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ORIGINAL ARTICLE

Photobiomodulation therapy on collagen type I and III, vascular endothelial growth factor, and metalloproteinase in experimentally induced tendinopathy in aged rats

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Abstract This study investigates the effect of photobiomodulation therapy (PBMT) on collagen type I and III, matrix metalloproteinase (MMP), and vascular endothelial growth factor (VEGF) in experimentally induced tendinopathy in female aged rats. Tendinopathy was induced by the Achilles tendoncollagenase peritendinous. Forty-two Wistar rats (*Norvegicus albinus*) were used; groups consisted of 36 aged animals (18 months old; mean body weight, 517.7 ± 27.54 g) and 6 adult animals (12 weeks old; mean body weight, 266 ± 19.30 g). The animals were divided into three groups: control, aged tendinopathy, and aged tendinopathy PBMT; the aged groups were subdivided based on time to euthanasia: 7, 14, and 21 days. PBMT involved a gallium-arsenide-aluminum laser (Theralaser, DMC®) with active medium operating at wavelength 830 ± 10 nm, 50 mW power, 0.028 cm^2 laser beam, 107 J/cm^2 energy density, 1.8 W/cm^2 power density, and an energy of 3 J per point. The laser was applied by direct contact with the left Achilles tendon during 60 s per point at a frequency of three times per week, until the euthanasia date (7, 14, and 21 days). VEGF, MMP-3, and MMP-9 were analyzed by immunohistochemistry, and collagen type I and III by Sirius red. PBMT increased the deposition of collagen type I and III in a gradual manner, with significant differences relative to the group aged tendonitis ($p < 0.001$), and in relation to VEGF ($p < 0.001$); decreased expression of MMP-3 and 9 were observed in group aged tendinopathy ($p < 0.001$). PBMT, therefore, increased the production of collagen type I and III, downregulated the expression of MMP-3 and MMP-9, and upregulated that of VEGF, with age and age-induced hormonal deficiency.

Keywords Calcaneal tendon · Tendinopathy · Collagenase · Photobiomodulation therapy · Aged

Introduction

Aging is accompanied by a decline in the homeostatic and regenerative capacity of all tissues and organs [1]. Tendon aging or chronic tendinopathy are often responsible for handicapping middle-aged and aging populations [2]. With aging, tendon tissue undergoes morphological and biochemical changes, involving both the cells and the extracellular matrix (ECM); the ECM increases in volume, causing a relative decrease of the number of cells per unit of tissue surface. During aging, parallel changes occur in the elastic fibers, which decrease in number and show structural alterations [3]. Aging and estrogen deficiency can reduce the constituents of the ECM. This decrease may compromise the tendons constitution and function, since they are formed of collagen structures, especially type I and III; the tendon is composed of fascicles, formed from collagen fibers. These collagen fibers are formed from continuous collagen fibrils, aligned to the tissue long axis for mechanical strength, and can therefore develop tendinopathy [4, 5].

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Tendon disorders are frequent and are responsible for substantial morbidity both in sports and in the workplace. Tendinopathy, as opposed to tendinitis or tendinosis, is the best generic descriptive term for the clinical conditions in and around tendons arising from overuse. Tendinopathy is a serious issue requiring lengthy management, and patients often respond poorly to treatment [6].

Several physical modalities have been developed to treat tendinopathy; however, they often fail to control the inflammatory process and pain. In recent years, phototherapy has been associated with other physical resources and has gained popularity in the treatment of tendinopathy demonstrating efficacy mainly in pain control [7, 8] and inflammation [9–11]. In particular, low-photobiomodulation therapy (PBMT) in early stages of the inflammatory process modulates the expression of pro- and anti-inflammatory cytokines [12–15], improves the production and differentiation of collagen [16–18], and shows positive clinical results [19–21]. Although some studies have evaluated the efficiency of PBMT in tendinopathy, aging conditions are associated with hormonal changes related to the estrogen production. Given the evidence that aging causes changes in cell structure and the tendon morphology, hormonal changes associated with aging complicates the resolution of tendinopathy. The purpose of this study was to verify the PMBT viability and normalize the tendinopathy process induced by collagenase in female aged rats.

Materials and methods

Animals and ethics

Forty-two female Wistar rats (*Norvegicus albinus*) were used, with groups of 36 aged animals (18 months old; mean body weight, 517.7 ± 27.54 g) and 6 adult animals (12 weeks old; mean body weight, 266 ± 19.30 g). Animals were acquired from the animal facility of the Universidade Nove de Julho (UNINOVE), where they were housed and kept under controlled light and temperature, with water and food given ad libitum. All experimental procedures were carried out in accordance with standards established by the Brazilian College for Animal Experimentation (COBEA). Animals were handled in compliance with national guidelines for the humane treatment of laboratory animals, and the Research Ethics Committee of the UNINOVE-AN00302014 approved all experimental procedures.

Experimental groups

Animals were divided into three experimental groups. The 36 aged animals were randomly divided into two groups (Tendinopathy aged group or Tendinopathy PBMT aged group—18 animals per group). An additional adult control group (with adult rats) with no PBMT irradiation and no injury included six animals.

1. Control group (adult)—no injury and no PBMT irradiation;
2. Tendinopathy aged group—injured and no PBMT irradiation;
3. Tendinopathy PBMT aged group—injured and with PBMT irradiation.

The aged injury groups were further divided into three subgroups based on time to euthanasia (7, 14, and 21 days).

Experimental model of collagenase-induced tendinopathy

Tendinopathy was induced using collagenase, an enzyme that degrades collagen and induces an acute inflammatory process. Collagenase (1 mg/ml; Sigma Chemical Co, Cat. C-6885) was dissolved at 100 mg/ml in sterile phosphate-buffered saline (PBS) containing 50 mM NaH_2P0_4 and 10 mM NaCl (pH 7.1). Skin was surgically prepared and collagenase was injected in the two legs (100 μg/tendon) percutaneously into the Achilles tendon, approximately 2 mm proximal to the osteotendinous junction under anesthesia. The same volume of PBS without collagenase was injected using the same procedure in a control group [22].

Photobiomodulation therapy

PBMT was applied using a gallium, arsenide, and aluminum (GaAlAs) laser (Theralaser, DMC®) with active medium operating at wave length of 830 nm (±10 nm), 50 mW power, laser beam 0.028 cm^2, energy density of 107 J/cm^2, power density 1.8 W/cm^2, and energy 3 J per point. The laser was applied by direct contact with the left Achilles tendon during 60 s per point, at three points (muscle-tendon, tendon, and bone-tendon). The animals were manually restrained for the procedure and irradiated at a 90° angle in relation to the tissue surface. Before the beginning of the experiments, the laser equipment was calibrated with a power meter (Model 13PEM001/J, Mellers Griot, Netherlands).

Treatment was initiated 12 h after injury, three times a week until the euthanasia time (7, 14, and 21 days).

Euthanasia

The animals were identified, weighed, and euthanized via intraperitoneal thiopental administration (THIOPENTAX - Cristália 100 mg/kg; DL) with lidocaine 10 mg/ml (Xylestesin-Cristália). Samples were then taken and sent for immunohistochemistry and morphometric collagen analysis. The tendon tissues were histologically analyzed by fixing with 10 % formalin, embedded in

paraffin, and sectioned. Sections (5-μm thick) were stained with hematoxylin-eosin and Picrosirius red.

Morphometric analysis of collagen types

Morphometric analysis was performed using image digitization and computational analysis with a specific image processing and analysis program (Image Pro plus 4.5). To quantify the areas representing collagen, five fields observed using a microscope that optically transmitted and polarized light (Carl Zeiss, Pol-Interferential Photomicroscope, Germany) were coupled to a color CMOS camera with 1280 × 1024 pixel resolution at ×400 magnification (Thorlabs GmbH, Germany), which was connected to a microcomputer equipped with a video board. All images were digitized before the quantification process, thereby standardizing the microscope light intensity and condenser height. Collagen areas were separated in the image, using color distribution as the discriminating parameter.

Picrosirius red, an anionic composite that distinguishes the collagen fibers thickness and density of through coloration emitted under polarized light, was used to estimate the percentage of collagen fibers. While thin dissociated fibers typical of type III collagen are greenish, the thickest strong associated fibers of type I collagen emit colors with longer wavelengths, such as red and yellow [23].

Immunohistochemistry

Paraffin was completely removed through a series of sequential xylene/ethanol/water washes to remove the wax and rehydrate the tissue. Sections were washed in PBS (6 × 5 min washes) and mounted with 1 % normal goat serum in PBS for 30 min. Subsequently, slides were incubated in the presence of primary antibody applied overnight at 4 °C. Primary antibodies used were mouse anti-rat VEGF antibody (VG-1; Abcam, Tokyo, Japan), rabbit anti-MMP-3 antibody (ab-53015, Abcam, Tokyo, Japan), and goat anti-MMP-9 antibody (sc-6840, Santa Cruz Biotechnology). After washing in PBS (six times, 5 min), sections were incubated with secondary antibody for 30 min. After washing in PBS (6 × 5 min washes), a coloring reaction was carried out with diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and nuclei were counterstained with hematoxylin. Areas of positive staining for each of the tags were observed under a light microscope (E200, Nikon, Japan), and images were captured by a microcomputer equipped with IC Capture 2.2 software (The Imaging Source, Germany). From each sample, four images were recorded of different view fields, including each stained area, using a ×10 objective lens to capture the length. Once captured, the images were analyzed using a software-based image analysis system (Image-Pro Plus® 4.5, Media Cybernetics, MD, USA) [24].

Statistical analysis

For collagen type and immunohistochemistry analyses, data were tabulated using Microsoft Excel 2007 software and initially assessed for normality using the Shapiro-Wilk test. Since a normal distribution was observed, two-way ANOVA with Bonferroni post hoc test was used for comparisons between experimental groups. All of the data are expressed as mean ± standard deviation. GraphPad Prism 5 software program was used (GraphPad Software Inc., San Diego, CA, USA) and significant differences were considered when $p < 0.05$.

Results

PBMT modulates collagen type I and III

The collagen content was assessed using light polarization, and we showed a reduced collagen type I expression 7 and 14 days after collagenase in the aged tendinopathy group compared to control animals (Fig. 1a, b). Moreover, the PBMT restored collagen type I levels in aged animals. Analyses carried out 21 days have shown an upregulation in collagen type I with PBMT application. As illustrated in Fig. 1a, c, tendinopathy was accompanied by upregulation of type III collagen content in tendon for all analysis times. On the other hand, PBMT blunted the type III collagen increases linked to tendinopathy.

Altered expression of the metalloproteinase 3 (MMP-3) and 9 (MMP-9) is affected by PBMT

We have applied immunohistochemistry assays to evaluate the PBMT role on MMP-3 and MMP-9 dysregulation. Thus, it can be seen in Figs. 2 and 3 that there was a substantial increase in MMP-3 and MMP-9 expression associated to tendinopathy. The main finding was reported to the PBMT effect, which attenuated the MMP-3 increases on the 7th and 14th day of the experiment. In addition, PBMT completely restored the levels of MMP-3 with 21 days. A similar pattern was notified in the MMP-9 analysis, in which PBMT inhibited the increase of this protease in all evaluation points (Figs. 2 and 3).

Vascular endothelial growth factor (VEGF) is upregulated by PBMT

On immunohistochemistry analysis, we observed that the tendinopathy triggered a significant VEGF reduction in the tendon for all analysis times (Fig. 4). An important observation was that the PBMT resulted in significant increase of the VEGF with 7 days of injury and normalized of protein content to baseline levels in the remaining periods of analysis.

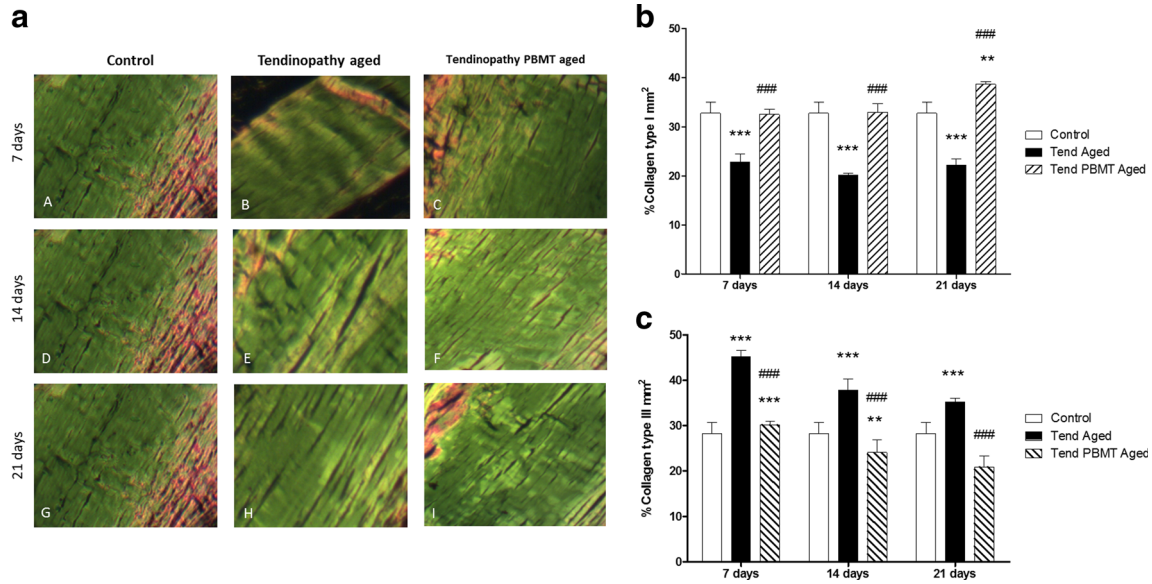

Fig. 1 Morphometric analysis of the percentage of collagen type I and III obtained from tendon and histological sections stained with Picrosirius red and observed under polarized light. **a** Panels representatively stained with Picrosirius red show the presence of the two types of collagen fibers as well as their interlacing at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, aged tendinopathy, and aged tendinopathy PBMT. Magnification ×40; *scale bar* = 20 μm. **b** Panels represent percentage of collagen type I at 7, 14, and 21 days after experimental induction. Data are presented as mean ± SD. **c** Panels represent percentage of collagen type III at 7, 14, and 21 days after injury. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ using Bonferroni test with comparisons against the control group. $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ using Bonferroni test with comparisons against the aged tendinopathy. Data are presented as mean ± SD

Discussion

The tendinopathy etiology remains unclear and many causes have been theorized, such as hypoxia, ischemic damage, oxidative stress, hyperthermia, impaired apoptosis, and inflammation. In tendon injury and healing, inflammatory mediators, fluoroquinolones, and MMP imbalances have been implicated as mechanisms of tendon degeneration. Histologically, tendinopathy is characterized by disordered and haphazard healing with absence of inflammatory cells,

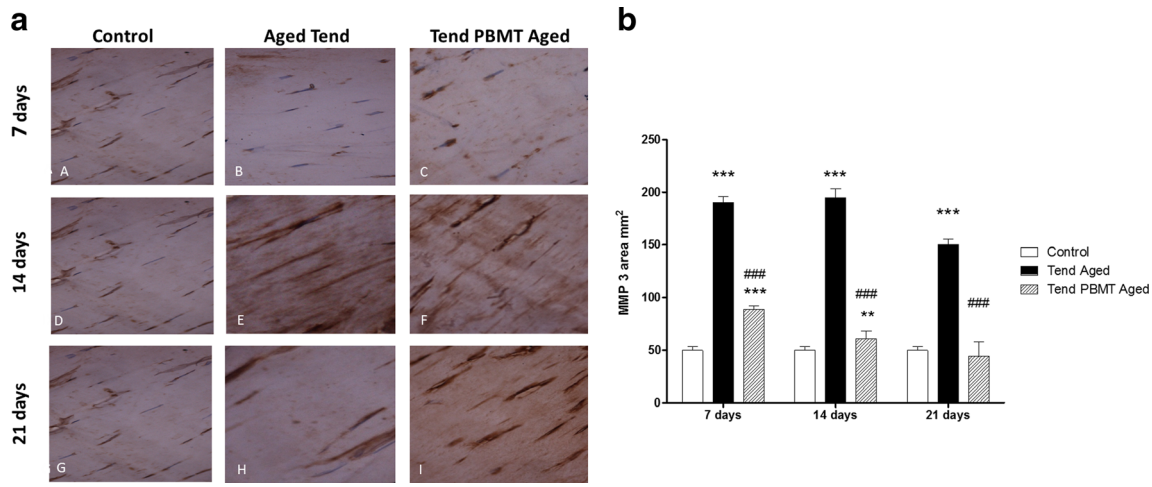

Fig. 2 Comparison of the mean and standard deviation of immunolabeled areas in square millimeter for technical MMP-3 by immunohistochemistry. **a** Panel representatively stained and immunolabeled for MMP-3 at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. Magnification ×20; *scale bar* = 20 μm. **b** Panel represents immunolabeled areas for MMP-3 at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ using Bonferroni test with comparisons against the control group; $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#p < 0.001$ using Bonferroni test with comparisons against the aged tendinopathy group. Data are presented as mean ± SD

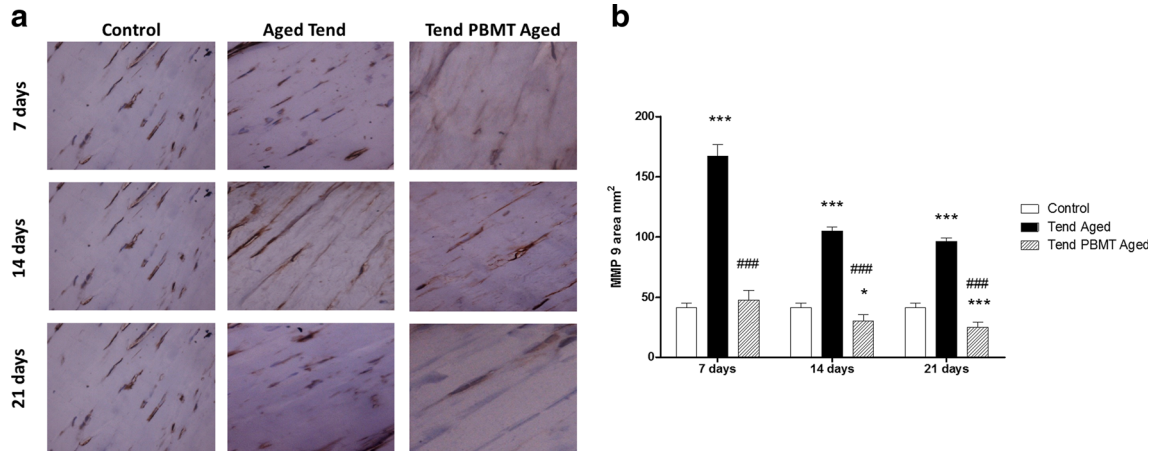

Fig. 3 Comparison of the mean and standard deviation of immunolabeled areas in square millimeter for technical MMP-9 by immunohistochemistry. **a** Panel representatively stained with immunolabeled MMP-9 at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. Magnification ×20; *scale bar* = 20 μm. **b** Panel represents immunolabeled areas for MMP-9 at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ using Bonferroni test with comparisons against the control group; $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#p < 0.001$ using Bonferroni test with comparisons against the aged tendinopathy group. Data are presented as mean ± SD

poor healing responses, non-inflammatory intratendinous collagen degeneration, fiber disorientation and thinning, hypercellularity, scattered vascular ingrowth, and increased interfibrillar glycosaminoglycans [6].

The sex difference in immune responses after injury is mediated in part by alterations in the circulating levels of gonadal steroid hormones through modulation of the production of inflammatory cytokines and immune regulation. For example, aberrant production of IL-6 is an important mediator of immunity after injury, and estradiol is a critical regulator of IL-6 production and overall immune function [25]. Therefore, the present study was designed with the purpose of investigating the chronic inflammatory process of tendonitis in older female animals when subjected to PBMT. Several studies have shown that PBMT may be a powerful therapeutic tool, throughout inflammatory processes of both tendinopathies [22, 26, 27] and in the repair of tendon damage [28]. However, hormonal changes that occur during aging, mainly related to estrogen, may be capable of interfering with any inflammatory process [29–31], often hampered by collagen deposition, angiogenesis, and imbalances in MMP action [32–34]. On this basis, the present study aimed to analyze the action of PBMT in

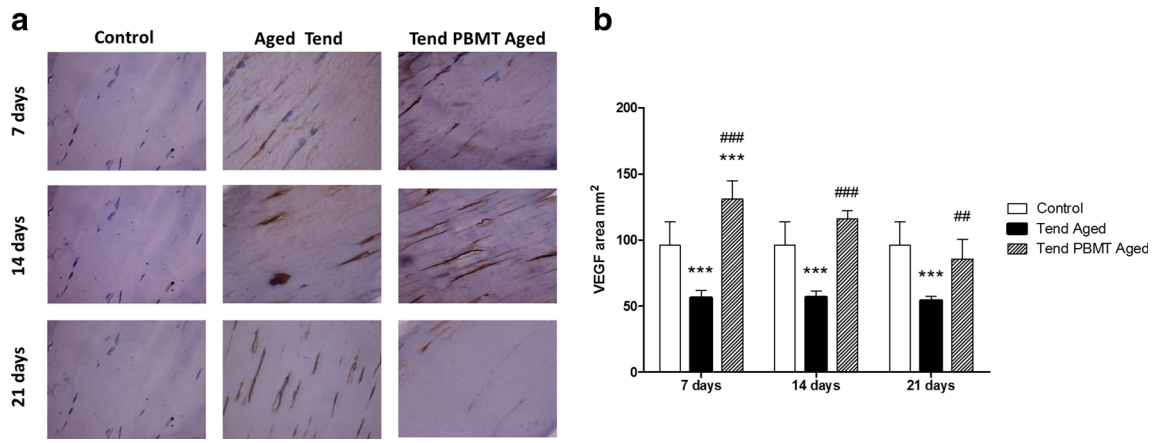

Fig. 4 Comparison of the mean and standard deviation of immunolabeled areas in square millimeter for technical VEGF by immunohistochemistry. **a** Panel representatively stained and immunolabeled for VEGF at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. Magnification ×20; *scale bar* = 20 μm. **b** Panel represents immunolabeled areas for VEGF at 7, 14, and 21 days after experimentally induced tendinopathy in rats in groups: control, tendinopathy aged, and tendinopathy PBMT aged. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ using Bonferroni test with comparisons against the control group; $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#p < 0.001$ using Bonferroni test with comparisons against the aged tendinopathy group. Data are presented as mean ± SD

experimentally induced tendinitis by collagenase in older rats by immunohistochemical analysis of VEGF, MMP-3, MMP-9, and collagen type III, and to verify the total collagen and collagen type I and III during chronic inflammation, using histological staining technique for Picrosirius red under polarized light in the timeline (7, 14, and 21 days).

In this study, we observed that PBMT at a 3-J dosage increased collagen deposition when compared with the group of older rats with tendonitis, and we demonstrated that collagen approached the values of our control group consisting of female adult rats. In addition to total collagen, we also assessed the percentage of collagen marked by Picrosirius red polarized light.

Few studies have focused on the effect of aging on tendon, and most investigated the mechanical features of tendons. Although studies show that dimensions or mechanical properties of tendons do not differ between young and old men, the collagen content is lower in older individuals, and the advanced glycation end marker accumulates markedly in the tendons of older subjects.

Type I and III collagen are the main components of ECM in tendons, corresponding to 65–95 % and 10 % of the composition, respectively. The synthesis of type III collagen increases during early stages of repair, and it is thought that when it decreases, type I collagen is synthesized and organized [35]. The analysis of tendinitis aged PBMT group at 7, 14, and 21 days showed a significant difference in the tendinitis aged group between type I and III collagen areas, similar to typical tendon healing. The same occurred with type I collagen in the control group. On the contrary, type I and III collagen in the areas tendinitis PBMT group at 14 and 28 days did not differ significantly, explaining the delayed healing. Our findings indicate that PBMT may lead to the improvement and acceleration of inflammation when compared to tendinopathy aged group.

In addition to aging, estrogen levels might also play a key role; women show a lower risk of tendinopathies during premenopausal years, whereas after menopause, this risk increases. Estrogen levels may influence tendon metabolism and, in addition to several growth factors, are important for the musculoskeletal tissues behavior and may affect tendon properties; postmenopausal estrogen deficiency seems to downregulate collagen turnover and decrease tendon elasticity [36]. Our results with respect to collagen are similar to findings by Guerra et al. [37], who used normal adult rats, carried out a partial tenotomy, and treated them with a GaAlAs laser with an 830-nm pulse with 4 J/cm^2, with the same dose at days 8 and 15 days after injury, and investigated collagen and MMP activity and collagen synthesis. The researchers concluded that pulsed PBMT improved remodeling of the ECM during the healing process in tendons through MMP-2 activation and stimulation of collagen synthesis. Collagen increment obtained by PBMT in [37] and in our study is in line with results from other researchers; in spite of not using models with aged animals and different tissue types, PBMT results are satisfactory in increasing collagen.

Collagen I is the most extensively examined in aged humans and the consensus is that aging confers a progressive decrease in collagen I synthesis, concurrent with an increase in collagen I degradation [38]. Production and reorientation of collagen are essential to restore the strength of the tendons and there are reports that effects of laser photobiomodulation on tendon healing may involve the promotion of ECM production and degradation [39]. Alves et al. [23] reported that the degradation of ECM components, such as collagen fibers, occurs as a result of MMP activity.

Healing MMPs modulators are important regulators of ECM remodeling, and their levels are altered during tendon healing. In the rat flexor tendon laceration model, the expression of MMP-9 and MMP-13 (Collagenase-3) peaked between days 7 and 14. Levels of MMP-2, MMP-3, and MMP-14 (MT1-MMP) increased after surgery, and remained high until day 28. These findings suggest that MMP-9 and MMP-13 participate only in collagen degradation, whereas MMP-2, MMP-3, and MMP-14 participate in both collagen degradation and collagen remodeling [25]. In the present study, we conducted a study of MMP-3 and MMP-9 over the same experimental periods; we used immunohistochemistry and showed high activity of these MMPs in the tendinitis aged group, suggesting that they had a key role in the collagen analysis presented above.

Aging might exert a negative effect on tendon structure or its healing process by a mechanism that involves increased MMP-2 and MMP-9 activities, and decreased proliferation of tenocytes [40]. Aro et al. [41] observed the presence of latent and active isoforms of MMP-9 during the first 7 days after tendon injury. This period is characterized by inflammation and MMP-9 is predominantly synthesized by inflammatory cells; it has an important role in the degradation of denatured collagen and other matrix components.

Our group treated with PBMT showed little markup for both MPPs, similar to the results obtained in the control group consisting of animals without changes due to age. Our results can also be endorsed by findings of Casalechi et al. [42] that used the same model to study the effect of PBMT, operating at 780 nm, 22 mW potency, and 1.54 J energy, investigating acute and chronic pathology, and determining the gene expression of MMPs and the reduction of MMP-1 mRNA expression in groups that had received PBMT in the initial phase of treatment. Silva et al. [43] conducted a study investigating the action of PBMT repair of skin wounds in diabetic rats; the authors correlate the increase of collagen type I and III with reducing MMP-2 and MMP-9 and regarding the authors description of MMP-9 gene expression, and a marked decrease was found in the diabetic group submitted to PBMT.

Tendinopathy or tendonitis is characterized histopathologically in the chronic stage by the disruption of fibroblast proliferation and apoptosis with arrays, and the proliferation of vessels and nerves containing sensory, nociceptive, and autonomic elements. This histopathology has been characterized as a "failed healing response" and has also been termed "angiofibroblastic tendinosis" to refer to the major finding of increased vascular fibroblastic and cellularity [44].

Regarding angiogenesis, VEGF plays a role in the formation and growth of new blood vessels, playing a substantial role in chronic inflammation and wound healing. VEGF is also known to increase vascular permeability, which can enhance clinical signs of overuse. The VEGF, von Willebrand factor, plays an important role in tendon hemostasis. VEGF is not highly expressed in adult tendon, but its expression is increased in several animal models of acute injury or mechanical loading [45]. Mechanical overload, inflammation and injury, hypoxic conditions, or a combination of the above could lead to increased expression of VEGF in tendon [46].

However, there is strong evidence that VEGF-induced angiogenesis might influence the course of degenerative tendon disease in another way. It is well known that ECM degradation is also required for the sprouting and invasion of new blood vessels. Vascular endothelial or smooth muscle cells are known to produce MMPs in response to VEGF stimulation [47].

Considering the evidence of the major role of VEGF in the tendinopathy healing process, PBMT action was also an important marker. We observed that in the older tendinitis group, expression by immunohistochemical labeling of VEGF had decreased compared to the other groups throughout the experimental periods; VEGF intra-group marking gradually rose over 7, 14, and 21 days but without approaching the normal control or tendinitis PBMT aged groups.

VEGF gene PBMT modulation and protein expression is common in the literature, and some groups have investigated the increase of VEGF expression under adverse conditions. De Oliveira et al. [47, 48] conducted a study using two types of mesenchymal stem cells from human and rat adipose tissues on nutritional deficiencies submitted to PBMT. VEGF and VEGFR2 were increased with PBMT action in both cell types; however, human cells at nutritional deprivation showed higher expression of VEGF and its receptor after irradiation with other laser doses. Casalecchi et al. [42] investigated the PBMT effect in an identical model to that used in this study and confirmed the early induction of VEGF mRNA expression in the PBMT groups. Savitskaya et al. [47] reported that chronic tendon loading causes mechanical trauma, with multiple microruptures of the microvasculature tendon. These microruptures initiate a cascade of VEGF-mediated vascular remodeling that may be chronically pathological, and chronic tendon pathology appears to be a highly active ongoing process of neovascularization. VEGF expression and neovascularization could be used in the clinical setting to monitor tendon degeneration.

In summary, our findings demonstrate that aging and hormonal factors related to gender affect the evolutionary process of tendinopathy, inferring in chronic inflammation phase and complicating its resolution. On the other hand, it was also observed that PBMT was effective in tendon treatment even in adverse conditions such as aging. This study suggests that PBMT obtained satisfactory results, where the collagen type III tendonitis PBMT elderly group resembled the control group at days 7 and 14 days, as well as collagen III at day 7. MMP-3 showed the best results in 21 days. MMP-9 showed the best results at 7 days. VEGF produced satisfactory results in 14 to 21 days.

Limitations to our study were the lack of estrogen measurement and the inability to investigate type I collagen by immunohistochemistry or another form of protein expression verification. Following on from our analysis of MMPs, we could expand this study to using markers TIMP II and I. It is noteworthy asserting that a group of aged animals not treated and without any damages could offer new comparisons, although at the present study it was not possible to use this group.

Compliance with ethical standards

Conflict of interest Professor Ernesto Cesar Pinto Leal-Junior received research support from Multi Radiance Medical (Solon, OH, USA), a laser device manufacturer. Multi Radiance Medical had a role in the planning of this study, and the laser device used was not theirs. They had no influence on study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remaining authors declare that they have no conflict of interests.

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