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ORIGINAL ARTICLE

Low-level laser therapy (LLLT; 780 nm) acts differently on mRNA expression of anti- and pro-inflammatory mediators in an experimental model of collagenase-induced tendinitis in rat

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Abstract Low-level laser therapy (LLLT) has been found to produce anti-inflammatory effects in a variety of disorders. Tendinopathies are directly related to unbalance in expression of pro- and anti-inflammatory cytokines which are responsible by degeneration process of tendinocytes. In the current study, we decided to investigate if LLLT could reduce mRNA expression for TNF-α, IL-1β, IL-6, TGF-β cytokines, and COX-2 enzyme. Forty-two male Wistar rats were divided randomly in seven groups, and tendinitis was induced with a collagenase intratendinea injection. The mRNA expression was evaluated by real-time PCR in 7th and 14th days after tendinitis. LLLT irradiation with wavelength of 780 nm required for 75 s with a dose of 7.7 J/cm^2 was administered in distinct moments: 12 h and 7 days post tendinitis. At the 12 h after tendinitis, the animals were irradiated once in intercalate days until the 7th or 14th day in and them the animals were killed, respectively. In other series, 7 days after tendinitis, the animals were irradiated once in intercalated days until the 14th day and then the animals were killed. LLLT in both acute and chronic phases decreased IL-6, COX-2, and TGF-β expression after tendinitis, respectively, when compared to tendinitis groups: IL-6, COX-2, and TGF-β. The LLLT not altered IL-1β expression in any time, but reduced the TNF-α expression; however, only at chronic phase. We conclude that LLLT administered with this protocol reduces one of features of tendinopathies that is mRNA expression for pro-inflammatory mediators.

Keywords LLLT · Tendinitis · Inflammatory mediators · RT-PCR · Rat

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Introduction

Tendinopathy (formerly known as tendinitis) is a common problem among athletes and workers [1, 2] and constitutes a high proportion of referrals to rheumatologists [3]. Tendinopathy can be disabling and frequently results in lost productivity, reduced physical activity, and early retirement from sports or labor [4–6]. Despite the prevalence and recalcitrant nature of tendinopathy, its pathogenesis remains poorly understood, since few studies have examined its earliest development [7]. 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 [8–11].

Inflammatory mediators can be produced by all cell types existing in the body with the purpose of regulating immune and inflammatory responses. There are a large

variety of cytokines that have pro- or anti-inflammatory activities. These proteins are regulators of inflammatory process in sense of counterbalancing the actions between anti- and pro-inflammatory mediators [12–14]. However, the tendinitis, like any inflammatory process, induces a disturbance in this immune balance which is characterized by significant production of cytokines in tissue injured. Regarding to tendinitis, the tendinocytes produces diverse chemical mediators. Moreover, some authors have reported that the symptoms of tendinitis can be driven to pro-inflammatory cytokine, as for instance TNF-α, and the IL-1β, IL-6 pro-inflammatory interleukins [15–18]. During the inflammatory process of tendinitis, the metabolites derived from enzyme cyclooxygenase-2 (COX-2) also contribute for development and maintaining of inflammation signals, principally the products of the isoforms COX-2, classified as inducible due fact that it arises in situations of tissue trauma or inflammation [19].

Tendon damage is a complex and highly regulated process that is initiated, sustained, and eventually terminated by a large number and variety of molecules [20]. Growth factors represent one of the most important of the molecular families that markedly up-regulated following tendon injury and are active at multiple stages of the healing process [21]. TGF-β is also active during inflammation, and has a variety of effects including the regulation of cellular migration and proliferation, and fibronectin binding interactions [22].

A variety of treatments is currently used or has been trialed, including steroid injections [23], ultrasound [24], nonsteroidal anti-inflammatory drugs [25], hyperbaric oxygen therapy [26], and extracorporeal shock wave therapy [27]; however, complete functional recovery of the damaged tissue is rarely seen. Although the role of inflammation is still debated, it has long been known that tendinopathies are primarily degenerative conditions—there is usually an absence of inflammatory cells in or around the lesion [25]. Consequently, it should be no surprise that treatment with anti-inflammatory drugs showed little benefit in controlled trials [28] In fact, there is remarkably little evidence that any conventional therapies are effective [29].

Low-level laser therapy (LLLT) incorporates visible and near-infrared irradiation with monochromatic or narrow-band wavelengths (600–1,000 nm). Clinical use of LLLT has been suggested for reduction of pain and morning stiffness in rheumatoid arthritis in a systematic review from the Cochrane Library [30]. Moreover, Demir and coworkers [31] demonstrated that LLLT relieved the acute phase of tendinitis and it increased either the level of hydroxyproline as the amount of fibroblast. These same authors found that LLLT increased collagen synthesis during the proliferative phase. Still on this context, Salate and colleagues [32] investigating the laser therapy effect on angiogenesis found that LLLT promotes good neovascularization. Although it (the cellular signaling mechanisms of laser therapy on damaged tissue) not only in experimental models but also clinical practice is poorly understood, it is believed to involve an anti-inflammatory action [33, 34]. To our knowledge, there are few (if any) studies investigating whether LLLT affects cytokine expression in vivo.

Herein, the present study was designed to investigate if the LLLT modulates the acute and chronic phase of collagenase-induced tendinitis in rat by interfering in mRNA expression for pro- and anti-inflammatory mediators.

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 42 male Wistar rats (180–200 g), with food and water ad libitum provided by 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 10% and xylazine 2%, both at a concentration of 0.1 ml.100 g^{-1} body weight. After the tendon cleansing (with 70% alcohol), the tendinitis was experimentally induced with a unique intratendinous injection of 0.1 ml of collagenase (1 $mg.ml^{-1}$; SIGMA, C6885) in the right Achilles tendon. Collagenase (1 mg) was dissolved in a sterile phosphate buffered saline (1 ml). Each rat was challenged with only a unique intratendinous injection of collagenase during all the experimental period. In order to determine the groups, the animals that were irradiated 12 h or 7 days after collagenase challenge are named as acute phase (AP) and chronic phase (CP), respectively. The animals were killed 7 or 14 days after induction of tendinitis.

Experimental groups The experimental groups consisted of 42 male Wistar rats randomly allocated into six groups: animals challenged with vehicle were considered as saline; animals challenged with laser (780 nm) were considered as laser; animals challenged with collagenase and killed in acute phase were named as AP; animals challenged with collagenase and killed in chronic phase were considered as CP; animals challenged with collagenase and treated with

laser (780 nm) in acute phase were named as AT; animals challenged with collagenase and treated with laser (780 nm) in chronic phase were named as CT.

Laser work The equipment used herein was a laser (MMoptics model) with wavelength of 780 nm, power of 22 mW, and energy density of 7.7 J/cm^2. The laser was applied in contact with the tendon of right calcaneus for 75 s and the irradiated area was the same size as the laser spot (0.2 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 experiments started, the laser equipment was checked with a power checker (13PEM001/J, Melles Griot, the Netherlands, Didara).

Therapeutical strategy The animals were irradiated according to phases of inflammation. Regarding the acute phase (AT_7), the treatment with laser was initiated 12 h after induction of tendinitis; in this condition the animals were irradiated on the second, fourth, and sixth days after induction of tendinitis, and then euthanized on the seventh day. In the acute phase, named as AT_{14}, the laser treatment was initiated equally to AT_7; however, the animals were irradiated on the first, third, fifth, ninth, eleventh, and thirtieth days after induction of tendinitis, and then euthanized on the fourth day. The group named CT_{14} received laser initially seven days after tendinitis induction; afterwards, the animals were irradiated on the seventh, ninth, eleventh, and third days after tendinitis, and then euthanized on the fourth day. The temporal profile of treatment with laser was designed in Table 1.

RNA extraction TNF-α, IL-1β, IL-6, COX-2, and TGF-β mRNA expression evaluation were performed from the animal groups mentioned above. The animals were killed by decapitation and the tendons were quickly dissected and frozen in dry ice, before being stored at –80°C. Thawed tissues were homogenized in 1 ml of TRIzol reagent (Gibco BRL, Gaithersburg, MD) and total RNA was isolated according 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 (RT) 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 reaction 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 triplicate for each data point. IL-1, IL-6, TNF-α, COX-2, and TGF-β mRNA abundance were quantified as a relative value compared to 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: rat IL-1 (GenBankTM accession number M98820), forward primer 5′- CACCTCTCAAGCAGAG CACAG-3′and reverse primer 5′- GGGTTCCATGGTGA AGTCAAC-3′; rat TNF (GenBankTM accession number X66539) forward primer 5′- AAATGGGCTCCCTCTA TCAGTTC-3′ and reverse primer 5′- TCTGCTTG GTGGTT TGCTACGAC- 3′; rat COX-2 (GenBankTM accession number J00691) forward primer 5′- TGTATGC TACCATCTGGCTTCGG- 3′and reverse primer 5′- GTTTGGAACAG TCGCTCGTCATC-3′; rat TGF-β1 (GenBankTM accession number NM_021578) forward primer 5′- TGGCGTTACCTTGGTAACC-3′and reverse primer 5′- GGTGTTG AGCCCTTTCCAG-3′; rat IL-6 (GenBankTM accession number E02522) forward primer 5′- TCCTACCCCAACTTCCAATGCTC-3′ and reverse primer 5′- TTGGA TGGTCTTGGTCCTTAGCC-3′. One microliter of RT reaction was used for real-time PCR.

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

Table 1 Temporal profile of tendinitis induction and laser treatment

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

values of the samples were determined by subtracting the average C_t value of IL-1β, IL-6, TNF-α, COX-2, and TGF-β 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 $2^{-\Delta Ct}$ 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 TNF-α, IL-1β, IL-6, COX-2, and TGF-β were obtained from R & D Systems (Minneapolis, MN, USA).

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 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

Effect of LLLT on TNF-α mRNA expression in rat tendon

Figure 1 shows the effect of the laser in different periods of treatment (12 h or 7 days after induction of tendinitis) on mRNA expression for TNF-α. Figure 1a shows the experiments performed in the acute phase 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 TNF-α mRNA expression between the saline group and group challenged with collagenase and killed 7 days after induction of tendinitis (AP_7). When we analyzed the TNF-α expression in group AP_7 and compared it to animals from the group challenged with collagenase and treated with laser (AT_7) it is possible to observe that LLLT did not alter the expression of mRNA TNF-α. Figure 1b is referent to experiments performed in the chronic phase of laser treatment, which is referent to treatment initiated 7 days after tendinitis induction. As in the acute phase, there is an increase of TNF-α expression in groups challenged with collagenase in comparison to the saline group; both groups were killed 14 days after tendinitis induction. Curiously, the LLLT was efficient only in chronic treatment when compared to animals with tendinitis but not irradiated.

Effect of LLLT on IL-β α mRNA expression in rat tendon

In Fig. 2, it is possible to verify that there is no difference between groups with saline and the groups challenged with tendinitis and killed 7 (Fig. 2a) or 14 (Fig. 2b) days after induction of tendinitis. Similarly, the IL-1β mRNA expression was not different in the group of animals challenged with collagenase and treated with laser either in acute phase as chronic phase.

Effect of LLLT on IL-6 α mRNA expression in rat tendon

Figure 3 represents the LLLT effect on IL-6 mRNA expression. In these experiments, the IL-6 expression was significantly increased after collagenase injection when compared to animals from the saline groups killed either on the seventh or fourteenth day. When compared to the IL-6 expression between groups challenged with collagenase (AP_7 and AP_{14}) and those treated with laser and killed 7 or 14 days after induction of tendinitis it is possible to observe that the laser therapy was markedly efficient in reducing the IL-6 mRNA expression. Similar results were obtained when comparing the IL-6 expression between groups challenged

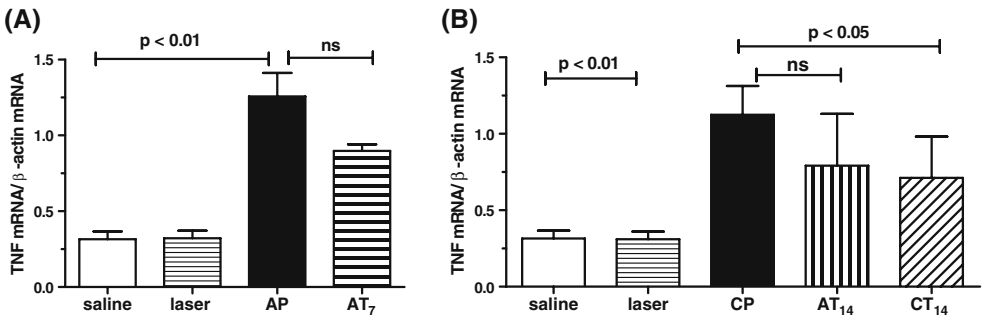

Fig. 1 LLLT on TNF-α mRNA expression in rat tendon. The rats were challenge with collagenase (1 $mg.ml^{-1}$) and treated with laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) post-challenge. Seven and 14 days after the rats were killed, TNF-α expression was measured by real-time PCR. Groups: saline – 7 days, AT_7-treated group 7 days, AT_{14} – group treated 14 days, CT_{14}-treated group from day 7 to day 14. *ns* not significant; a **p* value< 0.05 was considered significant

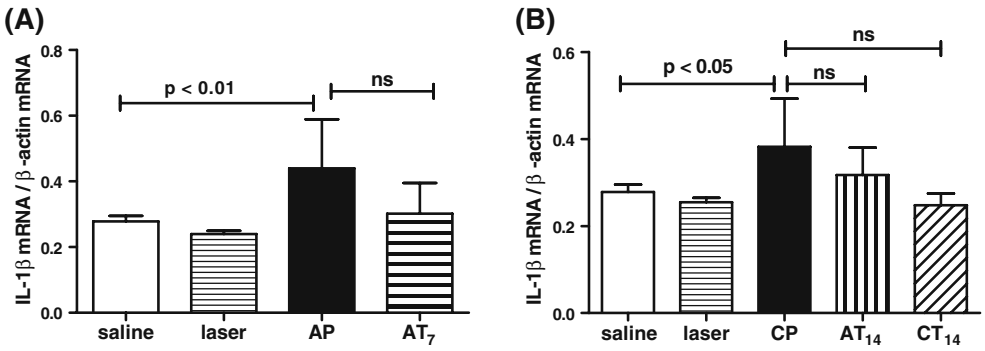

Fig. 2 LLLT on IL-1β mRNA expression in rat tendon. The rats were challenged with collagenase (1 mg.ml^{-1}) and treated with a laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) post-challenge. Seven and 14 days after the rats were killed, IL-1β expression was measured by real-time PCR. Groups: saline - 7 days, AT_7- treated group 7 days, AT_{14} – group treated 14 days, CT_{14}-treated group from day 7 to day 14. *ns* not significant; a **p* value<0.05 was considered significant

with collagenase and challenged with collagenase but treated with laser either acutely (AT_{14}) as chronically (CT_{14}).

Effect of LLLT on COX-2 mRNA expression in rat tendon

Figure 4 shows the participation of COX-2 in collagenase-induced tendinitis and the LLLT effect on this pro-inflammatory enzyme. The results demonstrated that in both groups treated at the period of 7 (AT_7) and 14 (AT_{14}) days the laser reduced profoundly the mRNA expression for COX-2 when compared to the groups of animals challenged with collagenase but not treated with laser therapy, AP_7 and AP_{14}, respectively.

Effect of LLLT on TGF-β mRNA expression in rat tendon

The effect of LLLT on TGF-β expression is illustrated in Fig. 5. These data showed that the LLLT reduced the TGF-β mRNA expression either in the group treated during the period of 7 (AT_7) and 14 (AT_{14}) days when compared to groups AP_7 and AP_{14}, respectively. Similarly, the LLLT was also efficient in reducing the TGF-β expression in the chronic phase of treatment in comparison to animals from group CP_{14}.

Discussion

Soft-tissue disorders are the third most common rheumatologic condition in the UK, with a reported prevalence of 18 cases per 1,000 people [35]. These disorders, which primarily affect tendons, are the main reasons for a musculoskeletal consultation with a general practitioner, and comprised 30% of all such consultations in a 1-year study [36]. This is probably an under-estimate of the scale of the problem, because only 40% of elderly individuals (over 70 years of age) with shoulder pain seek treatment [37]. Although many soft-tissue problems are treated by a general practitioner, often with NSAIDs, corticosteroid injection or physiotherapy, a substantial proportion of new

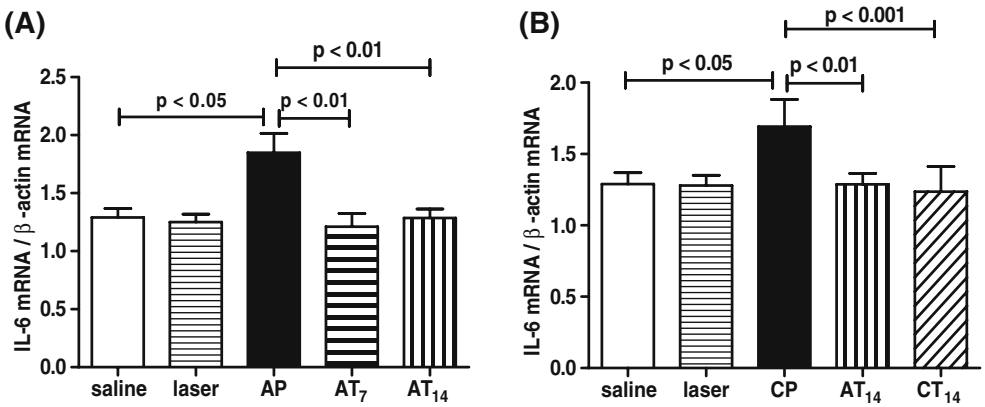

Fig. 3 LLLT on IL-6 mRNA expression in rat tendon. The rats were challenge with collagenase (1 mg.ml^{-1}) and treated with laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) post-challenge. Seven and 14 days after the rats were killed, IL-6 expression was measured by real-time PCR. Groups: saline - 7 days, AT_7-treated group 7 days, AT_{14} – group treated 14 days, CT_{14}-treated group from day 7 to day 14. *ns* not significant; a **p* value<0.05 was considered significant

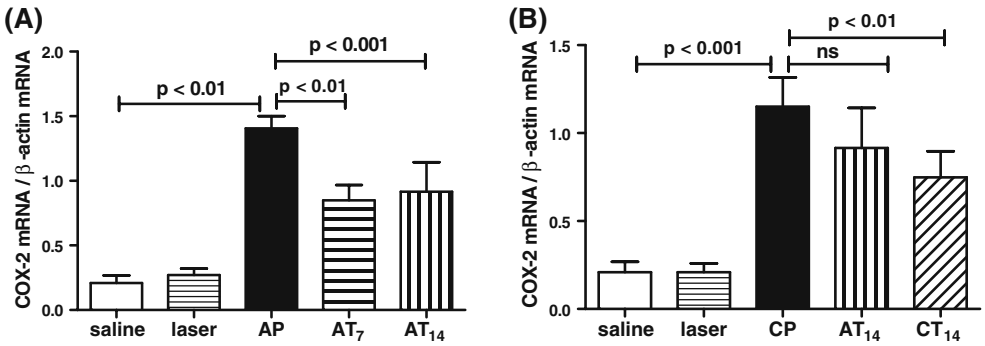

Fig. 4 LLLT on COX-2 mRNA expression in rat tendon. The rats were challenge with collagenase (1 mg.ml^{-1}) and treated with laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) post-challenge. Seven and 14 days after the rats were killed, COX-2 expression was measured by real-time PCR. Groups: saline - 7 days, AT_7- treated group 7 days, AT_{14} – group treated 14 days, CT_{14}-treated group from day 7 to day 14. *ns* not significant; a **p* value<0.05 was considered significant

patient consultations with rheumatologists are for soft-tissue rheumatism. Secondary referral rates vary widely, but one study reported that 17% of new patients seen in a rheumatology clinic had soft-tissue complaints [38].

A variety of treatments is currently used or has been trialed, including steroid injections [23], ultrasound [24], nonsteroidal anti-inflammatory drugs [25], hyperbaric oxygen therapy [26], and extracorporeal shock wave therapy [27]; however, complete functional recovery of the damaged tissue is rarely seen. Thus, from purpose of looking for alternative therapies that would presents a significant degree of efficacy, safe, absence of side-effects and low cost, the laser therapy has all characteristics. However, studies focuses in which the cellular signaling that trigger the LLLT anti-inflammatory action are necessaries in order to potentiate the LLLT effect [39, 40].

Herein we investigated the inflammation using a model in which the tendon of rat was damage with injection collagenase. The model of tendinitis we used is less invasive. Recently, Xavier and coworkers [41] showed that phototherapy reduced significantly the inflammatory influx and the mRNA expression to IL-1β, IL-6, and TNF-α in acute phase as well as in chronic phase. On the contrary, some authors have investigated the process of repairing tendinitis induced after total tenotomy. Using different wavelengths (685 and 830 nm) and fluences (3 and 10 J/cm^2), Carrinho and coworkers [32] demonstrated that both wavelengths and fluences used were efficient at accelerating the healing process of Achilles tendon post-tenotomy, particularly after the 685-nm laser irradiation, at 3 J/cm^2. It suggest the existence of a specific wavelength associated with a dose-dependency relation. Fillipin and colleagues [33] showed that the administration of LLLT for 14 or 21 days markedly alleviated histological abnormalities, reduced collagen concentration, and prevented oxidative stress in an experimental model of Achilles tendon injury induced by a single impact trauma. In rats, Dogan and coworkers [42] analyzed the histological alterations of tendon in a model of tenotomy and achilloplasty. Regarding the tendinopathies model, Koeke and coworkers [43] studied the deposition of collagen fibers in a model of tenotomy. Thus, the results described above evidence that

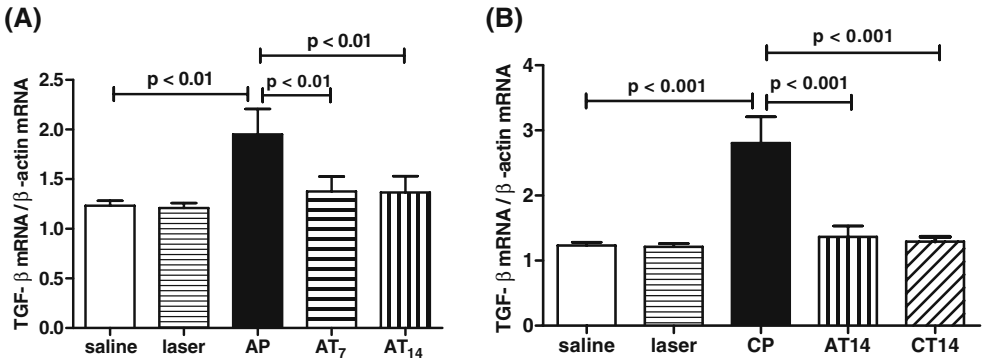

Fig. 5 LLLT on TGF-β mRNA expression in rat tendon. The rats were challenge with collagenase (1 mg.ml^{-1}) and treated with laser (780 nm) at an angle of 90° to the surface of tissue area at 12 h (**a**) or 7 days (**b**) post-challenge. Seven and 14 days after the rats were killed, TGF-β expression was measured by real-time PCR. Groups: saline - 7 days, AT_7-treated group 7 days, AT_{14} – group treated 14 days, CT_{14}-treated group from day 7 to day 14. *ns* not significant; a **p* value< 0.05 was considered significant

the choice of experimental model for induction of tendinopathy is based on which degree of lesion will be studied. Although of differences between animal species, the experimental models in which the method provoke tendon lesion by tenotomy (partial or total) present characteristics very similar to condition that occurs in human tendon lesions. Despite it is important notice that the collagenase-induce tendinitis present similar alterations those induced by experimental models with techniques more invasive. Considering that in the present manuscript the idea was to investigate the LLLT effect on inflammatory mediators without an analysis of morphological alterations, the collagenase-induced tendinitis model used herein is a trustworthy because it is reproducible. The model of tendinitis induced by collagenase (unique injection) also warrants the reliability to study morphological alterations.

The use of laser therapy comes increasing so much in clinical practice in last year's. It is a therapeutic modality commonly used for musculoskeletal disorders by it capacity of attenuating the symptoms of inflammation [44]. In fact, diverse types of lasers capable of inducing biomodulation have been studied [29]. Some authors have reported that lasers with a wavelength of 904 nm promote the relief of inflammation and increase collagen synthesis in different models of experimental tendinitis [31, 34]. The laser chosen here was based on the fact that it is considered as near-red in the electromagnetic spectrum. Moreover, there are a few studies using these parameters.

In the present manuscript we evaluated the efficacy of laser specifically on pro- and anti-inflammatory mediators. Naturally that a study in which the inflammation signals was investigated also are interesting; however the most authors that study the LLLT effect on muscle-skeletal disorders do not make an approach about which the cellular action mechanism responsible by anti-inflammatory activity. Herein, we did not explain exactly by which via the laser irradiation is able to alter the concentration of inflammatory mediators studied. Moreover, TGF-β, an anti-inflammatory mediator responsible for maintaining the immunological balance of inflammatory response was also investigated.

Regarding the pro-inflammatory cytokines, the LLLT was efficient in reducing almost all in groups of animals challenged with collagenase. Albertini and coworkers showed by the first time that LLLT is able to reduce the mRNA expression for COX-2 [45], in addition to TNF-α, IL-1β, and IL-6 [46]. Our findings are in accordance with this. A unique exception was the IL-1β. The LLLT did not reduce this cytokine in either the acute phase or the chronic phase during the different periods of treatment with laser. Asundi and Rempel [47] showed the participation of IL-1β protein in lesions produced in a model of cyclically loaded tendons. Although it occurred with a distinct experimental model, the importance is that it is also a model for tendinopathy. This result was curious because the LLLT significantly reduced the TNF-α mRNA expression. TNF-α is a pro-inflammatory cytokine derived from monocytes/macrophages [48]. This protein is considered one of the most important triggers of the acute inflammatory phase [49]. Moreover it is well known that TNF-α, a pro-inflammatory mediator important in development of inflammation, acts secondarily by stimulating the production of IL-1β in diverse biologic systems. In fact, Garbacki and colleagues [50] demonstrated that TNF-α and IL-1β act synergically in different inflammatory conditions and are able to modulate the inflammatory process through stimulating cellular migration and the release of chemical mediators into tissue damage. However, is important that TNF-α expression was reduced only in the chronic phase of treatment with the laser. Maybe it would to be some technical error during the measured of TNF-α; however, this hypothesis was discarded because there was significant difference in mRNA expression for all cytokines and COX-2 between the groups with tendinitis and the animals that received saline alone.

An excellent result was the effect of laser treatment on IL-6 mRNA expression. The chronic treatment with LLLT reduced the expression of cytokine in both phases (acute and chronic) of tendinitis. It is an important result for LLLT due the fact that IL-6 has both pro- and anti-inflammatory properties [51]. The IL-6 play an important role in the initial phase of healing due to the fact that they act on phagocytes migration and other inflammatory cells into inflamed tendon. After the process of inflammatory cell migration, it gives initial the proliferative phase characterized by presence of fibroblasts that contribute to tissue repair. However, it is important to consider that the increasing exacerbated activity in fibroblasts can also compromise the process of repairing. Although our results showed the participation of IL-6 expression 7 days after the induction of tendinitis, this pro-inflammatory cytokine has a key role in the chronic phase in almost all tendinopathies. Our results are in accordance with those performed by [52]. Histological studies are fundamental for showing the effect of laser treatment on damaged tendons. Maybe that improve in healing process of tendon caused by laser can be the result of laser effect in decreasing the IL-6 mRNA expression. In fact, we recently found that LLLT used with the same dosimetry adopted herein was able to attenuate the alterations in tendon after collagenase challenge (data not shown). Moreover, Oliveira and coworkers [53] applied lasers in a different dosing regimen on the process of tissue repair in partial lesion calcaneal tendon. These authors evidenced that LLLT was effective in the improvement of collagen fiber organization of the calcaneal tendon after undergoing a partial lesion. This result is in accordance

with most of the studies that reported the LLLT effect on experimental tendinopathy. In the present manuscript, the LLLT effect on wound healing was not evaluated. Most of the studies that investigate the LLLT effect place more emphasis on the analyses of wound healing and pain; obviously both are important features of tendinopathy and should be controlled, thus is not trivial to compare the effect of laser therapy in the present work with those obtained by authors mentioned above, due to the fact that we analyzed the interference of LLLT on inflammatory mediators. However, it is evident that a better use of LLLT on tendinopathy is based on the accumulating evidence regarding the beneficial effect and action mechanism of this therapy.

Another important protein involved in skeletal muscle disorders is the COX-2 enzyme [54]. The increased activity of COX-2 produces as metabolites a great variety of eicosanoids, among them the prostaglandins. The prostaglandins are particularly important in tendinopathies. Campana and coworkers [55] suggest that the inflammatory response can return to normal values or attenuated by acting of LLLT on COX-2. In fact, [56] demonstrated the participation of COX-2 in tendinocytes from rat Achilles tendon, since the treatment with celecoxib, a COX-2 specific inhibitor, inhibited the tendon cell migration and proliferation. Similar results were found using ibuprofen, a nonsteroidal anti-inflammatory drug [57]. Our results demonstrated that COX-2 seems to be related either acute as chronic phase of collagenase-induced tendinitis. Considering the importance of this enzyme for tendinopathies, our results show that LLLT reduces the COX-2 mRNA expression in all periods studied. These results are in accordance with those reported by [43] in which the laser therapy markedly reduced the COX-2 expression in paw edema induced by carrageenan. In addition, these authors found that LLLT also reduced the cell migration into inflamed paw [45]. Although the migration of inflammatory cells is not principally characteristic of collagenase-induced tendinitis, this LLLT effect can be considered beneficial. However, these results showed that the inhibition of COX-2, or of its own metabolic products by LLLT, reinforce the idea that the laser therapy acts essentially as a classic anti-inflammatory drug, as for instance dexamethasone, which is a potent inhibitor of protein synthesis. For reinforcing, Bjordal and colleagues [58] demonstrated that LLLT reduces not only the inflammatory process but also improves tissue repair in a manner more efficient than corticosteroids.

Regarding to inflammatory mediators, is important to consider that the RT-PCR technique permits the analysis of mRNA expression. It is not mean that the protein synthesis can be reduced by LLLT; anyway the present manuscript give support for thinking that the LLLT is a promising anti-inflammatory tool which can work combined with drugs of conventional treatment, principally due fact that laser do not present side-effects.

Regarding the anti-inflammatory mediator, transforming growth factor beta (TGF-β) is likely to be an important factor in tendon pathology since it plays a major role in tissue repair [59]. TGF-β has been shown to be upregulated in response to tissue damage in a number of connective tissue pathologies including tendinopathies [60]. One of its most important roles is the promotion of new matrix synthesis [61–64]. There are three mammalian isoforms of TGF-β (β1, β2, and β3) which are differently expressed in a variety of connective tissues, among them cartilage [65]. Moreover, TGF-β presents anti-inflammatory action on vascular smooth cells by inhibiting nitric oxide synthesis, which prevents the hypotensive shock induced by lipopolysaccharide from Gram-negative bacteria [66]. Our results demonstrated that collagenase-induced tendinitis raises the TGF-β mRNA expression when compared to saline groups. Maybe this could be explained by taking into consideration that the TGF-β is an anti-inflammatory cytokine; thus if collagenase provokes an increase in the TGF-β expression to values significantly higher than AP7, AP14, or CP14 groups, it is probably a response of TGF-β in trying to counterbalance the exacerbation of mRNA expressions for pro-inflammatory mediators. Our results corroborated with findings of Fenwick and colleagues [67]. Moreover, Opal and DePalo [51] reported that TGF-β has a pivotal role in impairing the synthesis of pro-inflammatory cytokines. Our data showed that LLLT reduced the mRNA expression for TNF-α, IL-1β, IL-6, and COX-2. If mRNA expression for these pro-inflammatory cytokine was reduced by LLLT, then it does not make sense that the tendinocytes continue to produce high levels of TGF-β. In fact, LLLT was extremely significant in reducing TGF-β mRNA expression in both periods of treatment with laser. Taking into consideration that the effect of LLLT does not seem to be specific for determined cytokines, the laser acts globally, i.e., the reduction of TGF-β induced by the laser is an indirect response to reduction of pro-inflammatory cytokines. In other viewpoint, the laser effect could be seen as a direct action on TGF-β expression. Pharmacological studies can give support to the hypothesis of a direct action of LLLT on TGF-β mRNA expression. Considering that the TGF-β is a cytokine responsible for the primary regulation of the inflammatory response, it is reasonable to suggest that mRNA expression for this cytokine arises either in the acute or chronic phase of tendinitis.

By the end, our results demonstrated that LLLT modulates the mRNA expression for pro- and anti-inflammatory cytokines in the model of collagenase-induced tendinitis in rat. Laser therapy was effective in reducing all pro-inflammatory cytokines, except for IL-1β. The LLLT effect occurred more significantly on IL-6

mRNA expression and TNF-α. The effect on TGF-β expression was considered an indirect action of anti-inflammatory activity of LLLT.

Conclusions

Taken together, our results show that LLLT has the competence to push down the expression of pro-inflammatory mediators in a model of collagenase-induced tendinitis in two moments distinct; in acute phase and in chronic phase of laser treatment. This makes LLLT a therapeutic alternative efficient in the treatment of tendinopathies and opens the window for studies focused on the use of LLLT combined with pharmacological therapy.

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References

1. Khan KM, Cook JL, Kannus P, Maffulli N, Bonar SF (2002) Time to abandon the "tendinitis" myth. BMJ 324:626–627
2. Corps AN, Robinson AH, Movin T, Costa ML, Ireland DC, Hazelman BL et al (2004) Versican splice variant messenger RNA expression in normal human Achilles tendon and tendinopathies. Rheumatol (Oxf Print) 43:969–972
3. Rees JD, Wilson AM, Wolman RL (2006) Current concepts in the management of tendon disorders. Rheumatol (Oxf Print) 45:508–521
4. Smidt N, Lewis M, Hay EM, Van der Windt DA, Bouter LM, Croft P (2005) A comparison of two primary care trials on tennis elbow: issues of external validity. Ann Rheum Dis 64:1406–1409
5. Coleman BD, Khan KM, Maffulli N, Cook JL, Wark JD (2000) for the Victorian Institute of Sport Tendon Study Group. Studies of surgical outcome after patellar tendinopathy: clinical significance of methodological deficiencies and guidelines for future studies. Scand J Med Sci Sports 10:2–11
6. Tallon C, Coleman BD, Khan KM, Maffulli N (2001) Outcome of surgery for chronic Achilles tendinopathy: a critical review. Am J Sports Med 29:315–320
7. Fredberg U (2004) Tendinopathy—tendinitis or tendinosis? The question is still open. Scand J Med Sci Sports 14:270–272
8. Jones GC, Corps AN, Pennington CJ, Clark IM, Edwards DR, Bradley MM et al (2006) Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human Achilles tendon. Arthritis Rheum 54:832–842
9. Puddu G, Ippolito E, Postacchini F (1976) A classification of Achilles tendon disease. Am J Sports Med 4:145–150
10. Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis. Ann Rheum Dis 53:367–376
11. Riley GP, Harrall RL, Constant CR, Chard MD, Cawston TE, Hazleman BL (1994) Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. Ann Rheum Dis 53:359–366
12. Riley G (2008) Tendinopathy—from basic science to treatment. Nature 4(2):82–89
13. Stone D, Green C, Rao U, Aizawa H, Yamaji T, Niyibizi C et al (1999) Cytokine-induced tendinitis: a preliminary study in rabbits. J Orthop Res 17:168–177
14. Chiu D, Ma K, Scott A, Duronio V (2005) Acute activation of Erk1/Erk2 and protein kinase B/akt proceed by independent pathways in multiple cell types. FEBS J 272:4372–4384
15. Hosaka Y, Kirisawa R, Ueda H, Yamaguchi M, Takehana K (2005) Differences in tumor necrosis factor (TNF)alpha and TNF receptor-1-mediated intracellular signaling factors in normal, inflamed and scar-formed horse tendons. J Vet Med Sci 67 (10):985–991
16. Fredberg U, Ostgaard R (2008) Effect of ultrasound-guided, peritendinous injections of adalimumab and anakinra in chronic Achilles tendinopathy: a pilot study. Scand J Med Sci Sports. 2008, May 19
17. Thampatty BP, Li H, Im HJ, Wang JH (2007) EP4 receptor regulates collagen type-I, MMP-1, and MMP-3 gene expression in human tendon fibroblasts in response to IL-1 beta treatment. Gene 386(1–2):154–161
18. Sivakumar B, Akhavani MA, Winlove CP, Taylor PC, Paleolog EM, Kang N (2008) Synovial hypoxia as a cause of tendon rupture in rheumatoid arthritis. J Hand Surg Am 33(1):49–58
19. Wang JH, Jia F, Yang G, Yang S (2003) Campbell BH, Stone D, Woo SL. Cyclic mechanical stretching of human tendon fibroblasts increases the production of prostaglandin E2 and levels of cyclooxygenase expression: a novel in vitro model study. Connect Tissue Res 44(3–4):128–133
20. Longo UG, Ronga M, Maffulli N (2009) Achilles tendinopathy. Sports Med Arthrosc Rev 17(2):112–126
21. Corps AN, Jones GC, Harrall RL, Curry VA, Hazleman BL, Riley GP (2008) The regulation of aggrecanase ADAMTS-4 expression in human Achilles tendon and tendon-derived cells. Matrix Biol 27(5):393–401
22. Moustakas A, Heldin CH (2009) The regulation of TGFbeta signal transduction. Development 136(22):3699–3714
23. Triantafillopoulos IK, Banes AJ, Bowman KF Jr, Maloney M, Garrett WE Jr, Karas SG (2004) Nandrolone decanoate and load increase remodeling and strength in human supraspinatus bio-artificial tendons. Am J Sports Med 32:934–943
24. Downing DS, Weinstein A (1986) Ultrasound therapy of subacromial bursitis. A double blind trial. Phys Ther 66:194–199
25. McLauchlan GJ, Handoll HH (2001) Interventions for treating acute and chronic Achilles tendinitis. Cochrane Database Syst Rev CD000232, 2001
26. Hsu RW, Hsu WH, Tai CL, Lee KF (2004) Effect of hyperbaric oxygen therapy on patellar tendinopathy in a rabbit model. J Trauma 57:1060–1064
27. Wiley P (2002) Low energy extracorporeal shock-wave treatment for tendinitis of the supraspinatus (Abstract). Clin J Sport Med 12:262
28. Almekinders LC, Temple JD (1998) Etiology, diagnosis, and treatment of tendonitis: an analysis of the literature. Med Sci Sports Exerc 30:1183–1190
29. Bjordal JM, Lopes-Martins RAB, Iversen VV (2006) A randomised, placebo controlled trial of low level laser therapy for activated Achilles tendinitis with microdialysis measurement of peritendinous prostaglandin E2 concentrations. Br J Sports Med 40:76–80
30. Demir H, Yaray S, Kirnap M et al (2004) Comparison of the effects of laser and ultrasound treatments on experimental wound healing in rats. J Rehabil Res Dev 41(5):721–728
31. Salate ACB, Barbosa G, Gaspar P et al (2005) Effect of In-Ga-Al-P diode laser irradiation on angiogenesis in partial ruptures of Achilles tendon in rats. Photomed Laser Surg 23(5):470–475

32. Carrinho PM, Renno ACM, Koeke P et al (2006) Comparative study using 685-nm and 830-nm lasers in the tissue repair of tenotomized tendons in the mouse. Photomed Laser Surg 24 (6):754–758
33. Fillipin LI, Mauriz JL, Vedovelli K et al (2005) Low-level laser therapy (LLLT) prevents oxidative stress and reduces fibrosis in rat traumatized Achilles tendon. Lasers Surg Med 37:293–300
34. Badley EM, Tennant A (1993) Impact of disablement due to rheumatic disorders in a British population: estimates of severity and prevalence from the Calderdale Rheumatic Disablement Survey. Ann Rheum Dis 52:6–13
35. McCormick A et al (1995) Assessing health needs in primary care. Morbidity study from general practice provides another source of information. BMJ 310:1534
36. Chard MD et al (1991) Shoulder disorders in the elderly: a community survey. Arthritis Rheum 34:766–769
37. Bamji AN et al (1990) What do rheumatologists do? A pilot audit study. Br J Rheumatol 29:295–298
38. Tumilty S, Munn J, McDonough S, Hurley DA, Basford JR, Baxter GD (2010) Low level laser treatment of tendinopathy: a systematic review with meta-analysis. Photomed Laser Surg 28 (1):3–16
39. Bjordal JM, Lopes-Martins RA, Joensen J, Couppe C, Ljunggren AE, Stergioulas A, Johnson MI (2008) A systematic review with procedural assessments and meta-analysis of low level laser therapy in lateral elbow tendinopathy (tennis elbow). BMC Musculoskelet Disord 29;9:75
40. Bjordal JM, Johnson MI, Iversen V, Aimbire F, Lopes-Martins RA (2006) Photoradiation in acute pain: a systematic review of possible mechanisms of action and clinical effects in randomized placebo-controlled trials. Photomed Laser Surg 24(2):158–168
41. Xavier M, Murilo Xavier, Débora Rodrigues David, Renato Aparecido de Souza, Arthur Nascimento Arrieiro, Humberto Miranda, Eduardo Tadeu Santana, José Antonio Silva JR, Regiane Albertini. Anti-inflammatory effects of low-level light emitting diode therapy on Achilles tendinitis in rats. Lasers Surg Med 2010 (Epub ahead of print)
42. Dogan A, Korkmaz M, Cengiz N, Kalender AM, Gokalp MA (2009) Biomechanical comparison of Achilles tenotomy and achilloplasty techniques in young rats: an experimental study. J Am Podiatr Med Assoc 99(3):216–222
43. Koeke PU, Parizotto NA, Carrinho PM, Salate AC (2005) Comparative study of the efficacy of the topical application of hydrocortisone, therapeutic ultrasound and phonophoresis on the tissue repair process in rat tendons. Ultrasound Med Biol 31 (3):345–350
44. Brosseau L, Robinson V, Wells G, Debie R, Gam A, Harman K, Morin M, Shea B, Tugwell P (2007) Low level laser therapy (Classes III) for treating osteoarthritis. Cochrane Database Syst Rev 18(1):CD002046
45. Albertini R, Aimbire F, Villaverde AB, Silva JA Jr, Costa MS (2007) COX-2 mRNA expression decreases in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation after low level laser therapy. Inflamm Res 56(6):228–229
46. Albertini R, Villaverde AB, Aimbire F, Bjordal J, Brugnera A, Mittmann J, Silva JA, Costa M (2008) Cytokine mRNA expression is decreased in the subplantar muscle of rat paw subjected to carrageenan-induced inflammation after low-level laser therapy. Photomed Laser Surg 26(1):19–24
47. Asundi KR, Rempel DM (2008) MMP-1, IL-1beta, and COX-2 mRNA expression is modulated by static load in rabbit flexor tendons. Ann Biomed Eng 36(2):237–243
48. Dunican AL, Leuenroth SJ, Grutkoski P et al (2000) TNFalpha-induced suppression of PMN apoptosis is mediated through interleukin-8 production. Shock 14:284–289
49. Aimbire F, Albertini R, Pacheco MT, Castro-Faria-Neto HC, Leonardo PS, Iversen VV, Lopes-Martins RA, Bjordal JM (2006) Low-level laser therapy induces dose-dependent reduction of TNFalpha levels in acute inflammation. Photomed Laser Surg 24(1):33–37
50. Garbacki N, Tits M, Angenot L et al (2004) Inhibitory effects of proanthocyanidins from *Ribes nigrum* leaves on carrageenin acute inflammatory reactions induced in rats BMC. BMC Pharmacol 4 (1):1–25
51. Opal SM, DePalo VA (2000) Anti-inflammatory cytokines. Chest 117:1162–1172
52. Fredberg U, Stengaard-Pedersen K (2008) Chronic tendinopathy tissue pathology, pain mechanisms, and etiology with a special focus on inflammation. Scand J Med Sci Sports 18(1):3–15
53. Oliveira FS, Pinfildi CE, Parizoto NA, Liebano RE, Bossini PS, Garcia EB, Ferreira LM (2009) Effect of low level laser therapy (830 nm) with different therapy regimes on the process of tissue repair in partial lesion calcaneous tendon. Lasers Surg Med 41:271–276
54. O'Connor JP, Lysz T (2008) Celecoxib, NSAIDs and the skeleton. Drugs Today 44(9):693–709
55. Campana V, Moya M, Gavotto A, Juri H, Palma JA (1998) Effects of diclofenac sodium 8 and HeNe laser irradiation on plasmatic fibrinogen levels in inflammatory processes. J Clin Laser Med Surg 16(6):317–320
56. Tsai WC, Hsu CC, Chou SW, Chung CY, Chen J, Pang JH (2007) Effects of celecoxib on migration, proliferation and collagen expression of tendon cells. Connect Tissue Res 48(1):46–51
57. Tsai WC, Hsu CC, Chen CP, Chen MJ, Lin MS, Pang JH (2006) Ibuprofen inhibition of tendon cell migration and down-regulation of paxillin expression. J Orthop Res 24(3):551–558
58. Bjordal JM, Klovning A, Ljunggren AE, Slørdal L (2007) Short-term efficacy of pharmacotherapeutic interventions in osteoarthritic knee pain: ameta-analysis of randomised placebo-controlled trials. Eur J Pain 11(2):125–138
59. Clark RAF, McCoy GA, Folkvord JM, McPherson JM (1997) TGF-beta 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event. J Cell Physiol 170:69–80
60. Shah M, Foreman DM, Ferguson MWJ (1995) Neutralisation of TGF-beta1 and b2 or exogenous addition of TGF-beta3 to cutaneous rat wounds reduces scarring. J Cell Sci 108:985–1002
61. Ignotz RA, Massague J (1986) Transforming growth-factor beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345
62. Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM et al (1986) Transforming growth-factor type-beta rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83:4167–4171
63. Wang X, Smith P, Pu L, Kim YJ, Ko F, Robson MC (1999) Exogenous transforming growth factor beta(2) modulates collagen I and collagen III synthesis in proliferative scar xenografts in nude rats. J Surg Res 87:194–200
64. Fukumura K, Matsunaga S, Yamamoto T, Nagamine T, Ishidou Y, Sakou T (1998) Immunolocalization of transforming growth factor-betas and type I and type II receptors in rat articular cartilage. Anticancer Res 18:4189–4193
65. Matsunaga S, Yamamoto T, Fukumurak K (1999) Temporal and spatial expressions of transforming growth factor betas and their receptors in epiphyseal growth plate. Int J Oncol 14:1063–1067
66. Tedgui A, Mallat Z (2001) Anti-Inflammatory mechanisms in the vascular wall. Circ Res 88:877–887
67. Fenwick SA, Curry V, Harrall RL, Hazleman BL, Hackney R, Riley GP (2001) Expression of transforming growth factor-beta isoforms and their receptors in chronic tendinosis. J Anat 199:231–240