Lasers Med Sci
DOI 10.1007/s10103-016-1918-7

ORIGINAL ARTICLE

Low-level laser therapy modulates pro-inflammatory cytokines after partial tenotomy

Flávia Da Ré Guerra[1] · Cristiano Pedrozo Vieira[2] · Letícia Prado Oliveira[2] · Petrus Pires Marques[3] · Marcos dos Santos Almeida[1] · Edson Rosa Pimentel[2]

Received: 4 May 2015 / Accepted: 1 March 2016

Abstract Tendon injuries give rise to substantial morbidity, and current understanding of the mechanisms involved in tendon injury and repair is limited. This lesion remains a clinical issue because the injury site becomes a region with a high incidence of recurrent rupture and has drawn the attention of researchers. We already demonstrated that low-level laser therapy (LLLT) stimulates the synthesis and organization of collagen I, MMP-9, and MMP-2 and improved the gait recovery of the treated animals. The aim of this study was to evaluate the effects of LLLT in the nitric oxide and cytokines profile during the inflammatory and remodeling phases. Adult male rats were divided into the following groups: G1—intact, G2— injured, G3—injured + LLLT (4 J/cm^2 continuous), G4—injured + LLLT (4 J/cm^2–20 Hz—pulsed laser). According to the analysis, the animals were euthanized on different dates (1, 4, 8, or 15 days after injury). ELISA assay of TNF-α, IL-1β, IL-10, and TGF-β was performed. Western blotting of isoform of nitric oxide synthase (i-NOS) and nitric oxide dosage experiments was conducted. Our results showed that the pulsed LLLT seems to exert an anti-inflammatory effect over injured tendons, with reduction of the release of proinflammatory cytokines, such as TNF-α and the decrease in the i-NOS activity. Thanks to the pain reduction and the facilitation of movement, there was a stimulation in the TGF-β and IL-1β release. In conclusion, we believe that pulsed LLLT worked effectively as a therapy to reestablish the tendon integrity after rupture.

Keywords i-NOS · Inflammatory · TGF-β · IL-1β · TNF-α

Introduction

Tendons are anatomic structures that generally promote the insertion of the muscles into bones [1–3]. However, the force generated by the muscles during intense activity as well as prolonged low-intensity activity can both lead to tendon degeneration and rupture [4].

Studies have shown that 44 % of tendon ruptures occurred during athletic activities [5], but this condition is not only restricted to athletes. In the general population, factors such as age, sex, obesity, or the presence of diseases, such as diabetes and rheumatoid arthritis, appear to be involved in injuries of the Achilles tendon. In the USA, musculoskeletal disorders are very common, it is estimated that approximately 315 million medical visits occur annually because of this type of injury [6].

After such injuries, the tissue undergoes a process of reorganization of the extracellular matrix (ECM) to recover the afflicted area [3, 7]. In all cases of tendon injuries, inflammation is the beginning of the tissue repair process. It is a normal response to reestablish the tissue after injury or infection. Symptoms such as pain, redness, and swelling at the site are characteristics of the inflammatory process [8].

According to Molloy, Wang, and Murrell [9], immediately after an injury, the formation of a clot around the wound

✉ Flávia Da Ré Guerra
dgflavia@yahoo.com.br; flavia.guerra@unifal-mg.edu.br

[1] Department of Anatomy, Institute of Biomedical Science, Federal University of Alfenas – UNIFAL-MG, 37130-000 Alfenas, MG, Brazil

[2] Department of Structural and Functional Biology, Institute of Biology, CP 6109, University of Campinas – UNICAMP, 13083-970 Campinas, SP, Brazil

[3] Department of Biochemistry, Institute of Biomedical Science, Federal University of Alfenas – UNIFAL-MG, 37130-000 Alfenas, MG, Brazil

Published online: 16 March 2016

occurs; then 1 day later, the first battery of growth factors and inflammatory molecules are produced by cells within the blood clot. Four days after the injury, the tissue is invaded by extrinsic cells, followed by a second battery of growth factors that stimulate fibroblast proliferation.

Wounding and inflammation provoke the release of growth factors, cytokines, and nitric oxide (NO) from platelets, polymorphonuclear leukocytes, macrophages, and other inflammatory cells. These substances induce neovascularization and chemotaxis of fibroblasts and tenocytes and stimulate the synthesis of collagen, the main component of the tendon [10].

Tendon lesions remain a clinical issue because the injury site becomes a region with a high incidence of recurrent rupture and have drawn the attention of researchers [11]. Trying to accelerate tendon repair, several physical agents such as ultrasound, electrical stimulation, and low-level laser therapy have shown beneficial effects [12–15]. Among those, low-level laser therapy (LLLT) has been fairly resorted by physiotherapists over the last 20 years, with significant effects, such as an increase in the proliferation of fibroblasts and collagen synthesis [16–18], cutaneous neovascularization [19], and tendon repair [13, 18, 20].

Houreld and colleagues [10] observed that wounded fibroblast cells exposed to 830 nm low-level laser presented a decrease in pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), and irradiation stimulated the NO release due to what appears to be a direct photochemical processes.

Previous studies from our laboratory demonstrated that a daily session using 4 J/cm^2 of pulsed LLLT stimulates the synthesis and organization of collagen I and metalloproteinases 9 and 2 (MMP-9 and MMP-2) and improved the gait recovery of the treated animals [18, 20]. These results suggest that the pulsed LLLT was effective, especially in the acute phase of healing. We believe that the functional result induced by the pulsed LLLT may indicate that pain severity was decreased through modulation of the inflammatory process [17, 21].

Tendon injuries give rise to substantial morbidity, and current understanding of the mechanisms involved in tendon injury and repair is limited. Besides, several studies have shown that the morphological and functional properties of a tendon after injury will never reach those of normal tissue. This loss may be caused by long-term immobilization resulting in the absence of mechanical load [22–24]. A more efficient treatment can reduce the pain and discomfort, effects of long-term immobilization.

Given the complexity of the regeneration and inflammatory processes, our group continued exploring the effects of LLLT, to unveil in more details of the molecular changes that take place in the tendon injuries. The understanding of such aspects may lead to future improvements in laser therapy or combination with other treatments.

Material and methods

Animal care was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and is consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA); the protocol was approved by the ethics Committee on Animal Experiments of the State University of Campinas, SP, Brazil (no. 1921-2).

Experimental groups

In this study, 85 male Wistar/Uni rats were used, with five animals per group per analysis, with a mean age of 60 days and weight ranging from 300 to 350 g. The rats were housed two per cage in a 12-h light–dark cycle at 23 °C, with free access to standard rat chow and water. The animals were divided into the following groups: G1—intact, G2— injured, G3—injured + LLLT (4 J/cm^2 continuous), G4—injured + LLLT (4 J/cm^2–20 Hz—pulsed laser). According to the necessity analysis, the animals were euthanized on different dates (1, 4, 8, or 15 days after injury).

Procedures for partial transection of the tendon

The animals were anesthetized with intraperitoneal injection of ketamine (90 mg/kg) and xylazine (12 mg/kg). After removing the skin, a transverse partial transection was performed in the tension region of the Achilles tendon, located at an approximate distance of 3 mm from the tendon insertion into the calcaneous bone [18, 20].

Laser therapy

The laser equipment used was a low-intensity GaAlAs laser (830 nm wavelength), programmed according to Brazilian medical equipment standards (NBR 60601-1, NBR IEC 60601-2-22 e IEC 825-1). The equipment was calibrated at 40 mW of power in the Biomedical Engineering Center at the State University of Campinas (UNICAMP). The animals were immobilized with containment equipment [25] and received a punctual application of 4 J/cm^2 once a day on the skin, directly over the lesion. Treatment with LLLT began the day after surgery and lasted until the day before euthanasia; that way, the groups euthanized 1 day after surgery received 1 application and 2 h later were euthanized; the groups euthanized 4 days after surgery received 2 applications; the groups euthanized 8 days after surgery received 6 applications; and the

groups euthanized 15 days after surgery received 13 applications. Each session of the continuous group lasts 16 s with a light intensity of 2500 W/m^2, and the pulsed group, 32 s with a light intensity of 1250 W/m^2. G1 animals were manipulated in the same way as the other groups, but with the equipment turned off (Table 1). After the last session, the animals were euthanized with deepening of anesthesia for the removal of the Achilles tendon [18].

Extraction procedures

The calcaneal tendon was removed and incubated in extraction buffer (50 mM Tris–HCl pH 7.4, 0.2 M NaCl, 0.1 % Triton X-100, 10 mM CaCl2, and protease inhibitor 100 μL/10 mL) at 4 °C for 3 h. Afterward, the material was centrifuged (13,000×*g*, 20 min, 4 °C), The supernatant was used for ELISA procedures and Western blotting for the inductive isoform of nitric oxide synthase (i-NOS).

ELISA procedures

The supernatant of the groups were utilized for this procedure. We performed ELISA analysis for interleukin-10 (IL-10) (R&D Systems, cat. N° R1000), interleukin-1β (IL-1β) (R&D Systems, cat. N° RLB00), TNF-α (R&D Systems, cat. N° RTA00), and transforming growth factor β (TGF-β) (R&D Systems, cat. N° MB100B) according to the manufacturer's instructions. The absorbance was measured at 450 nm.

Quantification of proteins

Samples of the extracts of each experimental group for the Western blotting procedures were used. Non-collagenous proteins were quantified according to the Bradford method [26] using bovine serum albumin as standard. The absorbance was measured at 595 nm.

Western blotting for i-NOS

For Western blot, 50 μg of total protein were mixed with reducing sample buffer (0.5 M Tris–HCl pH 6.8, 26 % glycerol, 20 % sodium dodecyl sulfate (SDS), and 0.1 % bromophenol blue). Proteins from the tendon subjected to electrophoresis on SDS-polyacrylamide (6 %) gels were transferred to nitrocellulose membranes, as described by Towbin and colleagues [27].

The membranes were transferred with the SNAP i.d. system (Millipore), blocked with Bløk-CH reagent for 15 s, and incubated with primary antibody (N9657, monoclonal anti-nitric oxide synthase inducible, produced in mouse, Sigma Aldrich) at a dilution of 1:200 for 10 min. The membranes were washed three times in TBS, incubated with secondary antibody (A0412, anti-mouse, Sigma Aldrich) for 10 min, and washed again. Finally, the Western blot signal was developed with dimethylaminobenzaldehyde. For the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used (sc-25778, rabbit polyclonal antibody and goat anti-rabbit IgG, A0545, Sigma Aldrich). Band densitometry was performed with the Scion Image Software Alpha 4.0.3.2 (Scion Corporation), and the results were expressed as the mean ratio in relation to the GAPDH band intensity.

Nitric oxide dosage

For this experiment, the blood plasma collected from the animals in vacuum tubes with sodic heparin was ultrafiltrated in Centricon tubes (AMICON), in which molecules with more than 30 kDa are separated. Before use, the tubes were previously centrifuged with a NaOH 0.1 M solution followed by three centrifugations with ultrapure water [28].

The total production of nitric oxide (NO) was evaluated by Griess method, where the NO concentration is indirectly measured in the determination of this final product, the nitrite. The absorbance was measured at 540 nm.

Statistical analysis

All results were expressed as the mean ± standard deviation. The results were analyzed by an analysis of variance (one-way ANOVA) followed by the Tukey test. The analysis was carried out in GraphPad Prism software.

Results

Figure 1 shows the quantification of TNF-α, IL-1β, and TGF-β1 using ELISA. The increase in the pro-inflammatory cytokine TNF-α in the transected group (G2) compared to the intact one (G1) is notable. One day after injury, statistically, it is not possible to say that both treatment groups (G3 and G4) are different from the injured group, but the mean value of TNF-α concentration in the last ones (G3: 26.35 ± 17.88 and G4: 22.96 ± 6.77) is less than half of the transected group value (57.04 ± 49.41) (Fig. 1a).

In the fourth day of treatment, a notable difference appears between the pulsed (9.06 ± 6.61) and continuous (40.04 ± 23.71) laser treatments. The pulsed treatment led to much inferior values of TNF-α count. There was no difference between the transected (88.17 ± 40.50) and the continuous laser-treated groups in this period (Fig. 1a).

The presence of IL-1β was prominent in the tendons treated with pulsed laser (2006.5 ± 85.55) when compared to the continuous treatment (1051.33 ± 360.93), 1 day after injury. On day four, there is no difference between these groups (Fig. 1b). It is interesting to note that, independent whether

Table 1 Parameters of irradiation

Parameters	Values for continuous groups	Values for pulsed groups
Laser operation	Continuous	Pulsed
Output power	40 mW	40 mW
Spot size area	0.1160 cm^2	0.1160 cm^2
Power density	2,500 W/cm^2	1,250 W/cm^2
Energy density	4.0 J/cm^2	4.0 J/cm^2
Time per point (seconds)	16	32
Number of points	1	1
Angle of application	90°	90°

or not the animals receive any treatment, the overall values of IL-1β are all similar after 4 days.

A marked increase in TGF-β1 concentration was detected in G4, reaching a mean value of 1464.35 ± 627.77 versus 92.11 ± 2.98 pg/g wet tissue in G1, 8 days after injury (Fig. 1c). On the 15th, the levels are still higher than the G3 group, but not as much discrepant.

IL-10 analysis did not detect any trace of the cytokine in any group.

The NO quantifications show a decrease in its levels after both laser treatments (G3: 4.1234 ± 0.9502 and G4: 5.7283 ± 2.9993), when compared to the transected group (6.9629 ± 1.2143), 8 days after injury. There was no difference between the treatments, though. On the 15th day after injury, the pulsed laser-treated group (3.6833 ± 0.4409) presented more NO concentration than the G3 (0.7111 ± 0.2405) and G2 (2.2875 ± 0.2994) groups (Fig. 2).

The presence of the isoform i-NOS, which catalyzes the production of high amounts of NO after inflammatory stimuli, was also analyzed in the different groups. Eight days after injury, i-NOS concentration was equally high in the transected (335.58 ± 110.93) and continuous laser groups (343.49 ± 80.42), but it was much lower when using pulsed laser treatment (69.94 ± 21.55) (Fig. 3). No statistical difference was found in the i-NOS levels in the groups in the 15th day, but a slight tendency of increase can be observed in G4 group (178.33 ± 79.51) (Fig. 3). That could mean a slight increase in activity of i-NOS in the pulsed laser-treated group.

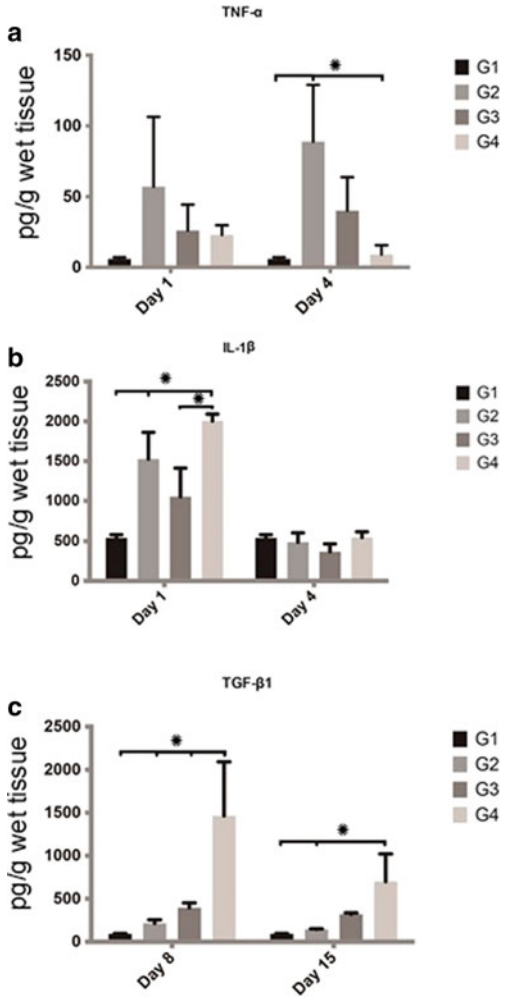

Fig. 1 Tendon extract ELISA tests results. **a** Results for TNF-α analysis. **b** Results of IL-1β analysis. **c** Results of TGF-β1. *Asterisk* indicates statistical significant difference

Discussion

Tendon is a crucial component of the musculoskeletal system. Tendons connect muscle to bone and transmit forces to produce motion. Tendon disorders are common, inflict a substantial effect on quality of life, and represent an important economic burden on healthcare systems. Chronic or acute injuries can occur in any tendon, but often affect major tendons with high in vivo loading demands, such as the Achilles, patellar,

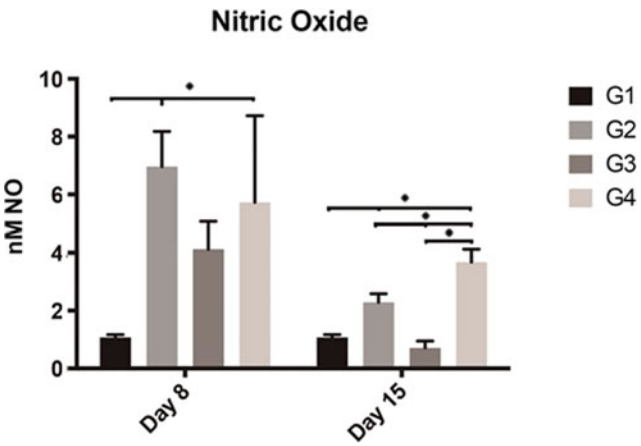

Fig. 2 Results of NO indirect dosage by the Griess method. *Asterisk* indicates statistical difference

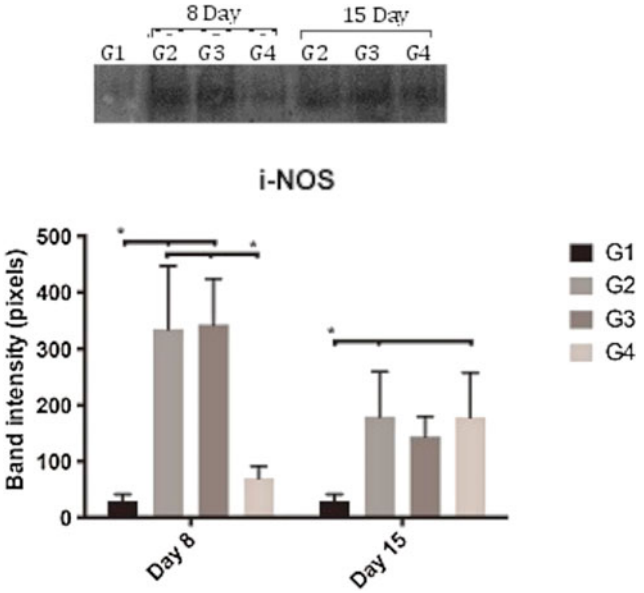

Fig. 3 Western blot bands of iNOS from the different groups. Graph of the band densitometry values. *Asterisk* indicates statistical difference

and rotator cuff tendons. Approximately 30 % of general practice consultations for musculoskeletal pain are related to tendon disorders [15].

Tendon rupture is followed by a natural healing process, in which inflammation is the beginning of the tissue repair process, although this healing is less efficient than in other components of the musculoskeletal system [4].

Increased production of inflammatory mediators leads to pain and swelling [29], hindering the talocrural articulation and feet support during gait after injury. In a typical rehabilitation protocol after a tendon injury, immobilization is performed to protect the injured tissue and prevent another possible rupture. However, prolonged periods of immobilization can cause damage, such as muscular atrophy, osteoarthritis, tendinocutaneous adhesion, and infections [22–24].

It is known that after lesion, the tendon is invaded by neutrophils, macrophages, and other inflammatory cells, which synthetize several pro- and anti-inflammatory cytokines and growth factor, influencing tendon components, like the collagen and glycosaminoglycans [30].

The cicatrization steps after tendon rupture are well described in literature [2, 4, 7, 9]. TNF-α, IL-10, and IL-1β are pro-inflammatory cytokines typical of the early inflammation phases, found from 4 h until the fourth day after lesion. On the other hand, TGF-β e NO can be detected in larger quantities from the beginning of the proliferative phase (8 days after lesion) until the 21st day. Based on that, the analysis dates were chosen.

The measure of the pro-inflammatory cytokine TNF-α reveals that a tendon injury can lead to a great increase in its concentration in just 1 day. Also, it is notable that only 1 day of treatment, following the calcaneal tendon transection, is enough to reduce the mean values of TNF-α concentration from 57.04 (control group—G1) to 26.0 or 22.96 pg/g of wet tissue (continuous—G3 or pulsed laser—G4).

Four days after injury, the TNF-α concentration remained high in the transected group. The continuous laser treatment kept the cytokine levels low, but no difference could be observed from 1 to 4 days of treatment following this protocol. The pulsed LLLT showed a different pattern, where the continuity of the treatment significantly decreased the pro-inflammatory cytokine, to values close to those of the control group.

Experiments show that TNF-α activates a cascade of cytokine release that leads to the development of sympathetic hyperalgesia. Its effects are similar to those of carrageenan (a known inflammatory agent). Hyperalgesia after the lesion is one of the main factors responsible for bad cicatrization [30].

Several studies have shown that the morphological and functional properties of a tendon after injury will never reach those of normal tissue. This loss may be caused by long-term immobilization [12, 23, 24] resulting in the absence of mechanical load [7, 31]. Recently, we demonstrated that the animals treated with pulsed laser show improvement on the functional properties, where the animals could move the injured joint and support the paw during gait [14]. The mechanical load resulted induces collagen synthesis and organization and enables faster recovery, thereby reducing the effects of long-term immobilization. Based on these results, we believe that this protocol has a high anti-inflammatory potential.

One day after injury and treatment, IL-1β concentration was lower in G3, than in G2 and G4, which had statistically the same values. After 4 days, all the groups, including transected, had basal levels of IL-1β, equals to the control group.

Recent data indicates that IL-1β has little influence over fibroblasts not submitted to some force of traction or stretch [32]. Such information supports the idea that the pulsed laser treatment possesses anti-inflammatory effect, reducing pain and supporting proper gait. With more regular walking, the tendon is submitted to traction, which would activate IL-1β. Previous studies made by our research group analyzed the gait of animals under tenotomy and treated with different LLL protocols. The results show that indeed, animals treated with pulsed laser supported the paws better during gait.

Another article states that IL-1β role in tendinopathies are questionable [33]; however, our results observed after just 1 day of treatment deserves attention, since the cytokine presence was higher while using pulsed laser treatment instead of the continuous one.

Apparently, contradictory data from TNF-α and IL-1β, with different behaviors in 1 day after pulsed and continuous laser treatments, can be due to some factor acting differentially

in the deacetylation of histones [34] and interrupting at different times, transcription factors to the cytokines genes.

TGF-β1 detection increased in the pulsed laser-treated group, especially after 8 days of injury (Fig. 1). On the 15th day, the concentration is still high, but it is not different from the tendons of the animals in the continuous laser-treated group. TGF-β1 is a well-known cytokine that regulates various processes in tendon healing, and its concentration increases in the tendon after lesions. Its expression is increased in the proliferative phase, around 7 days after injury. It modulates the inflammatory responses in early healing stages, participates in the intricate control of angiogenesis, regulates the proteoglycan deposition, and stimulates the production of collagens by tendon fibroblasts. The overproduction of this cytokine can be an indicator of the efficiency of the treatment.

There are three different forms of TGF-β and two specific receptors in humans. All three TGF-β isoforms could significantly increase collagen I and III production in cultured tendon fibroblasts. TGF-β1 induced a greater degree of contraction in tendon fibroblasts cultured in collagen gels as compared with TGF-β3 [35]. The literature is controversial when reporting the TGF-β roles: some researchers consider its gene associated with tissue fibrosis, related to scar and adhesions around tendons; others describe it accelerating collagen protein synthesis, cross-link formation, and matrix remodeling in tendon healing, thus enhancing mechanical strength. The fact is that different TGF-β isoforms may interact with one another to modulate the collagen synthesis in tendon healing [35–38].

Previous studies demonstrated that TGF-β1 can be released from cultured tendon fibroblasts in response to mechanical loading [39, 40]. Our experimental protocol allowed rats to move freely following surgery. Numerous studies have demonstrated that early mobilization plays an important role in tendon remolding and decreased adhesion, thus enhancing tendon healing [41, 42]. Our previous histological examination through polarization microscopy and birefringence analysis showed a much more organized and homogeneous pattern of collagen fibers in the pulsed group [20].

Nitric oxide (NO) is a short-lived free radical with biological functions in nervous, cardiovascular and immune systems, and tissue healing. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been identified, all requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor: the neuronal isoform (bNOS, NOS-I), constitutively expressed in discrete neuronal populations [4, 41, 42], the endothelial isoform (eNOS, NOS-III), present in endothelial cells of blood vessels [10, 12], and the inducible isoform (iNOS, NOS-II), expressed in various cell types including macrophages when activated. Previous studies show that all three NOS isoforms are expressed in tendon healing, each with its own unique temporal expression.

There was no difference regarding NO concentrations in tendons collected 8 days after injury between the two laser treatment protocols, but both had lower concentration than the injured group. On the 15th day, the group treated with pulsed laser presented higher levels of NO than both injured and continuous laser-treated groups. Previous birefringence studies reveal that pulsed laser treatment promotes a better organization of the collagen fibers. That goes in accordance with the general accepted idea that NO promotes better collagen organization [43].

The isoform i-NOS, which catalyzes the production of high amounts of NO in response to inflammatory stimuli [44], was also measured in the groups. The presence of i-NOS in tendons after 8 days of injury was equally elevated in the injured and continuous laser-treated groups. Nevertheless, the enzyme concentration was much lower in G4, which is an indication that the pulsed laser treatment is capable of reducing the inflammatory process in injured tendons. In the tendons of the animals euthanized 15 days after partial transection, no difference in the i-NOS presence was detected between the groups.

Lin et al. (2001) demonstrated that 4 days after injury, there were increases in the steady-state levels of niRNA for all three NOS isoforms, with peaks for the inducible isoform (iNOS) at day 4, the endothelial isoform (eNOS) at day 7, and the neuronal isoform (bNOS) at day 21. These findings indicate that all three NOS isoforms are expressed during tendon healing with differential expression patterns during the various phases of tendon healing. Our results indicate that pulsed LLLT decreased the inflammatory process anticipating the following tendon cicatrization steps. Authors believe the i-NOS peak occurred in the beginning of the inflammatory process and, afterwards, the NO production levels were kept by the other NOS isoforms.

The research group's previous studies demonstrated that variation of laser frequency parameter leads to very different results [18, 20]. Again, we investigate the effect of LLLT over injury inflammatory phase and its specific mediators obtaining different results.

The use of pulsed laser presented better results than the continuous laser protocol, modulating the release of inflammatory mediators along with metalloproteinases, acting directly in the inflammatory process, leading to the beneficial responses reported in our previous works, like greater and more organized collagen synthesis and improved gait. This protocol seems to exert anti-inflammatory effect over injured tendons, with reduction of the release of pro-inflammatory cytokines, such as TNF-α and the decrease in the i-NOS activity. Also, pain reduction and facilitation of movement stimulated the release of TGF-β and IL-1β, increasing the synthesis and organization of the main component of the tendon, collagen.

Compliance with ethical standards Animal care was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and is consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA); the protocol was approved by the ethics Committee on Animal Experiments of the State University of Campinas, SP, Brazil (no. 1921–2).

References

1. Latarjet M, Liard AR (1996) Anatomia Humana. 2ed. Editora Panamericana, pp.837-880
2. Maffulli N, Renström P, Leadbetter WB (2005) Tendon Injuries: Basic Science and Clinical Medicine. Springer-Verlag London Limited
3. Benjamin M, Kaiser E, Milz S (2008) Structure-function relationships in tendons: a review. J Anat 212:211–228
4. Nourissat G, Berenbaum F, Duprez D (2015) Tendon injury: from biology to tendon repair. Nat Rev Rheumatol 11:223–233
5. Cribb AM, Scott JE (1995) Tendon response to tensile stress: an ultrastructural investigation of collagen:proteoglycan interactions in stressed tendon. J Anat 187:423–428
6. Szaro P, Witkowski G, Smigielski R, Krajewski P, Ciszek B (2009) Fascicles of the adult human Achilles tendon - an anatomical study. Ann Anat 191(6):586–593
7. Sharma P, Maffulli N (2006) Biology of tendon injury: healing, modeling and remodeling. J Musculoskelet Neuronal Interact 6(2):181–190
8. Vieira CP, de Aro AA, de Almeida Mdos S, de Mello GC, Antunes E, Pimentel ER (2011) Effects of acute inflammation induced in the rat paw on the deep digital flexor tendon. Connect Tissue Res 53(2): 160–8
9. Molloy T, Wang Y, Murrell G (2003) The roles of growth factors in tendon and ligament healing. Sports Med 33(5):381–94
10. Houlred NN, Sekhejane PR, Abrahamse H (2010) Irradiation at 830nm Stimulates Nitric Oxide Production and Inhibits Pro-Inflammatory Cytokines in Diabetic Wounded Fibroblast Cells. Lasers Surg Med 42:494–502
11. Jarvinen TA, Kannus P, Mafulli N et al (2005) Achilles tendon disorders: etiology and epidemiology. Foot Ankle Clin 10(2):255–266
12. Chan HKF, Fung DT, Ng GY (2007) Effects of low-voltage microamperage stimulation on tendon healing in rats. J Orthop Sports Phys Ther 37(7):399–403
13. Oliveira FS, Pinfild CE, Parizoto NA et al (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
14. Aro AA, Simões GF, Esquisato MAM, Foglio MA, Carvalho JE, Oliveira ALR, Gomes L, Pimentel ER (2013) Arrabidaea chica extract improves gait recovery and changes collagen content during healing of the Achilles tendon. Injury 44(7):884–92
15. Kaux JF, Forthomme B, Goff CL, Crielaard JM, Croisier JL (2011) Current opinions on tendinopathy. J Sports Sci Med 10:238–253
16. Pinfildi CE, Liebano RE, Hochman BS, Ferreira LM (2005) Heliumneon laser in viability of random skin flap in rats. Lasers Surg Med 37:89–91
17. Chen M, Chun C, Meijuan Y et al (2008) Involvement of CAPON and Nitric Oxide Synthases in rat muscle regeneration after peripheral nerve injury. J Mol Neurosci 34:89–100
18. da Guerra FR, Vieira CP, Almeida MS, Oliveira LP, de Aro AA, Pimentel ER (2013) LLLT improves tendon healing through increase of MMP activity and collagen synthesis. Lasers Med Sci 28(5):1281–8
19. Pugliese LS, Medrado AP, Reis SR, Andrade ZA (2003) The influence of low-level laser therapy on biomodulation of collagen and elastic fibers. Pesqui Odontol Bras 17(4):307–313
20. Guerra Fda R, Vieira CP, de dos Santos Almeida M, Oliveira LP, Claro AC, Simões GF, de Oliveira AL, Pimentel ER (2014) Pulsed LLLT improves tendon healing in rats: a biochemical, organizational, and functional evaluation. Lasers Med Sci 29(2):805–811
21. Enwemeka CS, Reddy GK (2000) The biological effects of laser therapy and other modalities on connective tissue repair processes. Laser Ther 12:22–30
22. Wren TAL, Yerby SA, Beaupre GS et al (2001) Mechanical properties of human Achilles tendon. Clin Biomech 16:245–251
23. Chen CH, Tsai LL, Wang YH et al (2009) Low-level laser irradiation promotes cell proliferation and mRNA expression of type I collagen and decorin in porcine Achilles tendon fibroblasts in vitro. J Orthop Res 27:646–650
24. Demir H, Menku P, Kirnap M (2004) Comparison of the Effects of Laser, Ultrasound, and Combined Laser + Ultrasound Treatments in Experimental Tendon Healing. Lasers Surg Med 35:84–89
25. Request Privilege of Utility Model in Brazil, titled: Retainer of Rats For Laser Beam And Biocompounds Application, deposited inNational Institute Of Industrial Property–INPI, on 04.16.10, undern°MU9000622-4, authored by researchers Andrea Aparecida de Aro and Edson Rosa Pimentel, from Institute of Biology, UNICAMP
26. Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
27. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci 76(9): 4350–4354
28. Moro CF, Consonni SR, Rosa RG, Nascimento MA, Joazeiro PP (2012) High iNOS mRNA and protein localization during late pregnancy suggest a role for nitric oxide in mouse pubic symphysis relaxation. Mol Reprod Dev 79(4):272–82
29. Seibert K, Zhang Y, Leahy K et al (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proc Natl Acad Sci U S A 91:12013–12017
30. Schulze-Tanzil G, Al-Sadi O, Wiegand E, Ertel W, Busch C, Kohl B, Pufe T (2011) The role of pro-inflammatory and immunoregulatory cytokines in tendon healing and rupture: new insights. Scand J Med Sci Sports 21(3):337–51
31. Sharma P, Maffulli N (2005) Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg 87:187–202
32. Cao TV, Hicks MR, Campbell D, Standley PRJ (2013) Dosed myofascial release in three-dimensional bioengineered tendons: effects on human fibroblast hyperplasia, hypertrophy, and cytokine secretion. Manip Physiol Ther 36(8):513–21
33. Mobasheri A, Shakibaei M (2013) Is tendinitis an inflammatory disease initiated and driven by pro-inflammatory cytokines such as interleukin 1β? Histol Histopathol 28(8):955–64
34. Kersul AL, Iglesias A, Ríos Á, Noguera A, Forteza A, Serra E, Agustí A, Cosío BG (2011) Molecular mechanisms of inflammation during exacerbations of chronic obstructive pulmonary disease. Arch Bronconeumol 47(4):176–83
35. Chan KM, Fu SC, Wong YP, Hui WC, Cheuk YC, Wong WN (2008) Expression of transforming growth factor b isoforms and their roles in tendon healing. Wound Rep Regen 16:399–407
36. Chen CH, Cao Y, Wu YF, Bais AJ, Gao JS, Tang JB (2008) Tendon healing in vivo: gene expression and production of multiple growth factors in early tendon healing period. Hand Surg 33A:1834–1842
37. Hou Y, Mao Z, Wei XL, Lin L, Chen LX, Wang HJ, Fu X, Zhang JY, Yu CL (2009) The roles of TGF-b1 gene transfer on collagen

formation during Achilles tendon healing. Biochem Biophys Res Commun 383:235–239
38. Rocha Júnior AM, Vieira BJ, Andrade LCF, Monteiro F (2009) Low-level laser therapy increases transforming growth factor-_2 expression and induces apoptosis of epithelial cells during the tissue repair process. Photomed Laser Surg 27(2):303–307
39. Anaguchi Y, Yasuda K, Majima T, Tohyama H, Minami A, Hayashi K (2005) The effect of transforming growth factor-beta on mechanical properties of the fibrous tissue regenerated in the patellar tendon after resecting the central portion. Clin Biomech (Bristol, Avon) 20(9):959–965
40. Sporn MB (2006) The early history of TGF-beta, and a brief glimpse of its future. Cytokine Growth Factor Rev 17(1–2):3–7
41. Halikis MN, Manske PR, Kubota H, Aoki M (1997) Effect of immobilization, immediate mobilization, and delayed mobilization on the resistance to digital flexion using a tendon injury model. J Hand Surg [Am] 22(3):464–72
42. Aoki M, Kubota H, Pruitt DL, Manske PR (1997) Biomechanical and histologic characteristics of canine flexor tendon repair using early postoperative mobilization. J Hand Surg [Am] 22(1):107–114
43. Tomiosso TC, Nakagaki WR, Gomes L, Hyslop S, Pimentel ER (2009) Organization of collagen bundles during tendon healing in rats treated with L-NAME. Cell Tissue Res 337(2):235–342
44. Griffith OW, Stuehr DJ (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 57:707–36