According to Moriyama *et al*. (20), studies suggest that LLLT is beneficial to the inflammation process, wound healing and pain relief, but the molecular basis of this effect remains unclear. Recently, Marcos *et al*. (21) induced tendinitis using collagenase in Achilles tendons of rats, they found a reduction in the gene expression of COX-2 and PGE_2 following LLLT with wavelength of 810 nm, 100 mW power output, and power density of 3.57 W cm^{-2}. Their results suggest that LLLT is an important resource for improvement of tendon healing, modulating inflammatory mediators and preventing degeneration of tendon tissue.

In the present study, a significant increase in IL-1β expression was found in the control group at 24 h following injury of the Achilles tendon in comparison to the other control groups (6 and 72 h). In the laser group, however, the levels of IL-1β at 24 h were similar to those found at 6 h, demonstrating that LLLT inhibited the increase in this interleukin found in the corresponding evaluation period in the control group (Fig. 4).

The results of the present study demonstrate the ability of LLLT to reduce the number of inflammatory cells at the site of tendon injury. The anti-inflammatory effect of LLLT therapy may be related to the modulation of the inflammatory response in some steps of cell migration, suggesting that the reduction in the migration of inflammatory cells to the inflamed tissue leads to a reduction in the release of cytokines and other eicosanoids.

According to Andersen *et al*. (22) and Ghazizadeh *et al*. (23), IL-6 is involved in tissue remodeling and the early stages of the inflammatory response. This pro-inflammatory cytokine is mainly produced by T lymphocytes and aids in the activation of T, B cells, macrophages, neutrophils and eosinophils. Together, these aspects of the inflammatory response and cell antibodies provide immediate nonspecific (innate) and subsequently specific (adaptive) defense against infections and other attacks. According to Liechty *et al*. (24), the presence of pro-inflammatory cytokines, such as IL-6, leads to an increase in the number of fibroblasts and changes in the extracellular matrix of the tissue. Such changes may be related to the development of tendinitis (25). According to Schulze-Tanzil *et al*. (9), beyond the inflammatory phase, IL-6 may play a role in the proliferation stage *via* STAT3. In acute and chronic phases of collagenase-induced tendinitis, LLLT (780 nm) at a dose of 7.7 J cm^{-2} was able to reduce IL-6 and was considered an effective therapeutic alternative in the treatment of tendinopathy by Pires *et al*. (12).

Anti-inflammatory cytokines (IL-4, IL-10, IL-13 and tumor growth factor-β) reduce the inflammatory response by decreasing pro-inflammatory cytokines and suppressing the activation of monocytes. According to Reitamo *et al*. (26), IL-10 is produced by immune cells, such as macrophages, lymphocytes and dendritic cells, and may play a regulatory role regarding fibroblasts and chondrocytes. IL-10 accelerates the healing process through a reduction in inflammation and the maintenance of the mechanical and histological properties of the tissue as well as the inhibition of the expression of IL-6, IL-8 and IL-12 (27). Indeed, it appears that the best defined role of IL-10 is the inhibition of pro-inflammatory cytokines. However, Tanzil-Schulze *et al*. (9) state that it is still necessary to clarify how this cytokine contributes to the tissue repair process in tendons. Moreover, the hypothesis that IL-10 influences the remodeling of extracellular matrix is supported by the increase in elastin fibers and reduction in Type-I collagen in the presence of this cytokine (28).

Pires *et al*. (12) report that LLLT (810 nm) at a dose of 3 J produced a significant increase in the protein expression of IL-10 in an experimental model of collagenase-induced calcaneal tendinitis, thereby accelerating the inflammatory process, including the fact that this cytokine regulates the activity of certain MMPs. In the present study, a statistically significant increase in IL-10 occurred in the three laser-treated groups in comparison to the controls, with the most expressive difference found at 72 h following injury.

In this study, we observed that even when using a power density above that WALT guidelines recommends (29), LLLT was able to produce important effects on anti-inflammatory and pro-inflammatory cytokines release. Previously, Neves *et al*. (4) also observed positive results in accelerating tendon repair employing a laser device with power density of 3.5 W cm^{-2}. The authors recommend that further studies are necessary to clarify the validity of the 100 mW cm^{-2} limit stated in the WALT guidelines.

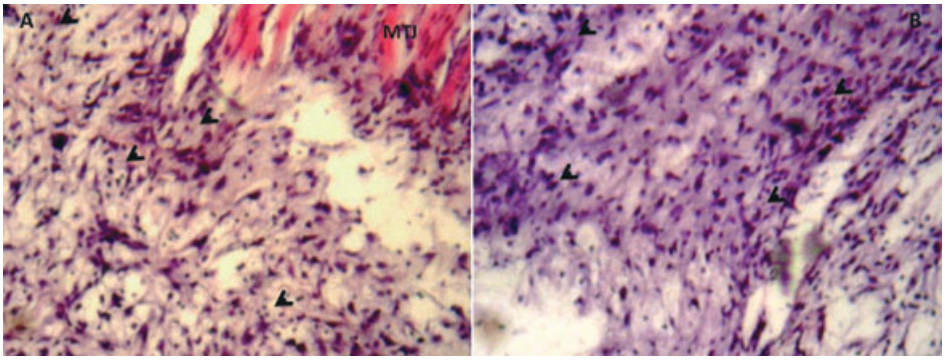

Figure 4. Photomicrograph 72 h after injury tendon. (A) Control group observed in inflammatory infiltrate in the junction region and myotendinous, intense mononuclear cell proliferation (arrowhead), (B) Low-level laser therapy group treated with 72 after induction of the injury, inflammatory cell proliferation observed (head arrow) and less-inflammatory infiltrate.

CONCLUSION

The results of the present study demonstrate that LLLT had a significant modulating effect on inflammatory mediators during the healing of incisions of the calcaneal tendon in rats, causing a decrease in the pro-inflammatory cytokines IL-1β and IL-6 as well as an increase in the anti-inflammatory cytokine IL-10.

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