

Low-level light-emitting diode therapy increases mRNA expressions of IL-10 and type I and III collagens on Achilles tendinitis in rats

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Received: 16 October 2012 / Accepted: 28 January 2013
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Abstract The present study investigated the effects of low-level light-emitting diode (LED) therapy (880 ± 10 nm) on interleukin (IL)-10 and type I and III collagen in an experimental model of Achilles tendinitis. Thirty male Wistar rats were separated into six groups ($n=5$), three groups in the experimental period of 7 days, control group, tendinitis-induced group, and LED therapy group, and three groups in the experimental period of 14 days, tendinitis group, LED therapy group, and LED group with the therapy starting at the 7th day after tendinitis induction (LEDT delay). Tendinitis was induced in the right Achilles tendon using an intratendinous injection of 100 μ L of collagenase. The LED parameters were: optical power of 22 mW, spot area

size of 0.5 cm², and irradiation time of 170 s, corresponding to 7.5 J/cm² of energy density. The therapy was initiated 12 h after the tendinitis induction, with a 48-h interval between irradiations. The IL-10 and type I and III collagen mRNA expression were evaluated by real-time polymerase chain reaction at the 7th and 14th days after tendinitis induction. The results showed that LED irradiation increased IL-10 ($p<0.001$) in treated group on 7-day experimental period and increased type I and III collagen mRNA expression in both treated groups of 7- and 14-day experimental periods ($p<0.05$), except by type I collagen mRNA expression in LEDT delay group. LED (880 nm) was effective in increasing mRNA expression of IL-10 and type I and III collagen. Therefore, LED therapy may have potentially therapeutic effects on Achilles tendon injuries.

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Keywords Inflammation · Light–tissue interaction ·
Tendinopathies and tendinitis · Tissue repair

Introduction

The term tendinitis was used to describe chronic pain referring to a symptomatic tendon, thus implying inflammation as a central pathological process [1]. An estimated 30 to 50 % of all sports-related injuries are tendon disorders [2]. The Achilles tendon is one of the most commonly injured tendons in the human body. The Achilles tendinitis (AT) is a common cause of disability and is clinically characterized by pain and swelling in and around the tendon, mainly arising from overuse [3].

Tendon matrix is rich in collagens, such as type I and III collagens. Type I collagen is considered to be responsible

for the mechanical strength of the tendon tissue, whereas that type III collagen has an important role in the healing process [4]. Type I collagen (thick fibers) is the primary collagen incorporated to the tendon structure, and increasing the production of type I collagen may enhance tendon healing [5].

Interleukin (IL)-10 is known as the most important anti-inflammatory cytokine and has been reported to be predominantly produced by immune cells such as macrophages, lymphocytes, and dendritic cells, but an increasing amount of evidence is indicating that IL-10 may also play a regulatory role in other connective tissue cells, such as fibroblasts or chondrocytes. However, the role of the immunoregulatory cytokine IL-10 in tendon still remains unclear and requires more detailed analyses [6–8].

A variety of treatments for tendinopathies is currently used or has been trialed, including ultrasound [9], extracorporeal shock wave therapy [10], steroid injections and non-steroidal anti-inflammatory drugs [11], and low-level laser therapy (LLLT) as well as low-level light-emitting diode therapy (LED therapy) [12, 13]. The use of visible or near infrared light for reducing pain, inflammation, and edema, promoting healing of wounds, deeper tissues, and nerves, and preventing cell death and tissue damage has been known for over 40 years since the invention of laser [14]. However, the therapeutic mechanism of LED therapy is not well understood.

The aim of this study was to investigate the effects of LED therapy on IL-10 and type I and III collagen mRNA expression by real-time polymerase chain reaction (PCR) evaluation in an experimental model of collagenase-induced AT in rats. PCR is one of the most widely used techniques in modern molecular biology allowing the reliable detection and quantification of nucleic acid sequences. Therefore, this quantitative assessment will be carried out aiming to add new insights to the growing body of knowledge regarding interaction of LED therapy and gene expression.

Methods

Animal care and study protocol

All experiments were carried out in accordance with the Research Ethics Committee of the Vale do Paraíba University for animal care (protocol number, A010/CEP/2009). The experiments were performed using male Wistar rats (220–260 g), 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, with four animals each, and maintained under standard conditions of temperature (22–25 °C), relative humidity (40–

60 %), and light/dark cycle with access to food and water ad libitum.

Experimental groups

The 30 animals selected for the study were randomly allocated into six groups, with five animals each: 1) control group (CONT)- animals without tendinitis induction, they received only saline solution; 2-3) groups TEND-7 and TEND-14, tendinitis induced animals, but not treated, for the experimental periods of 7 and 14 days, respectively; 3-4) groups LEDT-7 and LEDT-14, tendinitis induced animals and treated with LED 880 nm, for the experimental periods of 7 and 14 days, respectively; 5) group LEDT delay, animals with tendinitis induction and treated with LED 880 nm for an experimental period of 14 days, but treatment begins only after the 7th day from the application of collagenase.

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 in the concentration of 0.1 mL per 100 g body weight. Tendinitis was induced by an intratendinous injection of 100 µL of collagenase (1 mg/mL, Sigma, C6885, St. Louis, MO, USA), dissolved in a sterile saline phosphate buffer containing 50 mM NaH₂PO₄ and 150 mM NaCl at pH7.4.

LED therapy

LED therapy on infrared spectrum is a therapeutic modality commonly used for musculoskeletal disorders by its capacity of attenuating the symptoms of inflammation and increasing collagen synthesis in different models of experimental tendinitis. Moreover, there are a few studies using the parameters selected as described below. The equipment used in the study to undertake the phototherapy was a LED (880±10 nm) FisiLed® model (22 mW) presenting an acrylic tip with 0.5 cm² area. The irradiation was done at one point on the injured area, in direct contact with the right limb of the animals and at a 90° angle. Before the beginning of the experiments, the output power of the LED equipment was checked by using a power meter (13PEM001/J, Mellers Griot, Netherlands). The therapeutic procedure was begun 12 h after the induction and then repeated every 48 h. All animals were treated by the same way. For the irradiation procedure, the animals were positioned over a table in ventral decubitus and manually immobilized.

Quantitative real-time RT-PCR

IL-10 and collagen type I and III mRNA expression evaluations were performed from animal groups mentioned above. The animals were killed by decapitation, and the tendons were quickly dissected and frozen in dry ice, before storing at -80°C . Thawed tissues were homogenized in 1 mL of TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated accordingly to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis and real-time PCR gene expression analysis. Initially, contaminating DNA was removed using DNase I (Invitrogen) at a concentration of 1 unit/ μg RNA in the presence of 20 mM Tris-HCl, pH8.4, containing 2 mM MgCl_2 for 15 min at 37°C , followed by incubation at 95°C for 5 min for enzyme inactivation. Then, the reverse transcription (RT) was carried out in a 200- μL reaction in the presence of 50 mM Tris-HCl, pH8.3, 3 mM MgCl_2 , 10 mM dithiothreitol, 0.5 mM dNTPs, and 50 ng of random primers with 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The reactions conditions were: 20°C for 10 min, 42°C for 45 min, and 95°C for 5 min. The reaction product was amplified by real-time PCR on the 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBR Green core reaction kit (Applied Biosystems). The thermal cycling conditions were: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Experiments were performed in triplicates for each data point. IL-10 and collagen type I and III mRNA abundances were quantified as a relative value compared with an internal reference, β -actin, whose abundance was believed not to change between the varying experimental conditions. Primers used for real-time PCR are as follows:

- IL-10 (NM_012854) sense 5-AAAGCAAGG CAGTGGAGCAG-3 and antisense 5-TCAAAC TCATTCATGGCCTTGT-3
- COL1A1 (NM_053304) sense 5-GGAGAGAGTGC CAACTCCAG-3 and antisense 5-GTGCTTTGG AAAATGGTGCT-3
- COL3A1 (NM_032085) sense 5-AGGCCAATGGC AATGTAAAG-3 and antisense 5-TGTCTTGCTCC ATCACCAG-3

Quantitative values for the molecules mentioned above and β -actin mRNA transcription were obtained from the threshold cycle number, where the increase in the signal associated with an 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. ΔCt values of the samples were determined by subtracting

the average C_t value of IL-10 and collagen type I and III mRNA from the average C_t value of the internal control β -actin. As it is uncommon to use ΔCt as a relative data due to this logarithmic characteristic, the $2^{-\Delta\text{Ct}}$ parameter was used to express the relative expression data.

Reagents acepromazine, zolazepam chloride, and tiletamine chloride were purchased from Cristalia (São Paulo, Brazil). Collagenase was purchased from Sigma (St. Louis, MO, USA). The reagents for PCR of IL-10 and collagen type I and III were obtained from R&D Systems (Minneapolis, MN, USA).

Statistical analysis

Results are expressed as mean values \pm standard error of the mean (SEM). The data were compared by analysis of variance 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 Prism[®], version 5.0.

Results

Figure 1 displays the effect of LED therapy on IL-10 mRNA expression. It was observed that LED therapy was effective in promoting an increase of IL-10 in the experimental period of 7 days. Likewise, it is found that in the experimental period of 14 days, LED therapy also showed higher values of IL-10. However, the difference is not significant in the last case ($p > 0.05$).

Figures 2 and 3 show type I and III collagen mRNA expressions, respectively. An increase in type I and III collagen mRNA expression was observed in LEDT groups when compared to the TEND groups, for the 7-day experimental period. After 14 days, except for type I collagen mRNA expression in LEDT delay group, both treated groups showed higher values than TEND groups.

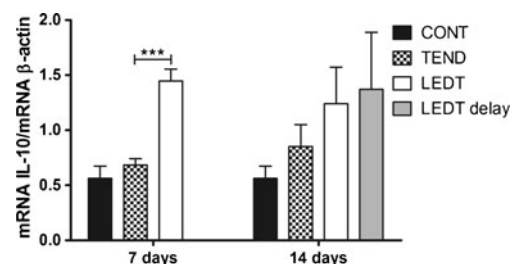


Fig. 1 LED therapy on IL-10 mRNA expression in rat tendon. The analysis was performed by RT-PCR. CONT 0.56 ± 0.11 , TEND-7 0.69 ± 0.06 , and LEDT-7 1.45 ± 0.11 in the experimental period of 7 days. CONT 0.56 ± 0.11 , TEND-14 0.85 ± 0.19 , LEDT-14 1.24 ± 0.33 , LEDT delay 1.37 ± 0.51 in the experimental period of 14 days. The values are means \pm SEM (** $p < 0.001$)

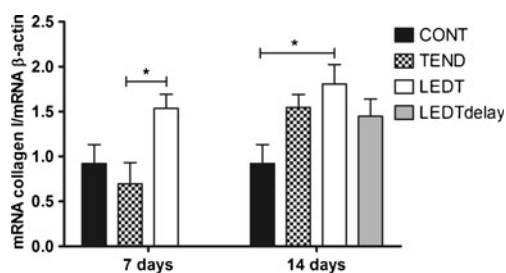


Fig. 2 LED therapy on collagen I mRNA expression in rat tendon. The analysis was performed by RT-PCR. *CONT* 0.92 ± 0.21 , *TEND-7* 0.69 ± 0.23 , and *LEDT-7* 1.53 ± 0.16 in the experimental period of 7 days. *CONT* 0.92 ± 0.21 , *TEND-14* 1.55 ± 0.15 , *LEDT-14* 1.81 ± 0.22 , and *LEDT delay* 1.45 ± 0.19 in experimental period of 14 days. The values are means \pm SEM ($*p < 0.05$)

Discussion

The use of phototherapy by laser is being extensively studied for the last decades and many effects were already reported, like angiogenesis [15], alignment of collagens fibers [16], prevention of oxidative stress, and reduction of fibrosis [17]. However, the use of phototherapy by using LEDs (LEDT) is recent and it is still unclear how exactly it stimulates tendon healing. Some authors showed that LEDT was capable to push down the expression of pro-inflammatory mediators in a model of collagenase-induced tendinitis in two distinct moments: in acute phase and in chronic phase of treatment [18].

In this study, LED therapy improves the IL-10 mRNA expression in the initial phase of tendon healing. Enwemeka and Reddy argue that the first few days are important to the quality of tendon repair, being the ability of phototherapy to increase IL-10 in acute phase of inflammation demonstrated by some authors like Laraia et al. that observed a significant increase of IL-10 at three experimental times (6, 24, and 72 h) after LLLT (660 nm) in acute inflammation induced by tenotomy of Achilles tendon in rats [19, 20]. In another study, it was found that the LLLT (660 nm) promotes an

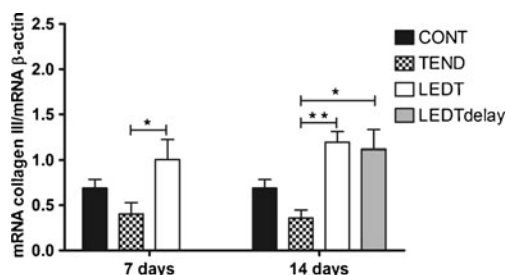


Fig. 3 LED therapy on collagen III mRNA expression in rat tendon. The analysis was performed by RT-PCR. *CONT* 0.69 ± 0.10 , *TEND-7* 0.40 ± 0.12 , and *LEDT-7* 1.01 ± 0.21 in experimental period of 7 days. *CONT* 0.69 ± 0.10 , *TEND-14* 0.36 ± 0.09 , *LEDT-14* 1.19 ± 0.12 , and *LEDT delay* 1.11 ± 0.22 in the experimental period of 14 days. The values are means \pm SEM ($*p < 0.05$; $**p < 0.01$)

increase in the levels of IL-10 mRNA expression on the acute lung inflammation induced by intestinal ischemia and reperfusion [21].

IL-10 has been shown to inhibit the migration of inflammatory cells to sites of injury by inhibiting the expression of specific chemokines. Some authors suggest that IL-10 overexpression would lead to decrease inflammation and to improve biomechanical as well as histological properties in adult healing tendon in comparison with that of the normal healing state [22].

Collagen is the main component of the extracellular matrix and it appears that the physiologic response of tendon cells to trauma induces production of both type I and III collagen [23]. In the present study, an increase in type I and III collagen mRNA expression occurs; however, no difference was found in collagen I mRNA expression on the LEDT delay group.

Type 1 collagen is responsible to produce mechanical resistance of tendon, and some authors suggest that when phototherapy starts immediately after injury, there is an increase in the tensile strength of the tendon, a larger deposition of collagen fibers at the injury site [24, 25], and a significant increase in tendon thickness [26].

During the healing phase after tendon injury, the expression of type III collagen, which is characterized by greater elasticity, is increased in the early phase compared with the normal tendon, and then, it is gradually substituted by type I collagen with time [27, 28].

Casalechi et al. observed that LED therapy reduced the number of fibroblasts in the initial phase and induces an improvement in the quality of tissue repair during the regeneration process of the tendon [13]. Similar effects between the LED therapy and the low-level laser therapy in the infrared radiation were reported on the repair of the Achilles tendon, such as the improvement of organization, aggregation, and collagen alignment [29].

Neves and coworkers [30] reported a study evaluating the effect of an 830-nm GaAlAs diode laser operating at output powers of 40, 60, 80, and 100 mW and energy density of 30 J/cm^2 on the repair of partial calcaneal tendon ruptures in rat. They found in their study that the phototherapy resulted in significantly greater amounts of type I collagen (output power of 80 mW) and type III collagen (output powers of 60 mW or higher) [30].

A study investigating the effect of the phototherapy on tendon healing by using only a laser at 830 nm or combined with the use of ultrasound was conducted by Wood and coworkers in the year 2010. They observed an increase in type I collagen on treated groups, but there were no differences in the amount of type III collagen between groups; however, the amount of type III collagen was significantly higher than the amount of type I collagen in all groups [31].

Recently, it has been described that phototherapy is able to modulate mRNA gene expression for pro-inflammatory mediators in similar experimental model of tendinitis adopted in the current study. Casalechi et al. showed that LLLT was able to modulate mRNA gene expression of IL-10, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMP1 and MMP13) both in acute than in chronic inflammatory phase [32]. Pires et al. showed that LLLT in both acute and chronic phases decreased IL-6, cyclooxygenase-2 (COX-2), and tumor necrosis factor-beta expression after tendinitis when compared to tendinitis groups. The LLLT does not alter IL-1 β expression in any time, but reduced the tumor necrosis factor-alpha expression only at chronic phase [12]. Using LEDT, Xavier et al. described decreases in the inflammatory cell influx and mRNA expression to IL-1 beta, IL-6, tumor necrosis factor-alpha, and COX-2 just in acute phase [18].

LEDT has been able to increase the mRNA expression of IL-10 and type I and III collagens. Thus, another possible mechanism of action of the LED therapy on the inflammatory process may be related to the modulation of the mechanism of production of anti-inflammatory cytokines.

This study investigated the effect of LED therapy on specific parameters of irradiation; however, further studies are needed to understand which of the best parameters of treatment and how exactly this therapy act on tendon repair.

Conclusion

This study seems to indicate that the LED therapy (880 nm) was effective in increasing mRNA expression of IL-10 and type I and III collagen; therefore, LED therapy may have potentially therapeutic effects on Achilles tendon injuries.

Acknowledgments M. Xavier thanks FAPEMIG (Minas Gerais Research Foundation) for the partial financial support (APQ-01733-11).

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