LOW-POWER LASER THERAPY ACCELERATES PERIPHERAL NERVES' REGENERATION

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SUMMARY

There are evidences that laser therapy may stimulate nerve regeneration and this hypothesis was tested in rats. A controlled crush injury was produced on the sciatic nerve of 20 Wistar rats, half of which submitted to effective Ga-As laser irradiation and the other half to simulated irradiation for 10 consecutive days beginning on the first postoperative day. Results were evaluated at three weeks postoperatively by measuring the sciatic functional index (SFI) at weekly intervals and the total number of nerve fibers and nerve fiber density of the sciatic nerve at three weeks ($p<0.05$). The SFI progressively improved for both irradiated and control nerves (69% and 45%, respectively) with a significant difference between them at two weeks ($p=0.04$). Nerve fiber density increased for the irradiated nerves and decreased for the control nerves, with significant differences between them ($p=0.001$). Low intensity therapeutic ultrasound accelerates nerve regeneration, as demonstrated with significance on the 21st postoperative day.

Keywords: *Nerve regeneration; Crush injury; Laser therapy; Sciatic functional index; Nerve fiber density.*

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INTRODUCTION

Many evidence lines have shown that peripheral nerves regeneration may be accelerated by physical agents, such as electric power, magnetic fields, and ultrasound. Laser therapy has also been studied regarding a potential positive role in this particular area, with first investigations focusing changes on nervous stimulus conveyance, with an electrophysiological demonstration of a reduced latency time, and increased conveyance speed in normal nerves, both in animals[1] and in human beings[2-4]. However, some authors did not find any nervous conveyance change in human beings[5] or animals[6-8], or neurotransmitter release on motor plate in rats[9], thus remaining the question about a potential stimulating role of laser on nerves.

In what concerns to regeneration after some kind of injury, beneficial effects have been reported, such as increased range of action potentials and reduced cicatricial tissue around the nerve[10], strong positive somatossensorial responses evoked, and a significant increase of the number and width of regenerated axons[11,12] as well as a quicker morphologic and functional recovery[13]. On the other hand, deleterious effects have also been demonstrated, such as lower regeneration percent rate translated by a less mature ultrastructural organization, with smaller cross-sectional areas and smaller number of myelinated axons in irradiated nerves compared to those submitted to sham irradiation[14], leading to the conclusion that irradiation with pulsed laser may have suppressive effects on peripheral nerves regeneration.

Despite of the current easy availability of therapeutic laser and its trend to be routinely employed in traumatic injuries of a number of tissues, its effects on peripheral nerve regeneration are still controversial and need to be precisely determined before it is used with no reserves. Thus, the purpose of the present investigation was to study the influence of low-power gallium arsenate (AsGa) laser irradiation on the regeneration of rats' ischiatic nerves, using a controlled model of serious crushing injury and comparatively assessing functional and morphological recovery.

MATERIALS AND METHODS

The experiment was approved by the Committee of Ethics in Experimental Use of Animals by the Ribeirão Preto Medical School. Twenty male Wistar rats weighting 325 g in average (range: 300 – 350 g) were used and kept in individual cages prior and subsequently to surgery, with water and food ad libitum. Prophylactic antibiotic therapy was provided as a single preoperative dose of Penicillin-procaine (400.000 UI) through subcutaneous injection. The animals were randomly divided into two groups, according to the procedure:

Group 1: crushing injury, sham irradiation with the laser ($n=10$);

Group 2: crushing injury, effective irradiation with the laser ($n=10$).

Pre-operative procedures: immediately before the surgery, rats were trained by repeated attempts to walk on a wooden runway for gait analysis (43 cm long, 8,7 cm high, with a small dark box at the end), until they were able to walk straight and fast towards the shelter box. Then, three pairs of footprints of rear paws were obtained on paper strips impregnated with bromophenol blue diluted in acetone at 1%, previously prepared, according to the method proposed by De Medinaceli et al.[15,16] and modified by Lowdon et al[17]. The paper impregnated with bromophenol blue turns yellow when dried, but becomes immediately and permanently blue when in contact with water or any aqueous solution. Instead of using water, rear paws were soaked into regular domestic detergent solution, which avoids footprint dispersion. Paper strips containing the footprints were allowed to dry and copied with a high-resolution scanner. The

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digitalized footprints were stored and analyzed on the computer by means of a graphic software especially developed to that end, allowing us to handle the footprints and to automatically calculate the Sciatic Functional Index (SFI)[18]. Footprints acquired in the preoperative period constituted the normal parameters for future comparison purposes (Figure 1).

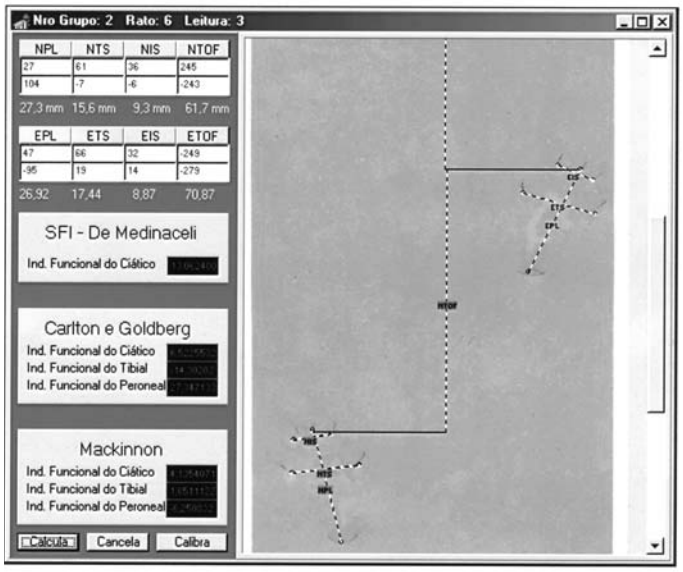

***Figure 1 -** Computer screen image, showing two footprints with parameters already marked on right paw. We can also see the windows with the three functional indexes, by De Medinaceli, Carlton and Goldberg, and Bain, MacKinnon and Hunter (left)*

Surgical procedure: under general anesthesia with a single intraperitoneal injection of sodium pentobarbital (Nembutal Abbott®, 60 mg/kg of body weight) and after routine preparation of the operation field (trichotomy, antisepsis with iodine alcohol solution, 20%), the right ischiatic nerve was exposed with a posterolateral longitudinal skin incision on the lateral surface of the right thigh and by blunt dissection of muscular planes, between gluteus maximum and quadriceps muscles. The nerve was detached from surrounding tissues only at its medial third, where a 5-mm long crushing injury was produced as rigorously as possible at 5 mm distal to its emergence, starting at a point marked by an epineural blue suture stitch for easier later identification (Prolene Ethicom®, 8/0) and ending at about 5 mm above its division on the three main stems (tibial, peroneal, and sural nerves). A stable load of 15,000 g was applied for 10 minutes, with spring tweezers especially developed and built for that purpose[19] and calibrated on a universal assay machine after consecutive use in five animals. The injury produced on the nerve was purposely serious and uniform, in order to make spontaneous regeneration more difficult and to facilitate comparisons between irradiated and non-irradiated nerves. The ischiatic nerve was carefully detached from tweezers and placed back to its original bed, and the wound was closed by layers. An antiseptic solution was sprayed on the wound, but no dressing was applied. All surgical steps were identical for both experimental groups.

Laser irradiation: A portable, commercially-available equipment for pulsed irradiation (wavelength: 904 nm, 20 W of peak power, 180 ns pulse range, 1 MHz frequency, and 4 J/cm2 dose) currently employed in clinical practice. Irradiation area was 0.07 cm2 for 10 minutes, in an intact skin region, as well as on the injured segment of the nerve. Irradiation was initiated on the first postoperative day, and repeated on a daily basis for 10 consecutive days, both on the right and left thigh, since left ischiatic nerves served as control. In Group 1 (sham irradiation), the equipment remained turned off throughout the application, while in group 2, it remained turned on since the beginning, so that the only effect they both had in common was tissue massaging.

Footprint acquisition and analysis: rear paws' footprints were captured at seven-day intervals, up to the 21st day. The SFI was automatically calculated by a dedicated software, as described in other publications[18-20], after the parameters proposed by De Medinaceli and cols[15,16] and modified by Bain et al.[21] were measured, as follows: 1) footprint length, or the maximum distance between the tip of the longest toe and the calcaneus (PL = print length); 2) total toe spread or the transversal distance between the first and the fifth toes (Ts =toe spread); and 3) intermediate toes, or the transversal distance between the 2nd and the 4th toes (IT, intermediate toes)(Figure 2). These values were inserted into the formula:

$$SFI = -38.3 \times \frac{EPL - NPL}{NPL} + 109.5 \times \frac{ETS - NTS}{NTS} + 13.3 \times \frac{EIT - NIT}{NIT} - 8.8$$

Comparisons were established between preoperative and postoperative prints and between normal and operated paws.

Histological and morphometric analysis of nerves: the animals were sacrificed with an intraperitoneal injection of an excessive dose of anesthetic agent (sodium pentobarbital, 120 mg/kg) and both ischiatic nerves (right and left) were removed through a posterolateral incision on the thigh. The right ischiatic nerve was divided into three 5-mm long segments, one proximal and the other distal to the injury area, and an intermediate, corresponding to the right injured segment serving as control. All segments were identified and individually fixated with buffered aqueous solution (phosphate buffer at 0.1 M) of glutharaldehyde 2.5% for two hours at 6°C and post-fixated with osmium tetroxide 2% for 12 hours at room temperature, after which they were dehydrated with ethylic alcohol aqueous solution of incremental strengths, from 25% to absolute, at 25% intervals, for one hour each. Then, the segments were included into epoxy resin (EPON-812) at 60°C for 72 hours. Each segment was entirely sliced as serial 5-μm thick portions with an ultra microtome (MT 6000-XL, RMC Inc.), so that about 1,000 slices were obtained from each; of these, 200 were examined (1:5) and 20 were effectively counted (1:50). Proximal and distal segments of right ischiatic nerves, as well as intermediate segments of left ischiatic nerves were submitted to the same procedure, but only 10 slices (1:100) were effectively counted. The slices were stained with toluidine blue 1% and examined under light microscope (Zeiss Axiophoto), with a built-in video camera (JVC-TK1270) connected to a PC equipped with a KS 400 Measure Interactive software, release 2.0.

The first step of the morphometric analysis consisted of capturing the image of each individual fascicle with the greatest possible magnification so that it could entirely fit the monitor screen, measuring then the fascicle area (magnification: objective lens 2.5X, optovar 1.6X, camera 0.5X); slices with rough technical characteristics (cracks, folds, poor color) were excluded in this phase. The next step consisted of the sequential capture of internal areas of each fascicle (magnification: objective lens 100X, optovar 1,6X, camera 0.5X), which were converted into binary images (black and white) and cleared of any blood vessel, degenerated fibers and artifacts. The individual myelinated fibers were counted and fiber density (fibers /mm2) were automatically calculated. Fibers density corresponded to the sum of densities in each fascicle, in each histological slide. A mean value was calculated for densities measured on the 20 slices of each nerve segment (10 on the left ischiatic nerve, control).

SFI values were submitted to statistical analysis by the Student's t-test, with a significance level of 5% ($p<0.05$), using the SigmaStat® software, release 2.03. Morphometry values were analyzed by the Mann-Whitney non-parametric test, at the same significance level.

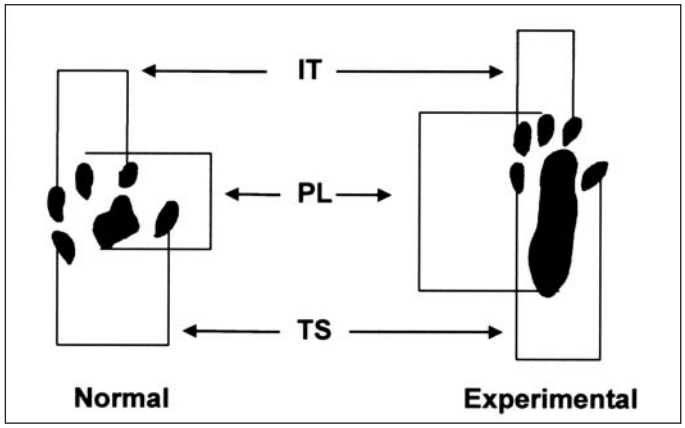

Figure 2- *Schematic illustration of the SFI parameters measured on a normal and an experimental footprint.*

RESULTS

Both on Group 1 (sham irradiation) and Group 2 (effective irradiation), the animals presented a serious equine-like deformity on the right rear paw, with very flexed toes, and they were unable to apply their load on it until the end of the first postoperative week, when they started to place partial load on the operated paw. The left rear paw obviously remained normal throughout the follow-up period. Both the appearance and support improved slowly on right rear paws throughout two consecutive weeks, but had not returned to normal levels until the 21st day, when animals were sacrificed and the ischiatic nerves were removed for histological and morphologic study purposes. The gross examination of the nerves showed that, although they have all recovered their original width, injury site could still be identified because of its appearance starting from the point where blue epineural suture (which could be easily viewed) had passed.

Sciatic functional index: 120 footprints were assessed, being 80 right rear paws and 40 left paws, including preoperative prints and of the 7th, 14th, and 21st postoperative days. The mean preoperative SFI for right rear paw was -4.88 (range: 019.69 to 8.23) on Group 1 (n=10), and 2.17 (range: -13.62 to 18.99) on Group 2 (n=10), with no significant difference between both groups (p=0.65).

On the 7th postoperative day, footprints were longer and narrower than the preoperative ones in both groups, with animals using both the calcaneus and shrunk toes to apply load. The mean SFI was -98.16 (range: -110.71 to -82.54) for Group 1, and -108.11 (range: -128.71 to -81.74) for Group 2, with no significant difference between both groups (p=0.107).

On the 14th postoperative day, footprints were slightly shorter and thicker, and the mean SFI was -79.50 (range: 96.41 to -59.83) for Group 1, and -70.04 (range: -78.46 to -48.18) for Group 2, but the difference between both groups was significant (p=0.04). Finally, on the 21st day, the prints became further shorter and thicker, although less than normal, and the mean SFI was -44 (range: -57.1 to -21.61) for Group 1, and -32.77 (range: -53.62 to -3.04) for Group 2, with no significant difference between both groups (p=0.065).

These results mean that, during a 3-week follow-up period, the SFI improved by 45% in Group 1, since it increased from -98.11 in the first week to -44 on the third week, while in group 2, the SFU increased from -108.11 to -32.77 in the same period, meaning an improvement of 69%, which is much superior to that seen for Group 1 (Figure 3).

Histological and morphometric studies: internal morphology was absolutely normal on left ischiatic nerves, with a typical aspect of myelinated fibers of several diameters distributed within two to four fascicles. The histological aspect was also essentially normal on proximal segments of the right ischiatic nerve on both experimental groups, with a regular distribution of narrow and wide nervous fibers and an apparently normal proportion between myelin sheaths thickness and fibers diameter, numerous non-myelinated fibers, intraneural blood vessels, and fibroblasts were also observed.

In the proximal and intermediate segments (injury site), blood vessels were more prevalent and thicker for Group 2 than for Group 1. Thick fibers with very thin myelin sheaths were prevalent in the intermediate segment in both groups. Schwamm cells with reactive-appearance nuclei, characteristic of synthesis activity, as well as typical images of axonal sprouting were more prevalent on group 2, while Wallerian degeneration was more evident on group 1.

For both groups, small-gauge fibers and thin myelin sheaths were prevalent, although thick fibers and thick myelin sheaths were also frequent on Group 2, next to a large number of Schwamm cells with reactive-appearance nucleus and images of axonal sprouting. In this segment, Wallerian degeneration was not so evident for Group 1 (Figure 4).

Morphometric results are summarized on Table 1. On Group 1, the average number of total counted fibers was 8,851, 7,057 and 6,268, on proximal, intermediate and distal segment, respectively. The mean fiber density on proximal, intermediate and distal segments was 21,268, 20,848 and 11,103 fibers/mm2, respectively, with a significant difference between proximal, intermediate and distal segments (p=0.04). On Group 2, the average total number of fibers was 9,528, 10,485 and 9,529, on proximal, intermediate and distal segment, respectively. The mean fiber density on proximal, intermediate and distal segments was 16,667, 20,727 and 21,588 fibers/mm2, respectively, with a significant difference between proximal and intermediate and distal segments (p=0.04) (Figures 5 and 6).

The differences found for total number of counted fibers and for fibers density between proximal segments of Groups 1 and 2 were not significant (p=0.23 and p=0.11, respectively). On intermediate segments (injury site), the total number of counted fibers was significantly higher in Group 2 (p=0.05), but not for fibers density (p=0.97). On distal segments, both the total number and the density of fibers were significantly higher in Group 2 (p=0.02 and 0.001, respectively).

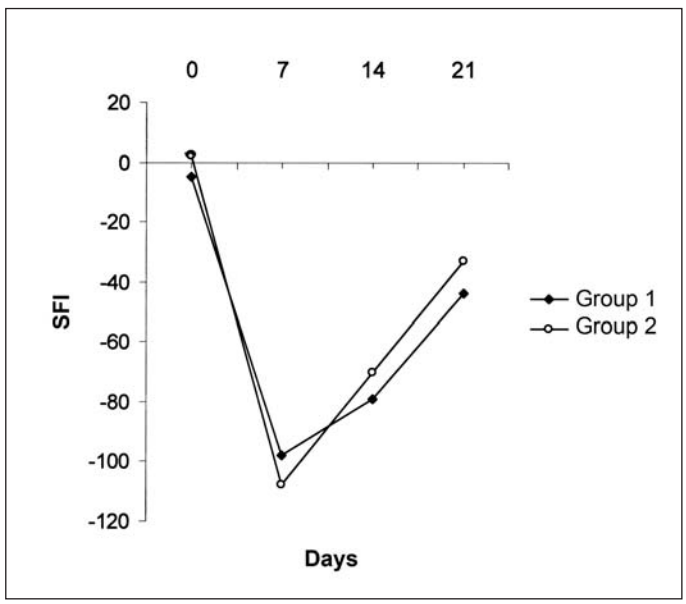

Figure 3 - *SFI behavior according to group. Preoperative SFI and on the 7th day was virtually the same for both groups, with evident improvement on Group 2 from the 14th day on, reaching 69% of improvement in this group, against 45% on Group 1 on the 21st day.*

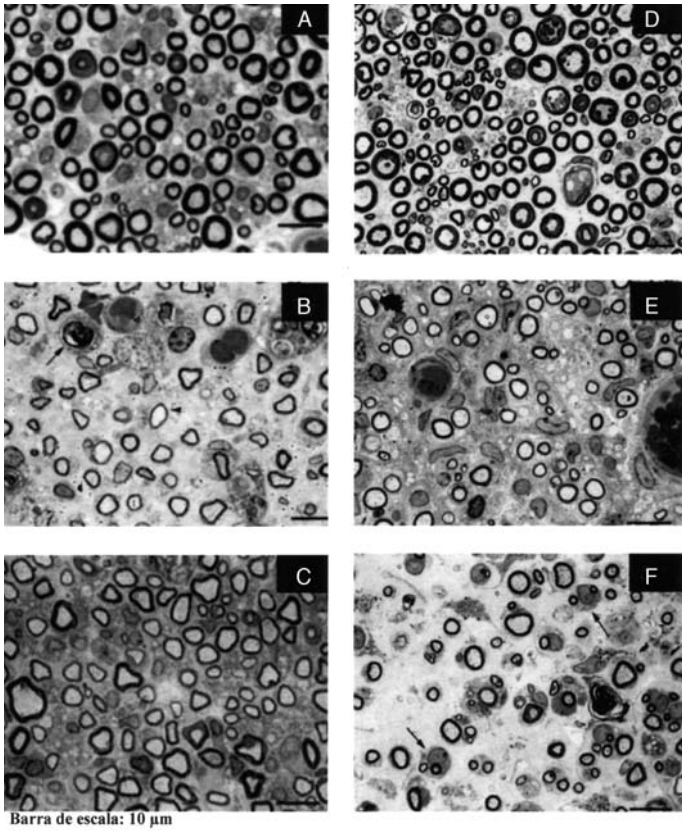

Figure 4 - *Photomicrograph of histological slices of proximal, intermediate and distal segments of nerves on Group 1 (A, B, C) and Group 2 (D, E, F), respectively. Normal aspect of proximal segments both in Group 1 (A) and Group B (D), with large and small-gauge nervous fibers with myelin sheaths of proportional thickness, and occasional blood vessels. Intermediate segments with prevalence of large-gauge nervous fibers and thin myelin sheaths in both groups (B and E), some of them experiencing Wallerian degeneration (B, arrow) and some showing rounded nucleus of Schwann's cells (E). Distal segment with smaller number of nervous fibers and prevalence of small-gauge fibers and thin myelin sheaths, both on Group 1 and Group 2 (C and F); a selected field (F) showing nervous sprout images on Group 2 [toluidine blue 1%; scale bar 10 µm].*

Table 1- *Total number and density of nervous fibers on proximal, intermediate and distal segments of the ischiatic nerve for Group 1 and 2.*

	Group 1			Group 2		
	prox.	interm.	dist.	prox.	interm.	dist.
Total number of fibers (mean ± sd)	8.851 ± 3.055	7.057 ± 3.283	6.268 ± 2.501	9.528 ± 2.220	10.485 ± 4.111	9.529 ± 3.290
Median	7.639	6.773	6.676	9.059	9.720	8.466
Fibers density (mean ± sd)	21.268 ± 8.309	20.848 ± 10.018	11.103 ± 3.115	16.667 ± 2.593	20.727 ± 7.098	21.558 ± 6.046
Median	18.116	19.184	11.120	16.308	17.391	21.618

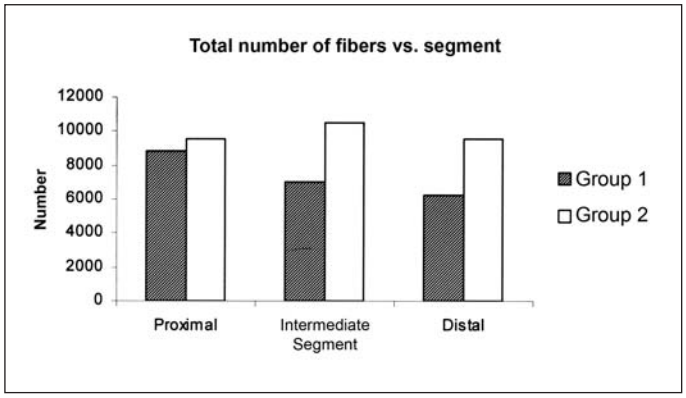

Figure 5 *- Distribution of the total number of nervous fibers according to nerve segment, on Groups 1 and 2.*

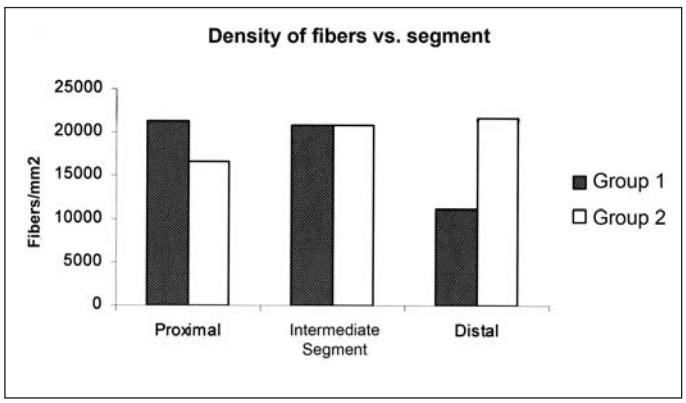

Figura 6 - *Distribution of nervous fibers density according to nerve segment, on Groups 1 and 2.*

For the intact left ischiatic nerves, myelinated fibers density was, in average, 20,256 fibers/mm2 (range: 15,702 – 23,554 fibers/mm2) on Group 1, and 21,403 fibers/mm2 (range: 14,667 – 23,786 fibers/mm2) on Group 2, with an apparently normal distribution of different width fibers, but with a prevalence of small-gauge fibers.

DISCUSSION

Compared to therapeutic ultrasound, very little is known about the role of laser irradiation in the rehabilitation of locomotive apparatus tissues. However, laser irradiation is widely employed to treat a number of pathological conditions of the musculoskeletal system, including peripheral nerves. The fact that laser irradiation somehow interferes with a nerve function seems to be well accepted, according to the demonstration that it reduces latency time and increases nervous conveyance speed[1,4]. Also, despite of few evidences on the contrary[14], there are experimental evidences that the laser has a positive effect on injured nerves' regeneration[10-13,22-24], which encouraged its use in human beings. However, the conclusions of such studies seem to be incomplete and controversial, which has encouraged us to develop the present investigation in order to assess if indeed low-power gallium arsenate laser irradiation can stimulate regeneration of an important nervous stem, the ischiatic nerve, using a serious injury model by crushing in rats, in whom natural recovery is very fast, and analyzing the results from a functional and morphometric perspective.

In an experimental study, regenerating peripheral nerves are usually assessed through histological, morphometric and electrophysiological studies, which do not anticipate any information about functional recovery. On the other hand, functional assessment in animals is always challenging, for obvious reasons, but the SFI developed by De Medinaceli et al.[15,16] and modified by Bain et al.[21] enables a

very accurate quantitative evaluation of the functional recovery of rats' ischiatic nerve, in almost all kinds of investigations. In addition, this is a method with which authors are very familiar for having had employed it in several previous investigations, through which a strong correlation with the results of morphometric studies became clear[18-20,25,26].

Compared to section followed by traditional neurorrhaphy, the crushing injury produced in the present investigation has the advantage of preserving, at least partially, the nerve support structure, thus favoring regeneration[15,18-20,27,28,29]. The 15,000 g load applied on the ischiatic nerve for ten minutes produces a serious injury, possible a Sunderland's type 4, of which regeneration is slow, difficult, and often incomplete; this is, thus, an appropriate injury for the purposes of the suggested investigation, with results to be assessed in the short term. Indeed, the differences between treated and untreated nerves are easier to detect in an early phase of recovery (21 days postoperatively), because the spontaneous recovery usually seen in rats tends to make any comparison more difficult in longer periods of time.

As expected, functional and morphometric results obtained with laser irradiation were not outstanding, yet better than those obtained without irradiation. In fact, SFI was virtually the same for irradiated and control nerves on the first postoperative week, but showed a stronger improvement in the previous on the two subsequent weeks, although the difference between them had been significant only after the second week. Anyway, a difference of 69% was found between the first and last assessment (1st and 3rd week) for irradiated nerves, in contrast with a difference of only 45% for control nerves.

The average total number of counted fibers progressively reduced for control nerves, from the first to the third week, but slightly increased from the first to the second week, being reduced from the second to the third week, while in irradiated nerves, it increased progressively. The increased fibers density on the distal segment of irradiated nerves followed SFI improvement, and can only be resultant from sprouting, probably as an effect of laser irradiation, which may also have stimulated the increase of the number of widely active Schwann's cells seen on these segments.

An intriguing fact was the evident lower fiber density on the proximal segment of irradiated nerves (16,667 fibers/mm^2), although not statistically significant. At first, we believed that it could be associated to the kind of animal used in the experiment, but the finding was explained by the relative enlargement of the section area on the nerve, due to an interstitial edema or congestion, because a higher number of thicker blood vessels than in non-irradiated nerves was found. Thus, what really happened on irradiated nerves was that a similar number of nervous fibers were distributed over a larger area, which ultimately reduced density. The increased number of blood vessels on irradiated nerves probably results from thermal effects of laser irradiation.

The histological study showed that small-gauge fibers and with similarly thin myelin sheaths were prevalent on the distal segment of irradiated nerves, next to large-gauge fibers with myelin sheaths of regular thickness, typical of degeneration. The very common finding of Schwann's cells with reactive nucleus was also important, showing strong regeneration activity, probably as a result of laser irradiation, although by a mechanism that is still unclear.

Peripheral nerves regeneration mainly depends on a neuronal response to trauma or disease, but axon is the way for an axoplasm produced by a cellular body to reach target organs (muscle fibers, sensitive ends, blood vessels) promoting its functional recovery. Crushing injuries, such as those produced in the present investigation, damage most of the axons, which are either restored or replaced by new axons during the natural regeneration process. The quicker regeneration seen with laser irradiation may be due to a local effect, accelerating the regeneration of the axon itself and its supporting structure, thus enabling the evolution of a regenerated axoplasm. Such effect is probably mediated by local-action growth factors, but the possibility that laser can also stimulate chemical and chemotaxis mediators release, which accelerate the axoplasm production itself on the cellular body cannot be excluded.

Although these findings about the potential mechanism of action are merely speculative, regardless of the mechanism involved, the authors conclude that low-power laser irradiation has positively influenced ischiatic nerve regeneration in rats after severe crushing injury. Thus, it can be useful for treating a number of pathological injuries of human peripheral nerves, at doses that must be studied yet, with the advantage that laser is almost free of deleterious effects.

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