Anti-Inflammatory Effects of Low-Level Light Emitting Diode Therapy on Achilles Tendinitis in Rats

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Background and Objectives: The present study investigated the effects of low-level light emitting diode (LED) therapy (880 ± 10 nm) on inflammatory process in a experimental model of Achilles tendinitis induced by collagenase.

Study Design/Materials and Methods: Fifty-six male Wistar were separated into seven groups ($n = 8$), three groups in the experimental period of 7 days and four groups in the experimental period of 14 days, the control group (CONT), tendinitis group (TEND), LED therapy group (LEDT) for both experimental periods, and LED therapy group 7th to 14th day (LEDT delay) for 14 days experimental period. The LED parameters was 22 mW CW of optical output power, distributed in an irradiation area of 0.5 cm^2, with an irradiation time of 170 seconds, the applied energy density was 7.5 J/cm^2 in contact. The therapy was initiated 12 hours after the tendinitis induction, with a 48-hour interval between the irradiations. The histological analysis and inflammatory mediators were quantified.

Results: Our results showed that LED decreases the inflammatory cells influx and mRNA expression to IL-1β, IL-6, tumor necrosis factor-α (TNF-α) in both phase, and cyclooxygenase-2 (COX-2) just in initial phase ($P < 0.05$).

Conclusion: Our results suggest that the anti-inflammatory therapy with low-power LED (880 nm) enhanced the tissue response in all groups. We can conclude that the LED was able to reduce signs of inflammation in collagenase-induced tendinitis in rats by reducing the number of inflammatory cells and decrease mRNA expression of cytokines. Lasers Surg. Med. 42:553–558, 2010. © 2010 Wiley-Liss, Inc.

Key words: calcaneous tendon; histological analysis; mRNA; RT-PCR; inflammatory mediators

INTRODUCTION

Calcaneous tendinitis (CT) is a common cause of disability and is clinically characterized by pain and swelling in and around the tendon, mainly arising from overuse and repetitive stretching triggered the release of pro-inflammatory mediators [1]. Over the past few years, various new therapeutic options have been proposed for the management of CT but the prescription of anti-inflammatory drugs (e.g., non-steroidal anti-inflammatory and corticosteroids) remains the therapy of choice. However, since pro-inflammatory mediators affect various cellular activities related to tendon healing, it is possible that anti-inflammatory agents might negatively affect tendon healing [2]. In addition, the CT is associated with disruption of collagen fibers, increase in non-collagenous matrix, haphazard proliferation of tenocytes, and subsequent decrease on biomechanical properties of tendon [3].

Thus, it is then reasonable that any modality that can enhance cell proliferation and not adversely affect inflammatory response may aid in the repair or recovery of the tendon. Most studies suggest that coherent light

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can start the modulation of physiological processes, by increment in the production of adenosine triphosphate (ATP), stimulates the microcirculation, increase collagen fiber deposition, promote higher fibroblast cell proliferation in the site of the lesion, and reduce the number of inflammatory cells [4–8].

Recently, several debates and studies have been generated about differences from and similarities to lasers [9–11]. Another potential resource of light therapy is the light emitting diode (LED), which has a longer life, is more economically accessible and can be efficient as laser light [12]. Casalechi et al. [13] demonstrated the effectiveness of the LED in the process of regeneration of the Achilles tendon, reducing the number of fibroblasts and improved quality of remodeling. Another study compared the effects of laser and LED in the tissue repair of diabetic rats and found similar effects, and at times the LED was more efficient than the laser in the reducing wound's diameters [10].

The number of researches with LEDs is still few, it is impossible to affirm or deny an efficient substitution of laser for LED. The aim of this study was to investigate the effect of LEDT (880 ± 10 nm) on collagenase-induced tendinitis of the Achilles tendon of rats through the real-time polymerase chain reaction (RT-PCR) evaluation of the inflammatory mediators (interleukin [IL]-1β, IL-6, cyclooxygenase-2 [COX-2], and tumor necrosis factor-α [TNF-α]) and histomorphometric parameters.

MATERIALS AND METHODS

Animals

All experiments were carried out in accordance with Research Ethics Committee of the Vale do Paraíba University for animal care (protocol number: A010/CEP/2009). The experiments were performed using males Wistar rats (220–260 g), supplied with food and water "ad libitum" provided by the Central Animal House of the Research and Development Department of the Vale do Paraíba University (UNIVAP). The rats were placed in appropriate cages and randomly divided into experimental groups with eight animals per group.

Experimental Groups

The 56 animals selected for the study were separated into seven groups, with 8 animals each, three groups in the experimental period of 7 days and four groups in the experimental period of 14 days, namely: control group (CONT), tendinitis group (TEND), LED therapy group (LEDT) for both experimental periods, and LED therapy group 7th to 14th day (LEDT delay) for 14 days experimental period.

Tendinitis Induction

Tendinitis was induced in the right Achilles tendon of the animals, previously anesthetized by intraperitoneal application of xylazine 2% and ketamine hydrochloride 10%, both at a concentration of 0.1 ml/100 g body weight. Tendinitis was induced by an injection of 100 ml of intratendinous collagenase (1 mg/ml; Sigma Chemical Co., St Louis, MO, C6885), dissolved in a sterile saline phosphate buffer containing 50 mM NaH_2PO_4, 150 mM NaCl, pH 7.4.

LED Therapy

The equipment used in the study was an LED (880 ± 10 nm) CW, FisioLed® model (22 mW) with an acrylic tip with 0.5 cm^2 area in direct contact with the right limb of the animal, on the lesion area. The application time was 170 seconds, with a dose of energy measured on the source of 7.5 J/cm^2 and applied on the injured area. Before the beginning of the experiments, the LED equipment was checked with a power checker (13PEM001/J, Melles Griot, The Netherlands, Netherlands Didam). The therapeutic procedure was begun 12 hours after the induction and was repeated every 48 hours. All animals were treated in the same way. For the procedure, the animals were positioned on a table in ventral decubitus and manually immobilized. The LED was used on their hind limbs, directly on the injury, at a 90° angle.

Histological Preparation and Analysis

The animals were killed on the 7th and 14th days. Before the rats were killed, the same sedation procedure was used as in the surgery, after which an intracardiac injection of sodium thiopental (Cristalia) (1 ml/100 g body weight) was given. The tendons were removed by dissection, from the calcaneal insertion to the myotendinous junction. They were fixed in 10% formaldehyde and sent for histological processing. After fixation, the tendons were dehydrated and embedded in paraffin, followed by microtomy in a semi-automatic revolving microtome to produce sections 5 μm thick, eight sections per animal.

Quantitative Real-Time Polymerase Chain Reaction

One microgram of total RNA was used for cDNA synthesis and RT-PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen, São Paulo, Brazil) at a concentration of 1 U/μg RNA in the presence of 20 mM Tris–HCl (pH 8.4), containing 2 mM $MgCl_2$ for 15 minutes at 37°C, followed by incubation at 95°C for 5 minutes for enzyme inactivation. The reverse transcription was carried out in a 200-μl reaction in the presence of 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxyribonucleoside triphosphate (dNTP), and 50 ng of random primers with 200 U of Moloney murine leukemia virus-reverse transcriptase (Invitrogen).

The reaction conditions were: 20°C for 10 minutes, 42°C for 45 minutes, and 95°C for 5 minutes. The reaction product was amplified by RT-PCR on the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBRGreen core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minutes. Experiments were performed in triplicate for each data point. The amounts of IL-1β, IL-6, TNF-α, and COX-2 mRNA were quantified as relative values compared with an

internal reference, β-actin, because its level was thought not to change despite the various experimental conditions. Primers used for RT-PCR were as follows: rat IL-1β (GenBank accession number: M98820): forward primer 5′-CACCTCTCAAGCAGAGCACAG-3′, reverse primer 5′-GGGTTCCATGGTGAAGTCAAC-3′; rat TNF (GenBank accession number: X66539): forward primer 5′-AAATGG-GCTCCCTCTATCAGTTC-3′, reverse primer 5′-TCTGCT-TGGTGGTTTGCTACGAC-3′; rat COX-2 (GenBank accession number: J00691): forward primer 5′-TGTATGCTAC-CATCTGGCTTCGG-3′, reverse primer 5′-GTTTGGAACA-GTCGCTCGTCATC-3′; rat IL-6 (GenBank accession number: E02522): forward primer 5′-TCCTACCCCAACTTC-CAATGCTC-3′, reverse primer 5′-TTGGATGGTCTTG-GTCCTTAGCC-3′.

Quantitative values for the molecules mentioned above and β-actin mRNA transcription were obtained from the threshold cycle number, which is where the increase in the signal associated with exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of β-actin expression as an endogenous RNA control. ΔC_t values of the samples were determined by subtracting the average $C1$ value of IL-1β, IL-6, COX-2, and TNF-α mRNA from the average $C1$ value of the internal control β-actin. As it is uncommon to use ΔC_t as a relative value due to this logarithmic characteristic, the $2^{-\Delta\Delta C_t}$ parameter was used to express the relative expression data.

Statistical Analysis

Results are expressed as mean values ± standard error of the mean (SEM). The data were compared by analysis of variance (ANOVA) and the Turkey–Kramer multiple comparisons test to determine the differences between groups. The level of significance was 5% ($P < 0.05$). The program used was GraphPad Prisma®, version 5.0.

RESULTS

Figure 1 shows the number of inflammatory cells; an increase of cells was observed in groups TEND when compared to the CONT and was decreased in the treated groups.

The tendons of the CONT groups had wavy collagen fibers with scattered rows of fibrocytes with elongated nucleus, the basic substance with scattered longitudinal fibers arranged and few blood vessels. As evidenced in the count of inflammatory cells, we observed an increase in cellular infiltrates in the areas of recovery of the tendon, foci of mononuclear inflammation, intra and peritendinous, intense fibroblastic activity, and moderate signs of neovascularization in the groups TEND. There was also a reduction in the leakage of inflammatory infiltrate, reduced fibroblast activity, rare neutrophils, reduction of inflammatory cells, production and reorganization of fibers in experimental periods of 7 and 14 days of the treated groups (LEDT and LEDT delay) (Figs. 2 and 3).

A decrease in IL-6 and TNF-α mRNA expression was observed in treated groups when compared to the TEND groups. However, the IL-6 mRNA expression of these groups still remained significantly different from the CONT groups (Figs. 4 and 5). Similarly occurred in IL-1β when comparing the group TEND to group LEDT; however, there was no difference between groups TEND and LEDT delay (Fig. 6).

The difference in Cox-2 mRNA expression between the groups was significant when compared TEND group versus LEDT group in experimental period of 7 days, all groups still remained different from the CONT groups (Fig. 7).

DISCUSSION

Several studies show promising technological advances in the field of phototherapy light sources, coherent or not [9,10,13]. The application of therapy with low-power laser in clinical practice has increased in recent years, a method commonly used for musculoskeletal disorders, pain, and

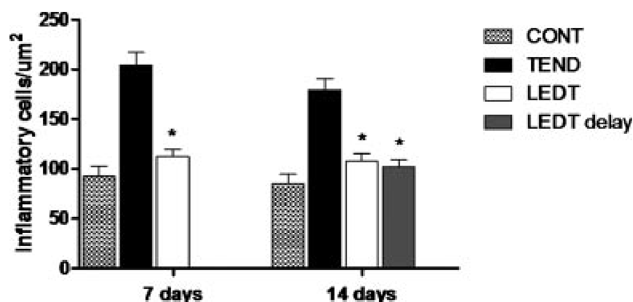

Fig. 1. Number of inflammatory cells. CONT (92.61 ± 10.35), TEND (204.28 ± 12.85), and LEDT (112.88 ± 7.09) in the experimental period of 7 days; CONT (85.32 ± 9.40), TEND (179.44 ± 11.20), LEDT (107.44 ± 7.87), and TEND delay (102.24 ± 6.86) in the experimental period of 14 days. The values are means ± SEM (*$P < 0.05$ vs. TEND).

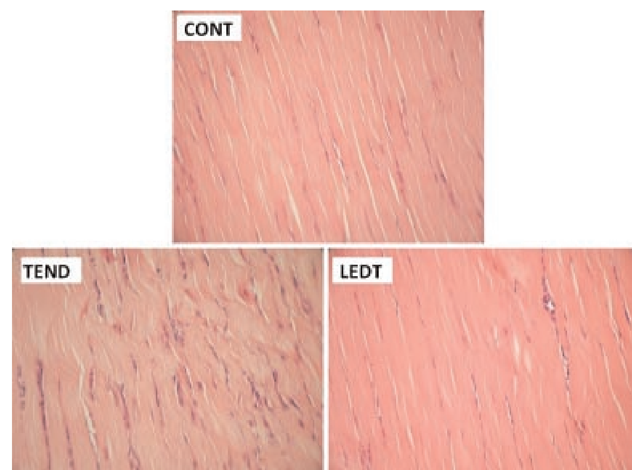

Fig. 2. Microscopy of Aquilles tendon section in different groups of 7 days experimental period. HE: 400×.

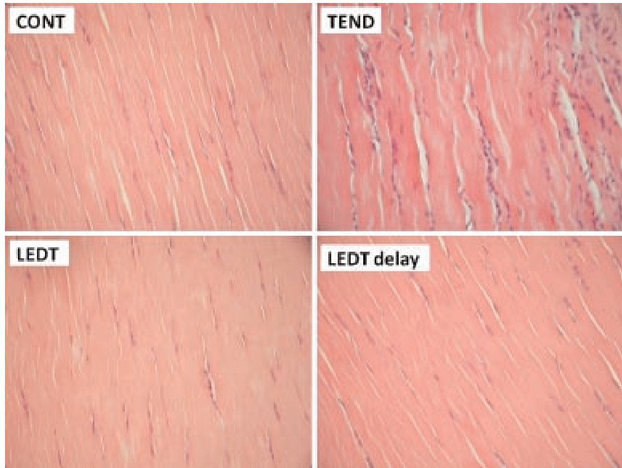

Fig. 3. Microscopy of Aquilles tendon section in different groups of 14 days experimental period. HE: 400×.

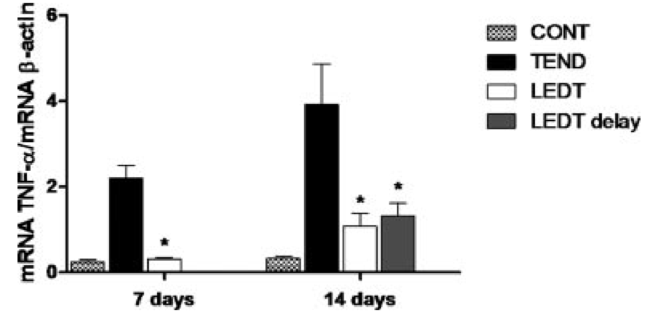

Fig. 5. Effects of LED therapy on TNF-α mRNA expression. The analysis was performed by RT-PCR. CONT (0.25 ± 0.04), TEND (2.20 ± 0.30), and LEDT (0.31 ± 0.03) in experimental period of 7 days; CONT (0.32 ± 0.05), TEND (3.92 ± 0.95), LEDT (1.08 ± 0.29), and LEDT delay (1.32 ± 0.29) in the experimental period of 7 days. The values are means ± SEM (*$P < 0.05$ vs. TEND).

inflammation. Recently, several studies were performed with light, pointing to a similarity of their effects with the laser, in addition to being low-cost equipment [7,10,14,15].

Our results demonstrated the ability of the LED (880 nm) to reduce the number of inflammatory cells at the site of the tendon. Casalechi et al. [13] observed through the application of LED to reduce the number of fibroblasts in the initial phase and an improved quality of tissue repair during the regeneration process of the tendon. Another study found similar effects between the LED and low-intensity laser with a wavelength in the infrared, the repair of the Achilles tendon, through the improvement of the organization, aggregation, and alignment of collagen [9].

It has been reported that one of the factors responsible for inducing inflammation is the presence of inflammatory cells at the injured site, caused by interactions between the inflamed tissue and circulating leukocytes. After inflammation is stimulated, the vascular endothelium begins to express adhesion molecules that facilitate the migration of inflammatory cells into the inflamed tissue [16,17]. In addition, inflammatory mediators such as prostaglandins (PGE2), thromboxane (TXA2), leukotrienes (LTD4), nitric oxide (NO), COX, TNF-α, and IL-1β and IL-6 are released by nearby cells. These mediators modulate vascular tone via vasodilatation, increased vascular permeability, and increased numbers of inflammatory cells (monocytes and neutrophils) at the site of injury [18].

Our results show that therapy with LED was able to reduce the expression of mRNA for IL-1β and IL-6 and TNF-α. These cytokines are triggering factors, participants, and perpetuating the inflammatory cascade. These results allow us to suggest that the anti-inflammatory effect of therapy with LEDs can be related to modulation of the inflammatory response in some of the steps of cell migration. Corroborating previous works claiming that the TLBP optimizes the process of healing of tendons [19,20],

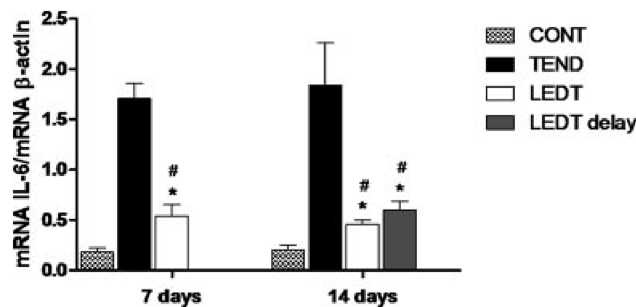

Fig. 4. Effects of LED therapy on IL-6 mRNA expression. The analysis was performed by RT-PCR. CONT (0.18 ± 0.04), TEND (1.71 ± 0.15), and LEDT (0.54 ± 0.11) in the experimental period of 7 days; CONT (0.20 ± 0.05), TEND (1.84 ± 0.42), LEDT (0.46 ± 0.04), and LEDT delay (0.60 ± 0.09) in the experimental period of 14 days. The values are means ± SEM (*$P < 0.05$ vs. TEND; $^{\#}P < 0.05$ vs. CONT).

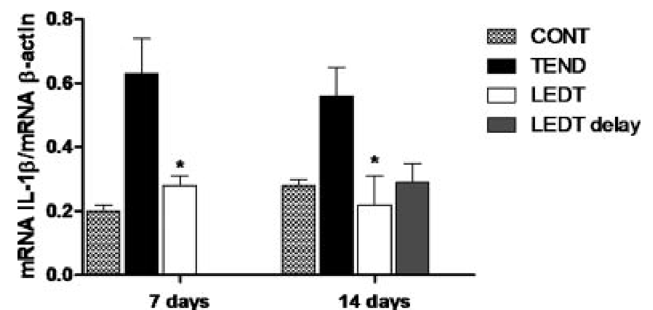

Fig. 6. Effects of LED therapy on IL-1β mRNA expression. The analysis was performed by RT-PCR. CONT (0.20 ± 0.02), TEND (0.63 ± 0.11), and LEDT (0.28 ± 0.03); CONT (0.28 ± 0.02), TEND (0.56 ± 0.09), LEDT (0.22 ± 0.09), and LEDT delay (0.29 ± 0.06). The values are means ± SEM (*$P < 0.05$ vs. TEND).

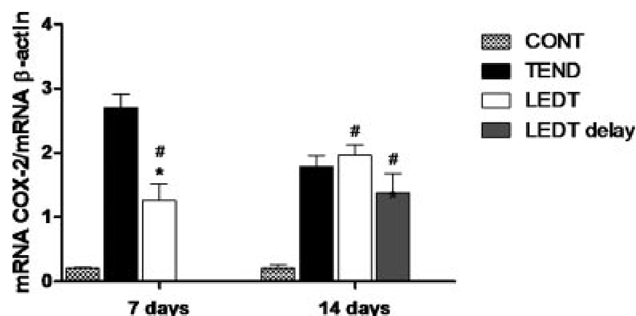

Fig. 7. Effects of LED therapy on COX-2 mRNA expression. The analysis was performed by RT-PCR. CONT (0.20 ± 0.02), TEND (2.70 ± 0.21), and LEDT (1.26 ± 0.26) in experimental period of 7 days; CONT (0.21 ± 0.05), TEND (1.79 ± 0.17), LEDT (1.96 ± 0.16), and LEDT delay (1.38 ± 0.30) in experimental period of 14 days. The values are means ± SEM ($^{*}P < 0.05$ vs. TEND; $^{\#}P<0.05$ vs. CONT).

Albertini et al. [21] also observed decreased expression of mRNA for the cytokines TNF-α, IL1-β, and IL-6, with low-level laser therapy. However, the mRNA expression of IL-1 was not statistically significant in the LED group delay. Enwemeka and Reddy [22] argue that the first few days are important to the quality of tendon repair.

The levels of IL-6 were higher in groups TEND, thereby demonstrating the action of this cytokine in tendinitis. The pro-inflammatory cytokines play important roles in the initial stage of wound healing, for working in the migration of phagocytes and other cells to the site of inflammation, initiating the proliferative phase, promoting the recruitment of cells needed for tissue repair [23]. However, the inflammatory action needs to be regulated and IL-6 has anti-inflammatory properties, and participate in the inflammatory process by controlling local and systemic acute inflammatory response [24].

The importance of reducing migration of monocytes and neutrophils to the site of inflammation is the fact that these cells, once attracted to the focus of inflammation secrete TNF-α, and other chemotactic factors, back-feeding the inflammatory process [25]. Thus, the LED therapy is contributing to the breakdown of positive feedback loop of inflammation.

The two important cytokines in relation to the expression of adhesion molecules during most inflammatory processes are TNF-α and IL-1β [26]. The main cellular source of both TNF-α and IL-1β is the monocytes/macrophages. Some authors have demonstrated that TNF-α and IL-1β act synergistically in several inflammatory conditions, being able to regulate inflammation through constant stimulus for cell migration and release of inflammatory mediators [27].

Considering our results showed that therapy with LED was able to reduce the expression of mRNA for the cytokines IL-1β and TNF-α, we suggest that chemotaxis to the inflammatory cells is also reduced, thereby reducing the density of cells in inflammatory site.

Our results showed a decrease in mRNA expression for COX-2 in the initial phase after therapy with LED. These results suggest that the LED can inhibit the chemotactic effects in the early stages of inflammation or may interfere with the chemical mediators by inhibiting COX. This is due to the fact that COX-2 is a progenitor of the enzymes of the inflammatory processes, thus showing their highest values in the acute phase of inflammation. LED therapy acts on the early stages of inflammation, as suggested by Campana et al. [28], since it was observed reduction of mRNA for COX-2. Another study showed that the LED low intensity was able to reduce mRNA expression for COX and their precursors as fospolipase A2, and reactive oxygen species [15]. Albertini et al. [29] also reported a significant reduction in mRNA expression for COX-2 after application of phototherapy in inflammation in rat paw.

LED therapy has been able to reduce the expression of mRNA, IL-1β, IL-6, TNF-α, and COX-2. These cytokines are triggering factors, participants, and perpetuating the inflammatory cascade as listed above. Thus, another possible mechanism of action for the LED on the inflammatory process may be related to modulation in the mechanism of production of inflammatory cytokines.

CONCLUSION

Our results suggest that the anti-inflammatory therapy with low power LED (880 nm) enhanced the response of tendon tissue in all treated groups, showing significant difference compared with control groups. We can conclude that the LED was able to reduce signs of inflammation in collagenase-induced tendinitis in rats by reducing the number of inflammatory cells and a decrease in mRNA expression of IL-1β, IL-6, TNF, and COX-2.

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