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Low-Level Laser Therapy and Spinal Cord Injury: Effects of 3 Different Fluences in the Intermediate Period of Repair in an Experimental Model in Rats

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HIGHLIGHTS

- LLLT produced an improved functional recovery.
- LLT promote a decreased CD68 immunoexpression after irradiation with the 3 fluences.
- LLLT at higher fluences modulated the inflammatory process after spinal cord injury.

Abstract: The aim of this study was to evaluate the effectiveness of 3 different fluences of low-level laser therapy (LLLT) in intermediate period of spinal cord repair using an experimental model in rats. Thirty two rats were randomly divided into four experimental groups: Control Group (CG); Laser treated group 500 J/cm² (L-500); Laser treated group 750 J/cm² (L-750) and Laser treated group 1000 J/cm² (L-1000). Spinal cord injury (SCI) was performed by an impactor equipment (between 9th and 10th thoracic vertebrae), with a pressure of 150 kdyn. Afterwards, the injured region was irradiated daily for 14 consecutive sessions, using an 808 nm laser, at the respective fluence of each experimental groups. Locomotor function and tactile sensitivity were performed on days 1 and 15 post-surgery. Animals were euthanized 15 days post-surgery and samples were retrieved for histological and immunohistochemistry analysis. Functional behavior and tactile sensitivity were improved after laser irradiation. Moreover, higher fluencies of LLLT reduced the volume of injury. Additionally, LLLT produced a decreased CD-68 expression. These results demonstrated that, for an intermediate period of SCI repair, LLLT at higher fluences, was effective in promoting functional recovery and modulating the inflammatory process in the spinal cord of rats after traumatic SCI.

Keywords: low-level laser therapy; spinal cord injury; neuronal plasticity.

INTRODUCTION

Spinal cord injury (SCI) is an interruption of the neural signal conduction along the axonal tracts due to traumas or diseases [1]. It is estimated that the number of people in the United States who currently live with SCI is around 253,000 with 11,000 new cases occurring each year [2-3]. SCI compromises motor, sensory, autonomic and reflex functions, leaving the patients with temporary or permanent residual disabilities, such as paralysis, chronic pain, urinary and respiratory problems and neurologic decline, leading to a significant decrease in quality of life [4].

The first phase after the occurrence of SCI is marked by significant hemorrhage, cell death, spinal shock, vasospasm and ischemia at the site of the lesion [5-6]. Moreover, astrocytes become highly activate and their hypertrophy and proliferation culminate in the gliotic scar (which may constitute a physical and chemical barrier to axonal regeneration) [7]. The intermediate phase is characterized by the continued maturation of the astrocytic scar and by regenerative axonal sprouting [6,8]. In addition, the chronic phase, which begins approximately 6 months following the injury, is marked by the maturation/stabilization of the lesion including continued scar formation and the development of cysts. Unfortunately, the lesion may not remain static and some patients with SCI can suffer with delayed neurological dysfunction (ascending paralysis, brainstem symptoms) and neuropathic pain [8].

In this context, therapeutic strategies aim to encourage regeneration/sprouting of disrupted axons and to promote plasticity [8-9]. Various innovative interventions have been examined for SCI repairing, including stem cells blockage of the endogenous growth inhibitory factors infusion of neurotrophic factors and transplantation of growth promoting cells [10-12]. Additionally, alternative treatments with the potential to stimulate tissue repair have been emerging such as the low-level laser therapy (LLLT). LLLT has been used to treat injuries and diseases in many tissues including the neural tissue (strokes, neurodegenerative diseases and brain injuries) [9,13-14]. LLLT acts after the absorption of the light by cells which culminate in accelerated metabolic chemical reactions, stimulating mitochondrial respiration and consequently, the production of molecular oxygen and ATP synthesis [15-16]. These effects can upregulate the expression of growth factors and cell-cycle regulatory proteins, therefore promoting cell proliferation [17]. Moreover, in biological tissues, LLLT presents anti-inflammatory and regenerative potential after an injury [15-16,18].

It has been demonstrated that the association of embryonal spinal cord nerve cells with laser irradiation promoted axonal sprouting and spinal cord repair in a SCI model in rats [19-23]. Moreover, Wu et al.[13] using 2 models of SCI (a contusion model and a dorsal hemisection model) verified that LLLT produced a longer length of axonal re-growth in the rats compared to control and improving functional recovery. Ando et al.[9] demonstrated that 808nm laser produced a significantly higher score of the functional test and in the locomotive function in SCI rats compared to SCI control animals.

Although the positive effects of LLLT, its effects on neural repair after an injury in the spinal cord are not well known. Then, it is necessary to investigate the effects of such treatment in in-vivo studies to determine their safety and efficacy. In this context, it was hypothesized the LLLT may positively interfere the regeneration of spinal cord after an injury, reducing the formation of the glial scar and improving the functional recovery. Consequently, the present study aimed to evaluate the effects of LLLT in a model of SCI.

MATERIALS AND METHODS

Experimental groups

Ten week female Wistar rats (weighing between 200 and 220g) were used as experimental animals. The study was approved by the Research Ethics Committee on Animal of the Federal University of São Paulo under the report 743029 and national guidelines for the care and use of laboratory animals were observed. Animals were distributed into 4 groups (8 animals each group in a total of 32): Control Group (CG): SCI animals without any treatment; Laser treated group 500 J/cm² (L-500): SCI animals treated with 500 J/cm²; Laser treated group 750 J/cm²: (L-750): SCI animals treated with 750 J/cm²; Laser treated group 1000 J/cm²: SCI animals treated with 1000 J/cm² (L-1000).

Traumatic Spinal Cord Injury Surgery

Before the surgery, all animals were anesthetized with ketamine (90 mg/Kg), xylazine (13 mg/Kg) and acepromazina (1 mg/kg). After the trichotomy and antisepsis of the dorsal region, an incision (~5 cm) was made in the skin at the dorsal midline. The muscles of the spinous processes were detached between the T9 and T11 vertebrae. Afterwards, the spinous processes and the laminas of T9 and T10 were carefully removed, using ophthalmic surgical scissors, without causing injury to the dural sac, exposing the spinal cord.

Then, animals were properly positioned on IH-0400 Impactor equipment (Precision Systems and Instrumentation, LLC, USA) for the injury induction. For this purpose, a cell load of 150 Kdyn was applied to the exposed spinal cord causing an incomplete traumatic injury in the spinal segment [23]. Thereafter, the wound was closed with resorbable Vicryl® 5-0 (Johnson & Johnson, St. Stevens-Woluwe, Belgium). To minimize post-operative discomfort, buprenorphine (Temgesic; Reckitt Benckiser Healthcare Limited, Schering-Plough, Hoddesdon, UK) was administered intraperitoneally (0.02mg/kg) directly after the operation and subcutaneously for 5 days after surgery.

Laser irradiation

The regime of treatment consisted of 14 sessions of laser irradiation, starting 15 minutes after the surgery. A laser equipment gallium-aluminum-arsenide (GaAlAs) diode laser (Photon Laser II, DMC® equipment Ltda, São Carlos, SP, Brazil) was used in this study. The laser parameters used in this treatment are shown in Table 1. The punctual contact technique was used and the irradiation was performed at a single point, above the area of the created injury [22].

One day after the final session of irradiation, animals were deeply anesthetized with urethane (3 mL/animal - Sigma-Aldrich Corp.®, St. Louis, MO, USA) and euthanized by transcardially perfusion with 100 mL isotonic saline at room temperature, followed by 500 mL of fixation fluid (4 ºC) over a period of 6 minutes. The fixative consisted of 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.4.

Functional Analysis: Locomotor function and tactile sensitivity evaluation

The motor functional behavior analysis was performed using the Basso, Beattie, Bresnahan (BBB) test for all animals in two set points: 24 hours and 15 days after the surgery. The BBB rating scale is a 21-point system based on operationally defined behavior features to follow up recovery progression from paralysis to normal locomotion. For this analysis the animals were individually placed on a circular plastic floor where their behavioral was observed for 5 minutes by two blind evaluators [23-24].

For tactile sensitivity test, evaluations were performed 24 hours and 15 days after the surgery (2 evaluations). For evaluating the tactile sensitivity, Von Frey monofilaments (Touch Test®) were used. The trial was double-blinded and the filaments applied in ascending order, starting with the thinnest (6 grams). The filaments were positioned until they were slightly curved. A full test consisted of five applications with 5 seconds intervals or as soon as the pelvic limb was properly supported on the platform. If the withdrawal did not occur in 5 applications of a given filament, the next one, following the order, was applied. If the pelvic limb was removed when stimulated by a particular strand five times the amount in grams of this filament was considered [25].

Histological procedures

Spinal cord samples were dissected at the site of injury, fixed for 5h in 4% paraformaldehyde and then 30% sucrose for approximately 15 days. Subsequently, samples were frozen and stored at -80 °C. Sections (30µm thickness) were prepared perpendicular using a cryostat (Leica CM 3000, Wetzlar, Germany).

Injury volume analysis

Eight sections of each specimen (representing 10 mm around the injury area) were stained with cresyl violet and eriochrome cyanine [26]. Digital images (magnification 2.5X) (AxioVision Rel. 4.8, Carl Zeiss) were obtained and, through a computer-based image analysis technique (Leica®QWin Pro-image analysis system, Wetzlar, Germany) the volume of the injury was measured. The following formula was used: V = section thickness (30µm) x sum of the areas of all cuts of injury (in mm^2).

Immunohistochemistry

CD-68 and glial fibrillary acidic protein (GFAP) expression was measured. For the immunostaining, antigen retrieval was performed by incubating slides in 10 mM sodium citrate, pH 6, for 5 min at 95 ºC, and then for 30 min at room temperature. The sections were pre-incubated for 5 min in 0.3% hydrogen peroxide in phosphate buffered saline (PBS) solution to inactive the endogenous peroxidase. Slides were then incubated for 1h at room temperature in blocking solution (5% normal donkey serum, 0.3% TritonX-100 in TBS). Primary antibody (IBA-1 and GFAP - Santa Cruz Biotechnology, USA) was added to the blocking

solution which was then incubated overnight at 4 ºC. Sections were incubated with biotinylated anti-goat-IgG for 2h followed by incubation with streptavidin-peroxidase conjugate (Dako) and developed with 3,3'diaminobenzidine (DAB, 0.6 mg/ml, Sigma) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.03% H_2O_2. Then, sections were counterstained with Harris' haematoxylin, dehydrated and mounted with coverslips using Permount. The slides were photographed in AxioVision (Carl Zeiss, Gottingen, Germany).The spinal cord lesion images were evaluated by placing a frame (100µmx100µm) randomly and counting the positive immunostained cells. Image J v.1.48 software (U.S. National Institutes of Health, Bethesda, Maryland,USA – available as freeware from http://rsbweb.nih.gov/ij/) was used.

Statistical analysis

All results were calculated as mean values ± SEM. The data were submitted to Shapiro-Wilk normality test. For data normal distribution data ANOVA with post-hoc Tukey was used. For non-parametric data Kruskal-Wallis test as applied and multiple comparisons were using Dunn´s test. The significance level was 5% ($p \leq 0.05$). All analyses were performed using statistical software GraphPad Prism version 6.01.

RESULTS

Functional analysis: Locomotor function tactile sensitivity evaluation

Before the surgery, intact animals presented a BBB score of 21 (data non-shown). One day after surgery, similar findings for BBB evaluation were observed for all experimental groups (Figure 1A). However, these values were lower compared to the values found before the surgical procedure. In the re-evaluation, BBB scores for all laser treated groups were higher compared to CG ($p = 0.026$ [L-500]; $p = 0.023$ [L-750]; $p = 0.0007$ [L-1000]). No other difference was observed.

Figure 1B demonstrates the values for the tactile sensitivity analysis. In the evaluation period, no statistical difference was observed between the groups. After 15 days post-surgery, lower values of tactile sensitivity for L-750 ($p = 0.021$) and L-1000 ($p = 0.004$) were noticed compared to CG.

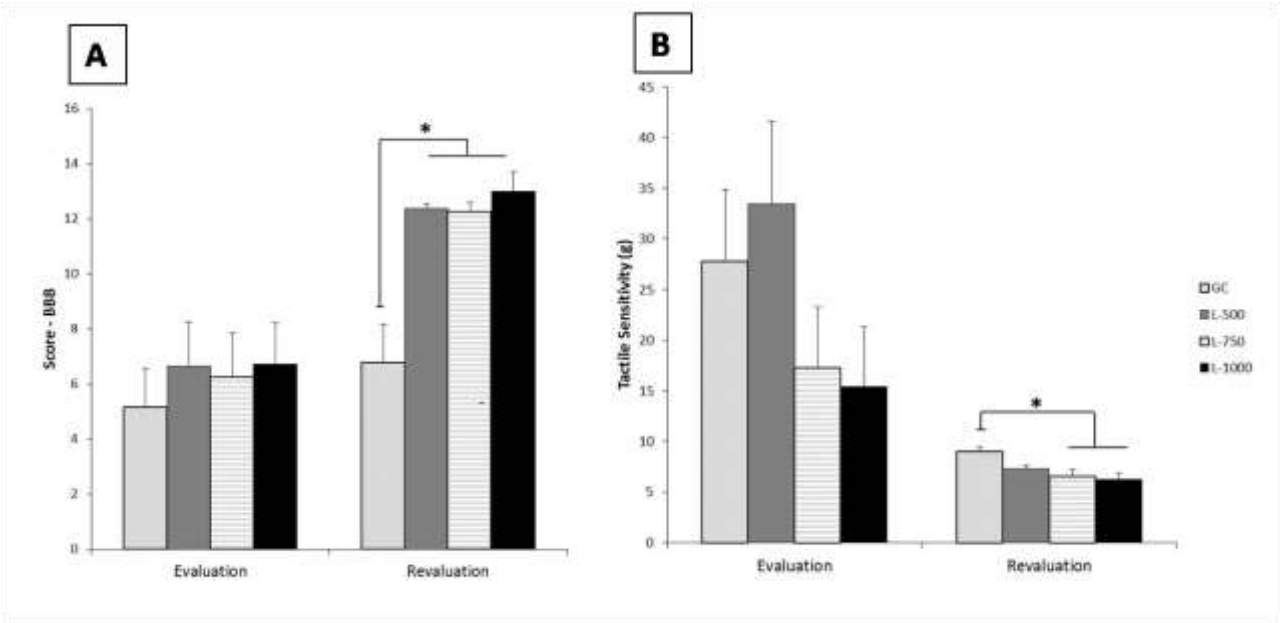

Figure 1. A) Basso, Beattie, Bresnahan Scale:Means and standard errors. Injured group control (CG); Injured group irradiated with 500 J/cm^2 (L-500); Injured group irradiated with 750 J/ cm^2 (L-750); Injured group irradiated 1000 J/ cm^2 (L-1000). **B)**Tactile sensitivity:Means and standard errors. Injured group control (CG); Injured group irradiated with 500 J/cm^2 (L-500); Injured group irradiated with 750 J/ cm^2 (L-750); Injured group irradiated 1000 J/ cm^2 (L-1000).

Injury volume

The volume of the injury was measured for all experimental groups, 15 days post-surgery. A lower injury volume (Figure 2) was presented by L-1000 compared to CG and the other laser treated groups ($p = 0.018$).

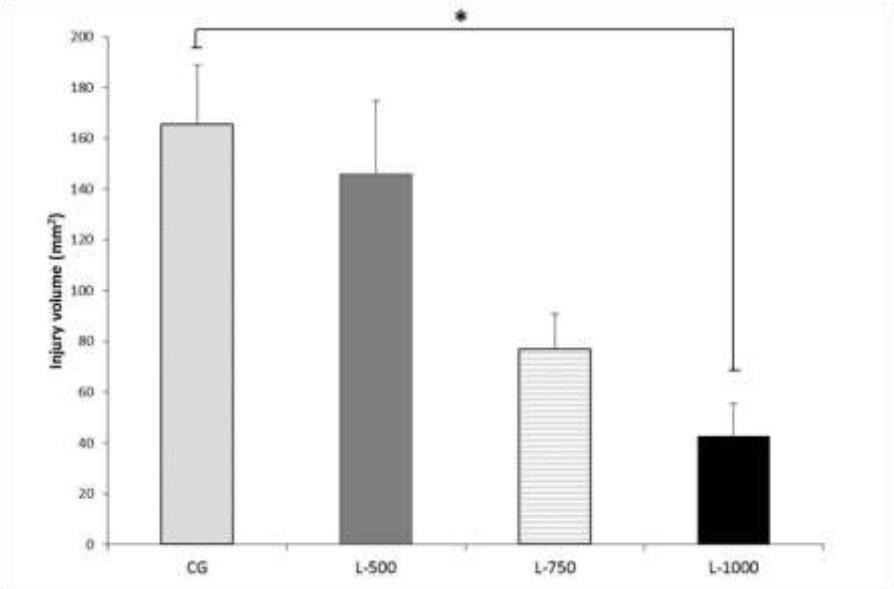

Figure 2. Means and standard errors of injury volume. Injured group control (CG); Injured group irradiated with 500 J/cm^2 (L-500); Injured group irradiated with 750 J/ cm^2 (L-750); Injured group irradiated 1000 J/ cm^2 (L-1000).

Immunohistochemistry - CD68 expression

To investigate the inflammatory process in the spinal cord lesion, the microglia/macrophage marker, CD68, was used. In Figure 3A, it was possible to observe CD68 marked cells mainly in the core of the lesion and around the edges on the 15th day after lesion. The cell quantification analysis shows that the groups treated with LLLT presented lower CD68 cells compared to the CG ($p = 0.0089$ [L-500];$p = 0.0037$ [L-750]; $p = 0.0012$ [L-1000]; Figure 3B).

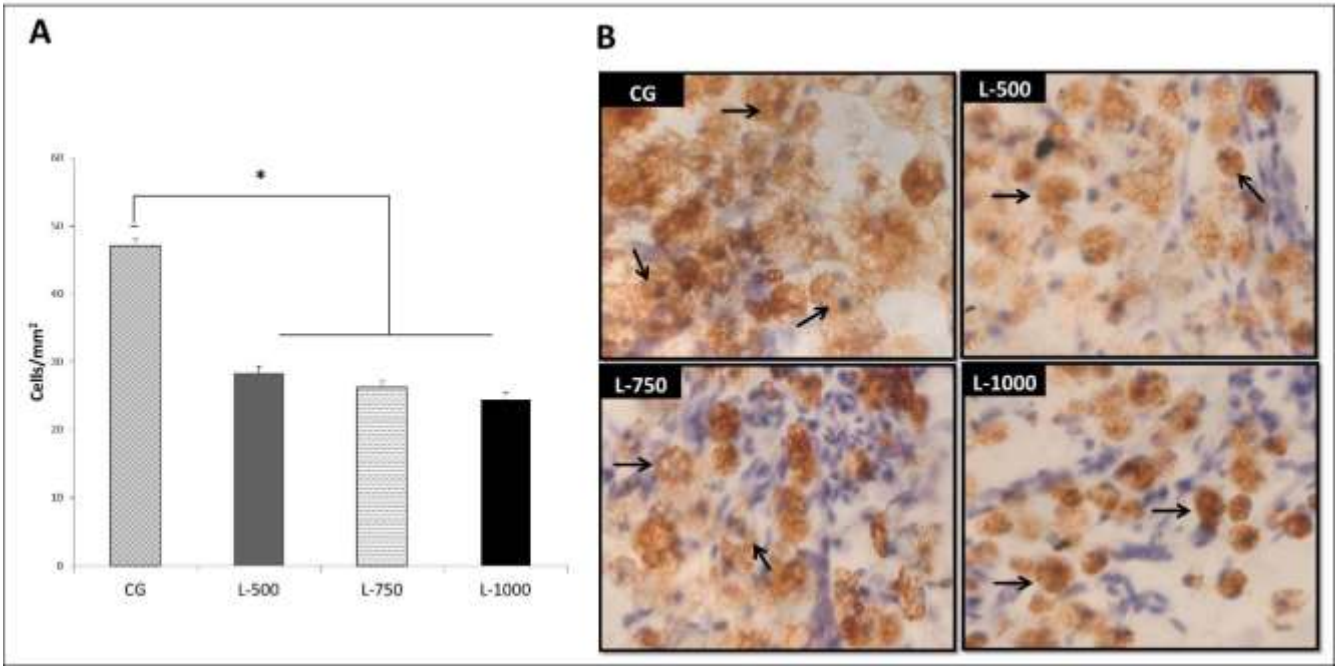

Figure 3. A) Representative spinal cord sections immunostained for microglia/macrophage by CD68 (bar = 100µm; objective 10x). CD68+ cells mainly in the core of the lesion and around the edges was observed in all groups on the 7th day after lesion (arrow). **B)** Means and standard errors of CD68+ cells. Injured group control (CG); Injured group irradiated with 500 J/cm^2 (L-500); Injured group irradiated with 750 J/ cm^2 (L-750); Injured group irradiated 1000 J/cm^2 (L-1000).

Immunohistochemistry - GFAP expression

Figure 4A demonstrated an overview of GFAP expression for all experimental groups. An intense GFAP immunolabelling was observed in CG, L-500 and L750. In addition, a moderate expression was demonstrated

in L-1000 compared to others groups. However, quantitative immunohistochemistry analysis demonstrated no difference in density of GFAP among groups (Figure 4B).

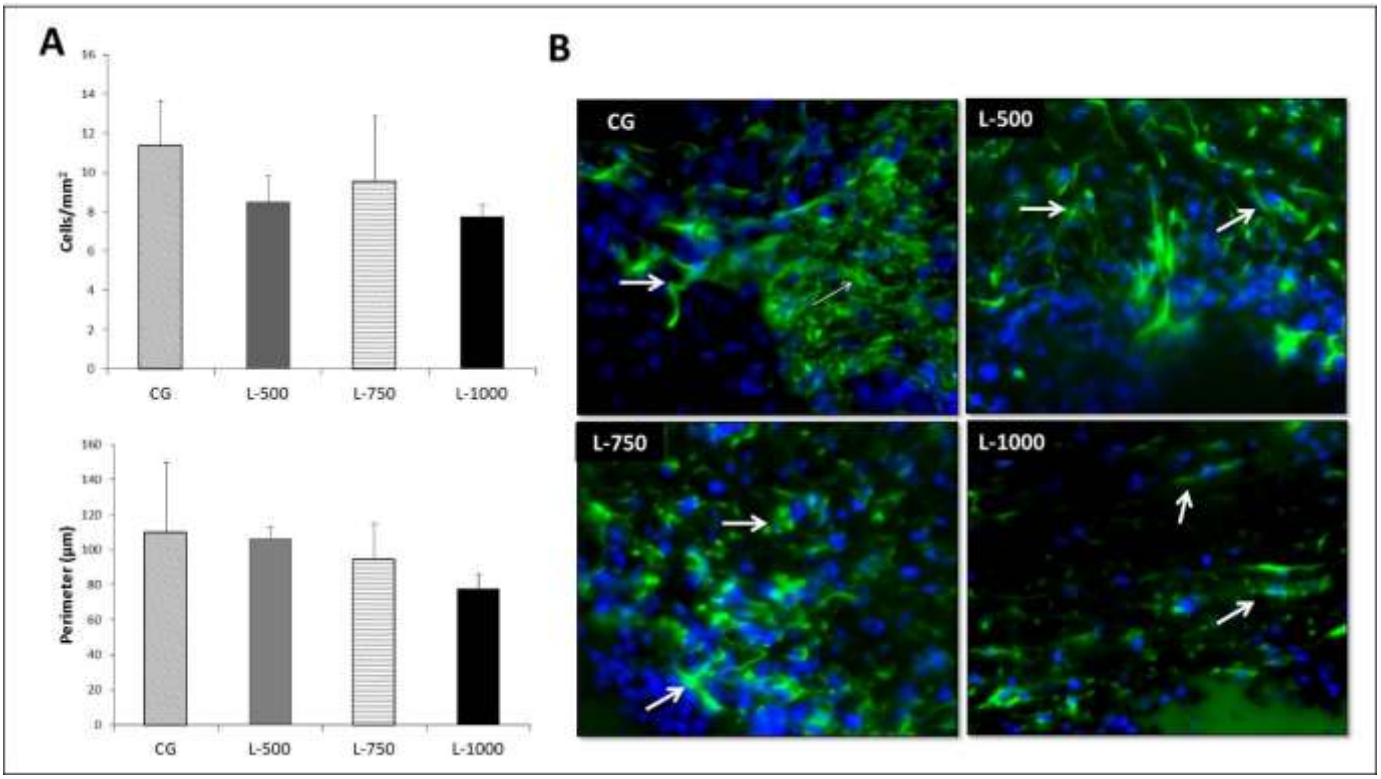

Figure 4. A) Representative spinal cord sections immunostained for microglia/macrophage by GFAP (bar = 100µm; objective 10x). GFAP+ cells mainly in the core of the lesion and around the edges was observed in all groups on the 7th day after lesion (arrow). **B)** Means and standard errors of GFAP+ cells. Injured group control (CG); Injured group irradiated with 500 J/cm^2 (L-500); Injured group irradiated with 750 J/ cm^2 (L-750); Injured group irradiated 1000 J/cm^2 (L-1000).

DISCUSSION

The present work evaluated the effects of LLLT on spinal cord injured rats in an intermediate period of recovery. Our results demonstrated that LLLT produced an improved functional recovery and a decreased CD68 immunoexpression after irradiation with the 3 fluences. Furthermore, tactile sensitivity was increased after 750 and 1000 J/cm^2 irradiation and injury volume was decreased after 1000J/cm^2 irradiation.

One day post-surgery, BBB scores for all injured animals were lower compared to the baseline values (before the surgery). These findings demonstrated the effectiveness of the experimental model used, with a marked reduction of range of movements in the hips, knees and ankles, associated to trunk instability and impaired coordination. In the reevaluation, all the irradiated animals demonstrated a significant functional recovery compared to control. The same results were observed by Byrnes and coauthors [27] using an experimental model of SCI, who also demonstrated a significant improvement in the locomotor function after laser irradiation. Similarly, significant improvements in laser treated animals (750 and 1000 J/cm^2) were observed in the tactile sensitivity evaluation. Possibly, laser therapy, at the higher fluences, was able of modulating the initial inflammatory process after SCI, decreasing neural tissue loss and necrosis at the site of the injury, creating a more appropriate environment for tissue organization, culminating in the improvement of functional activities.

Furthermore, the injury volume in the laser treated animals at 1000 J/cm^2 was statistically lower compared to other experimental periods, indicating the effectiveness of this amount of laser energy to reduce the related amount of damaged tissue due to the injury in the spinal cord. Possibly, the fluence of 1000 J/cm^2 produced an earlier recruitment of immune cells, modulating the inflammatory process and allowing axonal regeneration and sprouting. Many authors demonstrated that LLLT (especially at fluences above 1000 J/cm^2) acts positively in the acute phase of inflammation and stimulating the process of tissue repair [28-31]. Wu and coauthors [13] observed a significantly longer length of axonal re-growth and higher total axon number in the site of the SCI after LLLT (fluence of 1,589 J/cm²), contributing to the functional recovery of the animals.

In the present study, the immunohistochemistry analysis was used to evaluate some markers of neural tissue regeneration, including macrophage and astrocyte activity. Many studies have demonstrated that CD68 is a marker of active phagocytosis and for activated microglia which are still present at 6 weeks post-SCI in rats. In humans, activated microglia/macrophages, as detectable by CD68 immunostaining, are also present during months following the SCI [32]. The significant decrease of CD68 expression after LLLT, in all fluences used, suggests that the laser irradiation was able of modulating the inflammatory process. Similarly, Byrnes and coauthors [27] demonstrated a decrease in macrophage/activated microglia after laser irradiation in a SCI experimental model.

Also, glial scar formation is a reactive cellular process involving astrogliosis that occurs after the SCI. Reactive astrocytes are the main cellular component of the glial scar [33]. After the injury, astrocytes suffer morphological changes, stimulating the synthesis of GFAP, which is an important protein involved in the synthesis of cytoskeletal supportive structures and extend pseudopodia [1,29,34]. Interestingly, in this study, LLLT had no effect in GFAP expression. At this moment, it is difficult to explain these findings but possibly, the amount of energy of LLLT used was not able to inhibit reactive astrocytosis (astrogliosis) evaluated by GFAP expression in this phase of spinal cord repair.

Thus, the results of this investigation corroborate with our previous study which demonstrated that the fluence of 1000 J/cm^2 was more efficient to stimulate spinal cord repair in the inflammatory phase of repair (7 days) [22]. Interestingly, no positive effect was observed in the spinal cord repair after the irradiation with lower fluences (750 and 500 J/cm^2). In the present study, our aim was to progress the investigation of the effects of LLLT in the intermediate period of spinal cord repair and it was observed that, also, lower fluences had positive effects on the modulation of inflammation and functional and morphological aspects. As this study was limited to relatively short-term evaluation of the effects of LLLT, further long-term studies should be developed to provide additional information concerning the late stages of healing after the injury in the spinal cord, evaluating the effects of LLLT on spinal neuroplasticity after an injury.

CONCLUSION

Our results demonstrated that, for an intermediate period of SCI repair, LLLT at higher fluences, was effective in promoting functional recovery and modulating the inflammatory process in the spinal cord of rats after traumatic SCI. As SCI is a chronic disease, there is still a need to develop and to investigate the effect of this therapy on further stages of repair. Thus, the efficacy of LLLT on long-term studies remains to be determined.

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