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Effects of low-level laser therapy after nerve reconstruction in rat denervated soleus muscle adaptation

Efeitos do laser de baixa potência após reconstrução nervosa na adaptação do músculo sóleo de rato

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Abstract

Background: Peripheral nerve injury (PNI) rehabilitation remains a challenge for physical therapists because PNI effects are very disabling. Low-level laser therapy (LLLT) has been described as a physical resource that is able to influence enzymes called metallopeptidases (MMPs) associated with extracellular matrix (ECM) turnover, thus accelerating neuromuscular recovery after nerve crush injuries. However, the effects of LLLT in the treatment of severe nerve injuries and denervated slow-twitch muscles are still inconclusive. **Objectives:** The aim of this study was to evaluate the effects of different wavelengths and energy densities of LLLT irradiation, applied to a severe nerve injury after reconstruction, on denervated slow-twitch skeletal muscle adaptation. **Method:** Rats were submitted to a neurotmesis of the sciatic nerve followed by end-to-end neurorrhaphy. They received transcutaneous LLLT irradiation at the lesion site. The LLLT parameters were: wavelengths - 660 or 780 nm; energy densities - 10, 60 or 120 J/cm^2; power - 40 mW; spot - 4 mm^2. Sciatic functional index (SFI), histological, morphometric, and zymographic analyses were performed. One-way ANOVA followed by Tukey's test was used ($p \leq 0.05$). **Results:** An atrophic pattern of muscle fibers was observed in all injured groups. The MMP activity in the soleus muscle reached normal levels. On the other hand, SFI remained below normality after PNI, indicating incapacity. No difference was found among PNI groups submitted or not to LLLT in any variable. **Conclusions:** LLLT applied to the nerve post-reconstruction was ineffective in delaying degenerative changes to the slow-twitch denervated muscles and in functional recovery in rats. New studies on recovery of denervated slow-twitch muscle are necessary to support clinical practice.

Keywords: neurological rehabilitation; nerve injury; laser therapy; skeletal muscle; physical therapy.

Resumo

Contextualização: A reabilitaçao das lesões nervosas periféricas (LNP) ainda é um desafio para a fisioterapia. A terapia com o laser de baixa potência (LBP) é descrita como um recurso físico capaz de interagir com enzimas relacionadas à alteração da matrix extracelular. Denominadas metalopeptidases (MMPs), essas enzimas atuam durante a recuperação neuromuscular após LNP. No entanto, os efeitos da LBP no tratamento de músculos desnervados de contração lenta após LNP graves ainda são inconclusivos. **Objetivo:** Avaliar os efeitos de diferentes comprimentos de onda e densidades de energia de irradiação de LBP, aplicado sobre o local do nervo após LNP grave e reconstrução. **Método:** Ratos foram submetidos a neurotmese do nervo isquiático e neurorrafia término-terminal. Os parâmetros do laser são: comprimento de onda: 660 ou 780 nm; densidades de energia: 10, 60 ou 120 J/cm^2; potência: 40 mw; spot: 4 mm^2. O índice funcional isquiático (IFC) e análises histológicas, morfométricas e zimografia foram realizados. ANOVA one-way e teste de Tukey ($p \leq 0,05$) foram utilizados. **Resultados:** Um padrão atrófico das fibras musculares foi observado em todos os grupos com LNP. A atividade das MMPs no músculo sóleo alcançaram níveis normais. Entretanto, o IFC permaneceu inferior à normalidade após a LNP, indicando incapacidade. Não houve diferença entre os grupos de LNP submetidos ou não à LBP em qualquer variável. **Conclusão:** O LBP é incapaz de retardar alterações degenerativas em músculos sóleos desnervados e é ineficaz na recuperação funcional de ratos. Novos estudos sobre a recuperação do músculo de contração lenta desnervados são necessários para apoiar a prática clínica.

Palavras-chave: reabilitação neurológica; desnervação; laserterapia; músculo esquelético; fisioterapia.

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Introduction

Peripheral nerve injury (PNI) rehabilitation remains a challenge for physical therapists. This type of injury causes paralysis and causes profound degenerative alterations to skeletal muscle, leading to atrophy[1] and force deficits[2], thus impairing funcionality[3]. According to a Brazilian study, out of 456 cases analyzed, 41% of PNIs are neurotmesis[4]. In this type of PNI, there is nerve discontinuity as well as perineural disruption and in many cases loss of nerve tissue[5]. The patient is impaired both economically and socially in the occurrence of PNI, therefore post-operative treatment should aim for maximal restoration of patient functionality by stimulating neuronal growth and maintaining muscle trophism until reinnervation occurs.

The denervated skeletal muscle is a rich scenario of modifications that still has not been fully clarified. PNIs usually generate not only muscle fiber atrophy, but also incite alterations to the extracellular matrix (ECM) surrounding these fibers[6]. For example, denervated muscles have extensive endomysium and perimysium proliferation[4]. Often such proliferation can be associated with flexibility reduction, fibrosis, and deficits in the conduction of tension forces[7]. In this sense, ECM reorganisation is an important element to understanding the mechanisms of muscle adaptation in denervation.

MMPs are a zinc-dependent proteolytic enzyme family involved in the ECM remodeling process. They can be synthesized and secreted in the skeletal muscle by Schwann cells, satellite cells, and fibroblasts, specifically in the intramuscular nerves and the neuromuscular junction (NMJ)[8,9]. Among these enzymes, the MMP-2 (gelatinase A) and the MMP-9 (gelatinase B) are key to the ECM remodeling process in the skeletal muscle during changes in the intensity of physical activity or in cases of changes to task demands and to the process of injury repair[8].

These enzymes are known for acting on a non-fibrillar form of type IV collagen degradation and interstitial collagen hydrolysis[10]. The investigation concerning MMP activity is clinically relevant because MMPs act directly on collagen turnover and, therefore, on fibrosis formation, flexibility reduction, and mechanical force alterations in denervated muscles[7]. Furthermore, previous studies reported that MMPs can be involved in the reinnervation process of denervated muscle fibers[6,10] and probably allow axonal growth cones to advance into the muscle ECM.

In this context, the regulation of MMPs in denervated muscles has great importance to clinical practice. Understanding how the resources normally used by the rehabilitation team can affect MMP activation can provide a scientific basis for its use in humans. Among the possible candidates that promote neuromuscular recovery in PNI is low-level laser therapy (LLLT).

Recently, it was demonstrated that LLLT accelerates muscle fiber cross-section area (CSA) recovery in denervated fast-twitch muscles of rats when LLLT is applied to crushed nerves[3]. These authors concluded that LLLT irradiation accelerated neuromuscular recovery by increasing MMP-2 activation in the injured nerve and inhibiting the activation of MMP-9 and -2 in injured nerves and denervated muscles, respectively. These changes in MMP activation were also associated with walking recovery[3]. This study has brought subsidies for future indications of LLLT use in humans. However, studies are needed on slow-twitch muscle adaptation during severe nerve injuries.

The objective of this study was to evaluate the response of a denervated slow-twitch skeletal muscle (soleus) to LLLT irradiation applied to an injured nerve. A severe nerve injury model (neurotmesis), followed by end-to-end neurorrhaphy reconstruction, was used in the present study in an attempt to mimic clinical situations. Furthermore, special attention was given to the selection of irradiation parameters, muscle function and trophism, and ECM adaptation in denervated muscles. This work is relevant to neurological rehabilitation because it considers a common situation in physical therapy practice. Moreover, the use of the animal model in this study is justified due to ethical reasons surrounding the biopsy of denervated muscles in humans. Finally, the hypothesis of this study was that LLLT irradiation in injured-reconstructed nerves is able to accelerate nerve recovery and muscle reinnervation, improving function and reestablishing soleus muscle trophism via the regulation of MMP activity.

Method

Animal care and experimental groups – Sixty-four male 3-month-old Wistar rats (275 g) were used. The animals were housed in plastic cages in a room with controlled environmental conditions and free access to water and standard food. The Ethics Committee of Universidade Federal de São Carlos (UFSCar), São Carlos, SP, Brazil, approved the experimental procedures (Process 001/06), and the study was conducted in accordance with the national guide for care and use of laboratory animals.

Experimental groups

The animals were randomly divided into eight groups (n=8): (1) normal (N) in which the animals received no intervention and remained free in the cage for 84 days; (2) transected nerve and end-to-end neurorrhaphy (TT) with simulation treatment (placebo) for one minute; (3) transected nerve and end-to-end

neurorrhaphy irradiated with LLLT 660 nm 10 J/cm^2 (TT660 10); (4) transected nerve and end-to-end neurorrhaphy irradiated with LLLT 660 nm 60 J/cm^2 (TT660 60); (5) transected nerve and end-to-end neurorrhaphy irradiated with LLLT 660 nm 120 J/cm^2 (TT660 120); (6) transected nerve and end-to-end neurorrhaphy irradiated with LLLT 780 nm 10 J/cm^2 (TT780 10); (7) transected nerve and end-to-end neurorrhaphy irradiated with LLLT 780 nm 60 J/cm^2 (TT780 60); (8) transected nerve and end-to-end neurorrhaphy irradiated with LLLT 780 nm 120 J/cm^2 (TT780 120).

Surgery procedure

The animals were anesthetised with an intraperitoneal injection of a premixed solution containing ketamine (95 mg/kg) and xylazine (12 mg/kg). The skin was shaved and cleaned with 10% povidone iodine. A 2-cm-long incision was made on the skin through a gluteal approach and the left sciatic nerve was exposed. The sciatic nerve was cut and sutured with a nylon monofilament 8.0 in the epineural region only. This microsurgical procedure was performed by visualization of a surgical magnifying lens with 4x magnification. The nerves were kept moist with 37ºC sterile saline solution throughout the surgical intervention. After surgery[11,12], the animals were housed in single cages and fed rat chow and water ad libitum. For the first four days, acetaminophen (13.5 mg/100 mL) was added to the water for pain reduction. A single dose of the antibiotic Terramycin (1 mg/0.1 mL) was administered to prevent secondary complications related to possible infections.

LLLT protocol and experimental design

Biostimulation was carried out using a gallium–aluminum–arsenide laser device (TWIN LASER, MM Optics, São Carlos, SP, Brazil) with the following parameters: continuous radiation, wavelength: 660 or 780 nm, power: 40 mW, spot area: 4 mm^2, energy density at the point of entry: 10, 60 or 120 J/cm^2. The time of stimulation was predetermined by the device following the abovementioned parameters. All parameters were obtained from Gigo-Benato et al.[3] and are described in detail in Table 1. Calibration was performed by MM Optics (São Carlos, SP, Brazil). Briefly, a calibrated powermeter was used to verify the power of the laser device. This verification was approved only if the deviation was not higher than 20% of mean value.

Radiation was applied transcutaneously after shaving the skin over the site of the surgery (recognizable for the presence of the surgical scar) at two points along the sciatic nerve, one above and one below the scar site, and two centimetres apart. Applications were made daily for 10 consecutive days beginning on the first day after surgery and on alternative days for another month. The animals were handled with care. Laser biostimulation did not cause any pain or distress to the animals, therefore it was not necessary to use anesthesia.

Assessment of nerve function recovery

The assessment of nerve function recovery was carried out by calculating the sciatic functional index (SFI) as described by Bain, Mackinnon and Hunter[13]. Animals were tested in a confined walkway 42 cm long and 8.2 cm wide, with a dark shelter at the end. A white sheet of paper was placed on the floor of the rat walkway. The rats' hind paws were pressed down onto a finger paint-soaked sponge, and they were then allowed to walk down the walkway leaving their hind footprints on the paper. Three measurements were taken from the footprints: (1) the print length (PL), i.e. the distance from the heel to the third toe; (2) the toe spread (TS), i.e. distance from the first to the fifth toe; and (3) the intermediate toe spread (ITS), i.e. distance from the second to the fourth toe. All three measurements were taken from the experimental (E) and normal (N) sides. The SFI was calculated according to the following equation[14]:

$$SFI = -38.3[(EPL-NPL)/NPL]+109.5[(ETS-NTS)/NTS]+13.3[(EITS-NITS)/NITS]-8.8$$

Muscle evaluation

The right soleus muscles were carefully dissected to avoid mechanical injuries. The muscles were then divided in half at the middle of the belly. The proximal fragment was used for the histological and morphometric measurements. The distal fragment was immediately frozen in liquid nitrogen and stored at -80°C (Forma Scientific, Marietta, OH) for the zymographic analysis.

Afterwards, the proximal fragment was frozen in isopentane, previously frozen in liquid nitrogen. Muscle samples were placed in plastic tubes and stored at -80°C. Histological serial cross-sections (10 μm), cut transversely to the muscle main axis, were obtained with a HM 505E cryostat (Microm, Walldorf, Germany) at a level corresponding to the middle belly of the muscle. Muscle sections were stained with 1% toluidine blue/1% borax. Pictures from five different regions were obtained using a light microscope (Axiolab, Carl Zeiss, Jena, Germany) equipped with a digital camera (AxioCam HRc, Carl Zeiss, Germany). From each picture, the CSA of 70 randomly chosen fibers was measured using the software Axiovision 3.0.6 SP4 (Carl Zeiss, Jena, Germany).

Table 1. Parameters of LLLT application in different experimental groups.

Experimental Groups n=8	Wavelength (nm)	Power (mW)	Energy density (J/cm²)	Total energy emitted per point (J)*	Time ON (s)
N	-	-	-	-	-
TT	Simulated	Simulated	Simulated	Simulated	60
TT660 10	660	40	10	1.2	30
TT660 60	660	40	60	2.4	60
TT660 120	660	40	120	4.8	120
TT780 10	780	40	10	1.2	30
TT780 60	780	40	60	2.4	60
TT780 120	780	40	120	4.8	120

*Total energy for point (J) = Power (W) x Time on (s).

Zymography

Tissue extraction and zymographic analysis was performed according to current methodology[15,16]. The molecular mass of gelatinolytic activities was determined by comparison to reference protein molecular mass marker PageRuler Prestained Protein Ladder (Fermentas Life Sciences, Burlington, ON, Canada). Activity bands were identified following a previous description[17], according to their molecular weights (pro-MMP-2: 72 kDa; intermediate-MMP-2: 64 kDa; and active-MMP-2: 57 kDa and pro- MMP-9: 92 kDa; intermediate-MMP-9: kDa; active-MMP-9: 81 kDa). Densitometric quantitative analysis of the protein bands in the zymography was performed using the software GeneTools v3.06 (Syngene, Cambridge, UK).

Statistical analysis

The Shapiro–Wilk test and Levene's test were applied to evaluate the normality and homogeneity of the results, respectively. Repeated measures ANOVA was performed for the SFI. For the muscle-fiber cross-sectional area and the MMP activity variables, one-way ANOVA was used to identify possible differences among groups. When differences were observed, Tukey's test was performed. For all tests, the significance level was set at 5% ($p \leq 0.05$).

Results

Sciatic functional index (SFI)

The pre-neurotmesis (pre-TT) SFI values were considered normal (-7.44). No difference was found among the experimental groups in the pre-TT moment ($p>0.05$; Figure 1). As expected, on the first day after injury there was a reduction in the SFI compared with pre-TT in all injured groups ($p>0.05$; Figure 1). On the last day (84th day), a partial functional recovery was observed in all injured groups when compared to day 1 ($p<0.05$; Figure 1). Nevertheless, these values remained inferior to those values observed in the normal group ($p<0.05$; Figure 1), with no difference among injured groups on day 84 post-injury ($p>0.05$; Figure 1). No difference was detected among the groups at any point between the first and the last day (data not shown).

Muscle morphology and cross-sectional area (CSA)

The muscle morphology analysis showed an atrophic pattern of muscle fibers for all injured groups (LLLT irradiated or not) when compared to the normal group (Figure 2). Connective tissue proliferation was observed in the denervated soleus muscles (Figure 2B-G), specially surrounding the muscle fibers (endomysium) and fiber bundles (perimysium) compared to Normal group (Figure 2A).

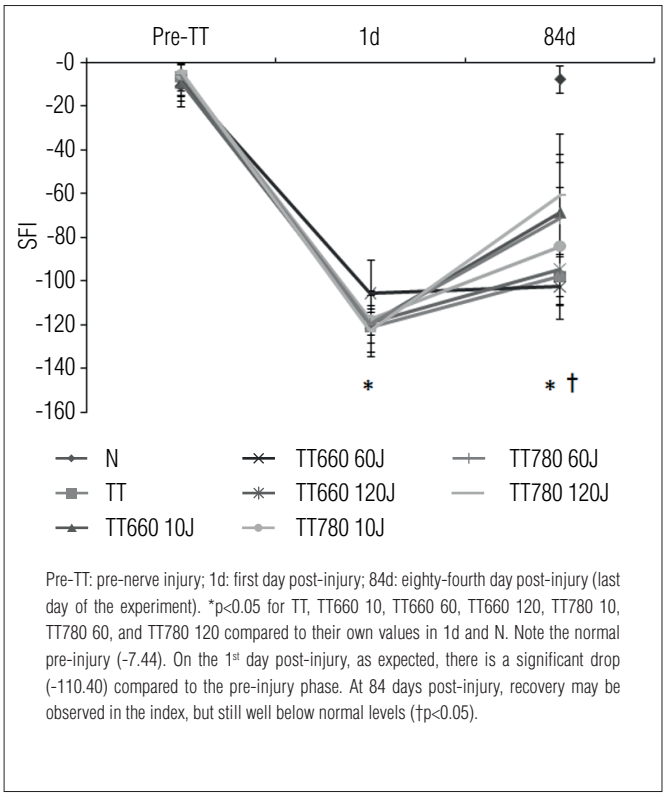
Moreover, angulated and degenerated fibers were observed in all denervated groups (Figure 2B-G). These characteristics are a direct indication of skeletal muscle modifications caused by the absence of innervation. In these groups, central nuclei were also observed, confirming the myopathic phenotype (Figure 2I).

Muscle fiber atrophy was confirmed by muscle fiber CSA measurement. All denervated groups showed smaller muscle fiber CSA than normal ($p<0.05$; Figure 3; TT: -69.2%, TT660 10: -61.5%; TT660 60: -46.1%; TT660 120: -64.1%; TT780 10: -51.3%; TT780 60: -53.8% and TT780 120: -52.6%). No difference was observed among denervated groups ($p>0.05$; Figure 3).

MMP activity in denervated soleus muscle

The MMP-9 activity was not detected in any samples of the soleus muscle. In comparison, three MMP-2 isoforms were located (pro, intermediate, and active) in all groups. A representative gel is shown in Figure 4A.

Densitometric analysis showed no difference among denervated and normal groups in any of the isoforms ($p>0.05$; Figure 4B-D).



Pre-TT: pre-nerve injury; 1d: first day post-injury; 84d: eighty-fourth day post-injury (last day of the experiment). *p<0.05 for TT, TT660 10, TT660 60, TT660 120, TT780 10, TT780 60, and TT780 120 compared to their own values in 1d and N. Note the normal pre-injury (-7.44). On the 1st day post-injury, as expected, there is a significant drop (-110.40) compared to the pre-injury phase. At 84 days post-injury, recovery may be observed in the index, but still well below normal levels (†p<0.05).

Figure 1. Sciatic functional index (SFI) in different experimental groups.

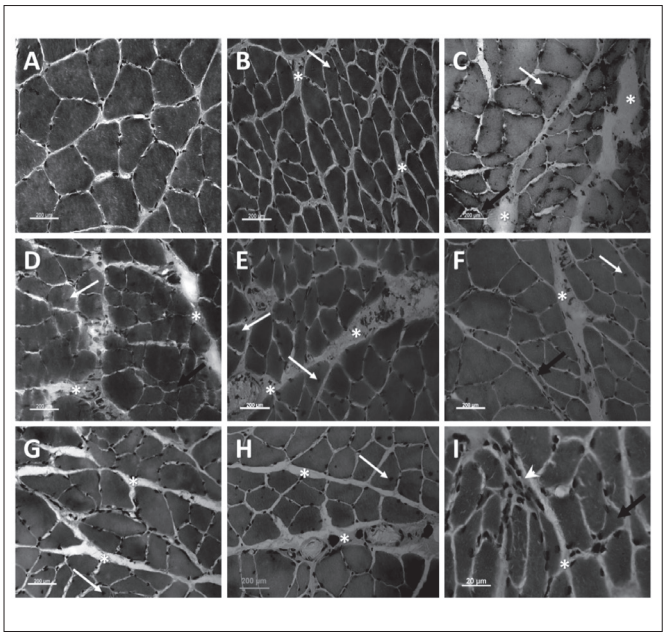


Figure 2. Soleus muscle cross-section of the different experimental groups stained with toluidine blue: A) N; B) TT; C) TT660 10; D) TT660 60; E) TT660 120; F) TT780 10; G) TT780 60; H) TT780 120; and I) TT660 120. White arrows indicate muscle fibers with centralized nuclei; black arrows indicate the angled fibers; asterisks indicate proliferation of connective tissue; and arrow heads indicate the degenerating fibers.

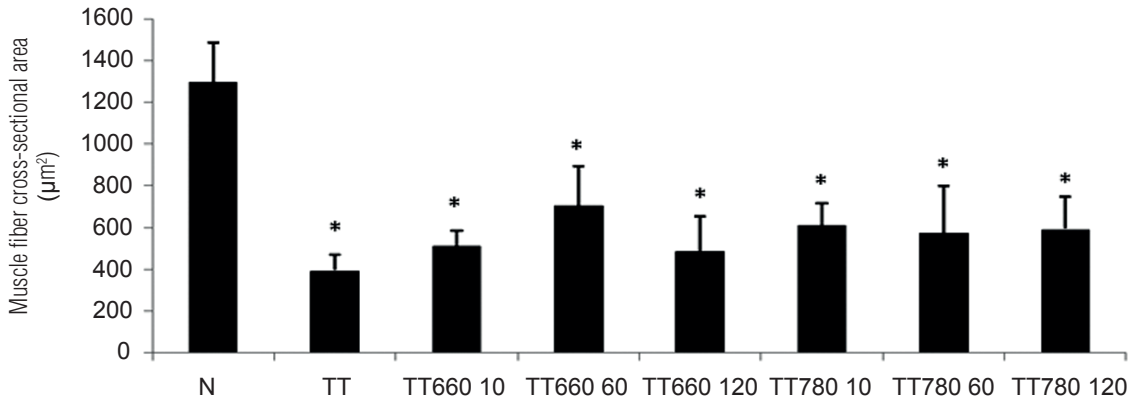
Discussion

The present study demonstrated that LLLT applied to reconstructed nerve with the parameters investigated here is unable to avoid degenerative modifications to denervated slow-twitch muscles and ineffective in recovering functionality in rats. The present results did not corroborate the findings observed in denervated fast-twitch muscles whose crushed nerves were irradiated with LLLT[3].

Recently, Gigo-Benato et al.[3] pointed out that LLLT irradiation of crushed sciatic nerves caused acceleration in nerve regeneration and contributed toward CSA recovery in the tibialis anterior (TA) muscles of rats. They also demonstrated that a wavelength of 660 nm, with energy densities of 10, 60, and 120 J/cm^2, was effective in increasing the MMP-2 activity of the injured nerves, possibly facilitating axonal growth through laminin, fibronectin, and type IV collagen degradation[18]. In addition, LLLT decreased the MMP-9 activity of these nerves, which probably helped to attenuate the inflammatory process. Finally, they demonstrated a decrease in MMP-2 activity in the TA muscles of the crushed groups irradiated with 660 nm LLLT. The factors that hinder a direct comparison between this and the present study are the differences between the experimental models, namely, the crushing model compared to the nerve section model and the different investigation times.

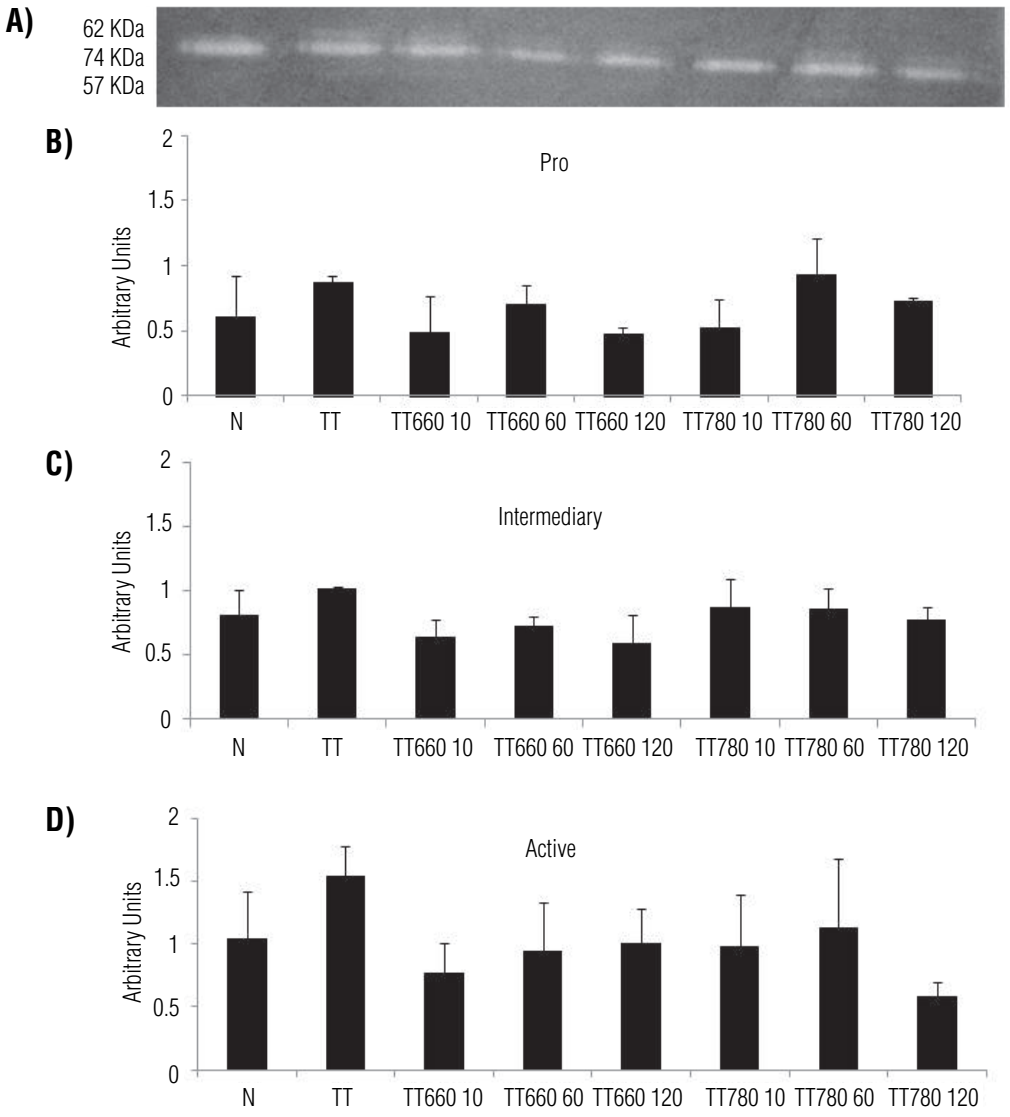
Another factor that may have influenced the findings related to muscle trophism is fiber composition. Gigo-Benato et al.[3] investigated fast-twitch muscles (TA) composed mainly of type II fibers. However, the present study focused on a slow-twitch muscle (soleus) composed mainly of type I fibers[19]. It has been well described in the literature that slow-twitch muscles exert antigravity function[20] and respond more to immobilization and disuse situations if compared to fast-twitch muscles. Furthermore, the reduction in oxidative capacity, and the increase in glycolytic metabolism in denervated slow-twitch muscles combined with muscle fiber phenotype transition (from type I to type II) can also interfere with muscular adaptative response[19,21].

Another difference between these studies lies in the fact that: first, slow-twitch muscle fibers are innervated by small motoneurons and fast-twitch muscle fibers are innervated by larger motoneurons[22-25]; and second, that after a nerve injury, Wallerian degeneration occurs from the proximal stump towards the distal followed by neuronal growth cone advance[5]. Thus, a hypothesis generated for the present study is that LLLT could selectively stimulate the neuronal growth cones of large motor units. This hypothesis can be corroborated by two other facts: 1) large motoneuronal growth cones have more mitochondria than those of small motoneurons[5]; and 2) there is evidence that LLLT can act selectively on oxidative metabolism by activating the mitochondrial respiratory chain[26]. LLLT acts



*p<0.05 compared to N. Note the decrease in CSA in all groups compared to denervated N. There was no difference between the TT groups irradiated or not with LLLT.

Figure 3. Cross-sectional area (CSA) of soleus muscles from the different experimental groups.



The bands representing MMP-2 gelatinase activity are shown at the top of figure (A). Activity isoforms pro (B), intermediate (C), and active (D). MMP-2 observed in different experimental groups. All experimental groups demonstrated similar values to group N (p>0.05).

Figure 4. MMP-2 activity in denervated soleus muscle.

on cellular metabolism increasing the size and amount of mitochondria, and oxygen consumption[27,28]. Thus, we believe that LLLT could stimulate the mitochondria of large motor units, facilitating growth cone advance. However, future studies must be performed to test this hypothesis.

Regarding MMP-2 activity, normal levels were found in all denervated soleus muscles. This can be attributed to timing. Demestre et al.[6] reported an increase in MMP-2 activity in denervated soleus muscles 20 and 40 days after crush nerve injury and a return to normal levels 63 and 72 days after injury, in accordance with the present study. Thus when comparing two types of injury, it is possible to reach the conclusion that MMP activity is normal in later phases of post-neurotmesis recovery.

In contrast, these normal levels of MMP-2 activity could be related to a state of balance that atrophic muscles can reach. Recent studies reported that other degradation pathways, i.e. autophagic pathway, can be involved in late denervated muscle atrophy. In addition, skeletal muscles have strategies to inhibit these pathways and preserve myofibrillar organization, such as the increase in Runx1 (Runt-related transcription factor 1) expression[29]. The present study corroborates this finding because the muscle fiber analysis of the denervated groups showed many signs of injury-regeneration cycles, such as central nucleus and fiber fragmentation. Angled fibers were also observed in all denervated groups, showing a homogeneous pattern in these muscles.

Considering functional recovery, it should be observed that the injury model used in the present study is slower than crush models, with worse prognosis and longer recovery. De Sá et al.[30] demonstrated that 80% of nerve function was recovered within 60 days of end-to-end neurorrhaphy. The authors suggested that in a few more weeks the repairing process could be complete. Hence, when evaluating the period of 84 days post-injury, we observed a significant, albeit incomplete, functional improvement in all injured groups (LLLT irradiated or not). New studies should investigate longer times after injury in order to determine the moment of total functional recovery.

It is also worth noting that the present study carried out a dose-response curve following recommendations of clinical protocols of LLLT application. For the wavelength, some studies demonstrated that either visible[31-33] or near-infrared lasers[34] are able to stimulate neuronal growth. Energy density is another important parameter to be considered when investigating LLLT effects. Analysis of published results showed that LLLT at different levels of energy density (ranging from <10 to 150 J/cm^2) are effective in promoting nerve regeneration. The same is observed for time of irradiation which varies from <1 min to 90 min. But despite the evidence that light can affect nerve recovery, the literature is still unclear on the best combination of parameters to improve the regenerative process. Finally, all published studies that reported good results in nerve regeneration used continuous LLLT[33,35,36]. The present study followed all of these recommendations.

The results of the present study have significant clinical considerations. Based on our findings, therapists should keep in mind that denervated slow-twitch muscles do not recover as quickly as fast-twitch muscles[3]. Future studies should focus on interventions not only for the injured nerve, but also for the denervated muscle. Muscle stretching, electrical stimulation, and LLLT have already shown significant results in PNI treatment[37-40], thus, they should also be considered in future studies for the recovery of denervated slow-twitch muscles.

The present study has some limitations that should be considered. A time-course curve to verify cellular and molecular modifications could provide information about the MMP activity pattern over time and possible correlations with morphological findings. Furthermore, a comparison between fast- and slow-twitch muscles is necessary to investigate the differences in the reinnervation process and MMP content/activity according to muscle type. Although the present study focused on muscle investigation, other studies should consider the evaluation of morphological and molecular nerve aspects to provide evidence of interaction between LLLT and peripheral nerves.

In conclusion, using a severe PNI standardized model and an LLLT protocol based on literature recommendations, we found that LLLT applied to injured nerves, regardless of the dose, was ineffective in accelerating functional recovery and improving denervated slow-twitch muscle trophism in rats.

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