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ORIGINAL ARTICLE

Low-level laser therapy combined with platelet-rich plasma on the healing calcaneal tendon: a histological study in a rat model

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Abstract The objective of this study was to investigate the effects of low-level laser therapy (LLLT) treatment alone (λ=660 nm and λ=830 nm) or associated with platelet-rich plasma (PRP). We used 54 male rats divided into six groups, with nine animals each: group 1, partial tenotomy; group 2 (GII), PRP; group 3 (GIII): λ660nm; group 4 (GIV), λ830nm; group 5 (GV), PRP + λ660nm; and group 6 (GVI), PRP + λ830nm. The protocol used was power density 0.35 W/cm^2, energy 0.2 J, energy density 7.0 J/cm^2, time 20 s per irradiated point, and number of points 3. Animals in groups GII, GV, and GVI received treatment with PRP, consisting of a single dose of 0.2 mL directly into the surgical site, on top of the tenotomy. Animals were killed on the 13th day post-tenotomy and their tendons were surgically removed for a quantitative analysis using polarization microscopy. The percentages of collagen fibers of types I and III were expressed as mean ± SD. Higher values of collagen fibers type I were obtained for groups GV and GVI when compared with all other groups ($p<0.05$), whereas groups GIII and GIV showed no significant difference between them ($p>0.05$). For collagen type III, a significant difference was observed between GII and all other groups ($p<0.5$), but no significant difference was found between GIII and GIV and between GV and GVI. Results showed that the deposition of collagen type I was higher when treatment with PRP and LLLT was combined, suggesting a faster regeneration of the tendon.

Keywords Low-level laser therapy · Platelet-rich plasma · Calcaneal tendon

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Introduction

Lesions of the calcaneal tendon (CT) are a common cause of disability and are clinically characterized by pain and swelling in and around the tendon, mainly arising from overuse [1]. These lesions are associated with disruption of collagen fibers, increase in noncollagenous matrix, haphazard proliferation of tenocytes, and subsequent decrease on biomechanical properties of tendon [2].

Tendon matrix is rich in collagens, such as types I and III collagens. Type I collagen is considered to be responsible for the mechanical strength of the tendon tissue and type III collagen has an important role in the healing process [3]. Type I collagen (thick fibers) is the primary collagen incorporated in the tendon structure, and increasing the production of type I collagen may enhance tendon healing [4].

Currently, a variety of treatments for lesions of the CT are used or have been trialed. However, there is little evidence that any conventional therapies are effective. In the last years, low-level laser therapy (LLLT) [4–12] and platelet-rich plasma (PRP) [13–21] have been used in orthopedics, traumatology, and sports medicine showing interesting results in modulation of calcaneal tendon repair. However, optimal parameters and mechanisms behind these effects are not fully understood.

It has been demonstrated that LLLT reduces inflammatory processes [5, 6] and promotes calcaneal tendon healing interfering with the production and realignment of collagen fibers [4, 7, 9–11], as well as enhancing biochemical and biomechanical parameters of the tendon [12]. On the other hand, the properties of high interest in PRP and calcaneal tendon repair are justified by a reduction in total time of recovery of tissue injury [16–18]. Besides, PRP is contributing to the tissue repair through stimulation promoted by the presence of chemotactic cytokines, chemokines, blood proteins, and growth factors present in the plasma [15]. Platelets act in homeostasis, wound healing, and reepithelialization, releasing several growth factors, which in turn stimulates angiogenesis, promoting fibroblast proliferation thereby increasing collagen synthesis [21]. Up to now, we found no study that associates the two therapies (LLLT and PRP) in an attempt to improve tissue repair.

Inserted in this perspective, the objective of this study was to investigate the effects of LLLT treatment (λ=660 nm and λ= 830 nm) alone or associated with PRP in tenotomies partial of the CT in Wistar rats. The qualitative and quantitative assessment will be carried out aiming to analyze the presence of fibers of collagen type I and type III in the histological slides.

Materials and methods

This work was developed in compliance with the Standards of Animal Experimentation and in accordance with the ethical principles of handling and care of laboratory animals recommended by the Brazilian College of Animal Experimentation. Standards for educational and scientific practice of vivisection of animals were observed at all stages of the study (Law 6638 of 08/05/1979). The approval of the research protocol is registered under the number CEP/UNIPAC 2011/6.

Animals and groups

This study was conducted with 54 male Wistar rats (*Rattus norvegicus* albinos), with 8 weeks of age, body mass of 200±12.3 g, and maintained at temperature range of 20–22 °C, from the Central Animal Facility at the University of São Paulo. The animals remained in the vivarium of the Laboratory of Physiology and Pathology, President Antonio Carlos University (Itajubá, Minas Gerais, Brazil) in seven standard polypropylene cages, kept in a controlled environment with 12-h light–dark cycle, and received water and food "ad libitum."

The animals were randomly divided into six groups with nine animals each: Group I (GI), partial tenotomy of the CT but received no treatment; group II (GII), partial tenotomy of the CT + PRP treatment; group III (GIII), partial tenotomy of the CT + LLLT λ660 nm; group IV (GIV), partial tenotomy of the CT + LLLT λ830 nm; group V (GV), partial tenotomy of the CT + PRP + LLLT λ660 nm; and group VI (GVI), partial tenotomy of the CT + PRP + LLLT λ830 nm treatment.

Procedure for preparation of PRP

Animals from groups GII, GV, and GVI, after being anesthetized, were subjected to a puncture of the caudal vein, and then 0.3 mL of blood was removed from each animal for PRP preparation. It is suggested in the literature that the amount of blood withdrawn should be not more than 6.4 % of the animal body weight [22]. The quantity of PRP obtained was approximately 10 to 15 % of the total volume of blood.

Most of the protocols for PRP production used a small fraction of blood (0.3–0.5 mL). This blood is first subjected to a 10-min centrifugation at 800 rpm, followed by another 20 min at 1,600 rpm [23, 24]. A 10 % calcium chloride activator was added in a ratio of 1:20 for obtaining the total volume of PRP. Platelet counts were performed to calculate the PRP concentrate, which should be around 400 % of the peripheral blood platelet count [25]. The platelet concentrate was stored at 20 °C until the exact time for use at the surgical site [22].

Procedure for the partial lesion of the CT

Rats were previously medicated with acepromazine (0.2 % Acepram, Univest SA) and butorphanol (Fort Dodge Lab Ltd.) at doses of 0.02 and 0.1 mL/kg, respectively, injecting intramuscularly in the region of the right *quadriceps* muscle.

After 15 min, anesthetic Zoletil 50® (Virbac) at a dose of 0.1 mL/kg was applied. Trichotomy was performed in the entire right thigh, and then a longitudinal incision was made 3 cm on the skin just above the origin and insertion of the CT. The partial tenotomy was performed with a scalpel blade number 11, with a cut of 2 mm in the middle third of the tendon, the medial to lateral. Then, the skin was sutured with nonabsorbable monofilament polyamide 4.0 (Ethicon, Johnson & Johnson) and subjected to local asepsis [26]. These procedures occurred with all animals.

PRP treatment

The animal in groups GII, GV, and GVI received treatment with PRP. Each animal received a single dose of 0.2 mL directly into the surgical site, on top of the tenotomy. The application of PRP was performed immediately after injury but before suturing the lesion [27].

Laser treatment

For laser therapy, the low-intensity laser device Laser Flash® DMC III (DMC Equipments Ltda, São Carlos, SP) was used which can be operated in two wavelengths: λ=660 nm (red laser, mid-activity: InGaAlP) that was used for groups GIII and GV and λ=830 nm (infrared laser, mid-activity: GaAlAs) applied to groups IV and VI [28]. Laser irradiations were made at the same day time (10:00 a.m.) leaving an interval of 1 day between applications. The animals were immobilized manually, exposing the right side of the thigh and leg. Animals from groups GIII, GIV, GV, and GVI were irradiated by the laser according to the protocol described in Table 1.

Tissue samples

All animals were killed at the 13th day after surgery, receiving an intracardiac application of anesthetic sodium thiopental (crystal) at a dose of 0.05 mL per 100 g body weight, followed by 19.1 % potassium chloride via intracardiac, with a single dose of 0.4 mL per 100 g body weight. After confirmation of euthanasia by verification of vital data and absence of reflexes, the entire *triceps surae* muscle was dissected and extirpation occurred at the calcaneal insertion and myotendinous junction.

Table 1 Protocol for the LLLT irradiation (λ=660 nm and λ=830 nm)

Parameters	Value
Laser operation	Continuous (cw)
Output power	100 mW
Spot size area	0.28 cm^2
Power density	0.35 W/cm^2
Energy	0.2 J
Energy density	7.0 J/cm^2
Time per point	20 s
Number of points	3
Angle of application	90°

Morphometrical analysis

The CT was fixed in 10 % neutral buffered formalin for 48 to 72 h and processed in routine histological processing order: dehydration, bleaching, paraffin inclusion, and dyeing [29]. Semi-serials cuts were obtained with 5 μm thick and stained with Picrosirius Red, which allows visualization of collagen fibers. The material was examined with a polarized microscope Olympus CX31 trinocular, YS100 model, equipped with digital camera Olympus SC20 and coupled to a microcomputer. Morphometric analysis of collagen fibers was performed according to Silva [29]: Collagen area=(Σ regions with fibers of the same pixel / area on the tendon)×100.

Statistical analysis

One-way analysis of variance was used for comparison between groups. The calculations were performed using the GraphPad Prism®. All statistical tests were performed at a significance level of $p<0.05$.

Results

Figure 1 shows histological analysis using the Picrosirius sections of calcaneal tendons with partial rupture showing the presence of type I collagen fibers, which are thick and yellow or red, and type III collagen fibers, which are thin and greenish.

Figure 2 shows the percentage of the type I collagen fibers. Type I collagen fibers were more frequent in all treated groups than in the untreated (group I). Both groups that associated LLLT and PRP treatments (V and VI groups) presented higher values ($p<0.05$) when compared with all other groups (I, II, III, and IV). Additionally, LLLT groups (III and IV) showed higher values ($p<0.05$) than PRP alone (group II). However, no difference ($p>0.05$) was found between LLLT groups (III versus IV).

Figure 3 displays the percentage of the type III collagen fibers. Type III collagen fibers were more frequent in untreated group (group I) than all treated groups. Both groups that associated LLLT and PRP (V and VI groups) presented lower values ($p<0.05$) when compared with the all other groups (I, II, III, and IV). Additionally, LLLT groups (III and IV) showed higher values ($p<0.05$) than PRP alone (group II). However, no difference ($p>0.05$) was found between LLLT groups (III versus IV) and between groups treated with LLLT plus PRP.

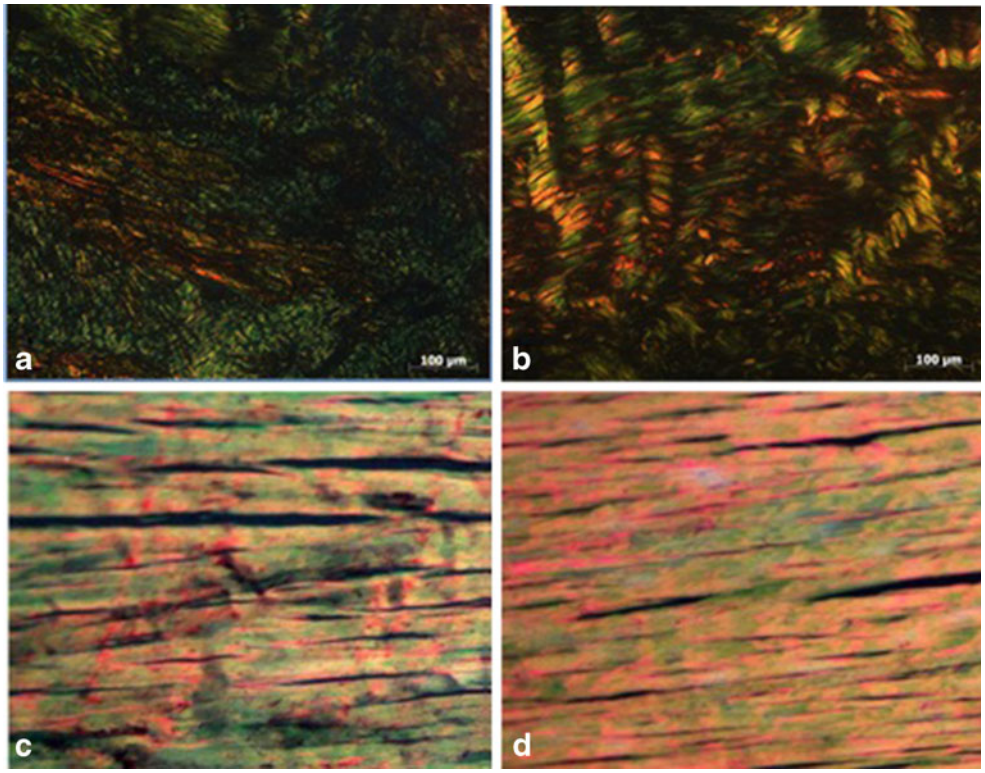

Fig. 1 Histological analysis using the Picrosirius sections of calcaneal tendons with partial rupture showing the presence of type I collagen fibers, which are thick and yellow or red, and type III collagen fibers, which are thin and greenish. *Bar scale* 1/4 100 μm. **a** GI, tenotomy; **b** GII, PRP; **c** GV, PRP + λ660 nm; and **d** GVI, PRP + λ830 nm. Representative histological sections (5 μm, Picrosirius Red under polarized light, ×10 objective) of the longitudinal axis of the central region of the tendon. The control group collagen fibers increased as evidenced by greenish areas in the image (**a**)

A predominance of fibers type III was observed for animals in groups GI (untreated), GIII, and GIV, a result that can be justified by the absence of treatment in GI and the low efficacy of only LLLT on groups GIII (λ660 nm) and GIV (λ830 nm).

Discussion

Defragmentation of the PRP promotes the release of various substances: platelet-derived growth factor, vascular endothelial growth factor, transforming growth factor beta-1, fibroblast growth factor, connective tissue growth factor, transforming growth factor such as insulin or stimulatory (IGF-1), epidermal growth factor, platelet thromboplastin, calcium, serotonin, and fibrinogen hydrolytic enzymes [30, 31].

The results show the percentage difference of the types of fibers of collagen type I and type III. The type I collagen presents closely packed, thick non-argyrophilic, strongly birefringent, yellow or red fibers and it is responsible for the tensile strength, whereas type III collagen (1 %) presents loose argyrophilic network of thin, weakly birefringent,

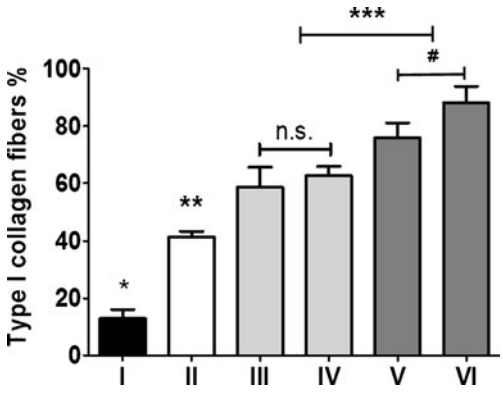

Fig. 2 Percentage of the type I collagen fibers. Results are expressed as mean ± SD. Statistically significant differences ($p<0.05$) when compared groups: *one asterisk* GI with GII thru GVI; *two asterisks*, GII with GIII thru GVI; *three asterisks*, GII–GIV with GV and GVI; *number sign*, GV with GVI; *n.s.* indicates no statistically significant difference ($p>0.05$)

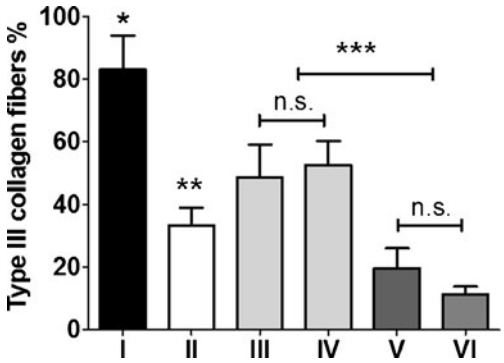

Fig. 3 Percentage of the type III collagen fibers. Results are expressed as mean ± SD. Statistically significant differences ($p<0.05$) when compared groups: *one asterisk*, GI with GII thru GVI; *two asterisks*, GII with GIII thru GVI, *three asterisks*, GII–GIV with GV and GVI; *n.s.* indicates no statistically significant difference ($p>0.05$)

greenish, reticular fibers and its main function is the structural maintenance in expansible organs [32, 33].

The fibers of type I collagen are responsible for tensile strength; this collagen type constitutes the major portion of the vertebrate body and they are the most abundant component of the tendons. The type I collagen in normal adult tissues appears in the form of thick (2–10 mm) fibers under the optical microscope. When these fibers are observed under polarized light, the enhancement of collagen birefringence promoted by Picrosirius staining is specific for collagen and discloses its distinct patterns of physical aggregation: type I collagen (thick fibers) displays a strong birefringence and are yellow or red [34]. It is suggested that the predominance of this type of fiber in the GV group (PRP + LLLT λ660 nm) and GVI (PRP + LLLT λ830 nm) is due to combination of LLLT and PRP.

On the other hand, fibers of collagen type III are responsible for maintaining the structure and this collagen type is usually found intermixed with type I collagen. It is present in many organs and is mainly, but not exclusively, related to smooth muscle cells [35]. Histochemical evidence has been presented suggesting that type III collagen appears by optical microscopy under the form of thin (0.5–2 mm) argyrophilic, weakly birefringent, greenish fibers. Polarized light microscopy of Picrosirius-stained sections has been widely used to quantify types I and III collagen [36]. A predominance of fibers type III was observed in GI (untreated) and GIII and GIV, being justified by the absence of treatment in GI and low efficacy of the proposed protocol for GIII (LLLT λ660 nm) and GIV groups (LLLT λ830 nm).

The study by Neves et al. [7], which aimed to evaluate the effect of the GaAlAs laser with λ=830 nm; a power of 40, 60, 80, and 100 mW; and an energy density of 20 J/cm^2 on the repair of partial lesions tendons of rats, showed that the fibers of type I collagen responded better for a laser power of 80 mW, whereas a better response was obtained for 60 mW for the fibers of type III collagen. The wavelengths of 660 and 830 nm were chosen because laser radiation at wavelengths between 660 and 840 nm is less absorbed by superficial chromospheres, resulting in better tissue penetration [36].

LLLT and PRP treatments separately showed positive results in the stimulation of healing of the Achilles tendon [37]. Studies involving treatment with LLLT and ultrasound combined showed positive results in the regeneration of the calcaneal tendon, with respect to the increase of type I collagen [38]. It is believed by some authors that the cellular response of any tissue, particularly the calcaneal tendon tissue, depends on the physical agent, a combination of parameters, and associations [37].

In the present study, the results showed that the treatment of animals with PRP or LLLT alone, groups GII (PRP), GIII (λ660 nm), and GIV (λ830 nm), has significant advantages over untreated animals ($p<0.05$), as the percentage of type I collagen fibers is concerned. Furthermore, it was found that the combined treatment with PRP and LLLT is even more efficient than when each of the two treatments is used alone. However, the treatments combining PRP and LLLT showed significant results between groups GV (PRP λ660 nm) and GVI (λ830 nm) ($p<0.05$). These encouraging results suggest a decrease in the time of tendon regeneration using the two therapies combined, accelerating the healing process. The inflammatory signals also showed rapid transition but were not measured in this study.

It is also interesting to observe that no significant difference is found ($p>0.05$) when animals are treated with either one of the two laser wavelengths λ=660 nm or λ=830 nm (groups III and IV).

Conclusion

The results showed the predominance of type I collagen fibers in groups treated with the combination of PRP with LLLT (λ=660 nm, λ=830 nm); nevertheless, further studies are necessary to identify which are the mechanisms by which this rapid regeneration occurs and the influence of LLLT on growth factors in PRP.

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Conflict of interest No competing financial interests exist.

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