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ORIGINAL ARTICLE

Analysis of photobiomodulation associated or not with platelet-rich plasma on repair of muscle tissue by Raman spectroscopy

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Abstract Treatment of muscle injuries usually results in the interruption of sports practice; thus, studies aimed at accelerating the return to activity, with proper tissue repair, are important. Therefore, this study aimed to evaluate the effects of photobiomodulation (PBM), associated or not with platelet-rich plasma (PRP), on the treatment of muscle injury. Thirty-five animals were used and divided into five groups ($n = 7$): control (C), control lesion (CL), lesion treated with low-level laser therapy (LLLT) (LLt), lesion treated with PRP (LP), and lesion treated with both techniques, LLLT and PRP (LLtP). Muscle injury was induced by stretching the gastrocnemius muscle, and the animals in the LLtP and LP groups received the application of PRP immediately following the injury. The LLLT was applied daily for 7 days. The animals were euthanized 7 days after the injury. Analysis of the NADH/NAD ratio and collagen was performed by Raman spectroscopy; in addition to which, histological analysis of the gastrocnemius muscle was performed. The LLtP group demonstrated a reduction in the area of injury, regenerating cells and a healthy appearance of muscle fibers. The Raman analyses showed a reduction in the NADH/NAD ratio in the CL group, demonstrating oxidative stress, and the collagen presented a reduction in the CL and LLt groups, when compared with the C group. It is concluded that either PBM or PRP, and the association of both, was able to reduce the oxidative stress promoted by injury and modulate collagen production at the site of the injury. Furthermore, although both treatments individually were effective for repairing the damage caused by muscle injury, the association of both demonstrated a better histological aspect.

Keywords Skeletal muscle · Injury · Low-level laser therapy · Platelet-rich plasma · Raman spectroscopy

Introduction

Muscle injuries are common in sports, affecting both professional and amateur athletes [1, 2], resulting in the interruption of sports activity for the treatment. In this sense, the greatest challenge of rehabilitation is to promote proper tissue repair in the shortest treatment period possible, enabling a quick return to the sport; this being important both for athletes in order that they do not lose performance and for amateurs so they do not stay away for prolonged periods from their healthy habits [3, 4].

In order to promote proper tissue repair and prevent possible complications such as recurrent injury and fibrotic processes and enable an early return to sports activities, photobiomodulation (PBM) and platelet-rich plasma (PRP) have been studied and present good results in the scientific literature [5–9].

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PBM is a widely used technique in physical therapy clinics for treatment of muscle injuries. Studies show that low-level laser therapy stimulates the muscle regeneration process, accelerating the formation of new fibers through the activation of quiescent satellite cells, which promote regeneration of the damaged muscle fibers. Furthermore, PBM operates in response to oxidative stress caused by the inflammatory process, enhancing the antioxidant activity with a consequent reduction in oxidative stress, increasing mitochondrial respiratory activity and ATP synthesis, and preventing fibrosis, resulting from the healing process of muscles, by inhibiting TGF-β expression [8, 10].

With regard to PRP, this technique is now widely used in the treatment of muscle, bone, and tendon injuries [11]. It consists of platelet concentrate which has regenerative properties and high concentrations of cytokines and growth factors, such as VEGF, which stimulates angiogenesis; insulin-like growth factor 1 (IGF-1), which promotes myogenesis; and TGF-β, among others [12, 13]. These elements have the ability to accelerate the muscle tissue regeneration process, especially in the first few weeks after injury [5, 14].

Muscle injury promotes a series of alterations in skeletal muscle tissue. These structural changes are visible when analyzed using histological slides under an optical microscope. However, it is difficult to quantify these changes, since this analysis is based on the observation of the raters. In this sense, Raman spectroscopy has been studied as it is an analytical tool, providing fast and reliable application that provides the molecular structure of chemical components in a heterogeneous sample [15].

Few studies analyzing the association of both techniques, PBM and PRP, were found in the scientific literature [9], a fact that motivated the development of this research, since both promote improvement in repair through distinct mechanisms, and there is no evidence that this association may promote adverse effects.

Given the above considerations, this research, based on the knowledge of the physiological effects generated by each technique, aimed to associate PBM and PRP in the repair of skeletal muscle, with the hypothesis that the association of both would promote quicker and better tissue repair, thus allowing the creation of an effective treatment protocol for muscle damage, improving the rehabilitation process.

Therefore, the aim of this study was to analyze the effects of low-level laser therapy, platelet-rich plasma, and the combination of both, on the repair of muscle tissue of rats after stretch injury, using Raman Spectroscopy.

Materials and methods

Thirty-five male Wistar rats of 150 days of age, which were provided by the Central Biotherium of the São Paulo State University (UNESP), Botucatu Campus (SP, Brazil), were used and maintained in the Faculty of Science and Technology, FCT/UNESP, Presidente Prudente Campus (SP, Brazil). The animals were kept in plastic boxes at a controlled temperature (22 ± 2 °C) and a 12-h light/dark cycle with free access to water and food (standard laboratory chow).

All procedures were previously approved by the ethics committee for animal use from FCT/UNESP, Presidente Prudente Campus (protocol 01/2013).

Experimental groups

The animals were randomly divided into five groups: control (C), control lesion (CL), lesion treated with low-level laser therapy (LLt), lesion treated with platelet-rich plasma (LP), and lesion treated with both techniques, LLLT and PRP (LLtP).

Experimental design

The animals were initially anesthetized with an intraperitoneal administration of ketamine (70 mg/kg) and xylazine (15 mg/kg) [16]. Next, the animals of the LP and LLtP groups underwent cardiac puncture for preparation of PRP and were then submitted to the muscle injury protocol. The other groups were submitted to the injury protocol immediately after confirmation of anesthesia. The C group was not submitted to any procedure.

After the lesion protocol, the PRP was immediately applied with the animals still under anesthesia as well as the first PBM session. The animals were not shaved prior to the treatments.

Collection of PRP

After anesthesia, the animals were submitted to cardiac puncture, using a disposable syringe containing 0.2 ml of sodium citrate (10 %); 4 ml of the blood was collected. Immediately after puncture, a physiological saline solution of the same volume as the blood which had been extracted was injected to restore blood volume.

The collected blood was centrifuged at 200*g* for 15 min. The sample was then fractionated into three components: red bottom fraction composed mainly of red blood cells, yellow-straw fraction (buffy coat) with a serum component and high concentration of platelets, and top fraction composed mainly of blood plasma [17]. The two top fractions, including the buffy coat, were pipetted. The pipetted contents were again centrifuged at 500*g* for 10 min, after which 0.2 ml from the bottom of the tube was pipetted [18].

Platelet count

Two blood samples and three PRP samples were analyzed in the laboratory of the Veterinary Hospital of the University of Western Paulista (UNOESTE, Brazil) by means of an automatic analyzer of blood cells (Poch 100iy Diff, the Sysmex brand) to assess the platelet concentration.

The platelet counting of PRP presented an average of 4999×10^3 platelets/μl in the blood, a number four times greater than that in the animal blood (1068×10^3 platelets/μl of blood).

Muscle lesion protocol

After the animals were anesthetized, two percutaneous electrodes were introduced to the right hind limb of the animals at regions corresponding to the insertions of the gastrocnemius muscle. Thereafter, the animals were placed in the damage inductor equipment, in the supine position, with the hip in slight flexion, knee in extension, and ankle in plantar flexion and with their right leg attached to the machine with adhesive tape (duct tape) (Fig. 1). Electrical stimulation was carried out with sufficient intensity for full contraction of the hind limb, inducing ankle plantar flexion, which then fired the trigger of the inductor damage equipment, promoting abrupt dorsiflexion movement, ceasing the electrical current shortly below. This procedure was repeated for a total of ten series, with 30-s intervals between series. In each series, 2.25 J was released, totaling 22.5 J of energy applied to the muscle injury. This protocol was adapted from Pachioni et al. [41].

PRP application

The PRP (100 μl) was injected into the animals of the LP and LLtP groups by means of a disposable syringe in the distal third of the tibia, in order to be applied to the gastrocnemius muscle belly. The application was performed immediately after the muscle injury protocol [6].

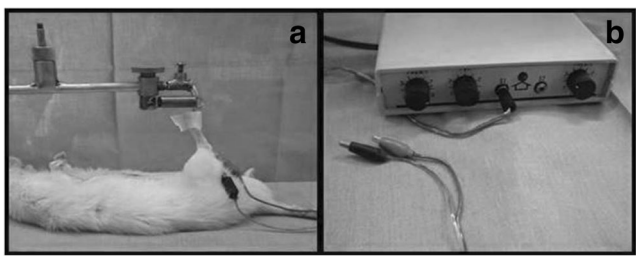

Fig. 1 *Left* the noninvasive induction of mechanical stretching injury. *Right* the electrical stimulator

PBM protocol

PBM was applied by means of diode laser equipment (Coherent, Laser Cube) and previously calibrated with a wavelength of 637 nm, power of 25 mW, beam diameter of 1 mm, and continuing emission for 10 s. Therefore, the dosage was 0.25 J/0.00785 cm^2 or just 31.85 J/cm^2. The laser was applied to a single point [19], daily, starting on the day of injury until the completion of seven applications. The laser was applied at the same point as the PRP injection, both to the muscle belly region.

Acquisition of tissue samples

After the experimental period, the animals were euthanized with an overdose of anesthetic xylazine and ketamine. Next, the right gastrocnemius muscle was removed from each animal and immersed in *n*-hexane; cooled in liquid nitrogen, using the freezing method for nonfixed tissues [20]; and stored in an ultra-low temperature freezer, Coldlab CL580-80V, at −75 °C. The samples were cut for making histological slides for analysis by Raman (20 μm) and histology (5 μm). The slides for histological analysis were stained with hematoxylin and eosin (HE) [21].

Histological analysis

Histological analysis was performed in a qualitative way on the slides stained with HE, using a Nikon Eclipse 50i microscope. The qualitative analysis was based on the morphology of muscle tissue, inflammatory infiltrate, and connective tissue (endomysium and perimysium) [21].

Raman scattering spectroscopy

The Raman spectroscopy measurements were obtained by means of a micro-Raman spectrograph (in-Via Renishaw). The laser used had a wavelength of 514 nm with power in the microwatt (μW) order in the sample and diffraction grating of 1800 lines per mm. The exposure time was set at 30 s, the amount of accumulation was equal to 3, and the spectral range measured was 400 to 3400 cm^{-1}. The analysis was performed on 20-μm sections without dye. The Raman analysis was performed in a 1-μm laser spot at six different areas in each sample.

Statistical analysis

The Shapiro-Wilk test was used to verify the normality of the data. When data showed normal distribution, the one-way ANOVA test was performed followed by Tukey's posttest. When the normality of the data was violated, the Kruskal-Wallis test was performed, followed by Dunn's posttest.

Analyses were performed using SPSS v.22 software, and for all analyzes, a significance level of 5 % was adopted.

Results

Histological analysis demonstrated a large lesion area in the CL group (Fig. 2b), with the presence of macrophages and inflammatory infiltrate between the cells and perimysium and muscle fibers with signs of stress, such as rounded and angular fibers. Furthermore, there was structural disarrangement of muscle tissue.

The LLt group presented less inflammatory infiltrate between the cells; however, there were few cells in regeneration, and the tissue was still disorganized (Fig. 2c). In the LP group, besides little inflammatory infiltrate, there was the presence of regeneration cells (Fig. 2d). In the LLtP group, the histological findings demonstrated superior improvement to the isolated treatments, presenting cells in regeneration and the greater presence of blood vessels, a lower area of injury, and muscle fibers with healthy appearance without the presence of signs of stress, such as the round fibers present in the other groups (Fig. 2e).

Figures 3 and 4 show the averages per group of the Raman spectrum. The analyzed bands were highlighted. The band of 998 cm^{-1} showed a peak at 1004 cm^{-1}, so the analysis was performed on that peak.

For the analysis of oxidative stress, the bands used were 1546 and 1688 cm^{-1} (referring to NADH) and 998 and 1032 cm^{-1} (referring to NAD). Using the peak values of these bands, the ratio of NADH/NAD was calculated (Table 1). The ratio of NADH/NAD decreased in the injury groups, revealing oxidative stress caused by muscle injury.

The analysis of the collagen was performed using the 1445 and 1662 cm^{-1} spectrum bands. In the 1171 cm^{-1} band, there was a decrease in intensity in the CL group compared to the control; the 1662 cm^{-1} band showed a decrease in the intensity when comparing group C with the CL and LLt groups (Table 2).

Discussion

This study aimed to analyze the effects of PBM, PRP, and the combination of both treatments on the repair of skeletal muscle, injured by stretching, in rats. The analysis showed that the damage promoted alterations in cell morphology as well as oxidative stress and reduced collagen content. Regarding the treatments, although both the PBM and PRP improved the condition of oxidative stress individually, the histological analysis demonstrated better tissue condition from both modalities of treatment in association.

Histological analysis showed great tissue destruction in the injury group (CL). However, in the treated groups (LLt, LP, and LLtP), the muscle presented better tissue organization and regeneration process, acting in the formation of new muscle fibers.

Muscle injury triggers the inflammatory process, which occurs in the following phases: damage, repair, and remodeling. In the first phase, degradation of the necrotic fibers occurs by means of neutrophils and macrophages, which perform phagocytosis of the damaged tissue, and released toxins, which promote the digestion of the damaged cells. However, this process ends up damaging some adjacent fibers; thus, there is motivation to modulate the inflammatory process to prevent damage to healthy structures [3].

The PBM parameters used were similar to those used in other studies [8, 22, 23], which show the effects of LLT in reducing reactive oxygen species, inhibiting TNF-β expression, improving mitochondrial activity and ATP synthesis,

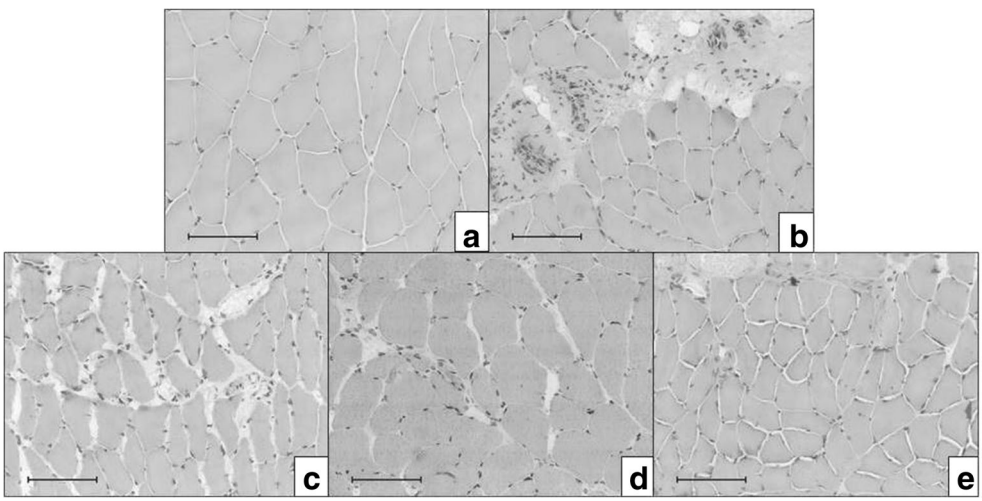

Fig. 2 Cross section of the gastrocnemius muscle, *scale bar* of 100 μm. Group C showed healthy muscle tissue (**a**). The CL group showed the presence of inflammatory infiltrate between the muscle fibers and perimysium (**b**). The lower presence of inflammatory infiltrate and better tissue organization in the LLt group (**c**), appearance similar to that seen in the LP, but with the presence of regeneration cells (**d**), and more advanced recovery process in the LLtP group, with better tissue organization, and muscle fibers without stress signals (**e**)

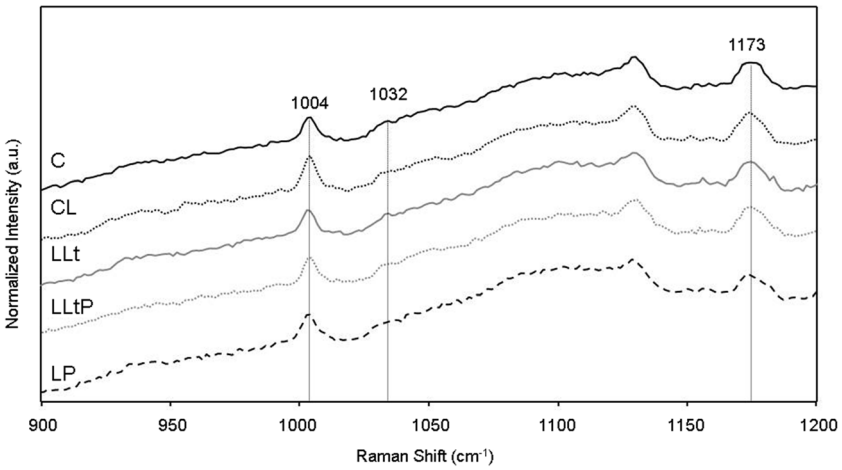

Fig. 3 Raman spectra of the gastrocnemius muscle (900–1200 cm^{-1}). The intensity is shown in arbitrary units (a.u.). Bands 1004 and 1032 cm^{-1} are associated with oxidative stress (NAD), and band 1173 cm^{-1} is associated with collagen. The intensity of $Y = 0$ (offset correction function) was normalized for better visualization of the bands. Excitation laser 514.5 nm

and reducing serum levels of creatine kinase (CK). In the present study, the group treated with PBM presented a lower injury area, with a significant reduction in the inflammatory infiltrate. Another study [24] revealed that low-level laser therapy has better effects than topical application of diclofenac and cryotherapy in reducing the levels of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, during the acute inflammatory phase. It is probable that the modulation of these cytokines promoted the regulation of the inflammatory process, reducing the damage to the muscle tissue in the present study.

The histological analysis of the group treated with PRP revealed a similar appearance to the findings of the LLt group. However, the LP group demonstrated the greater presence of regenerating muscle cells, suggesting a more advanced stage of recovery in the LP compared to the LLt. The PRP has many growth factors that assist the repair of damaged muscle tissue. Studies have shown that PRP increases the expression of cytokines that support muscle regeneration, among which are IGF-1, which stimulates the activation of satellite cells into myoblasts, and VEGF, which stimulates angiogenesis [5, 25].

To provide adequate cellular response to treatment with PRP, a concentration of platelets four times higher than the quantity found in the normal blood is required [26]. Therefore, the method described in the present study was effective in PRP preparation, as there were 4.68 times more platelets in the PRP sample than in the normal animal blood. According to the evaluation of the PAW, the platelet concentration of PRP used is classified as P4 [27]. Moreover, the PRP was applied immediately after the injury protocol [5, 6], as application on the day of the injury could improve the entire process of muscle repair, as the inflammatory process occurs in a cascade of events.

The combination of the treatments demonstrated better histological appearance, which was probably a result of the modulation of the inflammatory process, promoted by the PBM, and associated with the high regeneration potential of PRP [5, 24], allowing for better quality tissue repair, in a shorter recovery time. This combination resulted in a lower area of lesion, the presence of regeneration cells and blood vessels, better organization of muscle fibers, and healthy appearance of the muscle fibers.

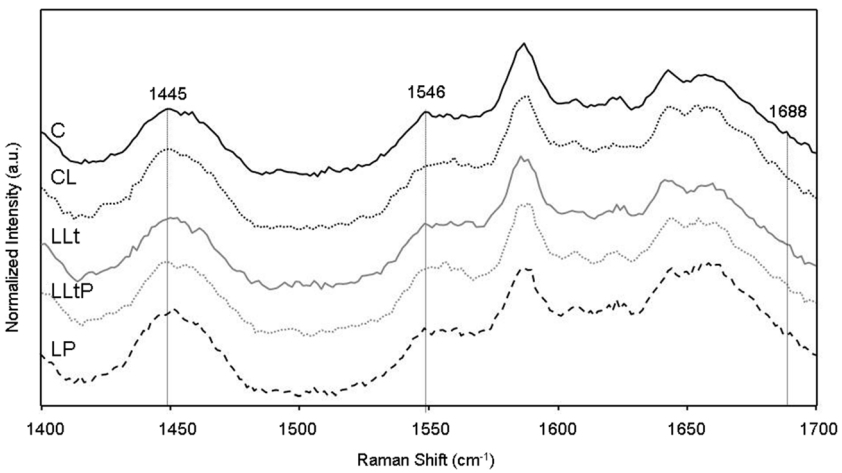

Fig. 4 Raman spectra of the gastrocnemius muscle (1400–1700 cm^{-1}). The intensity is shown in arbitrary units (a.u.). Bands 1546 and 1688 cm^{-1} are associated with oxidative stress (NADH), and band 1445 cm^{-1} is associated with collagen. The intensity of $Y = 0$ (offset correction function) was normalized for better visualization of the bands. Excitation laser 514.5 nm

Table 1 Mean values ± standard deviation of the relationship of the bands of NADH and NAD in arbitrary units

Group	Intensity			
	1546/998	1546/1032	1688/998	1688/1032
C	1.95 ± 0.11	1.79 ± 0.12	1.85 ± 0.11	1.70 ± 0.10
CL	1.68[a] ± 0.32	1.55[b] ± 0.33	1.68 ± 0.27	1.55 ± 0.29
LLt	1.81 ± 0.23	1.67 ± 0.22	1.76 ± 0.17	1.62 ± 0.17
LP	1.83 ± 0.11	1.69 ± 0.11	1.83 ± 0.09	1.68 ± 0.06
LLtP	1.88 ± 0.17	1.70 ± 0.17	1.85 ± 0.12	1.67 ± 0.14

[a] Statistically significant difference to group C, $p = 0.01$

[b] Significant difference to group C, $p = 0.03$

Raman spectroscopy has recently been studied in biological tissues as it enables quantification of several structures with little bias, since it uses the sample in a natural form, i.e., without the need for reagents, either for fixation or to identify the structures to be studied, allowing great data fidelity [28–32].

The method used in this study to analyze the oxidative stress was based on studies using Raman spectroscopy to quantify NAD and NADH alterations [28, 33]. These molecules are involved in the metabolism of muscle cells as well as in the control and production of reactive oxygen species (ROS) [28]. The NADH/NAD ratio decreased in the CL group compared to the C group; however, there were no significant differences in the treated groups, either using PRP, PRP, or a combination of both (LLtP group). Thus, it is evident that the treatments were effective in attenuating oxidative stress generated by muscle injury.

The inflammatory process triggered by muscle injury appears to be related to oxidative stress, since its exacerbation is usually accompanied by the increased production of reactive oxygen species [34]. The disruption of muscle fibers triggers the inflammatory process at the site of the lesion, which begins with the arrival of lymphocytes, especially macrophages of type I and neutrophils. However, during the digestion of necrotic fibers, reactive oxygen species are produced due to the release of cytotoxins [35]. In addition, a study relating the markers of oxidative stress, thiobarbituric acid reactive substances (TBARS); CK as an injury marker; and the marker of mitochondrial viability, methyl-tetrazolium (MTT), found that oxidative stress passes through two phases: the first one is related to mitochondrial damage that occurs shortly after the injury, after 30 min, and the second one is due to the inflammatory process itself, which is significantly increased from the day following the injury [36].

Table 2 Mean values ± standard deviation for Raman spectral intensities for the collagen in arbitrary units

Group	Intensity	
	1445 cm^{-1}	1665 cm^{-1}
C	1550.45 ± 256.20	2305.80 ± 365.99
CL	1250.86[a] ± 354.07	1566.63[b] ± 532.23
LLt	1260.6 ± 306.86	1818.33[c] ± 424.25
LP	1248.67 ± 133.12	1873.09 ± 158.61
LLtP	1367.4 ± 346.38	2019.36 ± 501.65

The letters indicate statistically significant differences from group C: a, $p = 0.02$; b, $p = 0.00$; and c, $p = 0.03$

Treatment by PBM has an antioxidant effect, which may be the result of improvement in the mitochondrial respiratory chain, which increases the amount of ATP available to the cell, thereby improving cell metabolism [37]. In addition, PBM has the ability to modulate the inflammatory process, reducing TNF-α and TGF-β expression, which act on the degradation phase of the damaged muscle tissue [7, 8, 36, 37]. Thus, PBM prevents oxidative stress acting on the metabolism improvement, both through the mitochondria respiratory chain and by modulation of the inflammatory process.

Regarding PRP, it acts by means of IGF-1, modulating the inflammatory process and prolonging the regeneration potential of muscle cells, thus accelerating the regeneration process [5]. Therefore, the reduction in oxidative stress is probably due to the shorter exposure time to the ROS for its ability to accelerate tissue regeneration [14, 38].

The analysis of collagen performed using bands related to type I collagen (1445 cm^{-1}) and nonspecific collagen (1665 cm^{-1}) [39] showed that the lesion reduced the quantity of collagen in the muscle tissue in the CL and LLt groups compared to the C group. Collagen is an important component of skeletal muscle tissue, being responsible for its structure and transmission of the contractile force generated by muscle fibers along the whole muscle, increasing its efficiency [4].

There is great concern with regard to fibrosis, during muscle injury repair, as the accumulation of connective tissue affects the function of the muscle, causing pain and making it more susceptible to recurrent muscle injuries. Several studies have shown increased collagen around 7 days after the injury [4, 8, 10], a fact contrary to what occurred in the current research. The accumulation of collagen after muscle injury is usually a prognosis of fibrosis after full recovery; however, a stretching injury has the characteristic of spreading damage to the structure of the muscle tissue and can cause disruption of the epimysium and bleeding between the fascia and the muscle, differing from a crushing injury, for example, in which the lesion occurs directly on the myofibril [3].

PBM has been used to prevent fibrosis after injury, as it has the ability to modulate the inflammation and reduce the expression of TGF-β [40], which could have caused a reduction in collagen in the LLt group. Regarding the PRP, this contains TGF-β in its composition, which could promote increased collagen in the muscle tissue [14]; however, there was no

significant difference in the amount of collagen in the LP group compared to the other groups, which may suggest a positive effect since stretch injury promotes disruption of the muscle tissue collagen structures.

This research presents methods that assist muscle regeneration, promoting a better morphological aspect, reduction in oxidative stress, and modulation of collagen. These treatments show promise for better and faster recovery of injured muscle, especially the association of PBM and PRP.

Regarding the limitations of this research, we should mention that the injury protocol used, besides mimicking the most common mechanism of muscle damage, allows for small variations due to anatomical differences among animals, such as an increased amount of adipose tissue and hind limb size. Furthermore, the analysis of collagen was performed only with a type I collagen band and another band of nonspecific collagen, and 7 days of recovery does not allow analysis of scarring as it usually starts to settle from 7 to 14 days after injury.

We suggest the application of these methods in clinical studies to validate these methods in humans. Furthermore, studies that quantify the gene expression and specific proteins of muscle regeneration (myofibrils (MyoD), collagen (TGF-β1), and blood vessels (VEGF)), and an assessment with a longer recovery time, would be useful to prove the effectiveness of the methods proposed in this research.

Conclusion

It concludes that the treatment methods were effective in repairing the structural damage of the muscle tissue. Furthermore, both PBM and the PRP and the combination of both were able to reduce oxidative stress caused by injury and modulate the production of collagen in this tissue, and the association of both showed better histological aspects.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All procedures were approved by the ethics committee for animal use from FCT/UNESP, Presidente Prudente Campus (SP, Brazil; protocol no. 01/2013). This article does not contain any studies with human participants performed by any of the authors.

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