LACK OF EFFECTIVENESS OF LASER THERAPY APPLIED TO THE NERVE COURSE AND THE CORRESPONDENT MEDULLARY ROOTS

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ABSTRACT

Objective: To investigate the influence of low intensity laser irradiation on the regeneration of the fibular nerve of rats after crush injury. Methods: Twenty-five rats were used, divided into three groups: 1) intact nerve, no treatment; 2) crushed nerve, no treatment; 3) crush injury, laser irradiation applied on the medullary region corresponding to the roots of the sciatic nerve and subsequently on the course of the damaged nerve. Laser irradiation was carried out for 14 consecutive days. Results: Animals were evaluated by functional gait analysis with the peroneal functional index and by histomorphometric analysis using the total number of myelinated nerve fibers and their density, total number of Schwann cells, total number of blood vessels and the occupied area, minimum diameter of the fiber diameter and G-quotient. Conclusion: According to the statistical analysis there was no significant difference among groups and the authors conclude that low intensity laser irradiation has little or no influence on nerve regeneration and functional recovery. ***Laboratory investigation***.

Keywords: Nerve regeneration. Rats. Crush injury. Fibular nerve. Laser therapy.

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INTRODUCTION

Peripheral nerve injury results in partial or total motor, sensory and functional loss, and alterations such as traumatic damage, diseases and tumors are common. The incidence or these injuries is estimated at more than 500,000 new patients per year.[1]

Different studies were conducted in search of improvement and/or acceleration of the recovery of injured peripheral nerves. The treatment methods studied include low intensity laser, whose use has increased in the last decade.[2,3] Authors[2,4-11] have concluded that this treatment promotes a positive influence on the regeneration of these injuries. However, Bagis et al.[12] and Comelekoglu et al.[13] did not observe any beneficial action.

Nevertheless, the experimental studies on regeneration of peripheral nerves do not follow appropriate standardization of the methodology used, presenting controversial aspects, as it is not always possible to establish a clear correlation between the results obtained by different methods in the same investigation, or between the results obtained by the same method employed in different investigations.[14]

The problems found in the regeneration of peripheral nerves, after the injury, reside in how to prevent the retrograde degeneration of the corresponding neurons in the spinal cord.[8] If nerve regeneration occurs, it develops slowly and is frequently incomplete.[2,9] Low intensity laser irradiation is usually applied to the site of the crush injury. Rochkind et al.[8] used laser irradiation at the root of the spinal cord, at the branch that corresponds to the sciatic nerve of the rat (L2), and thus observed an increase in the metabolism of the neurons and an improvement in myelin production bringing about an acceleration of the regeneration of the injured nerve. Anders et al.[2] described that the efficacy of the effect of low intensity laser irradiation on peripheral nerve recovery can be increased if the corresponding segment of the spinal cord is also irradiated.

The aim of this study is to investigate the influence of low intensity GaAlAs laser irradiation (830nm), applied to the medullary region corresponding to the sciatic nerve root, and subsequently to the injured nerve course, after crush injury of the common fibular nerve of rats, through the morphometric analysis and functional gait assessment.

All the authors declare that there is no potential conflict of interest referring to this article.

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MATERIAL AND METHODS

This project was approved by the Committee of Animal Experimentation and Research Ethics of Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo. The study subjects were twenty-five male Wistar rats with average weight of 250 grams. Divided into three groups: 1) Normal Group (n=5), not submitted to injury and not treated; 2) Control Group (n=10), injured and not treated; 3) Treated Group (n=10), injured and treated, at 2 points for each region, while in the spinal cord the application was centralized at points T13-L3 and in the lower limb at both ends of the scar. The animals were anesthetized with a mixture of 10% ketamine (0.1 ml/ 100g body weight) and 2% xylazine (0.07 ml/100g body weight), administered intraperitoneally, followed by trichotomy and antisepsis at the surgical site of the right lower limb to approach the common fibular nerve and to carry out the crushing technique. The crush forceps[15] produced a 5mm long lesion, with weight of 5Kgf and crushing time standardized at 10 minutes. The muscles were not sutured after this procedure, with suturing limited to the skin using 3-0 nylon thread finalized with the appropriate hygiene and antisepsis care. The procedures and allocation of the animals were performed at the Bioengineering Laboratory of Faculdade de Medicina de Ribeirão Preto/USP. The animals were kept in collective cages, containing no more than five animals each, receiving commercial feed and water *ad libitum*.

Low intensity laser therapy

The equipment used was the Ibramed Laserpulse of low diode intensity Gallium-Aluminum-Arsenate (GaAlAs), by the punctual transcutaneous method with contact, for 14 days running since the first postoperative day. The trichotomy, at the irradiation site, was executed repeatedly every 48 hours, using a razor blade, prior to the daily therapy with the laser. The laser had the following characteristics: wavelength of 830nm, continuous pulsed mode, power of 30mW and beam area of 0.116cm^2. Energy density of 10,34J/cm^2 was used at each point, and the energy delivered by point was 1.2J.

Functional gait assessment

A treadmill with controlled speed developed by Monte-Raso et al.[16] was used to capture gait for the functional assessment. The treadmill, with speed adjustment from 0 to 14 meters per minute, was made in acrylic, whose transparency allowed the filming of the animals' gait using a 1.3 megapixel webcam coupled to a portable computer. The captured images were analyzed using AFNP - Functional Analysis of Peripheral Nerves software,[17] which calculated the predetermined parameters for the functional gait assessment. (Figure 1) The animals were previously put to walk along the treadmill to adapt. The footprints were obtained on days 7 and 14 after the injury. The footprint parameters measured were: print length, or PL, maximum distance between the tip of the central toe and the heel); toe spread or TS, transversal distance between the tips of the first and fifth toes); and intermediate toes (or IT, transversal distance between the tips of the second and fourth toes). These data were entered in the formula of Bain et al.[18] which provided the Peroneal Functional Index (common fibular) - PFI.

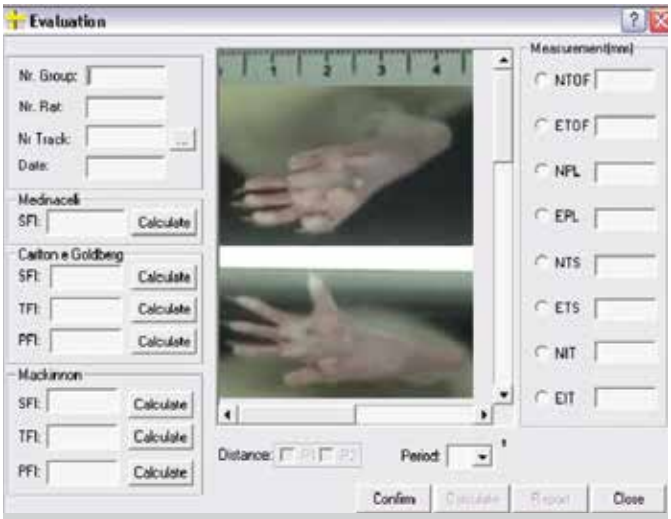

Figure 1. FAPN (Functional Assessment of Peripheral Nerves) program evaluation screen.

Morphometric analysis

On the 15th postoperative day, the animals were weighed and euthanized using an overdose of anesthetic, with the collection of the proximal region of the common fibular nerve for analysis. The samples were immersed in a fixing solution of 2.5% glutaraldehyde in a 0.025M sodium cacodylate buffer pH 7.4 for 48 hours, then were postfixed in 1% osmium tetroxide for 8 hours. The fragments were dehydrated in progressively increasing concentrations of ethanol, and subsequently immersed in an Epoxy resin (Epon 812) and propylene oxide mixture. The resin polymerization was carried out in an oven at 60C, for 72 hours. The nerves were included in silicone molds. The fragments were sectioned to 0.5μm of thickness in a microtome with glass knives and stained with 1% toluidine blue. The images were scanned using the following equipment: microcomputer, Zeiss Axiophoto light microscope, Zeiss motorized platinum and JVC TK1270 video camera. The macros used for acquisition, counting, adjustment and obtainment of measurements of the myelinated fibers were developed in the actual laboratory, using the KS 400 platform (Kontron 2.0). The specimen image was analyzed first, aiming to show the transverse area of the nerve and its constituent fascicle. After this the fascicle image was scanned and circled manually to obtain the fascicle area. The scanned image was used to obtain frames of 640 X 470 pixels with images of the myelinated fibers. In the morphometry the participants measured the total area of the nerve, and counted the quantity of myelinated nerve fibers, the quantity of Schwann cell nuclei and the quantity and area of the vessels in 100% of the frames. The KS 400 program randomly selected 30% of the frames, in which the minimum diameter of the axon, minimum diameter of the myelinated fiber and G-quotient were measured. The latter is obtained

by the ratio between the minimum diameter of the axon and the minimum diameter of the fiber. Histograms of frequency distribution of the diameters of the fibers and of the axons (separated in class intervals of 0.5μm) of the groups were obtained by the SigmaPlot application.

Statistical analysis

Functional and morphometric data were presented in addition to the mean and standard deviation. Since most of the variables do not present normal distribution and the groups are small, the Kruskal-Wallis nonparametric test, and when necessary Dunn's post hoc test, were applied to achieve the comparative objectives, adopting $\alpha \leq 0.05$ as a significance level.

RESULTS

No signs of infection and suture dehiscence were observed during the treatment. The animals tolerated the surgical procedure well and behaved well during the laser treatment.

Functional gait assessment

The study was conducted based on 50 footprint images in different periods, 7th and 14th postoperative days, and the values are in Table 1 and Figure 2. The Normal Group, in relation to the Control and Treated Groups, on the 7th and 14th postoperative days, presented a significantly greater difference ($p<0.001$ and $p=0.008$). No significant values were observed on the 7th and 14th postoperative days, when comparing the Control and Treated Groups.

Morphometric analysis

The morphometric data are in Table 2. In relation to the quantity of myelinated fibers and their density, the Normal Group, when compared with the other two groups, presented significantly greater difference ($p=0.02$ and $p=0.02$). However, when the Control and Treated Groups were compared, no significant value was observed. As regards the quantity of Schwann cell nuclei and minimum diameter of the myelinated fibers, when the three groups were compared and interspersed they did not present significant difference.

Table 1. Data of the functional gait assessment using the PFI, on the 7th and 14th postoperative days. Means and standard deviations of the groups.

	Normal	Control	Treated
PFI – 7th day	3.19 ± 5.71	-43.05 ± 9.34*	-42.50 ± 10.36*
PFI – 14th day	-0.26 ± 21.62	-39.85 ± 9.36*	-38.88 ± 12.99*

* Indicates significant difference when compared with the Normal Group.

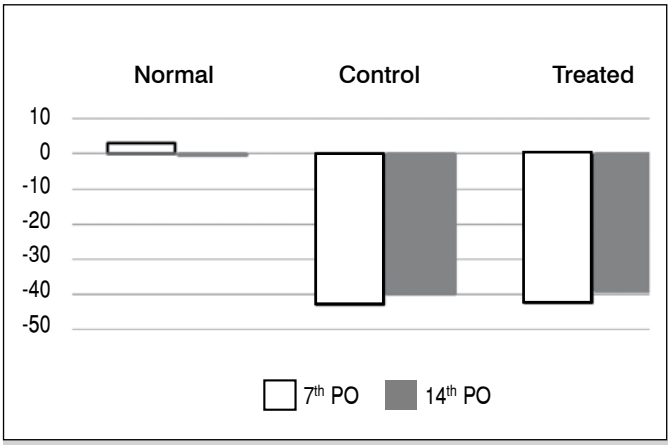

Figure 2. Functional gait assessment (PFI) on the 7th and 14th postoperative days.

Table 2. Data of the morphometric analysis. Means and standard deviations of the groups.

	Normal	Control	Treated
Myelinated fibers			
Numbers	1785 ± 170	889 ± 367*	899 ± 396*
Schwann cells	34.20 ± 5.93	56.11 ± 24.11	45.88 ± 19.10
Minimum diameter (µm)	398 ± 11	441 ± 58	464 ± 36
Density (fibers/mm^2)	12196 ± 1825	4242 ± 2184*	4552 ± 2129*
Capillary			
Number	9.60 ± 3.5	5.10 ± 2.8*	4.20 ± 2.3*
Area (µm^2)	533 ± 116	489 ± 403	259 ± 185*

* Indicates significant difference when compared with the Normal Group.

The distribution of the percentage of myelinated fibers and of axons (Figure 3) in relation to the minimum diameter values, when comparing and interspersing the three groups, presented significant differences between the Normal Group and the other two groups, with $p=0.001$ and $p=0.001$, respectively.

The histogram of distribution of the minimum diameter of the myelinated fibers of the Normal Group showed a tendency for unimodality, with peak at 5μm (8.88%) of diameter with extreme values of 1.5 and 12μm, showing a balance in the quantity of small and large fibers. The Control and Treated Groups also showed a tendency for unimodality, with peak of 2.5 and 3.0μm, frequency between 26.48% and 27.25%, respectively, and extreme values between 1 and 12μm, showing a large quantity of small fibers, and more accentuated disappearance of the large fibers. There was a tendency for the axon histogram to accompany the fiber histogram, yet with greater deviation to the left.

The histogram of distribution of the minimum diameter of the myelinated axons of the Normal Group showed a tendency for unimodality, with peak at 3μm (13.21%) of diameter with extreme values of 0.5 and 8.5μm, showing a large quantity of small axons and an average quantity of large axons. The Control and Treated Groups also showed a tendency for unimodality, with peak of 2μm, frequency between 26.51% and 28.91%, and extreme values between 0.5 and 9.5μm, showing a large quantity of small axons, and more accentuated disappearance of the large axons. (Figure 4)

The distribution of the percentage of fibers in relation to the values of the G-quotient, when comparing and interspersing the three groups, presented a significant difference ($p=0.02$) between the

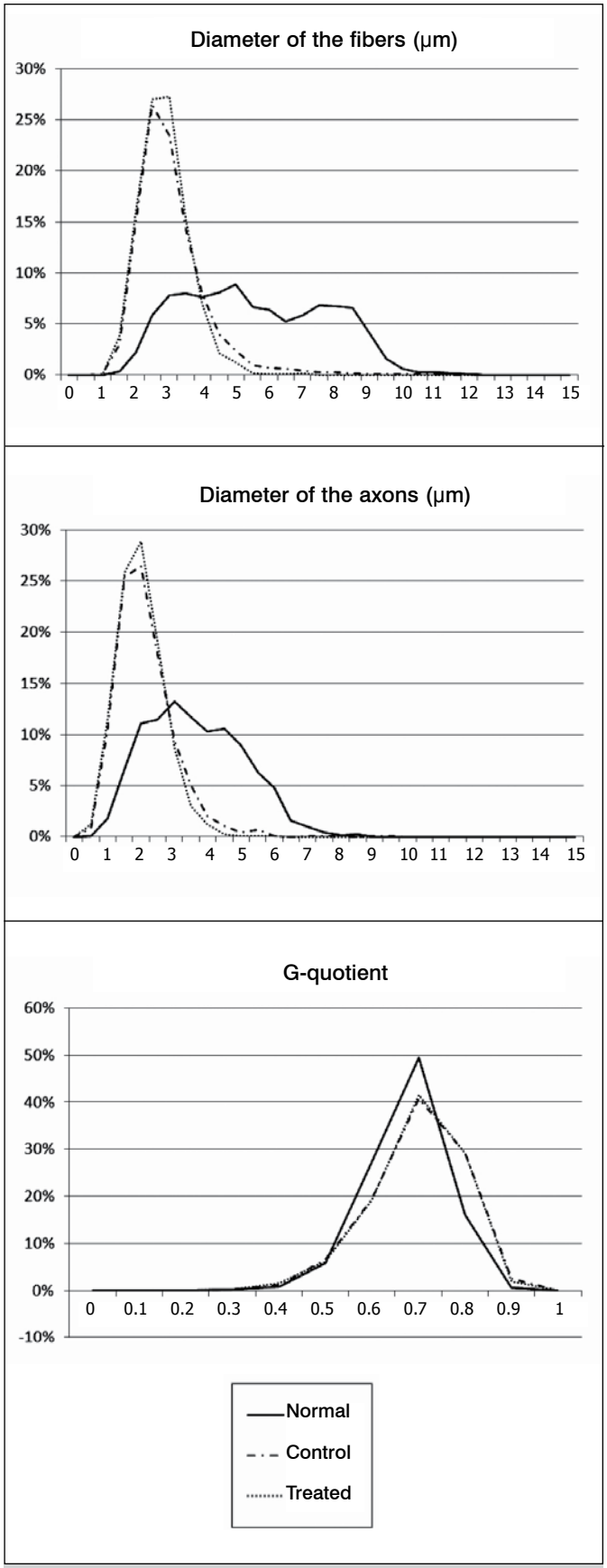

Figure 3. Distribution of the diameters of the myelinated fibers, diameter of the axons and G-quotient in the groups.

Normal and Control Group, and (p=0.002) between the Normal and Treated Group. All the groups presented a tendency for unimodality, with peak of 0.7, frequency of 49.50%, 40.76% and 41.54%, respectively, with extreme values of 0.3 and 0.9.

In relation to the quantity of vessels, when comparing the Normal Group with the other two groups, it presented significantly greater difference (p=0.004). The Control and Treated Groups did not present a significant difference between one another. In relation to the area of the vessels, the Normal Group, when compared with the Treated Group, presented a significantly greater difference (p=0.05).

DISCUSSION

The rat was the animal chosen for the study, as its peripheral nerves are similar to those of humans, as are the physiology and the biological processes involved in regeneration. For this reason it is the animal most frequently used for this investigation.[1] Pachioni et al.[19] concluded that there is no need for us to use loads above 5kg, as this is sufficient to injure the intraneural vessels, especially the endoneurial capillaries, thus producing an important lesion in the axon, characterizing the axonotmesic injury.

The publications whose treatments used the continuous--emission laser showed a positive result in peripheral nerve regeneration. This type of laser could be the first choice for the promotion of nerve regeneration.[3] However, Câmara et al.[20] used the GaAs pulsed-emission laser and concluded that it positively influences the regeneration of the sciatic nerve of Wistar rats, after axonotmesic injury, making nerve recovery faster and more efficient.

Authors[4,6,7,21-24] studied the influence of low intensity laser irradiation applied to the nerve course, on the peripheral nerve injury, and found positive effects for nerve regeneration. Other authors,[8,24] when studying spinal cord irradiation, also reported having found positive factors in recovery. Bagis et al.[12] when conducting a study with crushing of the sciatic nerve of rats, used spinal cord irradiation with the GaAs laser and declared that it is inefficient in the repair of nerve injuries. In the study by Bagis et al.,[12] two factors could have influenced the fact that no improvement was found in nerve regeneration: the probable short time of application of the laser, which was only seven consecutive days, and the pulsed emission of the low intensity laser.

Anders et al.[2] proposed that the effect of low intensity laser irradiation on peripheral nerve recovery can be increased if the corresponding segment of the spinal cord is irradiated in addition to the nerve course. Future studies investigated the influence of laser on nerve regeneration when irradiated at both sites, spinal cord and nerve, as did the studies by Rochkind et al.[25] on rats, and by Rochkind et al.[10] on humans, which showed an improvement in nerve regeneration, when compared with their respective placebos.

However, in this study, the irradiation time was 14 consecutive days, and the laser, of continuous emission, was irradiated at both sites, nonetheless no improvement was observed in the regeneration.

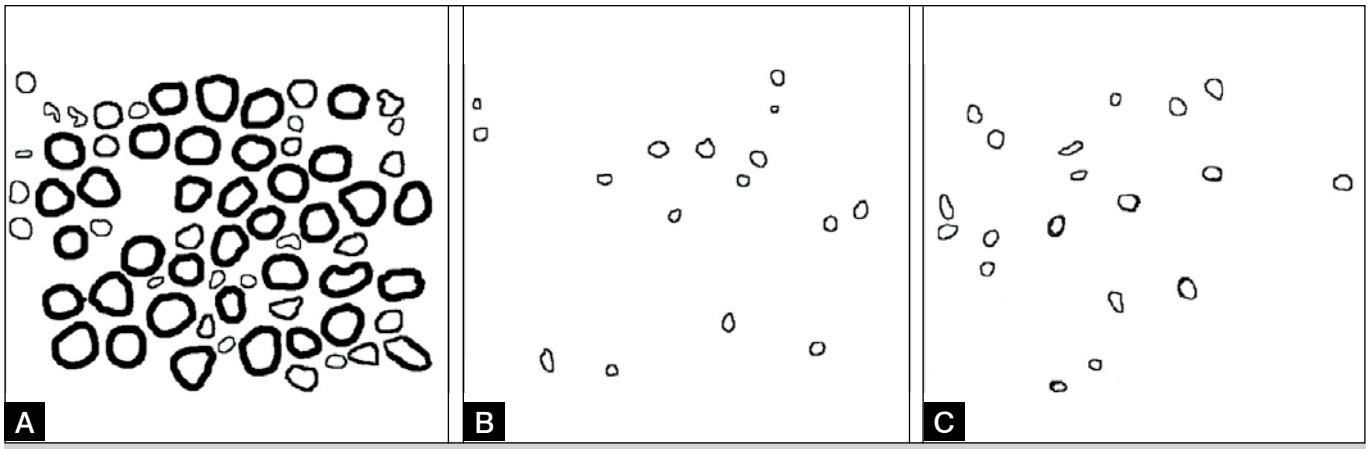

Figure 4. Binary frames of 640 x 470 pixels. Contour of the myelinated fibers of each group: Normal (A), Control (B) and Treated (C).

The regeneration of the peripheral nerves depends mainly on the neuronal response to the traumatism or disease, while the axon is the route for the axoplasm produced in the cell body to reach the target organs (muscle fibers, sensory terminations, blood vessels) and to promote their functional recovery. Crushing, as produced in this investigation, injures most of the axons, which are either restored or substituted in the natural regeneration process. The higher regeneration speed observed with the laser irradiation may have been owing to a local effect, accelerating the regeneration of the axon itself and of its supporting structure, thus allowing the progression of the regenerated axoplasm. Such an effect is probably mediated by growth factors of local action, but we cannot rule out the possibility that laser also stimulates the release of chemical and chemotactic mediators, which accelerate the actual production of the axoplasm in the cell body.[26]

The functional gait analysis has proven to be a safe functional evaluation method, keeping track from the nerve lesion through to repair,[27] having a strong correlation with the morphological evaluation,[27,28] and serving as an opportunity for us to evaluate the specific aspects of nerve recovery in a non-invasive manner.[18] Sousa et al.[24] concluded that low intensity GaAlAs laser irradiation (830nm) was able to accelerate and potentiate the peripheral nerve regeneration process of rats on the 14th postoperative day, according to the functional gait assessment, both for the group treated in the spinal cord and for the group treated in the nerve, without observing any improvement in the group treated in both places. No significant difference in peripheral nerve regeneration was found in this study through functional gait assessment either.

To carry out the morphometric analysis, different samples are frequently used. These include: selection of 15 random and distinct fields,[20] selection of 3 random and distinct fields,[29] and 30% of the frames.[11]

When Tomazini et al.[29] analyzed the quantity of fibers after the treatment with low intensity laser on the spinal cord, they concluded that peripheral nerve regeneration did not occur. In the same way as in this study, the quantity of myelinated nerve fibers was not sufficient to bring about nerve regeneration.

Câmara et al.[20] observed a significant difference between the control and treated groups in the injured limb, for the proliferation of neurons and Schwann cells on the 14th and 21st days of treatment, through laser therapy, demonstrating the efficiency of the treatment even over a short period of time. In this study, the three groups did not present significant differences in the quantity of Schwann cells. The predominant presence of fibers of reduced diameter, and similar appearance, in the groups that underwent nerve crushing, indicates that there was no positive action in the low intensity laser irradiation in the maturation process of the regenerating axons.[29] As observed in this study, the experimental groups presented a large quantity of small fibers, and more accentuated disappearance of the large fibers. Bae et al.[30] caused crush injury to the sciatic nerve of rats, carried out the 904nm GaAs low intensity laser therapy on the course of the injured nerve, and subsequently observed, at seven weeks of treatment, a greater quantity of axons and nerve fibers of considerable diameter, which indicates regeneration quality.

As regards the G-quotient, low values (around 0.4), generally indicate axonal degeneration, while high values (around 0.7) indicate degeneration of the myelin or regeneration.[28]

In the study by Bagis et al.[12] crush injury was produced in the sciatic nerve rats, submitted to the treatment with GaAs pulsed laser (980nm), for 7 consecutive days, at two points, one at the level of vertebra L2 and the other above the wound site. The contralateral side was used as control. Fourteen days after the end of the treatment, the samples did not present qualitative differences in the morphological pattern of the regenerated fibers, between the laser-irradiated and contralateral nerves (control). Histological sections of all the sciatic nerves revealed irregularities in the sizes and in axon degeneration, presenting an increase of vascularization in the regenerated regions of the sciatic nerve. This led to the observation that low intensity laser therapy was not able to produce detectable changes, and was not effective in nerve regeneration.

CONCLUSION

The GaAlAs low intensity laser irradiation (830nm), applied to the medullary region corresponding to the root of the sciatic nerve, and subsequently to the course of the injured nerve, was not effective, presenting little or no influence on nerve regeneration and functional recovery.

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