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Association of Swimming and Photobiomodulation to Alleviate Nociceptive and Motor Behavior in Rats with Neuropathic Pain

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Abstract: Several studies have shown that non-pharmacologic treatments such as photobiomodulation (PBMT) and swimming (SW) have been increasingly used as adjuncts to clinical practice. The aim of this work is to evaluate whether SW associated or not with PBMT has its effects potentialized on different kind of pain and motor aspects in a model of chronic constriction injury and if the treatments have any improvement on morphometry of the skeletal striated muscle. Our results have demonstrated a beneficial effect on pain and motor response for animals treated with the therapies isolated, and an even better response when we associate them. Regarding muscle tissue, we observed an improvement in inflammatory cells quantification and on the collagen expression on the muscle after each isolated treatment or in combination. These findings contribute to enhancing for individuals suffering from neuropathic pain conditions, offering a promising avenue for integrated care approaches that prioritize both symptom alleviation and functional recovery. Such insights are crucial for advancing treatment strategies in pain management since the currently available treatments are not completely effective to treat all the pain and also promotes several side effects on the patients' health.

Keywords: Pain; Swimming; Photobiomodulation; CCI; Neuropathic Pain; Muscle

1. Introduction

With increasing longevity, cases of neuropathic pain have increased, largely due to sedentary lifestyles, poor nutrition leading to conditions such as obesity, diabetes, and pre-diabetes, among other environmental or genetic factors.[1,2] Currently, the treatment of neuropathic pain is carried out through tricyclic antidepressant medications, anticonvulsants, local anesthetic administration, topical agents, opioid analgesics, antiarrhythmics, and even neurosurgeries [3–5]. Despite the range of pharmacological approaches, patients' response to these treatments is still not satisfactory, in addition to having a variety of adverse side effects, such as increased risk of heart attack, subacute myopathies, hypergastrinemia, gastrointestinal tumors, among others[3].

Non-pharmacological treatments are increasingly gaining ground due to their beneficial results, improvement in quality of life, and lack of adverse side effects. Thus, understanding the pathophysiology of pain and its processes is of huge importance for understanding the mechanisms that trigger painful processes and therefore choosing the best treatment for the patient [3,5–8]. Given this information and based on the literature, we know that neuropathic pain can directly and indirectly affects others tissues, including the skeletal muscle, modifying its function and structure through loss of mass, function, and muscle atrophy as shown in experimental models. However, the contributions regarding nerve injury and muscle atrophy remain uncertain [3,9].

Due to this, the muscle adjacent to the nerve injury may be affected by impaired nerve communication, which can potentially compromise its neuromuscular junction if denervation occurs, leading to a breakdown in communication between the nerve and the muscle [9–12]. Due to this, we

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chose as the main focus of this study the skeletal striated muscle tissue, specifically the gastrocnemius muscle, one of the main muscles innervated by sciatic nerve.

It is already proven in some studies that neuropathic pain contributes to atrophy and decreased muscle activation, often due to lack of use of the injured limb, which generates an increase in protein degradation, a decrease in protein synthesis, or both[9,13–15]. The effect of non-pharmacological treatments, such as swimming and photobiomodulation, on complications induced by the neuropathic pain model as chosen in this work is still unknown.

Physical exercise recruits many muscles of the human body, consequently increasing muscle activity through increased protein synthesis due to high muscular demand [16,17]. Exercise treatment is applied with the aim of reducing neuropathic pain and reducing the effects caused by muscle atrophy due to nerve constriction has been demonstrated in studies and has been increasingly studied[18–20].

Considering a more effective and less aggressive model suitable for the type of injury of our animals, we chose Swimming as treatment for this study because water induces less impact on animals, therefore making the treatment less painful. We aim to show the morphometric effects in the gastrocnemius muscle by the tissue analysis (macroscopic and microscopic). Some studies have shown that swimming training for four weeks attenuated the progression of thermal hyperalgesia as well as allodynia in rats with neuropathic pain, demonstrating higher expression of Hsp72 and lower levels of TNF-α or IL-1β in the spinal cord compared to the injured group [18,20]. From these studies, we can see that swimming may be an effective treatment for neuropathic pain, as it not only reduces pain behavior but is also an economical and safe therapy that has been increasingly studied for understanding its mechanisms [2,9,18,20].

In order to complement the effects of exercise, we used Photobiomodulation Therapy (PBMT) in this study. PBMT has been increasingly studied and has been showing beneficial effects for both chronic and acute pain [21–23]. Systematic review by Ibarra et al. (2021)[24] showing that neurogenesis stimulated by PBMT leads to neuroprotection activation through cell oxygenation. Pigatto et al. (2020)[25] observed in the spinal nerve injury model that the application phototherapy was able to reduce mechanical hypersensitivity and cold allodynia [26].

Therapeutic alternatives for treating neuropathic pain are necessary, as this type of pain does not satisfactorily respond to conventional interventions such as surgical and medication-based approaches. We can suggest that the association of PBMT and SW may present potentiated effects. Both techniques have shown effectiveness in improving pain symptoms, but there are few studies detailing and exclusively studying the muscular system in these animals. Our working hypothesis is that animals with neuropathic pain treated with either therapy, or with both, may exhibit improvements in pain behaviors, muscle atrophy, contractile and motor function, and potentially benefit from enhanced factors involved in skeletal muscle maintenance and regeneration. Thus, our approach has the potential to significantly advance clinical treatments for pain resulting from conditions such as nerve compression and carpal tunnel syndrome, as well as other compressive syndromes, by utilizing non-invasive, non-pharmacological, and cost-effective techniques for patients.

2. Results

Our analysis of the behavioral test results categorizes them into three distinct groups: Exercise groups (SW), Photobiomodulation groups (PBMT), and Association groups (SW + PBMT) according to the colors below. These groups were utilized as points of comparison in all behavioral analyses and light microscopy morphometry. It's important to note that our analyses encompassed both legs, the Experimental (Right; Leg-ipsilateral), and contralateral (Left Leg). However, as the contralateral leg did not exhibit any statistically significant differences, it is not showed in this article. In all behavioral test analyses, the following subtitle structure was employed for a better understanding:

M0 - Baseline measurements (before any procedure)

MI – Measurement after swimming habituation

M1- Post-surgical measurements (15 days after CCI or Sham - surgery)

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M2, M3, M4 – After treatments measures (First week of treatment – 22 post CCI/Sham; second week of treatment – 29 post CCI/Sham; third week of treatment – 36 post CCI/Sham), respectively.

The colors below represent, in the graph, the period in which the animals underwent treatment.

- Swimming Habituation
- Swimming Training (SW)
- Photobiomodulation Treatment (PBMT)
- Association Treatment (SW + PBMT)

2.1. Mechanical Hyperalgesia

The results regarding the behavioral analysis of mechanical hyperalgesia utilized the Randall & Selitto tests (Figure 1, Panels A-C) and the Electronic Von Frey Test (Figure 1, Panels D-F). From these tests, we observed that after the injury (CCI), there was a decrease in nociceptive threshold starting from the 15th day post-surgery (M1) and persisted throughout all measurements (M2, M3, and M4) (Figure 1). After swimming treatment (CCI + SW), we observed an improvement in the nociceptive condition from the second week of treatment, which persisted until the last session (M3; $p < 0.0126$ and M4; $p < 0.0081$) compared to the untreated CCI group (Figure 1 A). On the other hand, no alteration was observed in the response to mechanical hyperalgesia in the group treated only with PBMT (Figure 1 B). However, when animals were treated with both techniques (CCI + SW + PBMT), the improvement in the nociceptive condition was observed from the first session (M2; $p < 0.0007$; M3; $p < 0.0001$; M4; $p < 0.0015$) compared to the untreated group (CCI) (Figure 1 C).

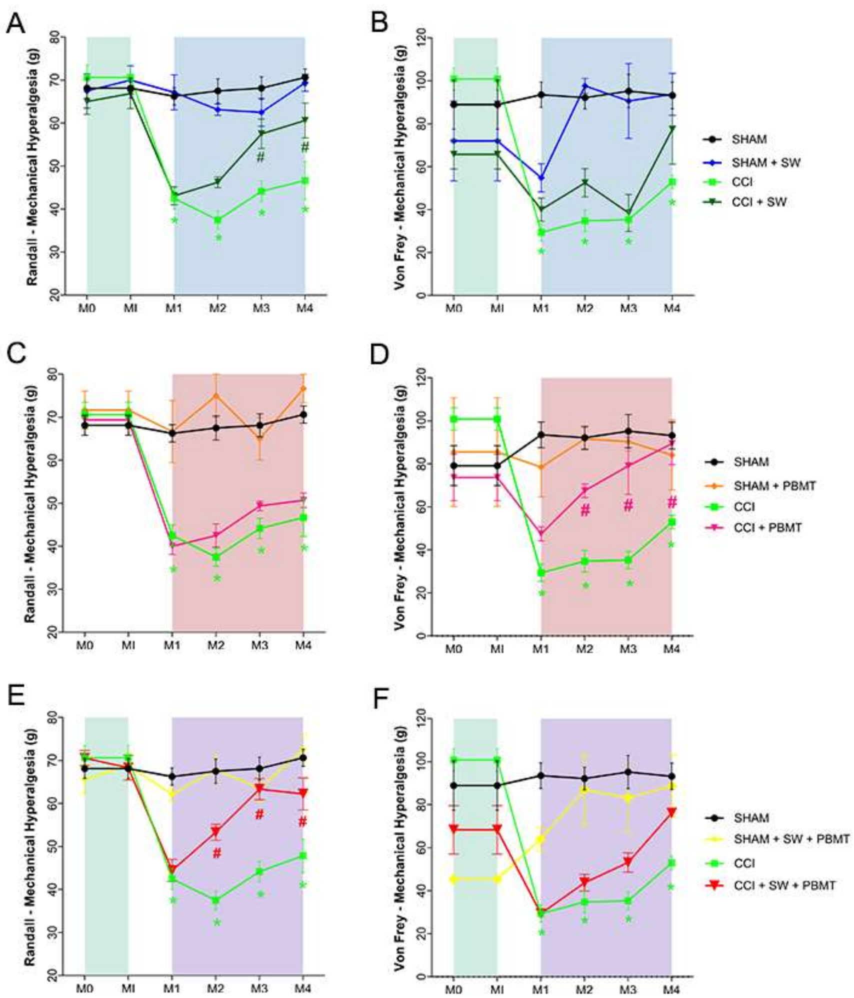

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Figure 1. Behavioral analysis of mechanical hyperalgesia using the Randall tests (panels A-C) and Von Frey tests (panels D-F). Both tests have their results expressed as force in grams (g). The force of the animals was defined before any procedure (initial measurement; M0), after the adaptation period (MI), 15 days after surgery (M1), and after treatments (M2 – first week – 22 days post-surgery), (M3 – second week – 29 days post-surgery), (M4 – fourth week – 36 days post-surgery). The results are presented as mean ± SEM. Two-way ANOVA followed by Tukey's post-test was performed. (*) symbols represent statistical difference between SHAM and CCI group; (#) symbol was used to show statistical difference between injured animals (CCI) and treated ones (CCI + SW; CCI + PBMT; CCI + SW + PBMT).

Regarding the Von Frey test, we observed a statistical difference only in the groups treated with photobiomodulation (CCI + PBMT) starting from the first week of treatment until the last (M2; p = 0.04; M3; p = 0.004; M4; p = 0.02) when compared to the CCI group (Figure 1E).

2.2. Thermal Sensibility

The results regarding the behavioral analysis of thermal hyperalgesia tests (Figure 2, Panels A-C) and cold allodynia (Figure 2, Panels D-F) demonstrated, after the injury (CCI), a decrease in nociceptive threshold starting 15th day after surgery (M1); this decrease in nociceptive threshold was maintained throughout all measurements (M2, M3, and M4) (Figure 2) when compared with control group [5]. Regarding the thermal hyperalgesia test (Hargreaves), it was possible to observe an improvement in the nociceptive condition in the group treated only with swimming (CCI + SW) compared to the untreated group (CCI) (Figure 2, Panel A). It is worth mentioning that the reversal of the nociceptive condition was only observed from the third week of treatment (M4; p < 0.0311). No statistical difference was observed between the CCI and CCI treated with PBMT groups (Panel B), or even when both treatments were used together (CCI + SW + PBMT - Panel C).

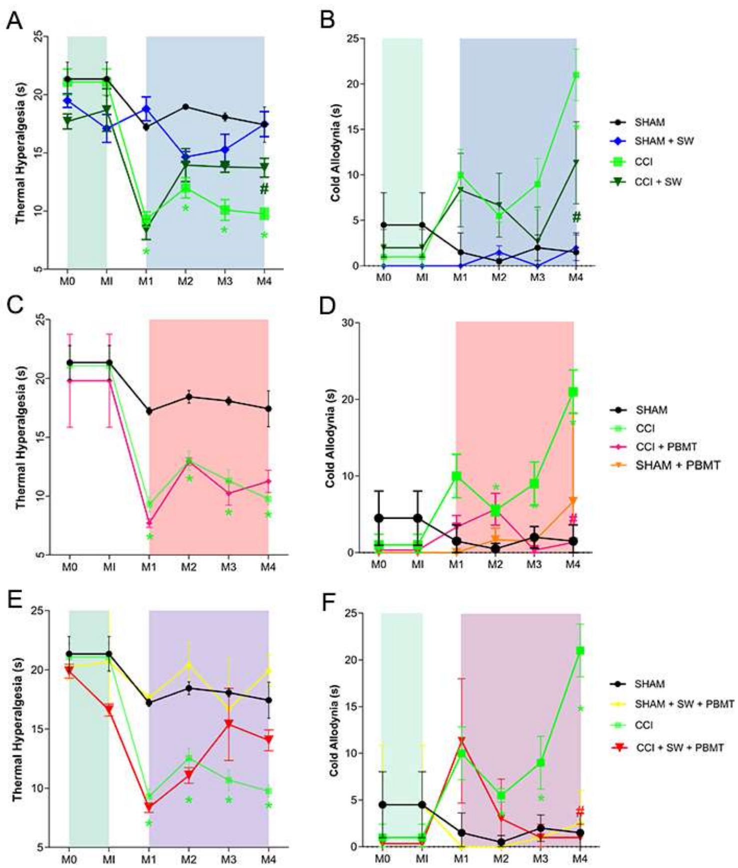

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Figure 2. : Behavioral analysis of thermal hyperalgesia (Panels A - C) and cold allodynia (Panels D - F). The results of both tests were expressed in seconds (s). Defined before any procedure (initial measurement; M0), after the adaptation period (MI), 15 days after surgery (M1), and after treatments (M2 – first week – 22 days post-surgery) and (M3 – second week – 29 days post-surgery) (M4 – fourth week 36 days post-surgery). The results are presented as mean ± SEM. Two-way ANOVA followed by Tukey's post-test was used. The symbols (*) represent statistical difference between SHAM and CCI group; The symbol (#) was used to show the statistical difference between injured animals (CCI) and treated ones (CCI + SW; CCI + PBMT; CCI + SW + PBMT).

Regarding the cold allodynia test, an improvement in pain condition was observed from the last week of exercise treatment (CCI + SW; Panel D) compared to untreated animals (CCI) (M4; $p < 0.0020$), we observed the same results when animals were treated with photobiomodulation (CCI+PBMT; Panel E (M4; $p < 0.0003$) and with the association of both therapies (CCI+SW+PBMT; Panel F (M4; $p < 0.0001$).

2.3. Incapacitancer Test (Static Weight Bearing)

The results regarding the analysis of the difference between the injured (ipsilateral) and non-injured (contralateral) limbs demonstrated that after the injury (CCI), there was a decrease in the difference between the limbs from the 15th day post-surgery (M1) (Figure 3). Regarding the groups treated with swimming, photobiomodulation and association (Panel A, B and C, respectively), a reduction in the difference between the limbs was observed in the second week of treatment (M3; $p < 0.0447$; Figure 3A; M3; $p < 0.0240$; Figure 3B; M3; $p < 0.0183$; Figure 3C) respectively (CCI + SW; CCI + PBMT; CCI + SW + PBMT).

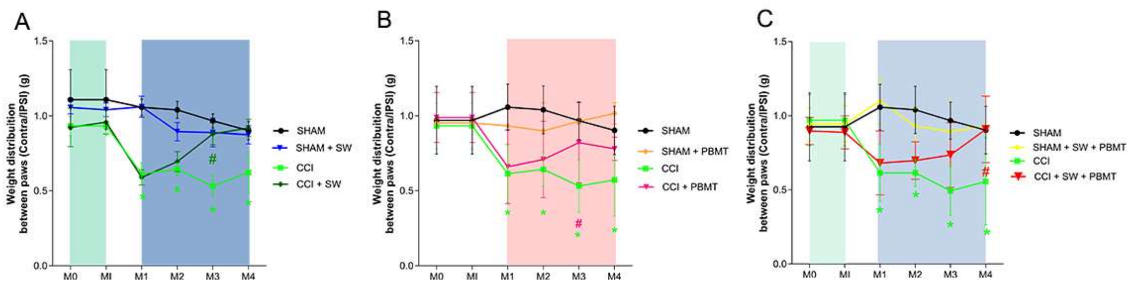

Figure 3. Behavioral analysis of animals paws incapacitance treated with swimming (Panel A), photobiomodulation (Panel B), and both techniques (Panel C). The results of the incapacitance tests were expressed as the difference between the ipsilateral limb divided by the contralateral limb. The baseline measurement of the animals was defined before any procedure (initial measurement; M0), after the adaptation period (MI), 15 days after surgery (M1), and after treatments (M2 – first week – 22 days post-surgery), (M3 – second week – 29 days post-surgery), (M4 – fourth week 36 days post-surgery). The results present the mean ± S.E.M. Two-way ANOVA followed by Tukey's post-test. The (*) symbol represents statistical difference between SHAM and the CCI group; The symbol (#) was used to show the statistical difference between injured animals (CCI) and treated groups respectively (CCI + SW; CCI + PBMT; CCI + SW + PBMT).

2.4. Hematoxylin and Eosin

After the evaluation of the behavioral tests and specific treatments, as previously mentioned, the animals were euthanized on the 37th day post-surgery, the ipsilateral gastrocnemius muscle was collected and kept frozen. The same sample was used for the Hematoxylin and Eosin and Picro Sirius Red colorings. It was observed that in the SHAM group, the nuclei were parallel to the muscle fibers, representing the standard morphology of the skeletal striated muscle tissue of the animals (Figure 4A). In CCI group, a massive accumulation of cellular nuclei and some fibers with centralized nuclei was observed in an attempt to recover these muscle fibers, in addition to the muscle fiber caliber (and

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the muscle itself) being smaller compared to the control group (Figure 4A). Regarding the injured animals, a greater accumulation of centralized nuclei was also observed due to the neuropathy induced by CCI. However, in the exercise-treated groups (CCI + SW), it is already possible to observe that the muscle cells are returning to their usual size, also showing signs of regeneration with circular aspects, and the nuclei in the region show dissipation compared to the CCI group. When the animals were treated with Photobiomodulation (CCI + PBMT) and the combination of both techniques (CCI + SW + PBMT), they presented a similar aspect to the control group, with the nuclei parallel to the muscle fibers, representing the standard morphology of the skeletal striated muscle tissue of the animals. However, the PBMT group presents a standardized aspect of circular fibers, still with some additional nuclei in the interstitial and where the cells are not well delimited as we can see in the control and combination groups (Figure 4A).

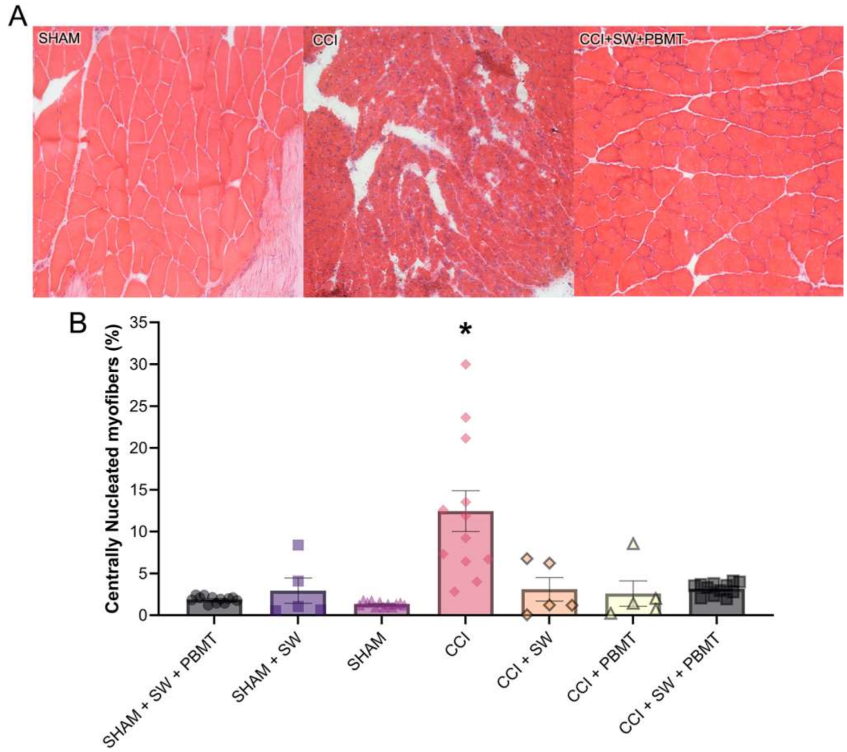

Figure 4. A) Cross-sections stained with Hematoxylin and Eosin technique. (Objective lens 10x); **B)** Bar graph representing the expression of inflammatory cells in the ipsilateral gastrocnemius muscle of animals treated with Swimming, with Photobiomodulation and with the association of the therapies. The results are presented as mean ± SEM. Two-way ANOVA followed by Tukey's post-test was conducted. The symbols (*) represent statistical difference between the CCI and other groups.

After taking pictures of our samples, our next step was to quantify the images. Our results demonstrate a 660% increase in inflammatory nuclei in the lesioned group (CCI) compared to the control (SHAM, considered 100%) (Figure 4B). After the different treatments, we observed a decrease in the percentage of these centralized nuclei compared to the CCI group. This decrease was 45% for the swimming group (CCI + SW), 67% for the Photobiomodulation group (CCI + PBMT), and 76% for the combination group (CCI + SW + PBMT) (Figure 4B).

2.4.1. Interstitial Collagen - Picro Sirius Red

Standard appearance of interstitial collagen between muscle fibers and the arrangement of these fibers and congruence were observed in control group. On the other hand, after injury (CCI), an abundant accumulation of collagen and fibrotic aspects with the discharacterization of muscle fibers and a decrease in their caliber were observed (Figure 5A). Treated groups showed a decrease in

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collagen expression and fibrotic aspects were observed, almost a complete reversal of the condition compared to the injured and control animals. It is also interesting to note that the treated groups (CCI + SW; CCI + PBMT; CCI + SW+ PBMT) showed a reduction in total collagen (%) and between muscle fibers compared to the SHAM and CCI groups (Figure 5A). The same was observed in the previous experiment. Our results demonstrate a 203% increase in collagen in the injured group (CCI) compared to the control (SHAM, considered 100%). After treatments, we observed a decrease in total collagen (Figure 5B). This decrease was 62% to the swimming treatment, 58% for the Photobiomodulation group (CCI + PBMT), and 66% for the association group (CCI + SW + PBMT) (Figure 5B).

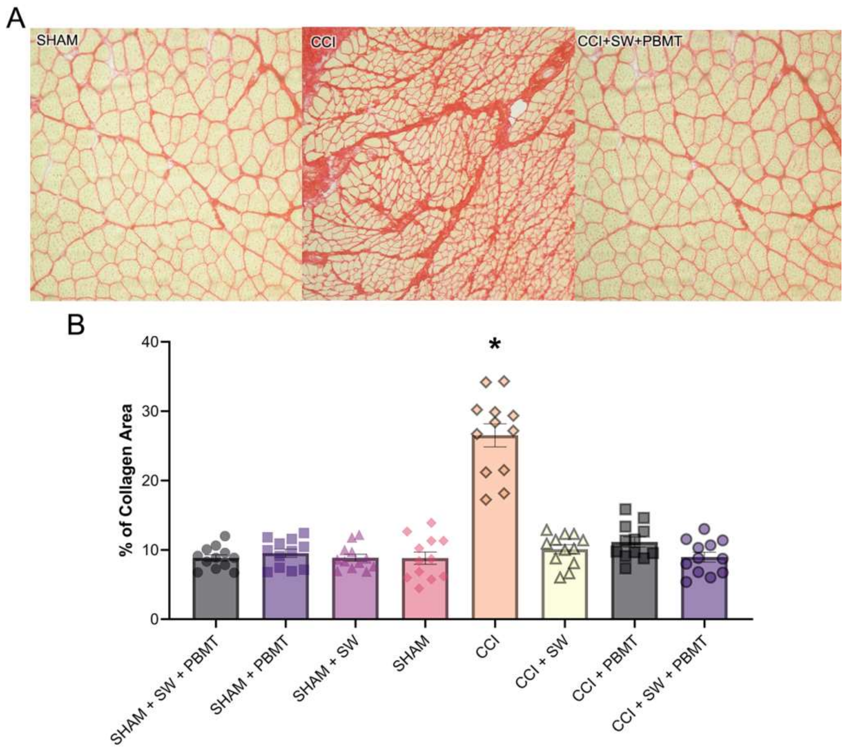

Figure 5. : A) Cross-sections stained with Picro Sirius Red technique. (Objective lens 10x); **B)** Bar graph representing the expression of collagen in the ipsilateral gastrocnemius muscle of animals treated with Swimming, with Photobiomodulation and with the association of the therapies. The results are presented as the mean ± SEM. Two-way ANOVA followed by Tukey's post-test was performed. The symbols (*) represent statistical difference between CCI and other groups.

3. Discussion

Neuropathic pain results from damage, disease, injury, or pressure affecting the peripheral nervous system (PNS), central nervous system (CNS), and somatosensory nerves. This chronic pain condition significantly impacts individual's quality of life and affects approximately 7-10% of the global population, with a higher prevalence in those over 50 years of age. Currently, neuropathic pain is challenging to treat and lacks a definitive cure. [2,3,27]

We observed that in the CCI model, literature had some works comparing the swimming training, as well as the PBMT as treatment, although we could not find any work that focus on the association between these two treatments. Our main aim of this study was to identify if the association between Swimming and PBMT had some beneficial on pain aspects as well as in the muscle.

Given the limitations of pharmacological treatments, which are often only partially effective and may lead to adverse side effects [7], non-pharmacological alternatives are crucial for improving patients' quality of life. Existing literature highlights the need for effective treatments that address both the pain and associated symptoms, such as muscle atrophy and disuse. In response, our research

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focuses on exploring two therapeutic approaches: Photobiomodulation and Swimming. We aim to assess the effects of these therapies both individually and in combination to provide a more comprehensive solution for managing neuropathic pain.

Initially, we evaluated each therapy individually and subsequently associated and compared each of them. Regarding the groups treated with swimming animals were submitted by 3 weeks of training based on the Fazard model [18], which on the first week of swimming, animals were submitted by times of 30, 45 and 60 minutes per day on the water. From the second to third week animals spend 60 minutes of swimming per day. Our results showed an improvement in pain response in the mechanical (Randall) and thermal (hot and cold) hyperalgesia assays. This improvement in nociceptive response was observed after the second week of treatment for the mechanical hyperalgesia test (Randall & Selitto)[28] and after the third week of treatment regarding the thermal hyperalgesia and cold allodynia.

Our results corroborate those obtained by Fazard et al. (2018) [18], as in their study, an improvement in the nociceptive profile regarding mechanical and thermal hyperalgesia was observed from the third week of swimming treatment in injured animals (CCI model). Furthermore, other studies also demonstrate the beneficial effect of swimming in reducing nociceptive response. For example, Kuphal et al. (2007) [19] observed that aquatic exercise reduced cold allodynia and thermal hyperalgesia.

Photobiomodulation has already shown positive results in various tissues and different conditions related to neuropathic pain [29–31]. Similarly, Swimming has demonstrated positive outcomes in literature concerning this type of pain[18,19,30]. However, little is known about the effect of these therapies on muscle tissue in the sciatic nerve constriction model. No studies were found where such therapies were associated and analyzed in this tissue. Therefore, our aim was to identify whether the combination of both therapies would have complementary effects on pain relief and a beneficial effect on muscle tissue.

When animals were subjected to Photobiomodulation therapy alone, no changes in sensitivity to mechanical stimulus were observed when using the Randall & Selitto test. However, we observed an increase in nociceptive threshold when using the Von Frey test. This improvement in pain response was observed from the first week of treatment. The same was observed when animals were exposed to thermal stimuli; we did not observe any statistical changes after the use of heat stimuli, but we observed a reversal of the pain profile when animals were exposed to cold stimuli. It's important to note that the Randall's test assesses withdrawal response when stimuli are applied to the anterior part of the hind paw, while the electronic von Frey test applies stimuli to the posterior part of the paw, also those test applies to different stimuli as showed by Deuis[32] which compares some different behavioral measurements in animals and supports our results being a little different in each test.

Previous studies conducted by our research group had already demonstrated the beneficial effect of PBMT in modulating nociceptive response using the same experimental model and in other models[21,33–35]. However, these studies observed a reversal of the nociceptive profile using three types of behavioral assays. These discrepant results may be due to the number of sessions applied per week. Additionally, our protocol presents a different total energy, varying the number of sessions and/or power density of the device, which may lead to different results within the same research group[29].

When we analyzed the animals subjected to the combination of both therapies, we observed improvement only when subjected to mechanical hyperalgesia and thermal stimulation. An interesting fact was that the improvement in pain profile after the combination was observed more recently, i.e., it started after the first week, demonstrating a beneficial effect when both therapies were combined. Regarding the evaluation of cold and thermal stimulation, an improvement comparable to that achieved with individual swimming treatment was observed.

These findings suggest that swimming therapy and photobiomodulation treatment may have complementary effects on pain relief, potentially offering benefits when used together. The differential effects observed between the two tests (Randall's and von Frey electronic) could indicate

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varying mechanisms of action or sensitivity to treatment modalities in different pain assessment contexts[32].

Regarding the evaluation of animal motor function, such as, the difference in weight deposited on the ipsi and contralateral hindpaws, performed in the incapacitance test, we observed an improvement from the second week of treatment in animals subjected to swimming or PBMT alone, and even during the association of both therapies. This result was somewhat surprising, as we anticipated a positive effect on nociceptive responses when both therapies were combined early, compared to individual treatments. However, our results are in line with those observed by Cobianchi et al. (2010) and Palandi (2020)[36,37], where they also observed an improvement in motor function after treadmill exercise.

There are no studies described in the literature with PBMT associated with Swimming in the model of neuropathic pain induced by sciatic nerve constriction, however, some results using other experimental models have shown beneficial effects in the combination of both therapies. For example, in the study by Beasi et al. (2021)[30], where the combination of these two therapies in the process of anterior tibial muscle regeneration after cryoinjury was shown to be more efficient than when both therapies are applied individually. Furthermore, the study by Malta et al. (2020)[38] suggested a beneficial effect of PBMT individually or in conjunction with creatine supplementation during a 12-week training program, resulting in significantly better muscle performance and lower levels of CK (a biochemical marker of muscle damage). Additionally, works by Farazi et al. (2022)[39] also demonstrated that the combination of PBMT and a recreational environment can be effective in reducing depression and anxiety-like behaviors in mice, possibly through modulation of corticosterone levels and the hippocampal BDNF/TrkB/CREB pathway. Dutra et al. (2022)[40] observed that, in humans, PBMT improves muscular endurance performance in single-joint exercises but is not effective for muscular strength performance.

When considered together, these data highlight the efficacy of both therapies not only in improving nociceptive behavior but also motor profiles, depending on the model and treatment employed. Additionally, it underscores the potential positive impact of these non-pharmacological therapies on patients' quality of life. [3,7,37]

The importance of histological assays, such as hematoxylin and eosin and picrosirius staining, is unquestionable in scientific and clinical research. These analyses provide a detailed view of the microscopic characteristics of tissues and organs, allowing the identification of morphological patterns, assessment of pathological changes, and understanding the effects of different treatments or experimental conditions. In the context of muscle regeneration, the application of these histological assays is crucial for examining tissue architecture and composition, as well as assessing the nature and extent of damage or repair. These techniques not only enrich scientific understanding but also play an essential role in the development of therapeutic strategies, offering significant insights for the optimization of medical interventions and regenerative therapies [41–43].

Regarding the stained with hematoxylin and eosin, a decrease in centrally located nuclei was observed in all groups treated individually or in combination compared to CCI animals. These showed massive accumulation of cellular nuclei and some fibers with centralized nuclei as an attempt to recover such muscle fibers. In the groups treated with exercise, it is possible to observe that muscle cells are returning to their usual size, presenting signs of regeneration with circular aspects. In animals treated with PBMT and the combination of both techniques, a similar aspect to the control group is presented, where the nuclei are situated parallel to the muscle fibers, being a standard aspect of the tissue morphology of skeletal striated muscle in animals.After image quantification, we observed an increased level of inflammatory nuclei and a decrease in inflammation by 45% in the Swimming, 67% in Photobiomodulation, and 76% for combination groups. Our results corroborate with several studies suggesting the beneficial effect of photobiomodulation [44] and swimming [18,37,45–47] in reducing inflammatory response and assisting in muscle regeneration.Regarding Picro Sirius Red staining, the standard aspect of interstitial collagen between muscle fibers and the arrangement of these fibers and congruence in the control groups was observed. When chronic neuropathic injury was performed, an abundant accumulation of interstitial collagen and fibrotic

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aspects with the discharacterization of muscle fibers were observed, now shown as muscle atrophy, characterized by a decrease in the caliber of muscle fibers. In groups treated with isolated or combined therapies, a decrease in the expression of total collagen in percentage, reduction of interstitial collagen between muscle fibers, and fibrotic aspects were observed. Our quantifications corroborate with our visual results, where an increase of 203% in total collagen was presented in injured group. After specific treatments, we could observe a decrease in this total collagen by 62% in swimming, 58% in photobiomodulation, and 66% for combination of both treatments. Similar results were also observed in the study by Shanti et al., 2013[48], that the combination of intramuscular collagen with an anti-inflammatory agent aided in reducing pain in a CCI model [48,49]. Thus, demonstrating that collagen, in addition to contributing to reducing the inflammatory response and muscle regeneration, also acts on improving the painful condition, even indirectly [43,49–52].

4. Materials and Methods

4.1. Animals

Male *Wistar* rats weighing 200–220 g (2 months old) were used in all experiments. Animals were housed in a temperature-controlled room under a 12:12 light-dark cycle with access to food and water *ad libitum.* Rats were allowed to habituate to the experimental room for 45 min per day during a week and before the behavioral experiments or treatments (photobiomodulation ,swimming and the association between them). All procedures were approved by the Institutional Animal Care Committee of the University of São Paulo (protocol number: 3110030221 – CEUA). All animals were monitored during the experiment and were divided into eight different groups with 8 animals per group. These groups were further categorized as: control [53], neuropathic pain (CCI), photobiomodulation treatment (SHAM + PBMT) or (CCI + PBMT), and swimming treatment (SHAM + SW) or (CCI + SW) either administered in isolation or in combination (SHAM + SW + PBMT) or (CCI + SW + PBMT).

4.2. Chronic Constriction of the Sciatic Nerve (CCI)

To mimic the neuropathic pain, it was used the technic of chronic constriction injury (CCI) which was performed on the sciatic nerve as previously described by Bennett and Xie (1988)[11]. Briefly, the rats were anesthetized using isoflurane (5% for induction and 1–2% for maintenance; Cristalia, MG, Brazil) to facilitate animal shaving and initiate the surgical procedure. The sciatic nerve was exposed at the mid-thigh level through dissection of the biceps femoris muscle. Approximately 7 mm proximal to the sciatic trifurcation, the nerve was cleared of adhering tissue, and four ligatures were created using sterile chromic gut sutures (cuticular 4-0 chromic gut, Ethicon, New Brunswick, NJ, USA), spaced 1 mm apart. In the Sham group, the sciatic nerves were also exposed and manipulated in an identical manner but were not ligated with gut sutures. Rats were carefully monitored during their recovery from anesthesia and were observed closely for the following 24 hours.[11,54]

4.3. Swimming Protocol

The swimming protocol was based on the method previously described by Fazard et al. (2018)[18]. The animals subjected to this protocol belonged to either the swimming treatment groups or the groups in which swimming was part of the treatment. We divided the protocol into two distinct phases: habituation to swimming and swimming training. In both phases, individual animals were placed in a container with a diameter of 50 cm and a depth of 65 cm, filled with warm water maintained at a temperature between 34 to 36°C. The duration of each phase of the protocol was gradually extended over the course of one week for habituation to swimming, followed by three weeks of swimming training (further details provided in the next section). It is noteworthy that the rats were continuously monitored throughout the experiment, and after each swimming session, the animals were gently dried with a towel.

4.3.1. Habituation to Swimming

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As previously described by Fazard [18] the habituation to swimming were performed by three days consisted by a 10 minutes sessions of exercise interspersed with a 5 minutes rest over a one week period (Figure 6).

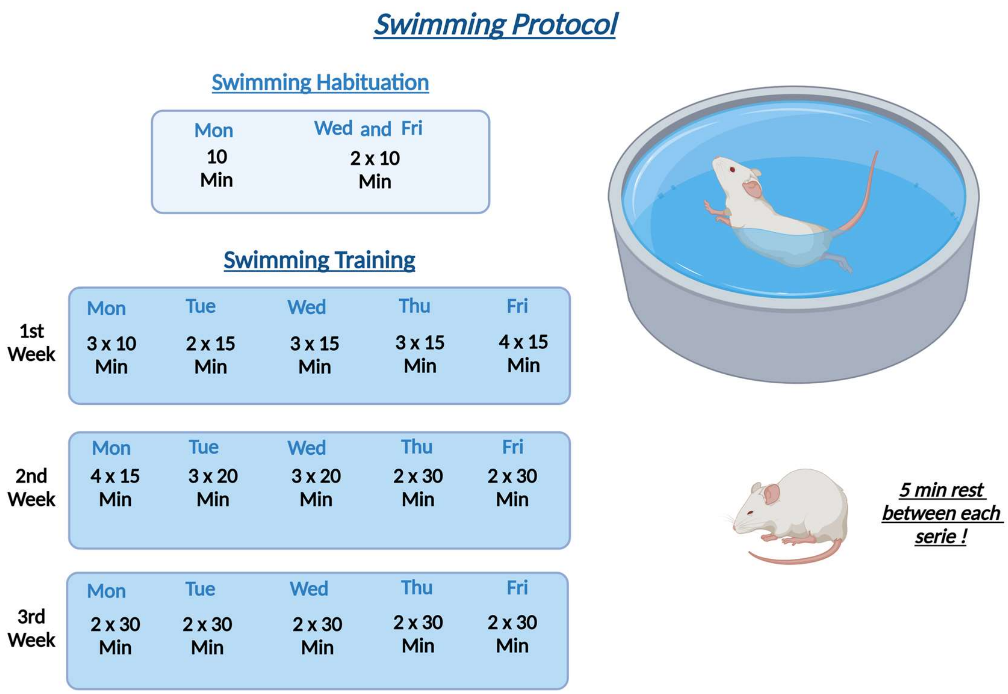

Figure 6. Swimming habituation protocol.

4.3.2. Swimming Training

The swimming training protocol were performed by a three-week training period gradually lengthened consisted by 5 times a week exercise (15 sessions) as shown in Figure 6 based on Fazard protocol .[18]

4.4. Photobiomodulation Treatment (PBMT)

Photobiomodulation protocol was previously described by Oliveira, ME (2020)[29], we irradiate the animals with a GaAs laser (Gallium Arsenide, laserpulse-laser, IBRAMED, Brazil) emitting a 904nm wavelength and 6J cm2 of energy density, the laser had a frequency of 9500Hz with a pulse time of 60ns in a 0,1cm2 spot area. The PBMT were performed three times per week, total of 3 weeks, 9 sessions. Each photobiomodulation session stimulated nine points that lasted 18s each as shown below in Figure 7.

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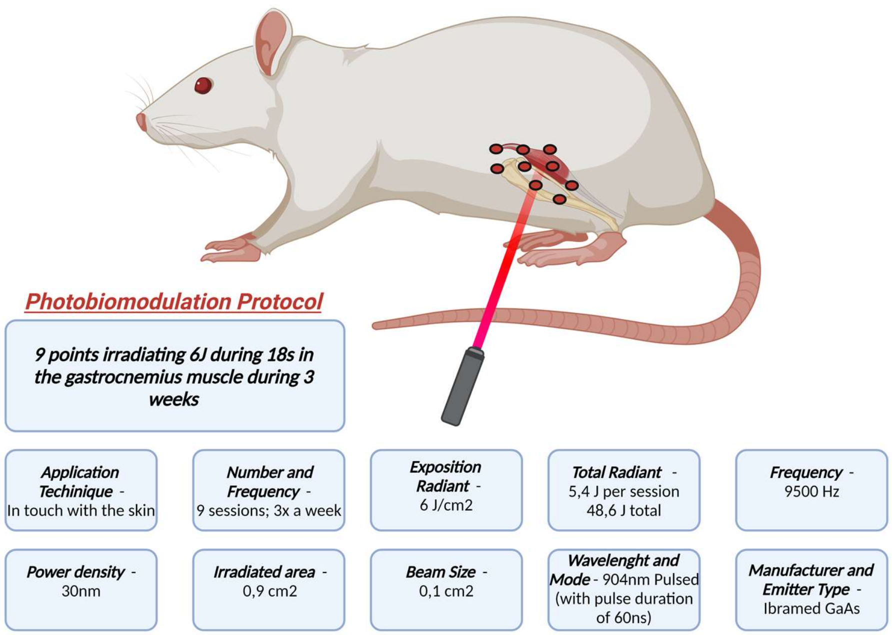

Figure 7. Photobiomodulation Specifications.

4.4. Experimental Design

The experimental design is illustrated in Figure 8. Initially, baseline behavioral responses to mechanical, cold, and heat stimuli were assessed. Subsequently, three sessions of swimming habituation were conducted over a one-week period. Following the habituation phase, either CCI (Chronic Constriction Injury) or Sham surgery was performed on the right sciatic nerve for the CCI groups, following the protocols described by Bennett (1988)[11] and Odem (2019)[55]. After surgery, a recovery period of fourteen days was allowed before post-surgery behavioral tests were conducted. The rats in the Swimming and Association groups underwent three weeks of swimming training, while the PBMT and Association groups received three weeks of PBMT, in both hindpaws. The corresponding SHAM groups also received the respective treatment during the three-week period as shown in Figure 8. All behavioral tests were repeated weekly over the three-week intervention period.

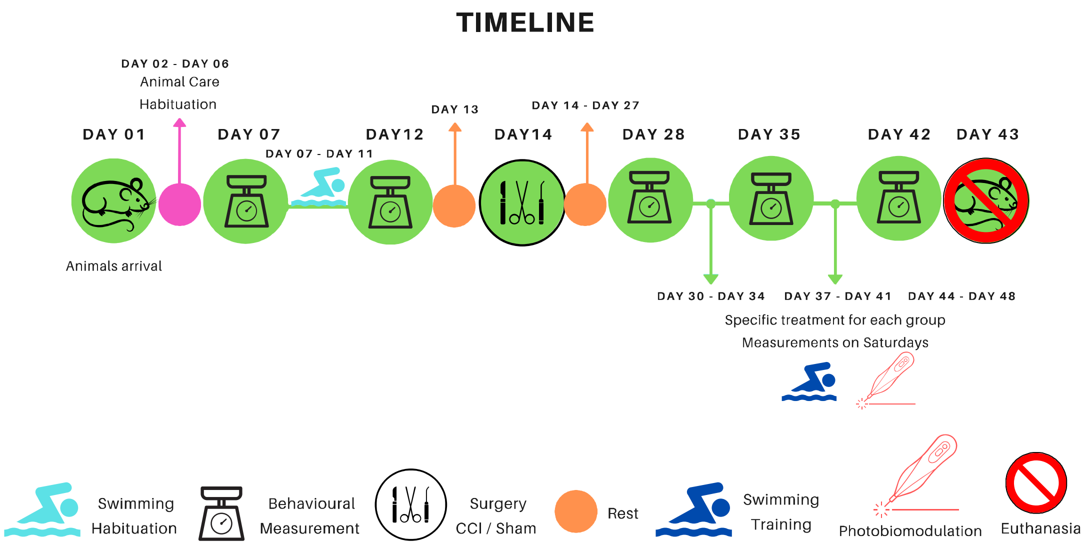

Figure 8. Timeline.

4.5. Behavioral Tests

4.5.1. Mechanical Hyperalgesia

For determinate mechanical hyperalgesia in our model, we used two different tests. The Randall-Selitto test was used to assessment of nociceptive thresholds by measuring the withdrawal threshold of the rat paw. It was previously described by randall and selitto (1957)[28]. This test involved application of an increasing mechanical force to the surface of the paw until withdrawal, vocalization or the limiar was achieved.

The Von Frey test was used to determinate mechanical hyperalgesia, rats were placed in acrylic containers with a wire mesh floor, they were allowed to acclimatize for 30 minutes before every session.[32] The von frey electronic has a single, unbending filament is applied perpendicularly to the surface of the paw of the animals, the force in grams is increased based upon the amount of pressure applied from 0.1 to 1000 g (Tena et al., 2012)[56] Guerrero et al. (2006) [57]by rotation of the handheld device until paw withdrawal occurs.

4.5.2. Cold Allodynia

The Cold Allodynia test was employed to assess cold sensitivity in rats. Each rat was placed individually in small acrylic containers with a grated floor, and a drop of acetone was applied to its hind paw. The nociceptive response, indicative of cold sensitivity, was measured by evaluating the reaction time. [22]

4.5.3. Thermal Hyperalgesia

The Hargreaves test was used to determinate thermal hyperalgesia. Rats were placed individually in small acrylic containers with a glass floor. An infrared heat source is focused on the plantar surface of the hind paw and the time, in seconds, taken to withdraw from the heat stimulus was recorded. [58]

4.5.4. Incapacitancer Test

The Incapacitancer test or the Static weight bearing test was used to determinate de weight difference between the control leg (left) and the experimental leg (right). The rat was placed in an inclined holder with the hind paws resting on two separate pressure sensors. Weight distribution between the hind paws was recorded. [32,59]

4.6. Light Microscopy Analysis

Following the assessment of behavioral tests and specific treatments, the animals were euthanized on the 35th day post-surgery and the ipsilateral gastrocnemius muscle was collected fresh, embedded, and frozen. Next, the material was sectioned and distributed on gelatin-coated slides for the Hematoxylin and Eosin technique. The cross-sections stained with HE were analyzed using an optical microscope for qualitative evaluation of tissue compromise and progression of cellular nuclei in the region for Picro Sirius Red as well as Hematoxylin and Eosin techniques. A light microscope (Axioskop 2®, Zeiss, Germany), was used to record the images (n = 12 per animal; 24-36 images per group) with 10× native microscope magnification. The images were analyzed by ImageJ® software (NIH, Bethesda, MD, USA). Transverse sections were stained and analyzed under an optical microscope for qualitative evaluation of the morphology of total collagen and interstitial collagen between the fibers, and for identification of regions with tissue fibrosis. To the picrosirius red the FD and lacunarity values were expressed as arbitrary units (AUs), and to quantify Hematoxylin-eosin we used as % of inflammatory cells that were analyzed by ImageJ®.

4.7. Statistical Analysis

Present data was analyzed by mean ± S.E.M. Statistical analysis was generated using the GraphPad Prism version 8 program (using GraphPad Software Inc., CA, USA). Statistical comparison between groups was performed using two-way analysis of variance (ANOVA) followed by Tukey's test. The significance index considered was $p<0.05$.

5. Conclusions

Our experimental model effectively induces neuropathy, and both individual and combined treatments improve pain symptoms in rats with peripheral neuropathy. All therapies enhance motor behavior in neuropathic animals, suggesting that swimming therapy and photobiomodulation may have complementary effects on pain relief. Differential results between the Randall's and von Frey electronic tests indicate varying mechanisms or sensitivities in pain assessment. Additionally, exercise helps maintain muscle mass, and all treatments impact muscle inflammation and collagen infiltration.

Overall, combining these therapies shows promise for improving the quality of life in patients with neuropathic pain. Being non-pharmacological, side-effect-free, cost-effective, and clinically applicable, even modest improvements from these therapies can significantly benefit patients, offering hope for better pain management and enhanced quality of life.

6. Patents

This section is not mandatory but may be added if there are patents resulting from the work reported in this manuscript.

Author Contributions: Conceptualization, M.C.; behavior experiments, LC and LCRB; surgical procedures LC; data analysis, MC, LC, LCRB and APC; data curation, MC, LC and APC.; writing—original draft preparation, MC, LC and APC ; supervision, MC; project administration MC.; funding acquisition MC. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines Ethics Committee on the Use of Experimental Animals at Institute of Biomedical Science (CEUA protocol number 3110030221, approval date 05/13/2021).

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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