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ORIGINAL ARTICLE

Low-level laser therapy in experimental model of collagenase-induced tendinitis in rats: effects in acute and chronic inflammatory phases

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Abstract A variety of treatments for tendinopathies is currently used or has been trialed. However, in fact, there is a remarkably little evidence that any conventional therapies are effective. In the last years, low-level laser therapy (LLLT) has been showing interesting results in inflammatory modulation in different musculoskeletal disorders, but the optimal parameters and mechanisms behind these effects are not fully understood. The aim of this study is to investigate if the LLLT modulates the acute and chronic phase of collagenase-induced tendinitis in rat by interfering in mRNA expression for matrix metalloproteinases (MMP13 and MMP1), vascular endothelial growth factor (VEGF), and anti-inflammatory mediator (interleukin (IL)-10). For such, tendinitis was induced by collagenase injection in male Wistar rats. Animals were treated with LLLT (780 nm, potency of 22 mW, 107 mW/cm^2, energy density of 7.5 J/cm^2, and energy delivered of 1.54 J) with different number of treatments in accordance with the inflammatory phase analyzed. LLLT was able to modulate mRNA gene expression of IL-10, VGEF, MMP1, and MMP13 both in acute than in chronic inflammatory phase ($p<0.05$). Our results suggest that LLLT with parameters employed in the present study was able to modulate IL-10, VEGF, MMP1, and MMP13 mRNA gene expression both in acute than in chronic tendon inflammation. However, further studies are needed to establish optimal parameters for LLLT.

Keywords LLLT · Tendinitis · Inflammatory mediators

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Introduction

Primary disorders of tendons are common and account for a high proportion of referrals to rheumatologists and orthopedic surgeons [1]. An estimated 30 to 50 % of all sports-related injuries are tendon disorders [2]. Historically, the term tendinitis was used to describe chronic pain referring to a symptomatic tendon, thus implying inflammation as a central pathological process [3]. Despite the prevalence and recalcitrant nature of tendinopathy, its pathogenesis remains poorly understood since a few studies have examined its earliest development [4].

Biopsy samples obtained at end-stage disease from patients undergoing surgery for longstanding tendon pain typically reveal variable tenocyte density, increased hyaluronan and chondroitin sulfate content, increased collagen turnover with decreased type I collagen, and neurovascular

proliferation [5]. Histologically, degenerative changes (classified as hypoxic, hyaline, mucoid or myxoid, fibrinoid, and fatty degenerations) are found in 90 % of biopsy specimens taken from symptomatic parts of the tendon [6].

It is known that matrix remodeling is enabled by the balance of matrix metalloproteinases (MMPs) and their inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs). Imbalance in the expression of MMPs and their inhibitors can contribute to tendon matrix degradation, as collagen degradation is initiated by MMPs. It is proposed that prior to tendon matrix degradation, subtle changes involving the release of inflammatory cytokines such as interleukin (IL)-1 by infiltrating macrophages/monocytes may occur. In addition, tendons in vivo are subjected to repetitive motion that may cause microinjuries, which is sufficient to induce endogenous IL-1β. The anti-inflammatory cytokine IL-10 has been reported to be predominately produced by immune cells such as macrophages, lymphocytes, and dendritic cells, but increasing amounts of evidence have suggested that IL-10 may also play a regulatory role in other connective tissue cells, such as fibroblasts or chondrocytes. The presence and function of the anti-inflammatory cytokine IL-10 in human tenocytes, however, has not yet been investigated [7–9].

The vascular function and angiogenesis are regulated by vascular endothelial growth factor (VEGF) and is not highly expressed in adult tendon but is increased in several animal models of acute injury or mechanical loading. VEGF mRNA levels are also elevated in homogenized Achilles tendinosis biopsies. VEGF, a potent angiogenic cytokine and signaling peptide with seven molecularly diverse isoforms, may play a role in this process. VEGF regulates many genes that drive the adaptive and angiogenic response to hypoxia or inflammation in pathologies such as soft tissue repair, promoting endothelial cell proliferation and migration [10].

The first phase of inflammation is thought to be highly catabolic. Accumulating leukocytes perform extensive phagocytosis and release a plethora of potentially damaging enzymes. Among them, MMPs are believed to induce nonspecific damages to the extracellular matrix (ECM). An increase in cytokine levels in response to repeated injury or mechanical strain may induce MMP release, with degradation of the ECM network and eventual tendinopathy. However, direct contribution of those enzymes to tissue injury is still a matter of debate. Different defense mechanisms are in place to minimize nonspecific damages to the ECM following an acute trauma. Indeed, TIMPs can counterbalance the actions of MMPs and limit ECM damages under proinflammatory conditions [10–12].

A variety of treatments for tendinopathies is currently used or has been trialed, including ultrasound [13], extracorporeal shock wave therapy [14], steroid injections and nonsteroidal anti-inflammatory drugs [15], and low-level laser therapy (LLLT) [5, 16].

However, in fact, there is remarkably little evidence that any conventional therapies are effective [17]. The use of low levels 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 lasers [18].

Pires et al. [5] showed that LLLT has competence to push down the expression of proinflammatory mediators in a model of collagenase-induced tendinitis in two moments distinct, in acute (AP) and chronic phases (CP) of laser treatment.

The present study was designed to investigate if the LLLT modulates the AP and CP of collagenase-induced tendinitis in rat by interfering in mRNA expression for MMP13 and MMP1, VEGF, and anti-inflammatory mediator (IL-10).

Materials and methods

Animals

All experiments were carried out in accordance with the guidelines of Vale do Paraíba University for animal care (protocol number: A034/2006/CEP). The experiments were performed using 30 male Wistar rats (180–200 g), with food and water “ad libitum” provided by the Central Animal House of the Research and Development Department of Vale do Paraíba University (UNIVAP). All rats were placed in common cages and randomly divided into groups of six.

Induction of tendinitis by collagenase injection

Before induction of tendinitis, the animals were pre-anesthetized with acepromazine (0.1 mg/kg, i.p.) and posteriorly anesthetized intraperitoneally with ketamine hydrochloride at 10 % and xylazine at 2 %, both at a concentration of 0.1 ml100 g^{-1} of body weight. After the tendon cleansing (with 70 % alcohol), the tendinitis was experimentally induced with an intratendinous injection of 0.1 mL of collagenase (1 mgmL^{-1}; SIGMA, C6885) in the right Achilles tendon. Collagenase (1 mg) was dissolved in a sterile phosphate-buffered saline (1 mL). In order to determine the groups, the animals were irradiated 12 h or 7 days after collagenase challenge are named as AP and CP, respectively. The animals were killed 7 or 14 days after induction of tendinitis.

Table 1 Temporal profile of tendinitis induction and laser treatment

	Initial of treatment	Period of treatment (days)	Euthanasia
AT_7	12 h after tendinitis induction	2nd, 4th, and 6th	7th day
AT_{14}	12 h after tendinitis induction	1st, 3rd, 5th, 7th, 9th, 11th, and 13th	14th day
CT_{14}	7 days after tendinitis induction	7th, 9th, 11th, and 13th	14th day

Experimental groups

The experimental groups consisted of 30 male Wistar rats randomly allocated into six groups: animals injected with vehicle were considered as saline; animals treated with laser at 780 nm were considered as laser; animals injected with collagenase and killed in AP were named as AP; animals injected with collagenase and killed in CP were considered as CP; animals injected with collagenase and treated with laser at 780 nm in AP were named as AT; and animals injected with collagenase and treated with laser 780 nm in CP were named as CT.

LLLT

The equipment used herein was a laser (MMoptics model) with a wavelength of 780 nm, potency of 22 mW, 107 mW/cm^2, energy density of 7.5 J/cm^2, and energy delivered of 1.54 J. The laser was applied in contact with the tendon of right calcaneus during 70 s and irradiated area was same size of laser spot (0.205 cm^2). For laser irradiations, the animals were immobilized by means of grip and were irradiated at an angle of 90° to the surface of tissue area. Before the beginning of the experiments, the laser equipment was checked with a power checker (13PEM001/J, Melles Griot, the Netherlands-Didara).

Therapeutic strategy

The animals were irradiated in according to phases of inflammation. Regarding to AP (AT_7), the treatment with laser was initiated at 12 h after induction of tendinitis; in this condition, the animals were irradiated in second, fourth, and sixth days after induction of tendinitis and then euthanized in the seventh day. In the AP named as AT_{14}, the laser treatment was initiated equally to AT_7; however, the animals were irradiated in first, third, fifth, seventh, ninth, eleventh, and thirteenth days after induction of tendinitis and then euthanized on the fourteenth day. The group named CT_{14} received laser initially 7 days after tendinitis induction, after that, the animals were irradiated in seventh, ninth, eleventh, and thirteenth days after tendinitis and then euthanized in fourteenth day (Table 1).

RNA extraction

IL-10, VEGF, MMP1, and MMP13 mRNA expression evaluation were performed from animal groups mentioned above. The animals were killed by decapitation, the tendon 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.

Quantitative real-time RT-PCR

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, pH 8.4, 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 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 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 SYBRGreen 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. MMp1, MMp13, IL-10, collagen types I and III, and VEGF mRNA abundance 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 (forward: 5-AAAGCAAGGCAGTGGAG CAG-3; reverse: 5-TCAAACTCATTCATGGCCTTGT-3), VEGF (forward: 5-TGGCTTTACTGCTGTACCTCCA-3; reverse: 5-TTTCTGCTCCCCTTCTGTCGT-3), MMP1 (forward: 5-GACGTGGACCGACAACAGTGA-3; reverse 5-GGGGAACATTAGTGCTCCTACATC-3), MMP13 (forward: 5-TGGTCCCTGCCCCTTCCCTA-3; reverse 5-CCGCAAGAGTCACAGGATGGTAGTA-3), and Beta actin was used as an internal control (forward: AAGATTTGGCACCACACTTTCTACA; reverse: CGGTGAGCAGCACAGGGT).

Quantitative values for the molecules mentioned above and β-actin mRNA transcription were obtained

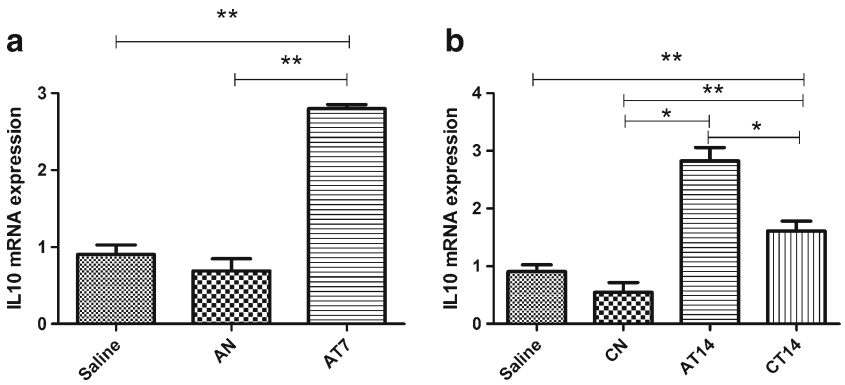

Fig. 1 LLLT in IL-10 mRNA expression in rat tendon. The rats were challenged with collagenase (1 mgml^{-1}) and treated with a laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) postchallenge. Seven and 14 days after the rats were killed, IL-10 expression was measured by real-time PCR. Groups—saline, 7 days; AT7-treated group, 7 days; AT14-treated group, 14 days; and CT14-treated group, from days 7 to 14 (*p<0.05; **p<0.001, levels of significance)

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 Ct value of MMP1, MMP13, IL-10, and VEGF mRNA from the average Ct value of the internal control β-actin. As it is uncommon to use ΔCt as a relative data due to this logarithmic characteristic, the $2^{-\Delta Ct}$ 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. Lois, MO, USA). The reagents for PCR of MMp1, MMp13, IL-10, Collagen types I and III, and VEGF were obtained from R&D Systems (Minneapolis, MN).

Data analysis

The data were expressed as mean ± SEM. The InStat program (GraphPad Software) was used for the statistical analysis. The data were examined by ANOVA followed by the Tukey's post hoc test to determine differences between groups, and the results were considered significant when p <0.05. For the construction of graphs, we used the program GraphPad Prism.

Results

Figure 1 shows the effect of the laser in different periods of treatment (12 h or 7 days after induction of tendinitis) on

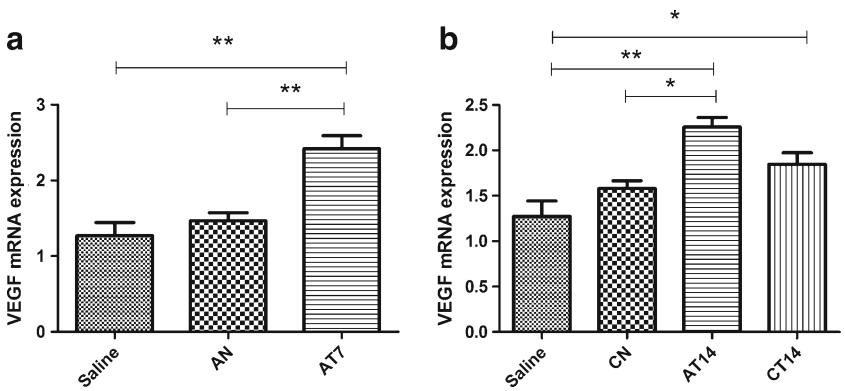

Fig. 2 LLLT on VEGF mRNA expression in rat tendon. The rats were challenge with collagenase (1 mgml^{-1}) and treated with laser (780 nm) at an angle of 90 ° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) postchallenge. Seven and 14 days after the rats were killed, VEGF expression was measured by real-time PCR. Groups—saline, 7 days; AT7-treated group, 7 days; AT14-treated group, 14 days; and CT14-treated group, from days 7 to 14 (*p<0.05; **p<0.001, levels of significance)

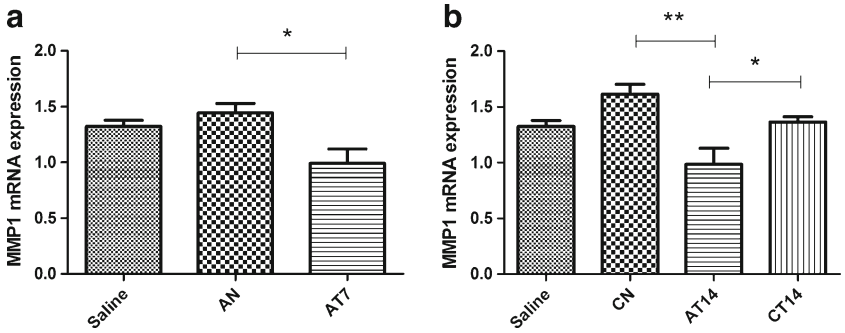

Fig. 3 LLLT on MMP1 mRNA expression in rat tendon. The rats were challenged with collagenase (1 mg ml^{-1}) and treated with a laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) postchallenge. Seven and 14 days after the rats were killed, MMP1 expression was measured by real-time PCR. Groups—saline, 7 days; AT7-treated group, 7 days; AT14-treated group, 14 days; and CT14-treated group, from days 7 to 14 (*p<0.05; **p<0.001, levels of significance)

expression for IL-10. Figure 1a shows the experiments performed in the AP of treatment with laser, which is referent to treatment that initiated 12 h after tendinitis induction. In this condition, there is a significant difference of IL-10 mRNA expression between the saline group and group challenged with collagenase and killed 7 days after induction of tendinitis (AP7). When we analyzed the IL-10 expression in AN7 group and compared it with animals from the group challenged with collagenase and treated with laser (AT7), it is possible to observe that LLLT alter the expression of mRNA IL-10. Figure 1b is referent to experiments performed in the CP of laser treatment, which is referent to treatment initiated 7 days after tendinitis induction. As in the AP, there is an increase in IL-10 expression in groups treated with laser in comparison to the saline group; both groups were killed 14 days after tendinitis induction.

The effect of LLLT on VEGF expression is illustrated in Fig. 2. These data showed that the LLLT increased the VEGF mRNA expression either in the group AN and CN, respectively.

In Fig. 3, it is possible to verify that there is no difference between groups with saline and the groups challenged with tendinitis and killed 7 (Fig. 3a) or 14 (Fig. 3b) days after induction of tendinitis. The MMP1 mRNA expression was different in the group of animals challenged with collagenase and treated with laser either in AP as CP.

Figure 4 represents the effect of LLLT MMP13 mRNA expression. In these experiments, the expression MMP13 increased significantly after irradiation with LLLT compared with animals from saline and control groups killed on the seventh or fourteenth day. However, we can observe that there is no difference between saline and control groups.

Discussion

Immediately after an acute injury, the body initiates a series of biological responses. The inflammatory reaction consists of both vascular and cellular events. It has been reported that one of the principal 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

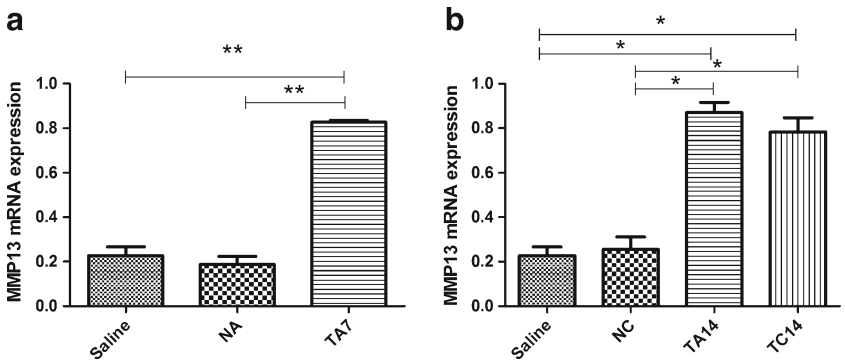

Fig. 4 LLLT on MMP13 mRNA expression in rat tendon. The rats were challenged with collagenase (1 mg ml^{-1}) and treated with a laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) postchallenge. Seven and 14 days after the rats were killed, MMP13 expression was measured by real-time PCR. Groups—saline, 7 days; AT7-treated group, 7 days; AT14-treated group, 14 days; and CT14-treated group, from days 7 to 14 (*p<0.05, level of significance)

vascular endothelium begins to express adhesion molecules that facilitate the migration of inflammatory cells into the inflamed tissue [19, 20].

The IL-10 is known as the most important anti-inflammatory cytokine. Previous data have shown that IL-10 is capable of blocking the inflammatory response induced by several inflammatory stimuli in different models [21]. However, the influence of LLLT on the presence and function of the anti-inflammatory cytokine IL-10 have to be investigated. Boschi et al. [22] did not observed action of laser on IL-10 in the early phase in pleurisy in rat. Mafra de Lima et al. [23] observed that the treatment with LLLT promote an increase in the level of IL-10 on animals with lung inflammation induced by intestinal ischemia and reperfusion.

Our results showed an increase in mRNA expression for IL-10 in the groups that laser therapy was initiated after the tendinitis induction in 7 and 14 days. These results suggest that the laser acts on the early stages of inflammation. However, the role of IL-10 in tendons remains still unclear; it is probable that co-stimuli are necessary for full effects [24].

The angiogenic factor VEGF is nearly completely downregulated in healthy tendons. However, its expression also occurs during various disease states in the tendon as well as during tendon healing [25]. In this study, the level of mRNA expression for VEGF increased in the groups that the laser therapy started early. Laser treatment on cutaneous wounds (670 nm) led to significantly higher expression of VEGF in irradiated tissues 6–24 h posttreatment [26].

A significant VEGF immune expression was detected in the laser-exposed group (660 nm) after 14 days when compared with the negative control group on the healing of second-degree skin burns in rats [27].

Secretion of matrix-degrading MMPs facilitated angiogenesis and VEGF stimulate MMPs expression in tendons. MMPs are the key players in physiological and pathological tendon ECM remodeling [24]. We observed a reduction of MMP1 mRNA expression in groups that have received LLLT in initial phase of treatment.

A study demonstrated that the 532 and 1,064 nm laser can markedly downregulate the expression of MMP1 and MMP2 in vitro and the infrared light was more effective than the red laser in inhibiting MMPs expression [28].

Another metalloproteinase which we evaluated was the MMP13; we found an augment in mRNA expression of MMP13 in all groups, indicating that the LLLT might modulate this proliferation. MMP13 is considered predominantly as an interstitial collagenase in the rodent [29]. An increased MMP levels might induce ECM degradation and remodeling and weaken biomechanical resistance of tendons [30, 31].

Anyway, the present study supports the thinking of LLLT as a promisor anti-inflammatory therapy.

Conclusions

We conclude that LLLT with parameters employed in the present study was able to modulate IL-10, VEGF, MMP1, and MMP13 mRNA gene expression both in AP than in CP inflammation. However, further studies are needed to establish optimal parameters for LLLT.

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