ABCS HEALTH SCIENCES

ORIGINAL ARTICLE

OPEN ACCESS

High energy photobiomodulation therapy in the early days of injury improves sciatic nerve regeneration in mice

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ABSTRACT

Introduction: Different studies have evaluated the effects of electrophysical agents on regeneration after peripheral nerve injury. Among them, the most used in clinical and experimental research is photobiomodulation therapy (PBMT). **Objective:** To analyze the effect of standard energy (16.8 J) of PBMT on peripheral nerve regeneration, applied at different periods after sciatic nerve injury in mice. **Methods:** Thirty male Swiss mice were divided into six groups: naive; sham; control; LLLT-01 (660 nm, 16.8 J of total energy emitted in 1 day); LLLT-04 (660 nm, 4.2 J per day, 16.8 J of total energy emitted in 4 days); LLLT-28, (660 nm, 0.6 J per day, 16.8 J of total energy emitted over 28 days). The animals were evaluated using thermal hyperalgesia, Sciatic Functional Index (SFI), and Static Sciatic Index (SSI). Data were obtained at baseline and after 7, 14, 21, and 28 days after surgery. **Results:** For the SFI and SSI, all groups showed significant differences compared to the control group, and the LLLT-04 group presented the best results among those receiving PBMT. In the assessment of thermal hyperalgesia, there was a significant difference in the 14th day of evaluation in the LLLT-04 group. **Conclusion:** The application of 16.8 J was useful in sciatic nerve regeneration with an improvement of hyperalgesia, with higher efficacy when applied in four days (4.2 J/day).

Keywords: low-level light therapy; lasers; crush injuries; nerve regeneration; sciatic nerve.

INTRODUCTION

Peripheral nervous system (PNS) injuries have an annual incidence of 3%, generating a cost of around US$150 billion in the United States. Although PNS injuries do not put the individual's life at risk, it affects its quality of life, as trauma results in motor and sensory disabilities[1-3].

The sciatic nerve injury model is highlighted in preclinical research and demonstrates its effectiveness in assessing the regeneration of the PNS, with this model different types

How to cite this article: Souza *et al.* High energy photobiomodulation therapy in the early days of injury improves sciatic nerve regeneration in mice. ABCS Health Sci. 2020;45:e020016. https://doi.org/10.7322/abcshs.45.2020.1345

Received: Oct 11, 2019
Revised: Jan 20, 2020
Approved: Mar 04, 2020

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Declaration of interests: nothing to declare

of injury are reproduced. To assess PNS regeneration, the crush injury is the most used, as it maintains the connective support of the tissue, generating an injury to the axons, allowing the connectivity of the proximal and distal portion to the injury to be maintained, which favors Wallerian degeneration[4,5].

Different electrophysical agents have been used to improve the neural regenerative process. Among them we have the use of: ultrasound[6,7], electrical stimulation[8], and photobiomodulation therapy (PBMT), with emphasis on the use of low-level laser (LLL)[9-15].

PBMT promotes the stimulation of microcirculation by paralysis of the pre-capillary sphincter, arteriolar and capillary vasodilation, vascular neoformation, which favor the increase of blood flow in the irradiated area[11]. It is also able to increase metabolism and cell proliferation, stimulating the production of adenosine triphosphate (ATP), triggered by the absorption of photons by cytochrome-c oxidase in the mitochondrial breathing chair[16-19].

In addition, it has analgesic properties, which occur through the modulation of anti-inflammatory chemical mediators and synthesis of β-endorphin, which tends to limit the excitability of nociceptive receptors and eliminate algogenic substances[20-22] thus the effects of LLL contribute to the acceleration of the PNS regeneration process.

Although different studies demonstrate the efficacy of LLL therapy (LLLT) in nerve regeneration, there is still a large therapeutic window, using several protocols with different dosimetric parameters that can vary according to the wavelength, power, total energy emitted, density, duration, pulsed or continuous application and different points of application[23,24].

The use of energy with known positive effects when applied in three to four weeks in previous studies, when concentrated in the first postoperative days may have photobiostimulatory or photobioinhibitory effects[25].

Thus, the aim of this study was to analyze the effect of photobiomodulation (16.8 J) on peripheral nerve regeneration, applied in different regimes after sciatic nerve injury in mice.

METHODS

Thirty adult male mice of the Swiss lineage, weighing 30-40 g, were obtained from the central animal facility from Universidade Federal de Santa Catarina (UFSC), Brazil. All procedures used were performed in accordance with the Principles of care for laboratory animals[26], and were approved by the UFSC Animal Ethics Committee (CEUA-UFSC, protocol number PP00956).

The mice were kept in cages, in groups of ten to twelve animals, in a controlled room temperature (22±2°C), with a light cycle divided into 12 hours of light, 12 hours of darkness, with free access to water and food. The animals were weighed and divided into six groups at random according to the procedures to be performed below: 1) Naive group (n=3): not submitted to the surgical procedure; 2) Sham Group (n=3): submitted to the surgical procedure without sciatic nerve crush and simulation of irradiation with LLL; 3) Control group (n=6): subjected to the surgical procedure associated with sciatic nerve crush and simulation of irradiation with LLL; 4) LLLT-01 Group (n=6): sciatic nerve damage and irradiation with LLL, total energy 16.8J emitted during 1 day; 5) LLLT-04 Group (n=6): sciatic nerve damage and irradiation with LLL during the first 4 days, with 4.2 J of energy/day and total final energy of 16.8 J; 6) LLLT-28 Group (n=6): sciatic nerve damage and irradiation with 0.6 J/day LLL energy and 16.8 J total energy emitted for 28 days.

Sciatic Nerve Injury Procedure

The animals were anesthetized with 2% xylazine (Syntec do Brasil Ltda, Rhobifarma™, Hortolândia, São Paulo, Brazil - 0.07 ml/100 g of body weight) and 10% ketamine (Syntec do Brasil Ltda, Rhobifarma™, Hortolândia, São Paulo, Brazil - 0.1 ml/100 g body weight) intraperitoneally.

Then, trichotomy and incision (scalpel number 15, Embramed Indústria e Comércio de Produtos Hospitalares™, São Paulo – São Paulo, Brazil) was performed on the side of the limb. To perform axonotmesis, a calibrated crushing device with a weight of 5000 g and an area of 0.5 cm^2 was used, which makes the crushing process practical and reliable[10]. The crushing point was defined 5 mm above the three main branches (sural, fibular and tibial) and the device was maintained for 10 minutes at the crushing point. At the end, the nerve was relocated to its original bed and a suture of muscles and skin was performed (Tecnew™, Quintino, Rio de Janeiro, Brazil) (Figure 1).

Photobiomodulation Therapy

Gallium-Aluminum-Indio-Phosphorus Arsenide diode (AsGaAlInP) of the brand (Ibramed Equipamentos Médicos™, Laserpulse, Amparo, São Paulo, Brazil) was used, the LLLT parameters used and dose are shown in Table 1.

The animals were physically contained manually and the low level laser was positioned at a 90° angle to the skin tissue, using the point contact technique at the central point of the surgical incision[27].

Figure 1: Surgical procedure for crushing the sciatic nerve and crushing forceps.

Table 1: PBMT parameters used in the study.

Parameters	LLLT-1	LLLT-4	LLLT-28
Wavelength	660 nm	660 nm	660 nm
Power	30 mW	30 mW	30 mW
Power Density	0.5 W/cm²	0.5 W/cm²	0.5 W/cm²
Beam Area	0.06 cm²	0.06 cm²	0.06 cm²
Beam	Continuous	Continuous	Continuo
Application days	1	4	28
Application time per day	560 s	140 s	20 s
Energy density	10 J/cm²	10 J/cm²	10 J/cm²
Total energy emitted per day	16.8 J	4.2 J	0.6 J

Evaluation

Sciatic Functional Index (SFI) and Sciatic Static Index (SSI)

Functional gait assessment provides the opportunity to assess specific aspects of sciatic nerve regeneration in a non-invasive way[28-32]. The results range from 0 to -100, which is an indicator of nerve function, where -100 represents complete nervous dysfunction and 0 represents the absence of dysfunction, where the results are reliable when comparing the histological analysis of the nerve.

The SFI consists of obtaining an image of the animal's footprint, captured by a camera, where an acrylic walkway was used to obtain the footprint, where the animal travels from side to side. The parameters analyzed were the footprint length, total finger opening and middle finger opening[32,33].

The SSI was used to perform the static functional assessment. Studies demonstrate the effectiveness of the SSI in the assessment of nerve regeneration in rodents, since, according to the authors, static evaluation reduces the deviations caused by the animal's speed in the SFI[34]. The results in the study by Smit et al.[30] also suggest more accuracy of the data obtained in the SSI than in the SFI.

For the acquisition of the footprints, a 13 megapixel camera (Sony™, Minato, Tokyo, Japan) was used, fixed under a transparent acrylic catwalk 43 cm long, 5.5 cm high and 8.7 cm wide, with a wooden box at the end. The videos were digitized by the Kinovea™ program. The images were analyzed using the Image J™ program to transform the pixels into millimeters and calculate the predetermined parameters for the SFI and SSI evaluation. The footprints were obtained preoperatively and at 7, 14, 21, and 28 days from the initial injury[31].

Thermal Hyperalgesia

To assess thermal hyperalgesia, the Hargreaves® device (Ugobasile, Comerio, Italy) was used. This emits an infrared light, which was radiated directly over the plantar region of the animal's right hind leg. The animals were housed in the test room one hour before the test. The paw withdrawal latency after the application of the thermal stimulus was automatically collected by means of a sensor, the time of 20 seconds was determined cut-off, in order to avoid possible tissue damage in the animals' paw[27,28].

Three measurements of response time were performed, being recorded at 20-minute intervals in order to determine the baseline threshold, all groups were assessed before the surgical procedure. The evaluation of thermal hyperalgesia was performed on days 0, 14, 21, and 28 post-surgery.

Statistical analysis

The results were expressed as mean ± standard deviation. Normality was assessed by the Shapiro-Wilk test and was analyzed by the two-way ANOVA test, followed by the Tukey post-test. Values of $p<0.05$ were considered to show significant differences between the means (GraphPad Prism® 8.0 software, San Diego, California, USA).

RESULTS

The results are shown in figures 2 to 5. The animals did not present postoperative complications, such as autotomy and dehiscence, and the success of the surgery model was evident due to the evolution of the groups operated on the 7th day, showing a difference when compared to the baseline. in the intragroup assessment and in the same intergroup assessment period for both SSI and SFI with a reduction in the values of both.

Figure 2 shows the results for the SSI and SFI where the groups submitted to sciatic nerve injury showed a significant difference between the baseline and day 7. Additionally, in the intra-group analyzes, LLLT-01, LLLT-04, LLLT-28 obtained significant difference between days 7 and 28. In relation to SSI, groups LLLT-04 and LLLT-28 showed significant improvement from day 14 and also for SFI, group LLLT-28 showed significant improvement from day 14. When comparing the baseline results, we can see that on day 21 the groups LLLT-04 and LLLT-28 did not show any more differences.

Figure 3 demonstrates the intergroup comparison of the SSI, where the LLLT-01 group demonstrated regenerative potential as of the 21st, but when compared to the other groups, the result is unsatisfactory. The groups LLLT-04 and LLLT-28, on the other hand, the effects appeared from day 14, with peak regeneration on day 21 showing a positive effect, however the group LLLT-04 was the group that showed the most evident initial recovery, with significant reduction since day 7 and day 14 showed results closer to zero when compared to the other groups, indicating functional improvement due to nerve regeneration.

Figure 4 shows the values obtained in the SFI, the groups LLLT-04 and LLLT-28 showed a significant improvement from day 14, showing better functional result when compared with other groups. However, only the LLLT-04 group showed a significant result on day 28 when compared to the control group.

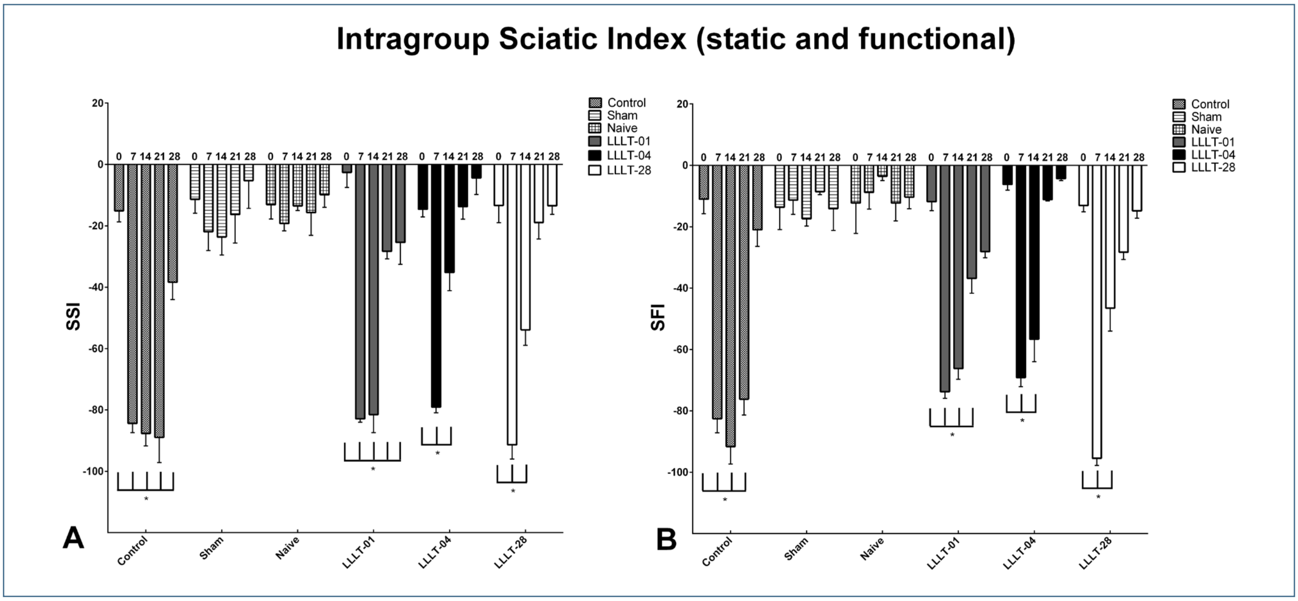

Figure 2: A) The results of the intragroup analysis of the static sciatic index (SSI). B) Sciatic Functional Index (SFI). *Statistical difference when compared to the baseline (p<0.05)

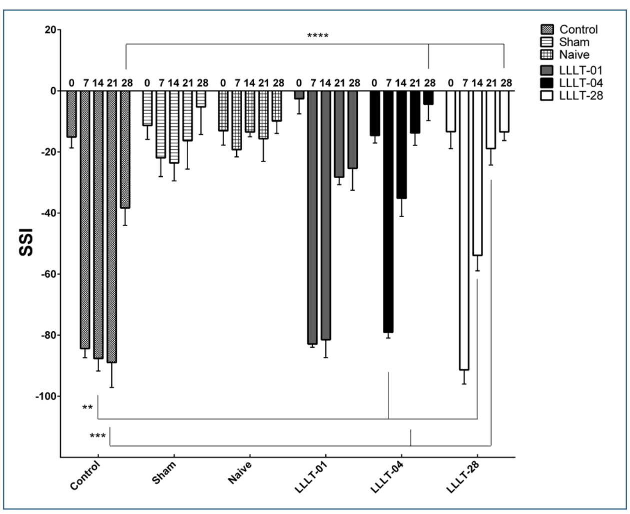

Figure 3: The results obtained from intergroup evaluation of the static sciatic index (SSI). Differences (p<0.05): **day 14; ***day 21; ****day 28.

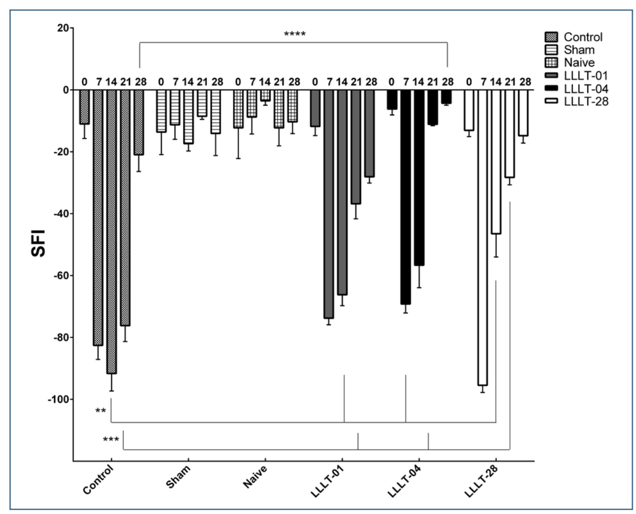

Figure 4: The results obtained from intergroup evaluation of the Sciatic Functional Index (SFI). Differences (p<0.05): **day 14; ***day 21; ****day 28.

Evaluation using thermal hyperalgesia to heat (Figure 5) showed significant differences only in the LLLT-04 group on the 14th day of the evaluation, which showed an increase in response time.

DISCUSSION

The crush injury model generates axonotmesis[35] preserving the neural support structure, favoring degeneration of the distal axon to injury[36]. In the present study, it can be seen that in the post-operative period the animals had difficulty walking, especially in the first week, with the paw in a flexor pattern, adduction of the fingers, inability to transfer load from one paw to another, compatible with the dysfunction of the sciatic nerve[33]. Studies show that the SFI has a clear correlation with the morphological and morphometric evaluation of the nerve and is a quantitative, reliable and reproducible method to assess the process of peripheral nerve regeneration, providing a numerical value to the function and allowing statistical analysis of the results[37-40].

The crush injury with a previously calibrated portable forceps was selected in this study, as it allows the lesion to be standardized and preserves the nerve support structure, in addition, the equipment provides results similar to dead weight equipment,

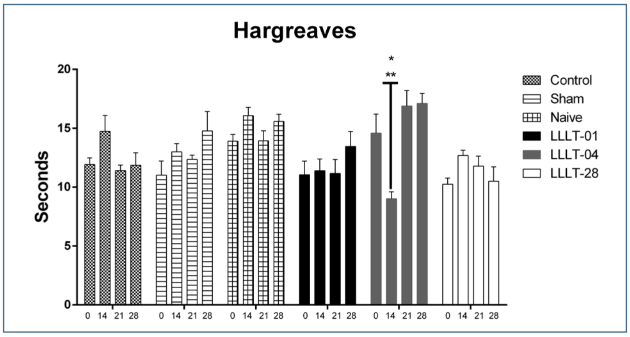

Figure 5: Thermal hyperalgesia using the Hargreaves device (in seconds). Differences (p<0.05): *between groups and **day 14 (p<0.05)

widely used in experimental research Souza et al.[9]. However, the portable clamp has simple application and handling compared to other equipment.

In studies of functional recovery of peripheral nerves different parameters of PBMT are used, and the definition of safer and more effective protocols is necessary[34].

Souza et al.[9] recently investigated the associative effects of photobiomoduction (660 nm, 10J/cm^2, 0.6 J, 16.8 J of total emitted energy, 20s) with dexamethasone (local injection of 2 mg/kg) in sciatic nerve crush injury in mice analyzed using the static and functional sciatic index. The animals were euthanized after 28 days and the results obtained with the analyzes were that the application of PBMT and dexamethasone were effective in nerve regeneration, being more satisfactory when photobiomodulation was associated with dexamethasone, corroborating our findings, since in the present study the same PBMT application parameter was used, in isolation and at different times, having positive effects for both LLLT-04 and LLLT-28.

Barbosa et al.[10] used the same lesion model and the same parameters (660 nm, 10J/cm^2, 0.6 J, 16.8 J of total emitted energy, 20s) 40 compared to the wavelength 830 nm, with positive results in nerve regeneration from day 14 on a protocol of 28 days of application. In the present study, the findings demonstrated that the application of the total dose of 16.8 J divided in the first four days (4.2 J/point) post-injury obtained significant results when compared to the other intervention groups, suggesting that the increase in energy applied in the initial phase of the injury may indicate a new perspective of treatment.

In addition, Almeida et al.[40] evaluated biochemical changes induced by LLLT after axoniotymesis, in this study a wavelength of 660 and 808 nm was used for 21 days, providing a total energy of 12 J per day. It was observed that in the PBMT group there was an increase in sphingophospholipids and collagen, constituents of the myelin sheath, and also that the wavelength of 660 nm was more effective than 808 nm in relation to cell proliferation and PNS repair. In the present study, we can observe that the LLLT-04 and LLLT-28 groups did not show any differences when compared to the baseline, starting on the 21st day of treatment, and that the LLLT-04 showed results closer to zero when compared to the other groups, suggesting that in 21 days the protocol was effective in treating nerve damage, in both groups with LLLT-04 being the most effective.

For the analysis of thermal hyperalgesia, measurements were not performed on the 7th postoperative day, as a pilot study showed an increase in the incidence of surgical wound dehiscence during the handgrip. In the assessment of thermal hyperalgesia, differences were presented only for the LLLT-04 group on day 14, not following an improvement pattern for the groups.

In the research that related the use of PBMT and nerve damage, there is a large therapeutic window of the parameters used. Thus, further studies are needed to verify the use of PBMT in early regeneration. The application of a high energy (J) in the first days after the traumatic injury appears to be a new perspective for treatment. Additionally, new pre-clinical and clinical studies are needed to verify functional restoration, improving the functional/sensory recovery process, in addition to speed in axonal regeneration.

From the above, it can be concluded that, in the sample analyzed, the PBMT protocol was effective in early nerve regeneration after sciatic nerve injury in mice, being more effective when the energy was applied during the initial four postoperative days (4.2 J/day).

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