

Photobiomodulation therapy increases collagen II after tendon experimental injury

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Summary. A tendon is a mechanosensitive tissue that transmits muscle-derived forces to bones. Photobiomodulation (PBM), also known as low-level laser therapy (LLLT), has been used in therapeutic approaches in tendon lesions, but uncertainties regarding its mechanisms of action have prevented its widespread use. We investigated the response of PBM therapy in experimental lesions of the Achilles tendon in rats. Thirty adult male *Wistar* rats weighing 250 to 300 g were surgically submitted to bilateral partial transverse section of the Achilles tendon. The right tendon was treated with PBM, whereas the left tendon served as a control. On the third postoperative day, the rats were divided into three experimental groups consisting of ten rats each, which were treated with PBM (Konf, Aculas - HB 750), 780 nm and 80 mW for 20 seconds, three times/week for 7, 14 and 28 days. The rats were sacrificed at the end of the therapeutic time period. The Sca-1 was examined by immunohistochemistry and histomorphometry, and COLA1, COLA2 and COLA3 gene expression was examined by qRT-PCR. COLA2 gene expression was higher in PBM treated tendons than in the control group. The histomorphometric analysis coincided with increased number of mesenchymal cells, characterized by Sca-1 expression in the lesion region ($p < 0.001$). PBM effectively interferes in tendon tissue repair after injury by stimulating mesenchymal cell proliferation and the synthesis of collagen type II, which is suggested to provide structural support to the interstitial tissues during the healing process of the Achilles tendon. Further studies are needed to confirm the role of PBM in tendon healing.

Key words: Tendon, Laser, Mesenchymal cell, Collagen II

Introduction

Best practices regarding the treatment of Achilles tendon rupture remain an ongoing topic of debate (Chiodo et al., 2010). Tendon pathologies range from chronic injury to acute injury with partial or complete tendon rupture that interrupt tendon continuity and lead to diminution or loss, respectively, of transmitted forces and potentially to loss of mobility (Voleti et al., 2012). Following acute rupture, the tendon undergoes a healing process involving the successive steps of inflammation, extracellular matrix (ECM) formation and remodelling.

Tendons and ligaments are basically composed of cells, the fibroblasts, and extracellular matrix, in which fibrous proteins (such as collagen and elastin), proteoglycans, glycoproteins, mucopolysaccharides and water are immersed. Collagen constitutes about 90% of tendon protein, or approximately 70% of the dry mass of the tendon (Kannus et al., 2000). Tendons and ligaments have a highly ordered fibrillary structure composed of bundles of proteins, predominantly type I collagen fibers. This collagen is described as a conventional molecule with little flexibility and high resistance (Fratzl et al., 2008). The molecular basis for the flexibility of type I collagen comes from sequences that lack the aminoacids proline and hydroxyproline. These sequences are the sites where bends can occur in the triple helix, based on electron microscopic images. Paterlini et al. (1995), suggest that sequences without proline and hydroxyproline are able to form internal loops that give these regions more flexibility than the other regions of the triple helix. The collagen triple-helix can be considered a composite of regions with varying

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degrees of stiffness; regions of the molecule devoid of proline and hydroxyproline appear to have the highest flexibility whereas regions with the sequence Gly-Pro-Hyp are very rigid (Silver et al., 2003). This variation in molecular flexibility affects collagen self-assembly as well as the resulting mechanical properties of tendons. The diameter of the fibril also appears to be inversely proportional to the molecular flexibility of the collagen. In tendons and ligaments, in addition to type I collagen there are present minor amounts of types II, III, IV and V collagen, suggesting that in these tissues the specific differences in the mechanical properties reflect different proportions of the collagen types (Silver et al., 2003). The multihierarchical structure contains collagen molecules arranged in fibrils then grouped in fibril bundles, fascicles and fiber bundles that are almost parallel to the long axis, making the tendon ideal for carrying and transmitting large tensile mechanical loads (Oakes, 2003). Tendon cells (tenoblasts, tenocytes and mesenchymal stem cells) (Tempfer and Traweger, 2015) reside between the strands of collagen fibers, where they can sense physical stimuli, such as fluid flow, and change their synthetic activities accordingly (Lavagnino et al., 2015).

The mechanisms of tendon degeneration and healing are not well understood. Tendon healing is characterized by a fibrovascular scar that never attains the mechanical properties of normal tendon, leading to a relatively high failure rate after repair (Galatz et al., 2015). The response to tendon injury can be divided into three overlapping stages. In the initial, inflammatory stage, which typically spans a few days, the wound site is infiltrated by red blood cells, white blood cells (leukocytes), and platelets equipped with important growth factors and endothelial chemoattractants. A fibrin clot is formed to provide temporary stiffness, macrophages digest necrotic debris, and tenocytes are recruited to the wounded area and stimulated to proliferate, particularly in the epitenon (Jozsa and Kannus, 1997). The second stage, known as the proliferative or repair stage, begins roughly two days into the injury response. This phase of healing is characterized by profuse synthetic activity and is directed by macrophages and tenocytes (Voleti et al., 2012). Macrophages, whose role shifts from phagocytic to reparative a few days after injury, release growth factors and direct cell recruitment (Massimino et al., 1997). Meanwhile, tenocytes deposit a temporary, mechanically inferior matrix composed mostly of type III collagen (Majewski et al., 2009). In the third and final stage, known as the remodeling phase, type I collagen synthesis begins to dominate, and the extracellular matrix (ECM) becomes more aligned. In addition, cell density and general synthetic activity are gradually decreased (Silver et al., 2003). This phase begins 1-2 months after injury and can last more than a year (Jozsa and Kannus, 1997). Unfortunately, tendon injuries are difficult to treat due to the healing process via formation of a fibrotic scar composed of a

disorganized matrix consisting largely of dense collagenous fibers. The repaired tissue appears scar-like and never completely regains the biomechanical properties it had prior to injury (Sharma and Maffulli, 2005). Moreover, tendon healing can be hampered by adhesion formation (Wong et al., 2009). The arrangement of collagen fibers (Miyashita et al., 1997) and fibril size (Battaglia et al., 2003) of fibrotic scar tissue are smaller than those of the uninjured tendon and cannot return to their original mechanical strength for a long time after injury (Miyashita et al., 1997) resulting in considerable dysfunction and disability. Therefore, tendon healing requires prolonged recoveries (extending for months or years) and the reconstructed tendons lose the mechanical properties of normal tendon, with only 60% of Achilles tendon patients operated on recovering their previous level of function (Wu et al., 2017).

Changes in collagen content and composition occur in tendon rupture, with an increase in collagen type III in human Achilles tendon (Eriksen et al., 2002). Collagen type III has also been shown to increase with ageing (Smith et al., 1999) and disease evolution (Goncalves-Neto et al., 2002). Degenerative changes are considered one of the most important risk factors for the total Achilles tendon rupture. This correlation is supported by studies of tendon morphology, which have revealed abnormal orientation of collagen fibers, changes in collagen type composition, mucoid-type degeneration and hypoxic changes (Jozsa and Kannus, 1997).

In some normal human tendons, such as the supraspinatus and biceps, there are variations in types I, II and III collagen across separate regions, suggesting adaptation to compressive loading (Buckley et al., 2013). Chronic or acute injuries can occur in any tendon but often affect major tendons with high *in vivo* loading demands, such as the calcaneal, patellar, rotator cuff and forearm extensor tendons (Sharma and Maffulli, 2005). The response of tendon to abnormal mechanical loading has an important role in tendon injury (Buckley et al., 2013).

Recently, several studies have been carried out with the aim of developing therapeutic modalities for the injured tendon to reduce the acute inflammatory response and accelerate the recovery phase, regeneration and scar formation (Speed, 2001; Shanks et al., 2010; Akamatsu et al., 2016; Jiang et al., 2019; Huegel et al., 2020).

Many therapeutic modalities, including medications (such as non-steroidal anti-inflammatory drugs and corticosteroid injections), physical treatments (such as therapeutic ultrasound, laser, manual therapy techniques, biomechanical alterations, and extracorporeal shock wave therapy) and numerous other drugs or substances (including heparin, dextrose, sclerosants, calcium gluconate, autologous blood injections and aprotinin) have been used in the treatment of tendon disorders. (Rees et al., 2006) Surgical techniques (mini-open, percutaneous repair and the open repair) (Hsu et al., 2015) are also reported. The outcomes of these

techniques described in the literature reinforce the idea that the treatments of these tissues do not always have the expected result. There is insufficient evidence from randomized controlled trials to determine which therapeutic method is the most appropriate for the treatment of acute or chronic Achilles tendinopathy. (McLauchlan and Handoll, 2001). In addition, many of these techniques cause initial weakness of the capsular and ligamentous structures, which require the use of immobilization over a long period of time (Wallace et al., 2001; Indelli et al., 2003).

Among several innovative techniques used to treat tendon injuries, is included photobiomodulation (PBM), also known as low-level laser therapy (LLLT). This therapy appears to be an effective tool to improve collagen fiber organization in experimental sheep deep digital flexor tendon injury because of the anti-inflammatory effect, with a reduction in cell number and decrease in vascularization (Iacopetti et al., 2015). The PBM consists of the application of light, usually using a low-power light source (laser or LED) (Chung et al., 2012). PBM exposes cells or tissue to low levels of red and near infrared (NIR) light, and is referred to as "low level" because of its use of light at energy densities that are low compared to other forms of laser therapy that are used for ablation, cutting, and thermally coagulating tissue (Chung et al., 2012). Because of the low power, (usually below 500 mW) the treatment with PBM causes no temperature rise in the treated tissue and, therefore, no significant change in the gross tissue structure (Caruso-Davis et al., 2011). Photobiomodulation has been previously used for successful disease management in wound healing (Posten et al., 2005; Kaviani et al., 2011), and tissue repair (Bjordal et al., 2003; Gigo-Benato et al., 2005; Christie et al., 2007; Jamtvedt et al., 2008).

The precise biochemical mechanism underlying the therapeutic effects of PBM are not yet well established. PBM has a wide range of effects at the molecular, cellular, and tissue levels, and its specific modes of action may vary among different applications (Grossman et al., 1998; Alexandratou et al., 2002; Lavi et al., 2003; Lubart et al., 2005; Pal et al., 2007; Zhang et al., 2008; Chen et al., 2011; Chung et al., 2012).

Unlike other medical laser procedures, PBM does not have an ablative or thermal mechanism but, rather, a photochemical effect, in which light is absorbed and causes chemical change (Huang et al., 2009). The aim of the current study is to evaluate the effect of AlGaAs laser (gallium arsenide) therapy after 7, 14 and 28 days of PBM on cell formation and organization and on collagen synthesis after Achilles tendon injury in rats.

Materials and methods

Animals and experimental protocol

This study was approved by the Ethics Committee for the Analysis of Research Projects Protocol 502/11.

All animals received humane care in compliance with the experimental protocols of the Ethical Principles in Animal Experiments adopted by the Brazilian Association of Animal Testing. In this study we chose to use the model of Achilles tendon injury in rats, previously described by our group (Akamatsu et al., 2014).

Sixty adult male *Rattus norvegicus albinus*, Wistar individuals weighing 250 to 300 g were housed in the São Paulo University School of Medicine's facilities. Five animals were housed per cage and were given food and purified tap water *ad libitum*. The animals were operated upon in the CEPEC (Center for Study and Research in Surgery, Department of Urology of the Faculty of Medicine). For the experiment, the right Achilles tendon was selected (PBM); the left Achilles tendons of the same animals were used as controls (CT). Rats were placed under general anaesthesia with 4% isoflurane and maintained under anaesthesia by mask using a 1.5-3% inspired fraction of isoflurane. After anaesthesia, the distal portions of the right and left legs of the animal were subjected to disinfection with 10 mg/L chlorhexidine gluconate solution and shaved.

The animals were placed in a sterile field on a heated surgery table and covered with a sterile surgical drape. The skin was cut longitudinally 0.5 cm from the calcaneal insertion lateral to the tendon; both the peritendons and tendons were exposed. The animals were surgically subjected to bilateral partial transverse sectioning of the Achilles tendon. The injury in this animal model was induced by dissection and transverse hemisection using a no. 15 scalpel blade perpendicular to the collagen fibers, 2.5 mm from the Achilles insertion on the lateral side of the Achilles tendon. We took care to separate the flexor hallucis longus, which is medially adjacent to the Achilles tendon. After transection, the skin was closed by a continuous suture with Prolene 6-0 (Ethicon). After surgery, the rats were kept warm until they regained consciousness. The rats were administered analgesics (200 mg/kg paracetamol) orally every 24 hours for three days and were left without cast immobilization. During the time they were kept housed, they were acclimatized to 12 hour light-dark cycles. On the third postoperative day, the rats were divided into three experimental groups and treated with a Konf AlGaAs semiconductor laser diode device, Aculas - HB-750, with wavelength 780 nm, power 80 mW, and a local beam applicator with 0.1 cm over the skin in continuous contact for 20 seconds three times weekly until they were sacrificed on the 7th, 14th or 28th days. The contact area for laser administration was circular with an area of 0.1 cm² and a diameter of 0.1 cm to contact the tendon of the animal. After treatment, the animals were euthanized using CO₂. The animals were placed in chambers previously filled with pure gas, or preferably, gas with 30% added oxygen to reduce stress caused by hypoxia. Because CO₂ is twice as heavy as air, it sits at the bottom of the chamber, and the chamber opening was oriented at the top. CO₂ lethality stems

from central nervous system depression. The animals were kept in the chamber for 10 minutes until their deaths were confirmed (Nolen, 2009).

Specimen preparation and histology

Achilles tendons were collected through dissection, with the animal placed in the prone position. The specimens were collected for histological, immunofluorescence, and immunohistochemistry analysis and histomorphometry of collagen and mesenchymal cells. To study the effects of laser administration on the matrix organization, the Achilles tendons were fixed by immersion in 10% neutral buffered formalin. After decalcification for 1 to 4 days with 7% nitric acid, the tissue was neutralized by soaking it in a sodium sulfate solution for one day and then washed for 10 hours under running water. After being embedded in paraffin, the tendons were cut from a longitudinal position. From each tendon, 6 slices were obtained at 4-5 μm thickness spaced at 50 μm between them. Subsequently, the samples were stained with Hematoxylin-Eosin (H&E), to evaluate cells inflammatory reactions under an optical microscope. Additional sections were used for immunohistochemistry and immunofluorescence.

Histopathologic Study - a grading system of injury

Histopathology was performed on all cases. Three or four blocks from each case's tendon, dependent on the gross presentation of the lesions, were sampled for histopathology, which included hematoxylin & eosin (H&E) staining. Two pathologists (VLC and WRT) experienced in using a semi-quantitative score reviewed each parameter to provide a final grading system. The following pathological changes, including basophilic degeneration, fragmentation of collagen, vascular proliferation, fibroblast proliferation, inflammatory infiltrate and ossification were observed after qualitative examination of the tendon histology. The extension and distribution of the above parenchymal alterations were not homogeneous along the tendon. Thus, the severity of the various pathological processes was rated semi-quantitatively according to the amount and severity of disease using a modified Bonar Score (Maffulli et al., 2008; Fearon et al., 2014). The review of the Bonar score and suggestions for changes to the scale were completed by consensus in the following grade:

- Grade 0: absence of tendon alterations;
- Grade 1: tendon alterations in 1 to 25% of the examined tissue;
- Grade 2: tendon alterations in 26 to 50% of the examined tissue;
- Grade 3: tendon alterations in 51 to 75% of the examined tissue;
- Grade 4: tendon alterations in 76 to 100% of the examined tissue.

The semi-quantitative scores of tendon lesions

represent the average of extension and intensity of lesions present in all histologic sections examined (three or four), which, in turn, represents the most compromised areas identified on gross examination of the tendons.

Collagen gene expression

For this analysis, total RNA of the tendon was isolated using an RNeasy[®] tissue kit (QIAGEN). The single-stranded cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Quantitative PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and threshold cycle numbers were determined using RQ Study Software (Applied Biosystems). Reactions were performed in triplicate, and threshold cycle numbers were averaged. The 50 μL reaction mixture was prepared as follows: 25 μL Platinum[®] SYBR Green Quantitative PCR SuperMix-UDG (Invitrogen[™] Life Technologies, Alameda, CA, USA), 10 μM of each primer (Table 1) and 10 μL cDNA (100 ng). The reaction was cycled with preliminary UDG treatment for 2 min at 50°C and a denaturation for 2 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing for 15 seconds, and primer extension at 72°C for 15 seconds. This was followed by melting point analysis of the double-stranded amplicons consisting of 40 cycles of 1°C decrement (15 seconds each) beginning at 95°C. The first derivative of this plot, dF/dT, is the rate of change of fluorescence in the reaction, and a significant change in fluorescence accompanies the melting curve of the double-stranded PCR products. A plot of $-dF/dT$ vs. temperature displays these changes as distinct peaks. The expression levels of *Col1A1*, *Col2A1* and *Col3A1* were examined and normalized to constitutive genes (β -actin), and the relative fold induction was calculated according to the formula $2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001) (Table 1).

Collagen II distribution and histomorphometry analysis

Immunofluorescence was performed to characterize

Table 1. Oligonucleotides employed for quantitative qRT-PCR.

Gene	Primer	Sequence (5' → 3')
β -actin	Sense	CGAAGAACCATCCGATTGA
	Antisense	GGCACTTCTGAAACCGACA
Col1A1	Sense	GCATGGCCAAGAAGACATC
	Antisense	GCCATTGTGGCAGATACAGA
Col2A1	Sense	AACCTGGTACCCCTGGAAAT
	Antisense	GCAAAGTTTCTCCACCAAG
Col3A1	Sense	GGCACAGCAGTCCAATGTAG
	Antisense	TGACATGGTTCTGGCTTCC

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the collagen II fibers in 4-5- μ m thick tendon sections attached to slides previously treated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO, USA). The reaction was initiated by deparaffinization and dehydrated in decreasing ethanol concentrations. For exposure of antigenic sites and retrieval, sections were digested with bovine pepsin (Sigma Chemical Co.; 10,000 ICU/mL) at a concentration of 8 mg/250 μ l in 0.5 N acetic acid for 45 minutes at 37°C, and nonspecific sites were blocked with 5% bovine serum albumin in PBS for 30 minutes at room temperature. The slides were incubated overnight with polyclonal anti-rabbit collagen II-N-terminal antibody 1:100 (Sigma Chemical Co.) in PBS solution and incubated for 90 minutes with anti-rabbit IgG antibody conjugated Alexa 488 (Invitrogen, Carlsbad, CA, USA) diluted 1:200 in PBS solution containing 0.006% Evans blue. Finally, sections were mounted with buffered glycerol solution, and examined under a fluorescence microscope (Olympus BX-51, Olympus Co., Tokyo, Japan). The histomorphometric analysis was performed to quantify the density of immunostained fibers of collagen type II using automated image analysis. The images were processed by software *Image-Pro plus 6.0* to quantify the green fluorescent immunostained fibers. Two observers blinded as to whether the specimen being analyzed came from a patient or a control randomly acquired 10 images per specimen at 400x magnification, and the area of each field was measured in μ m² (Alfredson, 2005). The average collagen content area was divided by the average of the total area analyzed, and the result was expressed as a percentage.

Mesenchymal cells immunohistochemistry and histomorphometry analysis

The sections were deparaffinized, and a 0.3% hydrogen peroxide solution was applied 4 times for 5 minutes to inhibit endogenous peroxidase activity. The antibody used in the immunohistochemical reactions was Sca-1 monoclonal antibody (Abcam Ab 124648), 1:800 dilution. Tissue sections were treated in citrate buffer solution at pH 9.0 and heated in a Pascal pressure cooker (125°C for 1 minute) to un-mask the epitopes. Sections were treated with the non-specific immunoglobulin blocker (CAS-Blok code 008120-Zymed) for 20 minutes. The sections were incubated with the primary antibody overnight at 4°C. The reaction was revealed using a biotin-streptavidin-peroxidase kit (Vector PK 4001) according to the manufacturer's instructions. The 3,3 diaminobenzidine (Sigma Chemical, St Louis, MO) was used as a chromogen. The sections were counterstained with Harris haematoxylin (Merck, Darmstadt, HE Germany). For negative controls, the primary antibody was replaced with PBS.

Morphometric analysis was performed using the *Image-ProPlus 6.0* system composed of an Olympus camera (Olympus Co, St Laurent, Quebec, Canada) coupled to an Olympus microscope (Olympus BX51), from which the images were sent to an LG monitor by means of a digitizing system (Oculus TCX, Coreco, Inc., St. Laurent, Quebec, Canada) and downloaded to a computer (Pentium 1330 Mhz). The area fraction of mesenchymal cells in tendon were determined at 1000x magnification in 10 random positive cells/field and expressed in percentage.

Table 2. Histopathology grading system of tendon lesion (Bonar score modified).

Grade		CT 7 days (N=10)					CT 14 days (N=10)					CT 28 days (N=10)				
		0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Variable	FD	0	0	1	5	4	0	1	3	5	1	0	0	2	6	2
	Frag	0	0	2	6	2	0	0	3	7	0	0	0	5	5	0
	VN	0	0	5	5	0	0	0	7	3	0	0	4	4	2	0
	Fibro	0	2	7	1	0	0	3	7	0	0	0	3	7	0	0
	CD	0	5	4	1	0	0	2	5	3	0	0	1	6	3	0
	II	0	0	2	5	3	0	0	4	6	0	0	0	4	6	0
Grade		PBM 7 days (N=10)					PBM 14 days (N=10)					PBM 28 days (N=10)				
		0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Variable	FD	0	5	5	0	0	0	4	6	0	0	0	5	5	0	0
	Frag	0	4	5	1	0	0	5	5	0	0	0	5	5	0	0
	VN	0	4	3	3	0	0	3	6	1	0	0	1	6	3	0
	Fibro	0	0	3	7	0	0	0	4	4	2	0	0	4	5	1
	CD	0	0	3	5	2	0	0	4	4	2	0	0	4	4	2
	II	0	4	5	1	0	0	6	4	0	0	0	5	5	0	0

A four-point semi-quantitative severity-based scoring system was used. The pathologic findings were graded as: grade 1: tendon alterations in 1 to 25% of the examined tissue; grade 2: tendon alterations in 26 to 50% of the examined tissue; grade 3: tendon alterations in 51 to 75% of the examined tissue; and grade 4: tendon alterations in 76 to 100% of the examined tissue. Abbreviations: FD: fibrillary degeneration; Frag: fragmentation; VN: vascular neoformation; Fibro: fibroblasts; CD: collagen deposition; II: inflammatory infiltrate.

Collagen quantification using 4-hydroxyproline

The collagen content was determined by measuring the 4-hydroxyproline (Bondjers and Björkerud, 1973). Briefly, the samples (n=40) were freeze-dried, weighed and hydrolysed at 100°C for 22 hours. The absorbance of each standard and sample was measured at 560 nm by spectrometry and the results expressed in µg of 4-hydroxyproline/mg of dry tissue.

Statistical analysis

For quantitative variables, the means±standard errors of the mean results from replicated samples, or from combined independent experiments when between-experiment variation allowed the reliable combination of raw data, were compared. Comparison of multiple groups was performed using analysis of variance (ANOVA) followed by Bonferroni post hoc tests or the Kruskal Wallis test to compare tendons treated with laser with non-treated tendons. The statistical procedures were performed with SPSS statistical software, version 22 (SPSS Inc., Chicago, Ill., USA).

Results

Effect of PBM administration on healing tendons

Fig. 1 shows the H&E staining of tendon tissues

from lesion-only and PBM treated groups. The groups treated after 7 days (PBM 7) displayed normal architecture, characterized by parallel or linear orientation of collagen bundles, a low degree of inflammatory infiltrate, and discrete increase of fibroblast coincident with the maintenance of their architecture (Fig. 1D). In contrast, the CT specimens displayed remarkable architecture distortion, which was modified by large basophilic areas with the disruption of the normal linear orientation of collagen bundles and characterized by a wavy pattern of fibrils. Nevertheless, we can identify a great change in the orientation of the collagen fibers exhibiting a heterogeneous and deformed pattern. We can also note the presence of an intense inflammatory infiltrate involving the region of the injury (Fig. 1A).

Fig. 1E, shows a large reduction of inflammatory cells in the injured region and an intense change in the orientation of collagen fibers when compared with their respective control at 14 days. This effect was reached 28 days after treatment with PBM, as shown in Fig. 1F, when compared to their respective 28-day control (Fig. 1E). However, no alterations in H&E coloration were observed between the PBM groups at any time studied. (Fig. 1D-F).

The Table 2 shows the histopathologic grading analysis in the CT and PBM animals groups and expressed in Mean±Standard Error. Compared to controls, tendons from PBM-7 days group demonstrate a

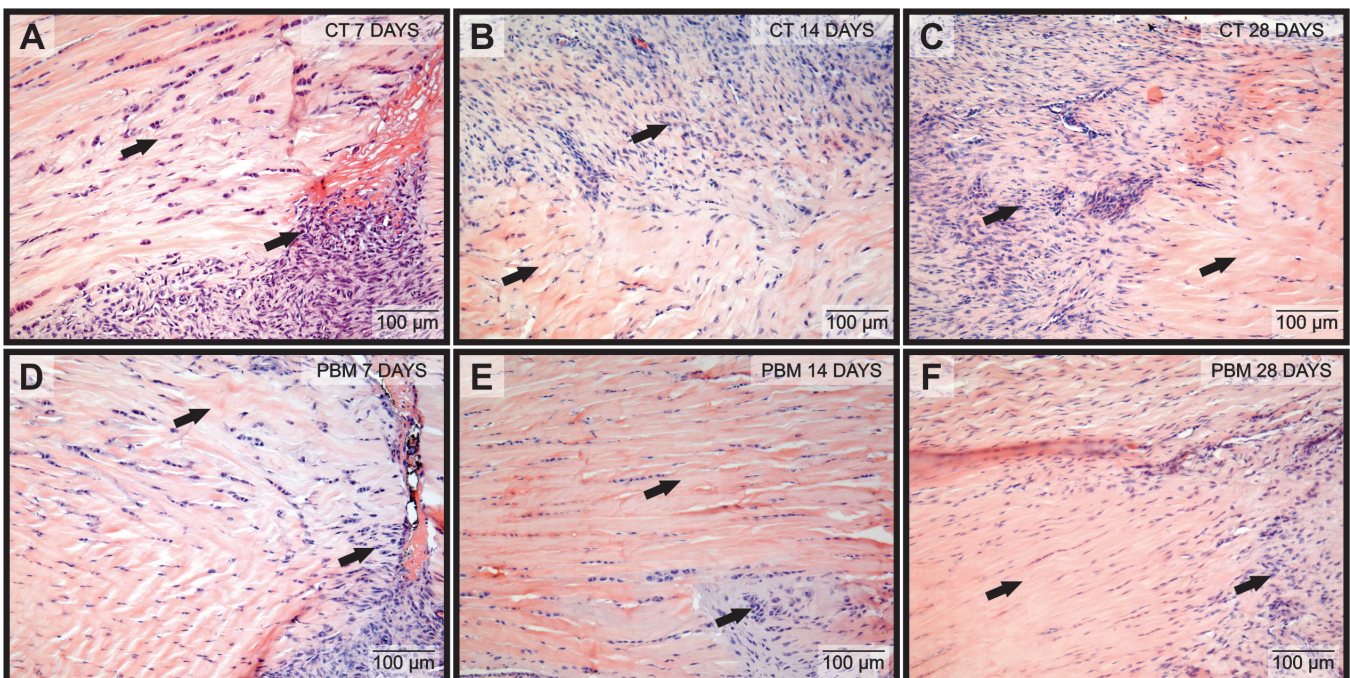


Fig. 1. Histological sections of tendon tissue after injury (CT) and treated with Photobiomodulation (PBM), stained with HE. In (A), tendon tissue with lesion after 7 days (CT 7 days) shows inflammatory infiltrate and collagen fibers (arrows). In (B) and (C), tendon with lesion after 14 and 28 days, respectively, shows an increase of inflammatory cells throughout the tissue and wavy patterns of collagen (arrows). In (D), tendon after 7 days of treatment with PBM shows reduction of inflammatory infiltrate (arrows) in relation to its respective control of 7 days. In (E), tendon after 14 days of treatment shows a linear pattern of collagen fibers (arrows). In (F), tendon after 28 days of treatment shows tissue repair and decrease of inflammatory cells (arrows) in relation to their respective control of 28 days.

Photobiomodulation therapy increases collagen

decrease in the basophilic degeneration of collagen fibers (3.30 ± 0.21 vs 1.50 ± 0.16 ; $p<0.0001$), collagen fiber fragmentation (3.0 ± 0.21 vs 1.7 ± 0.21 ; $p<0.0001$) and inflammatory infiltrate (3.10 ± 0.23 vs 1.7 ± 0.21 ; $p<0.0001$). Additionally, we recognize an increase of fibroblasts (1.90 ± 0.17 vs 2.70 ± 0.15 ; $p<0.0001$) and collagen deposition (1.60 ± 0.22 vs 2.80 ± 0.25 ; $p<0.011$) in tendons from PBM-7 day group, but no statistical difference was observed for vascular neoformation in tendons from PBM-7 day group (2.50 ± 0.16 vs 1.90 ± 0.15).

The tendons from PBM-14 day group shows a decrease in the basophilic degeneration of collagen fibers (2.60 ± 0.26 vs 1.60 ± 0.16 ; $p<0.0001$), collagen fiber fragmentation (2.70 ± 0.15 vs 1.50 ± 0.16 ; $p<0.0001$) and inflammatory infiltrate (2.60 ± 0.16 vs 1.4 ± 0.16 ; $p<0.0001$). Additionally, we recognize an increase of fibroblasts (1.70 ± 0.15 vs 2.80 ± 0.25 ; $p<0.0001$). No statistical difference was observed in vascular neoformation (2.50 ± 0.16 vs 1.90 ± 0.15) and collagen deposition (2.10 ± 0.23 vs 2.80 ± 0.25) in tendon from PBM-14 day group.

Compared to controls, the tendons from PBM-28

day group demonstrate a decrease in the basophilic degeneration of collagen fibers (3.00 ± 0.21 vs 1.50 ± 0.16 ; $p<0.0001$), collagen fibers fragmentation (2.50 ± 0.16 vs 1.50 ± 0.17 ; $p<0.0001$), inflammatory infiltrate (2.60 ± 0.16 vs 1.5 ± 0.17 ; $p<0.0001$) and collagen deposition (2.60 ± 0.20 vs 1.5 ± 0.16 ; $p<0.0001$).

Table 3. Expression of the CT and PBM group genes after 7, 14 and 28 days.

Groups	Genes		
	Col1A1	Col2A1	Col3A1
7 PBM	11.12±2.52	4.32±0.56**	4.58±0.10
7 CT	11.71±3.15	0.66±0.06	5.33±0.81
14 PBM	11.70±2.63	2.16±0.33*	4.46±0.87
14 CT	14.49±1.38	1.05±0.09	3.86±0.20
28 PBM	10.74±2.98	2.06±0.60*	3.73±0.35
28 CT	9.15±1.59	0.60±0.03	3.58±0.59

The groups were compared using ANOVA followed by Bonferroni post hoc tests or Kruskal Wallis test. * $p<0.05$ ** $p<0.01$. The values were expressed in fold change from constitutive gene beta-actin.

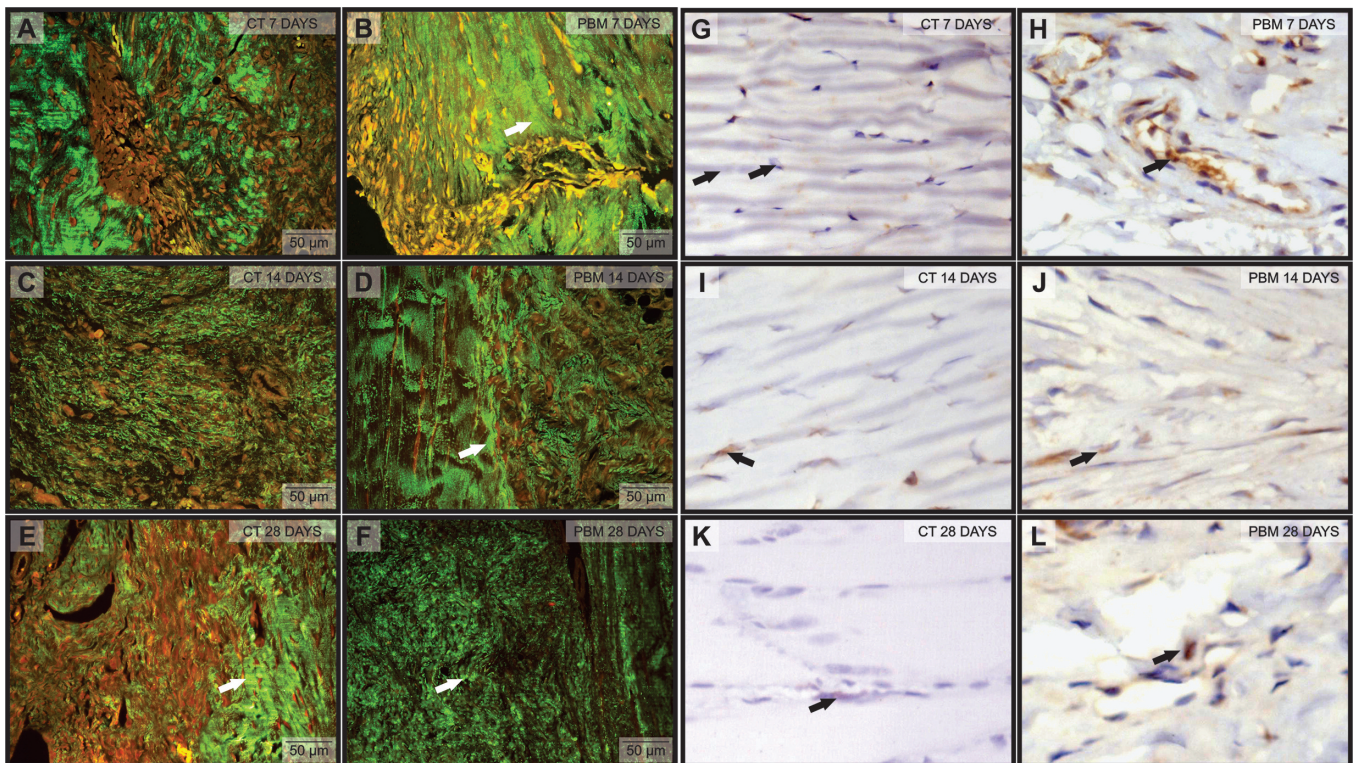


Fig. 2. Immunofluorescence for type II collagen (A-F) and immunolabelled for Sca 1 (G-L) in the CT and PBM tendon tissue after 7, 14 and 28 days. (A) shows a weak expression of collagen II after 7 days compared to (B) 7 days after treatment (arrow). In (C), tissue after 14 days, we observed low fluorescence intensity compared to that of PBM treated tissue after 14 days (arrow) (D). (F) shows an increase of the fluorescence intensity (arrow) in the tissue 28 days after treatment in relation to its respective control (arrow) (E). In (G), CT 7 days, we observed low expression of Sca 1 (arrows) in relation to treated tissue after 7 days (arrow) (H). (J) shows treated tissue after 14 days with increased expression of Sca 1 (arrow) relative to its respective 14 days (arrow) (I) control. (L) shows increased expression of Sca 1 (arrow) in treated tissue after 28 days relative to its respective control (arrow) (K).

Additionally, we identify an increase of fibroblasts (1.70 ± 0.15 vs 2.70 ± 0.21 ; $p<0,0001$). No statistical difference was observed in vascular neoformation (1.80 ± 0.25 vs 2.20 ± 0.20) in tendons from PBM-28 days group.

Discussion

In this work, it appears that PBM has an effect on early tendon healing, with increased proliferation of mesenchymal cells, improved collagen fibre alignment, reduced inflammation and increased production of collagen II. We suggest that these changes are beneficial in tendon healing because the type II fibril is likely to be more stable than the collagen type I fibril due, in part, to a higher crosslinking capability. The greater potential for interfibrillar crosslinking in type II collagen may also result in a more complex and possibly more stable network-like tissue organization (Antipova and Orgel, 2010).

Type II collagen adds structure and strength to the connective tissues that support the body's muscles, joints, organs, and skin (Fukuta et al., 1998; Buckley et al., 2013; Taylor et al., 2020). Type II collagen is found primarily in cartilage, a tough but flexible tissue that makes up much of the skeleton during early development.

Type II collagen is typically associated with tissues that experience compressive loads, including growth plate and articular cartilage, where type II collagen comprises nearly 80% of total collagen (Watanabe et al., 1998). This collagen also forms strong fibrils, but these fibrils are typically smaller in diameter than collagen I fibrils in tendons and ligaments (Mow and Huiskes, 2005), are present in tendons only in small amounts and

concentrated near the bone insertion (Adamczyk et al., 2008).

Despite the existence of controversial studies using PBM therapy for tendon recovery (Bjordal et al., 2003; Tumilty et al., 2010), we found another effect of PBM therapy after Achilles tendon lesion at 7, 14 and 28 days after injury. PBM was able to activate protein expression of collagen II, which probably resulted from stem cell stimulation, as indicated by increased COLA2 gene expression. PBM administration remains speculative due to contro-versial and varied results (Freitas and Hamblin, 2016) and especially due to the lack of an application protocol. The biochemical mechanisms remain poorly understood and the large number of parameters such as the wavelength, fluence, power density, pulse structure, and timing of the applied light must be chosen for each treatment (Sommer et al., 2001; Pereira et al., 2002; Sutherland, 2002; Huang et al., 2011). To our knowledge, this study presents the first information about the enhancement of type II collagen after PBM application on Achilles tendon lesions. Most studies demonstrated that PBM causes an increase in the proliferation rate of the stem cells (Abrahamse, 2012; Ginani et al., 2015; Freitas and Hamblin, 2016).

It appears that stem cells are particularly sensitive to light (light in the red or near-infrared region) (Huang et al., 2009). PBM induces stem cell activity, as shown by increased cell migration, differentiation, proliferation and viability, as well as by activating protein ex-pression (Abrahamse, 2012). Mesenchymal stem cells, usually derived from bone marrow, dental pulp, periodontal ligament and adipose tissue, increased cell proliferation after PBM irradiation (Ginani et al., 2015). PBM positively influences the “*in vitro*” proliferation of stem cells in experiments (Freitas and Hamblin, 2016). Since

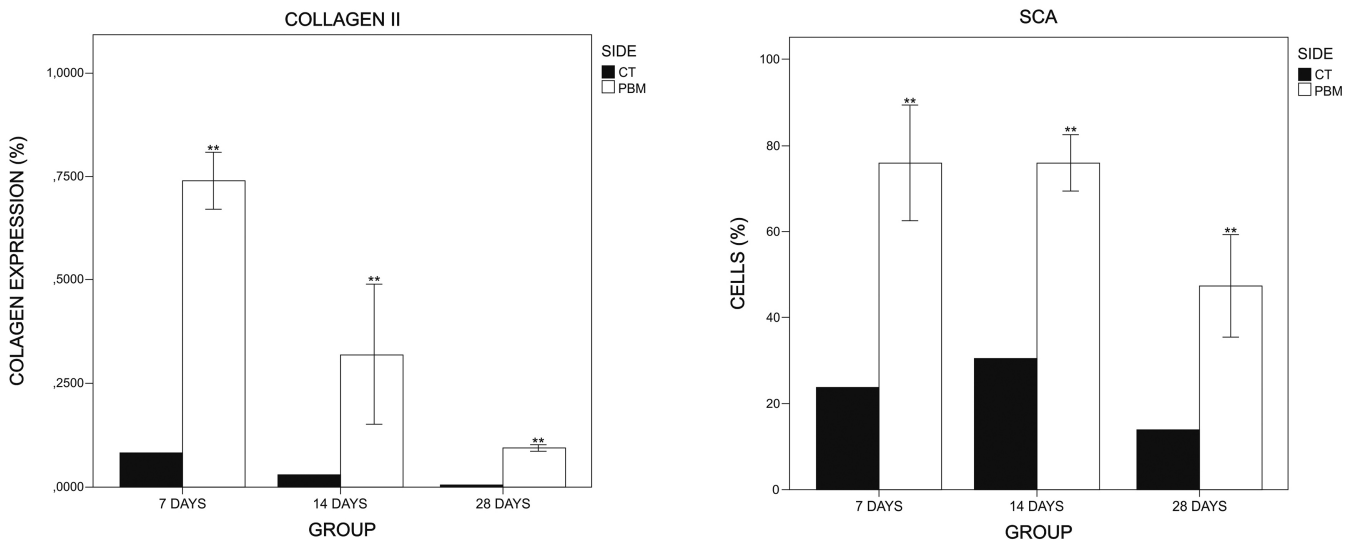


Fig. 3. Graphical representation of the quantification of type II collagen expression (A) and Sca-1 expression (B) in CT and PBM tendon tissue after 7, 14 and 28 days. Values are mean \pm of 10 animals. All values were quantified in 6 random fields in the tendon compartment. The groups were compared using ANOVA followed by Bonferroni post hoc tests or Kruskal Wallis test. ** $p<0.05$ *** $p<0.001$.

stem cells in their undifferentiated form show a lower rate of proliferation, this may be a limiting factor for the clinical effectiveness of stem cell therapies; PBM offers a viable alternative to promote the translation of stem cell research into the clinical arena (Ginani et al., 2015).

The Achilles tendon's function is to transmit loads. Mechanical loading constantly acts on Achilles tendon and withstands large forces (Komi et al., 1992). There is some collagen II in certain tendons, mainly in compressive regions where tendons go around bony pulleys or bear weight and in the fibrocartilage zone (Sun et al., 2010). Experimental studies have identified that the expression of collagen II is significantly reduced with limb suspension, to the point of nearly complete inhibition. This finding suggests that collagen II might be a more sensitive marker of tendon mechanical load deprivation (Sun et al., 2010). The load has deprivation as the animal does not put the normal weight on the legs subjected to surgical injury, so collagen II should decrease and that is not what happened. There was an increase in its expression with PBM.

This study has only identified that COL2 has increased. COL2 gene expression was higher in the PBM group than in the CT group. Interestingly, the histomorphometric analysis coincided with an increased number of mesenchymal cells, characterized by Sca-1 expression in the lesion region.

We observed enhanced type II collagen expression and production around the site of the lesion following lesion creation. This may represent a response of PBM therapy after Achilles tendon lesion. Type II collagen in tendons is typically restricted to areas of compressive loading, and in the human supraspinatus tendon, type II collagen was found in the medial portions of the tendon (Buckley et al., 2013). Thus, all regions of the supraspinatus tendon may experience some degree of compressive strain and contribute to this possible adaptation. Here, we found a similar result, but after lesion creation, it is possible that the augmented type II collagen in the Achilles tendon represents an attempt to prevent compressive strain.

We found augmented type II collagen in PBM group at all the times studied, suggesting that, after lesion creation, the Achilles tendon became susceptible to compressive strain, making this event part of the regeneration process during tendon healing. Type II collagen may assist in maintenance of flexibility and resistance in recently injured tendons (Silver et al., 2001, 2002; Holmes and Kadler, 2006; Sun et al., 2010).

We can infer that the effect of PBM is not similar to that of non-ablative radiofrequency (Akamatsu et al., 2016) and pulsed electromagnetic field (PEMF) therapy (Strauch et al., 2006; Huegel et al., 2020). Non-ablative radiofrequency and PEMF seem to increase tissue stiffness by enhancing resistance to stretch, while low level laser therapy seems to structure the tissue to resist compression. This is consistent with the collagen fibers and fibrils organized as parallel strands along the loading axis resulting in anisotropic tissue matrix with high

mechanical strength during axial stress (Maffulli et al., 2005) and reduced strength under compression or shear (Zhang and Fu, 2013). Histopathologic descriptive analysis showed that the groups treated after 7 days (PBM 7) displayed normal architecture, characterized by parallel or linear orientation of collagen bundles; a more normal alignment would be consistent with the structure of normal tendon.

What does not seem to be so attractive is the application time of PEMF, which can be up to thirty minutes (Strauch et al., 2006), one, three to 6 hours (Huegel et al., 2020) of daily application.

However, our study had several limitations. The lack of evaluation in more advanced remodeling periods is an important limitation of this work when considering the formation of collagen II. There was no use of a naive control group to assess the level of Col2a1 expression in an un-injured animal, so it could not be determined whether the increase of Col2a1 expression was to a level greater than that of normal tendon in an un-injured control. Even with work limitations, we believe that the increase of collagen II must contribute to understanding tendon regeneration and that the gain of this collagen in the lesion area might be a compensatory result of the tendon regeneration. Alternatively, mineralization may be occurring in an attempt to provide greater load-bearing capacity to the tissue as has been noted in avian tendons (Landis and Silver, 2002).

Cells such as fibroblasts, keratinocytes, lymphocytes, and osteoblasts have shown increased proliferation when subjected to laser irradiation, although little is known about the effects of PBM on stem cells (Ginani et al., 2015). It is evident that there exists a need for further studies analysing the effect of laser therapy on cells derived from other sources in addition to tendon tissue. It is necessary to standardize the parameters in order to obtain the desired effects by the use of PBM, which would also allow a more accurate comparison of results.

This study presents the first quantitative measurements of type II collagen in experimental Achilles tendon lesion with the use of PBM therapy for tendon recovery. We suggest this may represent an adaptation to mechanical loading following PBM therapy in Achilles tendon lesion. Further biomechanical testing in this experimental model would be beneficial to quantify the effects of PBM.

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