Lasers Med Sci
DOI 10.1007/s10103-011-0923-0

ORIGINAL ARTICLE

Effects of 940 nm light-emitting diode (led) on sciatic nerve regeneration in rats

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Received: 20 September 2010 / Accepted: 6 April 2011

Abstract The objective of the present study was to evaluate the effect of 940 nm wavelength light emitting diode (LED) phototherapy on nerve regeneration in rats. Forty male Wistar rats weighing approximately 300 g each were divided into four groups: control (C); control submitted to LED phototherapy (CLed); Sciatic Nerve Lesion without LED phototherapy (L); Sciatic Nerve Lesion with LED phototherapy (LLed). The lesion was caused by crushing the right sciatic nerve. A dose of 4 J/cm^2 was used for ten consecutive days beginning on the first postoperative day. Groups C and L were submitted to the same procedure as the LLed group, but the equipment was turned off. The LED phototherapy with 940 nm wavelength reduced the areas of edema, the number of mononuclear cells present in the inflammatory infiltration, and increased functional recovery scores at 7, 14 and 21 days. The results suggest that the use of phototherapy at 940 nm after nerve damage improves morphofunctional recovery and nerve regeneration.

Keywords LED · Phototherapy · Nerve regeneration

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Introduction

Peripheral nerve damage is a common type of clinical disorder that can result in long-term functional deficits [1]. Peripheral nerves are highly vulnerable to pressure and the extent of the damage depends on the nerve involved, the magnitude and type of pressure and the length of time the nerve was compressed. One of the causes of nerve compression is strangulation, and several animal models have been used as valid methods for studying this condition [2, 3]. Such damage can be caused experimentally in several ways, including crushing (compression), transection, stretching and freezing. Several studies have proposed the use of crush injury models to evaluate both Wallerian degeneration and functional recovery [4–11]. These models have been useful for evaluating the effects of therapeutic methods on the morphofunctional recovery process of the peripheral nervous system.

The degeneration of nerve fibers is accompanied by an infiltration of mononuclear cells, including macrophages and lymphocytes. Resident macrophages, or those recruited by the local inflammatory process, phagocytize the cellular debris of axons and myelin sheaths and concomitantly produce growth factors that induce the proliferation of Schwann cells and fibroblasts [12, 13]. Studies indicate that the presence of lymphocytes and macrophages in the inflammatory infiltrate is crucial for the regeneration of injured nerve tissue [12].

During the process of nerve degeneration and regeneration, T helper cells that produce Th1, Th2 and Th17 cytokines migrate to the lesioned area [14]. Although T helper cells are essential for nerve regeneration, the persistent presence of inflammatory infiltrate can compromise the morphofunctional recovery of nerve tissue, causing loss of function, painful sensitivity and neuroma

Published online: 06 May 2011

formation [15]. The presence of Th1 cytokines, such as Interleukin-1 (IL-1), interferon gamma (INFγ) and tumor necrosis factor alpha (TNFα), and Th2 cytokines (IL-10) during the Wallerian degeneration process in the sciatic nerve of rats was observed to be associated with a macrophage infiltration up to five weeks [14]. The expression of these cytokines, particularly TNFα, promotes chronic macrophage recruitment, oxidative stress injury, fibrosis and neuroma formation [14, 15].

Among the various methods proposed for improving nerve regeneration, phototherapy has received increasing attention over the two past decades, and has demonstrated satisfactory results in animal models treated with low-level laser (Light Amplification by Stimulated Emission of Radiation) therapy [16, 17]. The use of lasers in the treatment of peripheral nervous system injuries has shown anti-inflammatory, anti-algic, neurostimulating and neuroinductive effects in both animal models and humans studies [17–20]. New radiation sources such as Light Emitting Diodes (LED) have recently attracted attention for their potential to expand the applicability of phototherapy [16, 19, 21–25].

Light sources from LED or laser diodes can be employed to produce radiation energy. The laser, however, has a sophisticated structure that includes a resonant optical cavity. The effectiveness and applicability of LED arrays for injuries have been partially studied in vitro [21] and in vivo [22] and suggest that LED radiation has many biological effects similar to those of lasers. Nevertheless, the efficacy of LED phototherapy on functional nerve performance and morphological recovery has yet to be proven conclusively. Although the efficiency of lasers (630 to 780 nm) for accelerating nerve regeneration has been demonstrated in animal models [17–20, 23–28], the effects of LED phototherapy are still underexplored.

The objective of this study, therefore, was to evaluate the effect 940 nm LED phototherapy on morphological and functional recovery and in the recruitment of mononuclear cells in a traumatic lesion of the sciatic nerve.

Materials and methods

All procedures described herein are conformed to the Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) [resolution 592 26/06/1992 of the Federal Counsel of Veterinary Medicine and law no.9605 (regulated by Decree nº 3179, 21/12/1999)], and were approved by the Committee for Ethics in Animal Experimentation of the Universidade Estadual de Londrina, (protocol CEEA 5/2010 37359). Forty male *Wistar* rats, approximately 300 g, were supplied by the Animal Resources Center of the Universidade Estadual de Londrina. The animals were kept in collective cages (six per cage) under controlled temperature (~22°C) with a 12 h light-dark cycle, and were fed normocaloric rodent chow (Nuvilab CR1, Nuvital Nutrientes S/A, Colombo - Brasil) *ad libitum* until the day they were sacrificed (21st post-operative day). The experiment was carried out at the Histology Laboratory of the Universidade Estadual de Londrina. The animals were divided into four groups (n=10):

* Control (C): animals not submitted to LED phototherapy and without sciatic nerve lesion.
* Control LED phototherapy (CLed): animals submitted to LED phototherapy and without sciatic nerve lesion.
* Sciatic Nerve Lesion without LED phototherapy (L): animals submitted to sciatic nerve lesion without phototherapy treatment.
* Sciatic Nerve Lesion with LED phototherapy (LLed): animals submitted to sciatic nerve lesion and submitted to LED phototherapy.

Initially, the animals were trained for ten consecutive days on a walking track constructed according to parameters described by De Medinaceli et al. [6]. This track was used to facilitate sampling of the hind footprints, which was necessary to calculate the Sciatic Functional Index (SFI). The footprints were obtained in the pre-operative period, and after seven, 14 and 21 post-operative days. The footprints were collected using stamp ink. The ink-soaked paws left footprints on strips of paper cut to the same dimensions as the track (43×8.5 cm), three footprints per paw on average. The formula proposed by Bain et al. (1989) was used to calculate SFI. We measured the distances between the second and fourth distal phalanges, between the first and fifth distal phalanges and between the proximal edge of the foot and the third distal phalanx. Negative scores represent the percentage of functional loss. The functional recovery was analyzed by comparing scores of non-lesioned and lesioned animals, with or without LED phototherapy.

For the experimental lesion of the right sciatic nerve, the animals were anesthetized with an aqueous solution of xylazine hydrochloride (0.02 g/kg, Virbaxyl® 2%, Virbac do Brasil, São Paulo, Brasil) associated with ketamine hydrochloride (1 g/kg, Francotar ® 10%, Virbac do Brasil, São Paulo, Brasil). After verifying the state of consciousness, each animal was positioned in the ventral decubitus position and the right hind paw was submitted to trichotomy followed by local asepsis with iodated alcohol. An incision was then made in the skin from above and medial to the greater trochanter of the femur until close to the popliteal fossa. This was followed by a layer-by-layer dissection until the sciatic nerve was exposed. The nerve was compressed at a proximal segment 5 mm from its bifurcation for 30 seconds using hemostatic forceps with

20 g constant force. The incision was then sutured using 2–0 mononylon thread. After suturing, local asepsis was carried out, and 0.2 ml of metamizole sodium (50 mg/kg) and ceftriaxone sodium (1 mg/kg) were administered intraperitoneally.

The LED irradiation had a 940 nm wavelength, a 45 nm bandwidth, an optical power output of 9.5 mW, and a beam area of 1 cm^2. The distance between the radiation source and the skin was one centimeter. The radiation source was attached to a support, kept perpendicular to the skin surface, and a single point in the middle of the surgical incision was irradiated (transcutaneous method). The treatment was applied immediately after the sciatic lesion until the tenth post-lesion day. The irradiation was applied for seven minutes, delivering an energy intensity of 4 J/cm^2 and a power density of 9.5 mW/cm^2 to 1 cm^2 of area. The light source employed in this study included a single commercial LED and was specially developed for the experiment by the Optical and Optoelectronic Laboratory of the Department of Physics of the Universidade Estadual de Londrina. The optical output power of the light source was verified prior to experimental procedures using a broadband power and energy meter (Standard Photodiode Sensor PD 300; Ophir Optronics, Jerusalem, Israel). The C and L groups were submitted to the same procedure, but the equipment was turned off.

Functional recovery was evaluated according to the Sciatic Functional Index (SFI) after seven, 14 and 21 postoperative days, as described by DeMedinacelli et al. [6] using the formula modified by Bain et al.[8] After the course of procedures had concluded (21 days), the animals were euthanized by inhalation of a lethal dose of diethyl ether. The sciatic nerve and the adjacent muscle were then removed and immediately fixed in aqueous Bouin's fixative solution. Twenty-four hours later, the sections were placed in 70% alcohol and submitted to routine histological procedures.

Histological sections (7 μm) were stained with hematoxylin-eosin, and were analyzed using an optical microscope. Ten images of each animal were captured using a Moticam imaging device (Motic group, Xiamen, China) and analyzed with Motic Image Plus 2.0 software (Motic, Xiamen, China). The images were sampled from areas near the crush lesion segment in fields equivalent to 100 μm^2, separated by intervals of 100 μm from the distal segment of the nerve. Areas of edema were analyzed and expressed as the relative area of degraded myelin sheaths and myelin debris. Inflammatory infiltrate was composed mainly of mononuclear cells and recorded in each image.

Bartlett's test was used to evaluate the normality of distribution for areas of edema, mononuclear cells counts, and SFI scores. The data were expressed as mean and standard deviation (parametric data) or median and quartiles (non-parametric data). The differences in SFI scores between LLed and L animals at 7, 14 and 21 days were analyzed using the Mann-Whitney U test. Differences in SFI scores on different days were analyzed using the Friedman test. The difference between the edema level and the number of mononuclear cells was evaluated using the ANOVA with *post-hoc* Tukey's test. Differences were considered significant when $P<0.05$.

Results

The functional recovery process (SFI score) was accelerated in the LLed group compared to untreated animals (L) at day 7 (−44.6±15.3 X −108.9±23.0; $P<0.05$), 14 (−31.2±12.5 X −92.5±27.5; $P<0.05$) and 21 (−12.0±5.1 X −59.2±26.8; $P<0.05$). LLed animals presented a significant decrease in SFI scores compared to pre-operative evaluation (−11.3± 7.4) only at day 7 ($P<0.05$). L animals presented decreased SFI scores after 7 ($P<0.005$) and 14 days ($P<0.01$) compared to pre-operative values (−4.3±7.7). After 21 days, SCF scores were not significantly different from pre-operative values in both groups (Fig. 1).

The microscopic evaluation showed no morphological differences between C and CLed groups. More organized myelin sheaths and reduced areas of myelin debris were observed in LLed group compared to non-treated injured

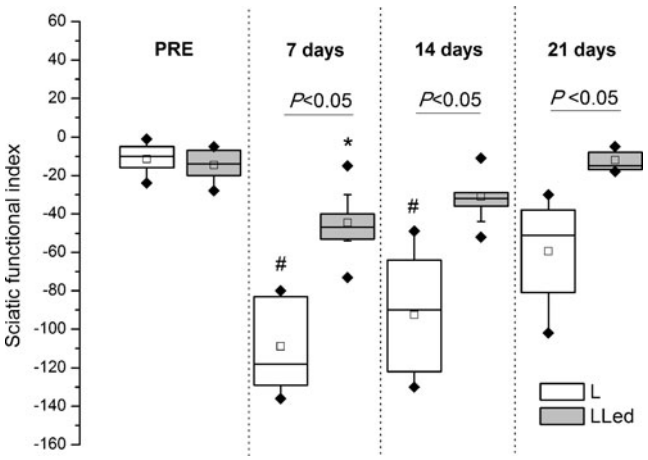

Fig. 1 Functional index of the sciatic nerve (SFI) of Wistar rats submitted to sciatic nerve crush lesion without treatment (L, white columns) and submitted to LED phototherapy at 940 nm for 10 days (LLed, grey columns). LLed animals presented better functional scores after 7, 14, and 21 days of treatment than the L group ($P<0.05$, Mann-Whitney U test). LLed group presented decreased functional scores in relation to pre-operative period (PRE) at 7 days for (* $P<0.05$, Friedman test and *post hoc* Dunn test). L group presented decreased functional scores at 7 and 14 days (# $P<0.05$, Friedman test and *post hoc* Dunn test). The values are expressed in quartiles (Box), median (horizontal bar) and mean (□). The vertical bars represent the values raging from 1 to 99%, and (♦) represents extreme values

animals (L) (Fig. 2). Morphometric analyses demonstrated few areas of degraded myelin in C (0.008±0.01%) and CLed (0.008±0.01%) groups. Sciatic lesioning induced the degeneration of myelin sheaths and increased areas of edema (48.4±8.04%, $P<0.05$) in comparison to C group. However, the areas of edema and degeneration in the LLed group (34.7±5.8%) were smaller than those of the L group ($P<0.05$) (Fig. 3).

Few mononuclear cells were observed in C (0.7±0.8 cells/5×10^4 μm^2) and CLed (0.4±0.6 cells/5×10^4 μm^2) animals. More mononuclear cells were observed in injured animals (69.1±8.4 cells/5×10^4 μm^2;$P<0.05$) than controls. The LLed group presented significantly fewer infiltrated mononuclear cells (48.8±10.0 cells/5×10^4 μm^2; $P<0.05$) in Wallerian degeneration areas than the L group (Fig. 4).

Discussion

Functional recovery of the sciatic nerve was expected on the 21st post-lesion day [1, 6–8, 38], and this occurred in non-treated (L group) animals. However, the application of 940 nm LED phototherapy for ten consecutive days reduced this time by 7 days, leading to a recovery of motor function on the 14th day, which suggests that the treatment may have accelerated the neuroregenerative process. The histological assessment corroborated the functional recovery findings. LLed animals presented better organized myelin sheets with fewer areas of myelin debris and less mononuclear cell infiltration than non-treated animals, which suggests that LED phototherapy controlled the local inflammatory process and improved tissue regeneration.

The efficacy of laser phototherapy on nerve regeneration has already been demonstrated [39–41], and we had similar findings when using LED irradiation in the present study. Several events can contribute to the acceleration of morphofunctional regeneration of the photo-irradiated nerve, such as an improvement in energy metabolism, accelerated impulse conduction, improved cytoplasmatic calcium turnover, accelerated wound repair, increased scar tissue resistance and pro-inflammatory actions [19–41]. The disadvantages on employment of laser irradiation are the heat generated by the laser beam that can damage biological tissues, and the concentrated light can accidentally injure the eyes [41]. The main differences between LED and laser technology are that an LED light source emits noncoherent, rather than coherent radiation, without generating significant amounts of heat. A coherent beam does not seem to be

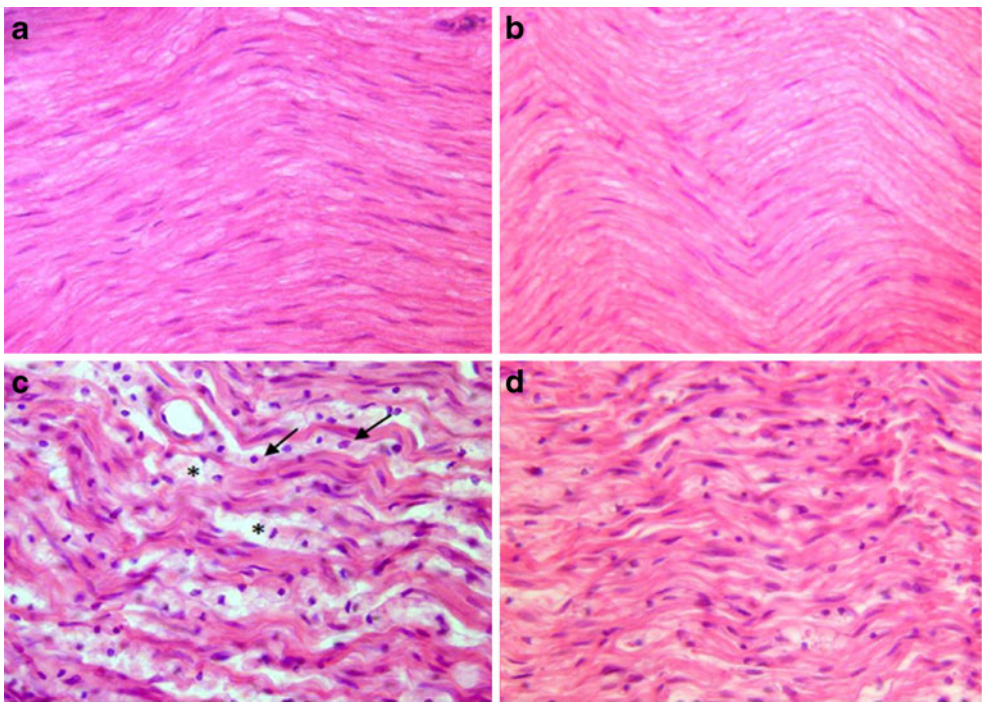

Fig. 2 Sciatic nerve of Wistar rats. **a**) Control group animals (C) were not submitted to sciatic lesion and did not receive LED phototherapy. **b**) Unlesioned control group that received LED phototherapy (CLed). The application of LED phototherapy at 940 nm for 10 days did not provoke morphological alterations in unlesioned sciatic nerves **c**) Sciatic nerve crush legion group (L) without treatment. After 21 days, large areas of edema and myelin degeneration were observed (*). An increased number of mononuclear cells (arrows) was still observed in the myelin sheaths debris. **d**) Sciatic nerve crush lesion group submitted to LED phototherapy at 940 nm (LLed) for 10 days. After 21 days, a significant reduction in area of edema and number of mononuclear cells was observed in the regenerating nerve. 400X magnification, HE stain

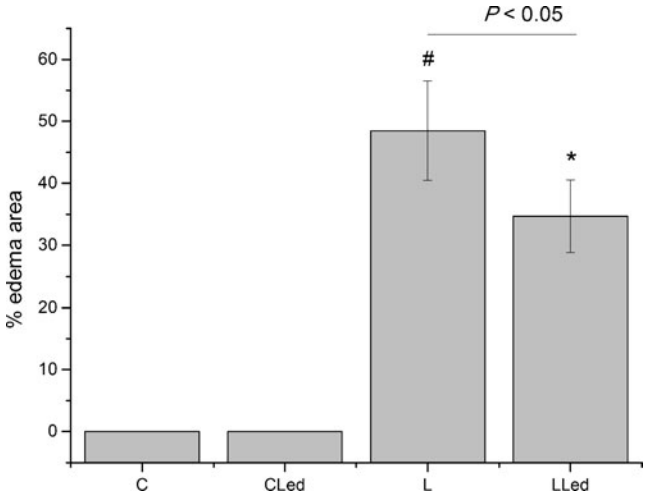

Fig. 3 Percentage of areas of edema and nerve degeneration in the sciatic nerve of Wistar rats. (C) Control animals. (CLed) Non-injured animals submitted to LED phototherapy. (L) Animals that underwent crush lesion without treatment. (LLed) Animals submitted to crush lesion and LED phototherapy. A significant reduction in areas of edema was observed after 21 days in the animals that received LED phototherapy ($P<0.05$, ANOVA and *post hoc* Tukey test). # $P<0.05$, in relation to C group. * $P<0.05$, in relation to CLed group

essential for modulating biological phenomena influenced by phototherapy, since luminous radiation loses its coherence upon contact with living tissue [41, 42].

According to DALL AGNOL et al. (2009), an LED source can reach the same level of biomodulatory effects on living tissue as a laser source [24], which suggests that LED phototherapy is a viable and safe alternative of treatment. Other advantages of LED arrays are that they can be built with more than one light source emitting different wavelengths simultaneously and can cover more extensive areas [40].

The effects of low-level laser irradiation on nerve regeneration are well known. Irradiation ranging from 632 to 901 nm with low-level laser light modulated, dose-dependently, the proliferation of Schwann cells in in vitro rats, enhanced nervous cell differentiation, increased axonal growth and myelination and improved functional and morphological recovery in experimental sciatic nervous lesions [17, 19, 26–28, 42–44]. However, the application of similar wavelengths using a LED source has not yet been investigated.

Studies in animal models have demonstrated that the efficiency of LED phototherapy in controlling the inflammatory process is similar to that of laser irradiation in that it, too, reduces edema, the migration of inflammatory cells, and the production of inflammatory cytokines, as well as accelerates the regeneration of connective tissues [16, 24, 29, 30]. Moreover, in vivo and in vitro experiments have demonstrated that 780 nm radiation emitted by LED arrays presents immunomodulatory effects by increasing the circulation of T-helper cells that produce anti-inflammatory cytokines [31]. The radiation of lymphocytes with 901 nm wavelength LED was proven efficient for increasing the availability of intracellular ATP, which demonstrates that this wavelength has a stimulating effect on lymphocytes [32]. The same wavelength applied with a laser diode can inhibit the in vivo migration of white blood cells during the first stages of the inflammatory process [33], the production of reactive oxygen species [34, 35], and stimulate tissue regeneration [36]. Considering that the initial inflammatory process and the infiltration of macrophages and lymphocytes are crucial steps in the process of nerve regeneration, the anti-inflammatory and immunomodulatory effect of LED phototherapy applied immediately after injury can alter and possibly contribute to the nerve regeneration process.

Studies in animal models have demonstrated that the inflammatory process is immediately initiated in damaged nerve and key inflammatory molecules were locally produced during the first week [24, 37]. Applying LED phototherapy immediately after nerve injury and during the early establishment of inflammatory response may alter macrophage activation and the nerve regeneration process. In the present study, LED phototherapy was applied for ten consecutive days after a nerve lesion in order to achieve its effects on initial inflammatory and immune response to nerve lesion and on initial steps of nerve regeneration (Schwann cell proliferation and axonal growth).

Photobiomodulation also presents anti-inflammatory effects, and can act on the recruitment and activation of inflammatory cells. Activated macrophages produce fewer

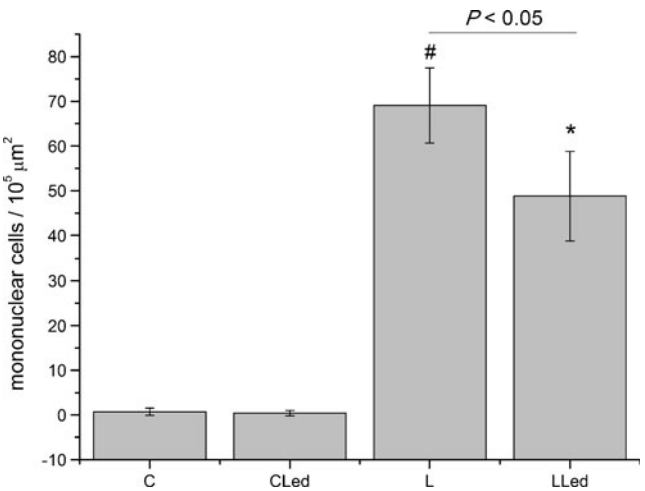

Fig. 4 Infiltration of mononuclear cells in the sciatic nerve of Wistar rats. (C) Control animals. (CLed) Non-injured animals submitted to LED phototherapy. (L) Animals that underwent crush lesion without treatment. (LLed) Animals submitted to crush lesion and LED phototherapy. The mean number of cells counted in images captured at 1000X (approximately 5×10^4 µm^2) after 21 days was significantly lower in animals that received LED phototherapy ($P<0.05$, ANOVA and *post hoc* Dunn test). # $P<0.05$, in relation to C group. * $P<0.05$, in relation to CLed group

pro-inflammatory cytokines (TNFα, IL-1, IL-6, monocyte chemoattractant protein I) when treated with 780 nm wavelength [45, 46]. In animal models, the effect of 635 nm phototherapy indicated that treatment could modulate the production of inflammatory cytokines and the activation of lymphocytes in a dose- and time-dependent form [47]. The treatment of spinal crush lesions in mice with 810 nm wavelengths accelerated nerve regeneration and reduced the production of inflammatory cytokines in the injured nerve tissue [48]. Recently, XAVIER et al. (2010) demonstrated that LED phototherapy at 880 nm wavelength can inhibit the migration of inflammatory cells and the production of cytokines and inflammatory mediators in an experimental model of tendinitis in rats [29]. Taken together these results indicate that the use of wavelengths above 630 nm can promote anti-inflammatory and immunomodulatory effects that, potentially, could contribute to the nerve tissue repair process.

The results of present study demonstrated a significant improvement in the functional recovery score after seven days, suggesting that LED phototherapy at a wavelength of 940 nm accelerates the nerve regeneration process. The reduction in both number of inflammatory cells and areas of edema suggests that 940 nm phototherapy presents an anti-inflammatory effect that may improve nerve regeneration.

Conclusion

The results indicate that the immediate application of LED phototherapy at a wavelength of 940 nm after nerve damage improves the regeneration process. The morphological analysis of the nerve indicated that phototherapy can reduce the migration of mononuclear cells to damaged tissue, which reduces areas of edema and fiber degeneration. A 940 nm LED phototherapy source seems to favor the early functional recovery of the injured sciatic nerve.

Acknowledgments The authors would like to thank Total Vet – Londrina and Virbac do Brasil, for the donation of the reagents used in the study.

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