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ORIGINAL ARTICLE

Pulsed LLLT improves tendon healing in rats: a biochemical, organizational, and functional evaluation

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Abstract In the last decades, the tendon injuries have increased substantially. Previous results suggested that low-level laser treatment (LLLT) promotes synthesis of extracellular matrix and improves the functional properties of the tendon. The aim of this study was to evaluate the effects of different protocols of LLLT on partially tenotomized tendons. Adult male rats were divided into the following: G1—intact, G2—injured, G3—injured+LLLT (4 J/cm^2 continuous), G4—injured+LLLT (4 J/cm^2 at 20 Hz). G2, G3, and G4 were euthanized 8 days after injury. G5—injured, G6—injured+LLLT (4 J/cm^2 continuous), and G7—injured+LLL (4 J/cm^2 at 20 Hz until the seventh day and 2 kHz from 8 to 14 days). G5, G6, and G7 were euthanized on the 15th day. Glycosaminoglycan (GAG) level was quantified by dimethylmethylene blue method and analyzed on agarose gel. Toluidine blue (TB) stain was used to observe metachromasy. CatWalk system was used to evaluate gait recovery. Collagen organization was analyzed by polarization microscopy. The GAG level increased in all transected groups, except G5. In G6 and G7, there was a significant increase in GAG in relation to G5. In G3 and G4, the presence of dermatan sulfate band was more prominent than G2. TB stains showed intense metachromasy in the treated groups. Birefringence analysis showed improvement in collagen organization in G7. The gait was significantly improved in G7. In conclusion, pulsed LLLT leads to increased organization of collagen bundles and improved gait recovery.

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Introduction

Among tendons in the lower extremities, the Achilles tendon is most commonly injured by athletes and it has been described as the tendon most likely to rupture spontaneously [1]. This tendon is subjected to extensive static and dynamic loads, and it can be subjected to loads up to ten times body weight in certain athletic activities [2–4]. Studies have shown that 44 % of ruptures occurred during athletic activities [5]. 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 [6].

Tendon healing is a slow and complex process because of the high level of organization of the components in its extracellular matrix (ECM), and it is aggravated by poor vascularization [3, 4]. Collagen is the most abundant protein in tendons. Type I collagen provides tensile stiffness to the tissue, and type III is distributed among collagen I bundles. Both are fibril-forming collagens with the ability to assemble into highly orientated supramolecular aggregates that are responsible for the properties of the tissue [7–9].

In the tendon-healing process of rats, there are several events that can be divided into distinct but overlapping phases: an inflammatory phase, which extends from 1 to 7 days after injury; a proliferative phase, which begins around day 8 and extends up to 14 days; and a remodeling phase, which begins around day 14 and reaches its peak around day 21 [3, 4, 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 [10, 11].

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 [9, 12, 13]. Thus, if the duration of healing process could be decreased, complications could be reduced [14].

Low-level laser therapy (LLLT) has attracted considerable attention because of its success in tissue repair and its broad spectrum of applications, but there is controversy about specific protocols [12, 14–17]. Previous studies in our laboratory exposed rats with partial tenotomy of the Achilles tendon to different LLLT protocols. The results showed for the first time that the pulsed frequency accelerated the repair process through the increased activation of MMP-2 and -9 which were responsible for the replacement of degraded collagen for intact collagen I that only 15 days after injury equaled the normal tendon [18].

Several studies have shown that after injury, the morphological, biochemical, and functional properties of the tendon are never again identical to normal tissue [16, 19, 20]. Knowing that collagen I is mainly responsible for the mechanical properties of the tissue, our previous results were promising and suggested that LLLT may improve the collagen organization and the functional properties of the tendon. Therefore, this study aimed to analyze the effects of different LLLT protocols in the early phases of wound healing after tendon transection to determine the most appropriate postoperative protocol for tendon repair.

Materials 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, 105 male Wistar/Uni rats with a mean age of 60 days and weight ranging from 300 to 350 g were used. 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 analyzed in the inflammatory and proliferative phases and were divided into seven groups: G1—intact, G2—injured, G3—injured+LLLT (4 J/cm^2 continuous), G4—injured+LLLT (4 J/cm^2–20 Hz), G5—injured, G6—injured+LLLT (4 J/cm^2 continuous), and G7—injured+LLLT (4 J/cm^2–20 Hz until the seventh day and 2 kHz from 8 to 14 days). G2, G3, and G4 were euthanized on day 8 after injury, and G5, G6, and G7 were euthanized on day 15.

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, 21, 22].

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) at 40 mW of power. The animals were immobilized with containment equipment [23] and received a punctual application of 4 J/cm^2 once a day. Treatment with LLLT began the day after surgery and lasted until the day before euthanasia; that way, the groups euthanized 15 days after surgery received 13 applications, and the groups euthanized 8 days after surgery received six applications. Each session of the continuous group lasts 16 seconds with a light intensity of 2,500 W/m^2, and the pulsed group, 32 seconds with a light intensity of 1,250 W/m^2. After the last session, the animals were euthanized with deepening of anesthesia for the removal of the Achilles tendon [18].

Agarose gel electrophoresis

The fragments of the tendons were dehydrated, and sulfated glycosaminoglycans (GAGs) were released from proteoglycans by digestion with a papain solution (Merck) (40 mg/g of dry tissue) containing 100 mM sodium phosphate buffer, pH 6.5, 40 mM EDTA, and 80 mM β-mercaptoethanol (Sigma). The GAGs were separated by agarose gel electrophoresis (0.6 %) in 0.05 M propylenediamine (PDA) (Sigma) [24].

Quantification of sulfated glycosaminoglycans

The content of GAGs was determined by the dimethylmethylene blue method [25], using chondroitin sulfate as standard. The absorbance was measured at and 540 nm.

Morphological and birefringence analysis and measurements

The tendons were fixed using a 4 % formaldehyde solution in Millonig's buffer (0.13 M sodium phosphate, 0.1 M NaOH–pH 7.4) for 24 h at 4° C and washed in water, ethanol dehydrated, diaphanized with xylene and paraffin-embedded. Longitudinal serial sections of 7 μm were stained with hematoxylin–eosin (HE) and toluidine blue (TB) and analyzed under an Olympus BX 60 light microscope.

Birefringence properties were studied using an Olympus BX51-P BX2 polarizing microscope and an image analyzer (Image-Pro Plus 6.3, Media Cybernetics, Inc.—Silver Spring, MD, USA).

Since birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits=1 pixel). The major tendon axis was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringences: intrinsic birefringence (Bi) and form or textural birefringence (Bf) [26, 27], total birefringence (sum of Bi and Bf) was used in this study. The measurements of the transected region of the tendons in each experimental group were made after immersing the sections in water [26, 27].

Evaluation of the maximum contact intensity of the rat paw after partial transection

The CatWalk system (Noldus Inc., The Netherlands) was used to analyze the gait recovery of the animals. In this protocol, the rats crossed a walkway (100 cm length×15 cm width×0.6 cm thickness) with a glass floor illuminated from the long edge in a dark room. Data acquisition was performed with a high-speed camera (Pulnix TM-765E CCD), and the paw prints were automatically classified by the software. The paw prints were obtained during the 2 days before the partial transection of the tendons to assess the normal standard gait of the animals, and they were collected again after the lesions [21].

Postoperative data were assessed on the second, fourth, and sixth days following surgical lesion for the groups that were sacrificed 8 days after surgery, and on the 2nd, 4th, 6th, 8th, 10th, 12th, and 14th days following surgical lesion for the groups that were sacrificed 15 days after surgery. The parameters used were "Max Contact Intensity", corresponding to the pressure exerted by the paw on the glass floor during gait. The intensity of magnification can vary from 0 to 255 pixels.

Statistical analyses

All results were expressed as the mean±standard deviation. For biochemical analysis, data from different experimental groups were analyzed by analysis of variance (one-way ANOVA) followed by the Tukey test. For the max contact intensity of the rat paw, the two-way ANOVA was performed. The level of significance was $p<0.05$. The Mann–Whitney test was used only for analysis of the birefringence measurements.

The analysis was carried out in GraphPad Prism® 3.0 program (GraphPad Software, La Jolla, CA, USA), version 3.0.

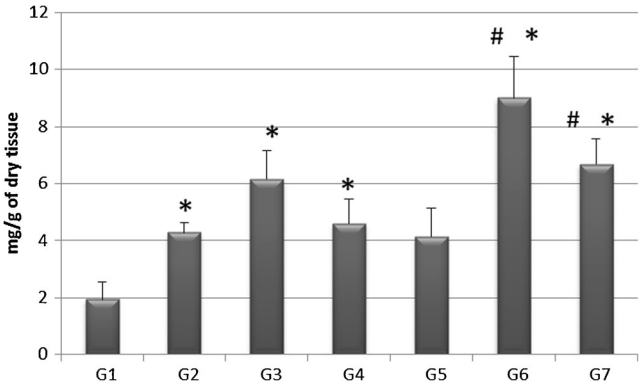

Fig. 1 Concentration of sulfated glycosaminoglycans (mg/g of dry tissue) in the different groups. *Asterisk* indicates significant difference in relation to G1 and *number sign* indicates significant difference in relation to G5

Results

The GAG levels (Fig. 1) in all groups except G5 (4.1298±0.9940) were increased over the G1 levels (1.9439±0.5890). Groups G6 (8.9815±1.4629) and G7 (6.6635±0.8984) showed higher GAG values than G1 and G5.

Analysis of GAG by agarose gel electrophoresis (Fig. 2a, b) showed only the presence of dermatan sulfate (DS), which is characteristic of the tension region of the tendon. The tenotomized groups showed a higher DS content than the control group G1. However, G4 and G7 showed an apparent decrease in DS content when compared to G3 and G6.

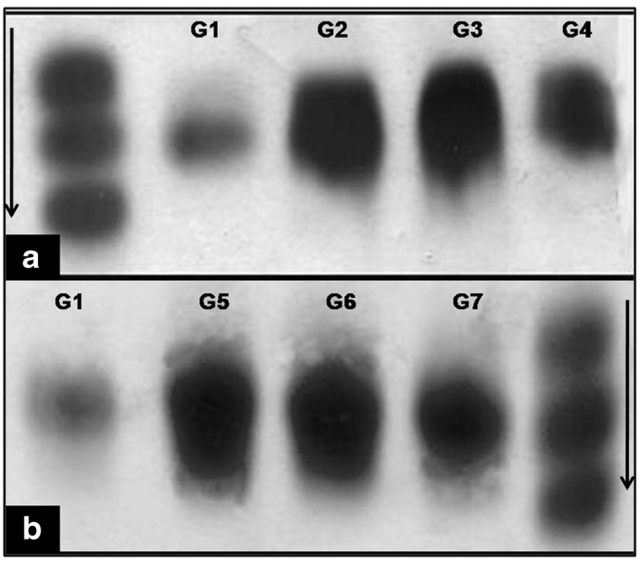

Fig. 2 Agarose gel electrophoresis. Standards are in the right: heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS). **a** Groups euthanized at 8 days after lesion. **b** Groups euthanized at 15 days after lesion. Observe the presence of DS in all analyzed groups, which showed a more intense band in the transected groups. Pulsed groups (G4 and G7) present a less marked band when compared to the other transected groups. *Arrow* indicates the direction of electrophoretic race

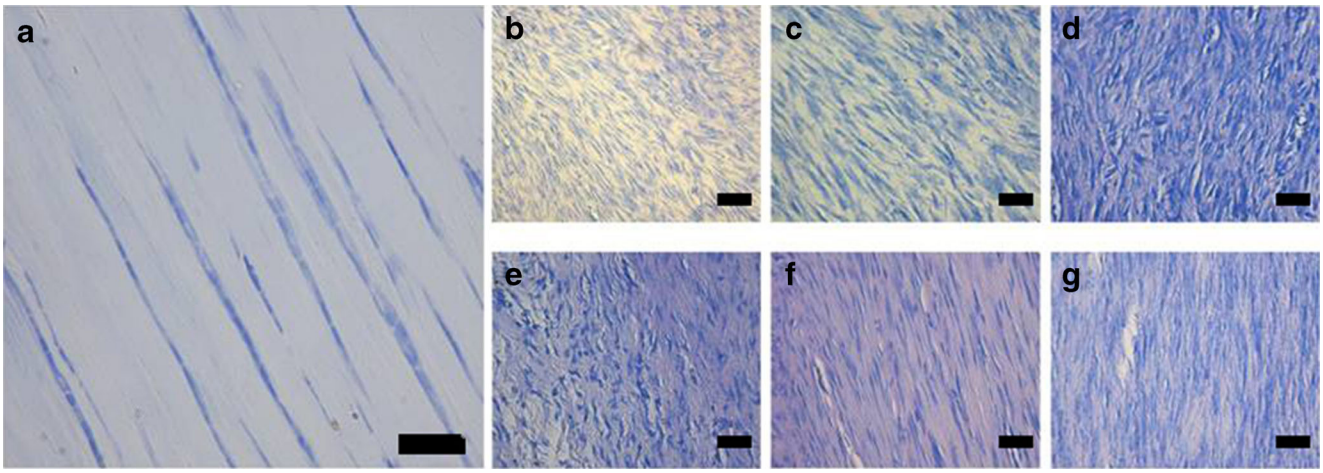

Fig. 3 Toluidine blue (TB) stains, where **a** G1, **b** G2, **c** G3, **d** G4, **e** G5, **f** G6, and **g** G7. Observe the bigger number of cells and intense metachromasy in the transected groups, especially in the groups euthanized on the 15th day after lesion. *Bar* 20 μm

Overall, the HE-stained sections showed marked differences between the different time periods analyzed (data not shown). We observed high cellularity in the repair region of the transected tendons, with total disorganization of the matrix in all groups. In the 14-day groups, cellularity was also high and the ECM was in the process of remodeling; it showed greater matrix orientation than at 7-day post-injury, although there were no differences between the groups, which are consistent with data reported in the literature.

TB staining (Fig. 3) was observed in all tenotomized groups but especially in the groups euthanized 15 days after transection, which showed an intense metachromasy indicating a high level of glycosaminoglycans.

When tendons are observed in a polarizing microscope, a normal tendon displays high gloss, a characteristic of a highly organized tissue. With transection, the fibers lose their organization and appear darker when observed under a polarizing microscope. The groups euthanized 8 days after transection (G2, G3, and G4) were highly disorganized. It was observed that G7 displayed greater organization than the other groups euthanized after the same period (Fig. 4).

From the images captured of the groups euthanized at 15 days after transection, birefringence measurements were performed as a way to evaluate the organization of the tissue compared to healthy tissue (Fig. 4). Table 1 shows the results of these measurements.

All groups were significantly different when compared with each other. G6 showed lower values than G5, indicating that its fibers were highly disorganized. In contrast, G7 was more organized than G5 and G6, but none of the injured groups reached a value approximating that of the normal group (G1).

Our results obtained with the CatWalk system showed a positive functional response to pulsed LLLT. G4 and G7 showed higher values, corresponding to the maximum

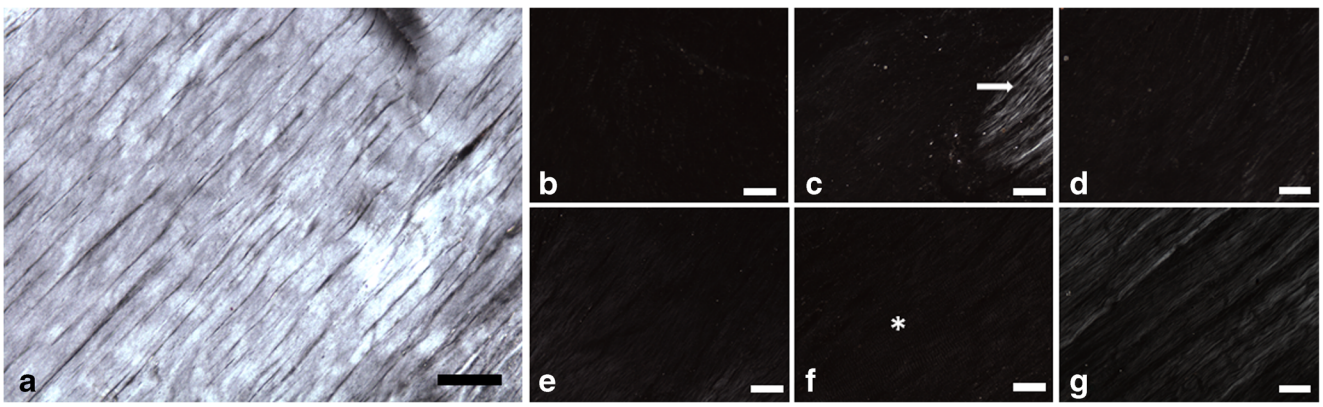

Fig. 4 Longitudinal sections of the tendons from the different groups observed by polarization microscopy. Analysis of birefringence where the biggest tendon axis is positioned 45° in relation to the polarizators, where **a** G1, **b** G2, **c** G3, **d** G4, **e** G5, **f** G6, and **g** G7. Observe the biggest glow in G1 when compared to the other groups. **c** *Arrow* indicates the portion of tissue around the transected region. *Asterisk* indicates the presence of crimps. Observe in 15 G that the area is apparently more organized when compared to the other transected groups. *Bar* 40 μm

Table 1 The largest axis of the tendon was positioned at 45° with respect to the crossed polarizers. The number of measurements (100) chosen at random in 12 sections from four tendons of each group

Groups	TR (GA median)	Comparisons	Mann–Whitney test (p)
G1	210.10	*	
G5	68.37	G5×G6*	0.0001
G6	14.39	G5×G7*	0.0001
G7	72.55	G6×G7*	0.0001

*$p<0.05$

TR transection region, *GA* gray average

intensity of paw contact (pixels) on the platform during walking, than G2, G3, G5, and G6; moreover, G4 and G7 showed values close to the tendons without transection. In contrast, G3 and G6 showed significantly lower values when compared to other groups (Fig. 5a, b). Among the 15-day groups, G7 displayed better results, especially in the first (inflammatory) phase of the injury. However, in the second (proliferative) phase, the results in all groups were similar to samples collected prior to surgery (Fig. 5b).

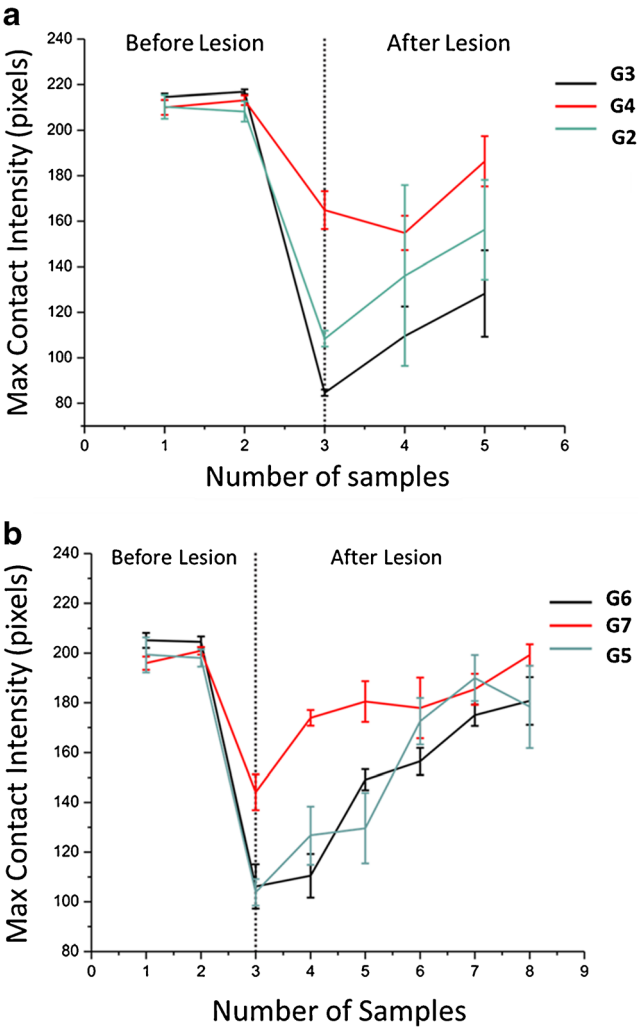

Fig. 5 Max contact intensity during gait of the rats obtained by the CatWalk system. **a** Measurements performed on animals 8 days after injury. Observe the higher values ($p<0.05$) of paw pressure during the gate of the animals in group G4 when compared to G2 and in special G3 (*asterisk*). Measurements were made on the second, fourth, and sixth days after injury. **b** Measurements performed on animals 15 days after injury. The G7 group had higher values when compared to G5 and in special G6 (*asterisk*). Observe that the differences between the groups are concentrated in the first phase of treatment, in agreement with the analysis of the groups euthanized at 8 days after transection. With the end of the inflammatory phase, the results become more similar. Measurements were made on the 2nd, 4th, 6th, 8th, 10th, and 12th days after injury

Discussion

Based on promising results reported in the literature, including less pain and inflammation, increased cell proliferation and synthesis of ECM components, stimulated biochemical reactions [12–14, 16], increased MMP activity and collagen synthesis [18, 28]; we subjected animals with partial tenotomy of the Achilles tendon to different LLLT protocols. The animals were then evaluated 8 and 15 days after injury to analyze the effects of laser treatment on the inflammatory and proliferative phases of injury.

GAG analysis revealed that all groups euthanized 8 days after injury had a significant increase in GAG content when compared to G1. These results corroborate previous studies [29, 30], reporting increased GAG content after tendon injury. Increased GAG is observed in tissues that are undergoing a healing process. It was also observed, though not with statistical significance, that the GAG values of the continuous group were higher than in G2 and G4. These results were also confirmed by TB stain analysis.

At 15 days, GAG analysis revealed that G5 had similar GAG values to G1 while GAG levels in G6 and G7 were significantly higher than in G1 and G5. These results suggest that LLLT increased synthesis of this component of the tendon.

Analysis of GAG by agarose gel electrophoresis showed only the presence of DS, which is characteristic of the tension region [31, 32]. The tenotomized groups clearly had a higher DS content than the control group G1. Previous studies [29, 33] have demonstrated that DS content increases after tendon injury, especially in the inflammatory phase, indicating the involvement of sulfated glycosaminoglycan in deposition and fibrillogenesis of collagen in tendon repair [34]. G3 showed a more pronounced band than the other groups, indicating that in this case, the laser promoted increased DS content.

The strongest DS bands in comparison to G1 were observed in the 15-day tenotomized groups. G7 showed an apparent reduction in DS when compared to G5 and G6, indicating that at 15 days, the synthesis of GAG is no longer occurring in this group.

Unlike collagen, GAG represents a small portion of the ECM of tendons but it is essential for the regeneration of this tissue. GAGs are attached to a protein core to form proteoglycans, which play an important role in cell migration and differentiation in addition to their regulatory role in collagen fibrillogenesis [35, 36].

Previous studies from our laboratory demonstrated that LLLT stimulates the synthesis of collagen I. We also showed that, in the inflammatory phase, there were high levels of active MMP-9 and MMP-2; these levels were higher during the proliferative phase in animals treated with pulsed LLLT than in animals that received continuous LLLT [18], suggesting that pulsed LLLT has a more potent effect on the process of tendon remodeling [18, 21].

Birefringence analysis revealed that the groups analyzed 8 days after injury had highly disorganized tissue. This result is consistent with expectations, as it marks the end of the inflammatory phase and the beginning of angiogenesis, stimulation of tenocite proliferation, and recruitment of inflammatory cells. At this time, type III collagen synthesis is initiated but neither collagen I synthesis nor collagen organization has yet occurred [11, 37]. At 15 days, the birefringence measurements revealed that the groups had not recovered their organization. However, G7 proved to be significantly more organized than G5 and G6. These results are in agreement with our preliminary findings with respect to collagen I and III and the high activity of MMP-2 and -9 [18], and are consistent with the higher GAG concentration observed in this group.

The results of functional analysis showed that after surgery, G4 had a higher value (corresponding to the maximum intensity of paw contact on the platform) than other groups. This result indicates greater pressure from the paw of the animal during walking, and G4 displayed values close to non-transected animals. In contrast, G3 showed the lowest value. The groups treated for 15 days were also subjected to the CatWalk analysis during walking. Again, the results of the first phase of the lesion were more dramatic, showing that G7 was significantly better than the other groups. After the inflammatory phase of injury, animals showed a slight improvement; on the last day of data collection, the results were very similar to results obtained before surgery. This result suggests that the pulsed LLLT was effective, especially in the acute phase of healing, in recovering the animals' gait after tendon injury.

Increased production of inflammatory mediators leads to pain and swelling [38], hindering talocrural articulation and paw support during gait after injury. We believe that the functional result for the pulsed LLLT group, in which animals more strongly supported the injured paw during gait, may indicate that pain severity was decreased through modulation of the inflammatory process [39, 40]. Recent studies showed that laser light, when applied in a pulsed mode, is capable of inducing metabolic processes through a convective mechanism called "light-cell pump" [41]. Through this mechanism, there is a periodic transport of water from the intracellular space to the outside (light on) and vice versa (light off). During the reflux phase, small molecules are pulled together with water into the cell. Among these molecules, it is possible that some proteins of the ECM and anti-inflammatory cytokines are present. This may be especially true in the acute healing phase, in which there is a high level of inflammation. In contrast, the continuous LLLT was not able to modulate the inflammatory response in tissue, with consequent persistence of possible adverse effects such as pain and edema.

Several studies have shown that the morphological and functional properties of a tendon after injury will never reach to those of normal tissue. This loss may be caused by long-term immobilization [11, 20] resulting in the absence of mechanical load [9, 12, 13]. With reduced pain and discomfort, the animals could move the injured joint and support the paw during gait [21], which may have aided collagen organization and enabled faster recovery, thereby reducing the effects of long-term immobilization.

Furthermore, our results suggest that during the application of LLLT, energy density should not be the only parameter taken into consideration. By altering the frequency parameters and thereby modulating the energy that is transmitted to the tissue, we obtained different results for different treatment groups, as previously demonstrated for other parameters [18].

In conclusion, we believe that this protocol can be adapted for use in rehabilitation as a way to reduce the immobilization period, accelerate repair, and enhance the functional characteristics of the tendon, allowing better recovery and healing for patients who have suffered tendon rupture and reducing the recurrence of injury.

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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