LOCAL LOW POWER LASER IRRADIATION ACCELERATES THE REGENERATION OF THE FIBULAR NERVE IN RATS

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ABSTRACT

Objective: To study the influence of low power GaAsAl laser irradiation on the regeneration of a peripheral nerve, following a controlled crush injury. Material and methods: The right common fibular nerve of 30 Wistar rats was submitted to a crush injury with an adjustable load forceps (5 000 g, 10 minutes of application). The animals were divided into three groups (n=10), according to the postoperative procedure (no irradiation; sham irradiation; effective irradiation). Laser irradiation (830 nm wave-length; 100 mW emission power; continuous mode; 140 J/cm^2) was started on the first postoperative day and continued over 21 consecutive days. Body mass, time spent on the walking track and functional peroneal index (PFI) were analyzed based on the hind footprints, both preoperatively and on the 21st postoperative day. Results: Walking time and PFI significantly improved in the group that received effective laser irradiation, despite the significant gain in body mass between the pre- and post-operative periods. Conclusion: Low Power GaAsAl laser irradiation, with the parameters used in our study, accelerated and improved fibular nerve regeneration in rats.

Keywords: Nerve regeneration. Rats. Crush syndrome. Peroneal nerve.

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INTRODUCTION

In spite of adequate surgical treatment and rehabilitation, traumatic injuries of the peripheral nerves do not always evolve satisfactorily, with return of motor and sensation functions to useful levels. Physical resources, such as electricity,[1] electromagnetism, therapeutic ultrasound[2] and laser,[3] have been employed to stimulate the regeneration of the peripheral nerves, with results that are still controversial, but tending positive. Of these resources, laser is perhaps the least well known, as regards its effects on the biological tissue[4,5] and the parameters of use, such as dose, wavelength, continuous or pulsed mode,[4-6] treatment duration[3] and application site.

Gigo-Benato et al.[7] applied lower power laser irradiation (wavelengths of 808 NM and 905 NM, and doses of 29 J/cm^2 and 40 J/cm^2, respectively) directly on the median nerve, sectioned and repaired by lateral terminal neurophy of the distal segment in the intact ulnar nerve, in rats; the treatment was performed over three weeks, since the first postoperative day and, in a control group, there was sham treatment. They demonstrated the occurrence of mielinization and recovery of muscle mass more quickly in the treated group, with significant improvement of function, in comparison to the control group.

Endo et al.[8] employed lower power laser irradiation (gallium arsenide, GaAs), in pulsed mode (wavelength of 904 nm, peak power of 20 W, pulse width of 180 ns, frequency of 1 MHz and dose of 4 J/cm^2) on the sciatic nerve submitted to crush injury, in rats. The results were evaluated by the functional gait analysis and by morphometry of the nerves treated, demonstrating that these showed better recovery, with a larger number of regenerated nerve fibers, reflecting functional improvement. The authors concluded that the low power laser effectively accelerates the regeneration of injured nerves, with potential for clinical application in humans.

The functional evaluation became one of the study methods of regeneration of a peripheral nerve in animals, since the description of the sciatic functional index by De Medinaceli et al.[9,10], later modified by Bain et al.[11] This method, which has the advantage of not being invasive, is closely correlated to the degree of morphologic regeneration, measured by the morphometry, and can be employed as a substitute of invasive methods, like morphometry itself and correlated methods, in longitudinal studies, as they do not require the animals' euthanasia.[12] For its execution, it is necessary to obtain the hind footprints of the animals, in which some parameters are measured, introduced in a mathematical

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formula, whose result is the sciatic functional index (SFI), which is a negative indicator of nerve function, ranging from zero (0) to -100, with zero meaning absence of dysfunction and -100, complete dysfunction.[1,12-14]

This investigation was aimed at analyzing the influence of laser irradiation (gallium-aluminum-arsenide, GaAlAs) on regeneration of the fibular nerve submitted to crush injury, through the functional gait analysis, including the measuring of gait speed, in rats.

MATERIAL AND METHODS

The experiment was approved by the Commission of Ethics in the Experimental Use of Animals of the institution where the investigation was performed. Thirty-five male Wistar rats were used in the study, with approximate age of three months and mean weight of 220 g (range: 190 - 250 g). The animals were kept in collective cages, with five animals each, receiving standardized ration and water *ad libitum*. The animals were divided into four experimental groups, according to the procedure performed:

Group 1: injured nerve, without treatment (n=10);

Group 2: injured nerve, sham treatment (placebo) (n=10);

Group 3: injured nerve, effective treatment (n=10);

A control group was also formed with five intact animals without any treatment.

Preoperative procedures

In the three days that preceded the operation, the rats were trained to walk along a wooden gait analysis track that was 43 cm long by 8.7 cm wide, with a dark hutch at the end, which served as shelter and where a few grains of feed were placed to serve as an attraction. The walking track was also equipped with two movement sensors, one at the entrance and the other at the door to the hutch, both connected to an electronic chronometer, to measure the walking time. (Figure 1) As soon as the animals were walking along the track without hesitation, the participants obtained the normal hind footprints, which were to serve as a normal parameter for future comparisons. The footprints were obtained on strips of paper with the track dimensions, impregnated with bromophenol blue diluted at 1% in acetone, previously prepared, according to the method proposed by De Medinaceli et al.[9,10], Khullar et al.[15], Rochkind et al.[16] and modified by Rochkind et al.[17] and Lowdon et al.[18] The paper impregnated with bromophenol blue exhibits a yellow-orange hue when dry, but becomes permanently blue in contact with aqueous solutions. For the purposes of this study, domestic detergent was used to dampen the hind feet of the animals, as it presents the advantage of avoiding dispersion and smudging of the ink. After they dried, the paper strips were copied with a high resolution scanner and the scanned images were stored and analyzed in the computer by means of a graphic program especially developed for this purpose, allowing footprint handling and automatic calculation of the PFI.[19]

Operating procedure

The animals were operated under general anesthesia, with a mixture of 5% Ketamine and 2% Xylazine, in the proportion of 1:4, with the administration of doses of 0.10 to 0.15 ml/100 g of body weight of the animal, by intraperitoneal route. The animal was positioned in prone on an appropriate table, with hind and fore feet fixed in abduction, and the operating site on the animal's right thigh was prepared routinely, with trichotomy, antisepsis (20% iodized alcohol) and placement of a surgical drape. The sciatic nerve was approached through a longitudinal cutaneous incision about 3 cm long, from the trochanter major to the knee, followed by blunt dissection between the gluteus maximus and the quadriceps muscles. The nerve was traced up to its division at the three main branches (tibial, fibular and sural), with isolation of the fibular nerve, subject of the study, in which a lesion was produced by crushing around a segment 5 mm in length, immediately distal to its emission, applying adjustable forceps produced for this purpose and calibrated for a static load of 5,000 g, for ten minutes. (Figure 2) The calibration of the tweezers was performed upon every five consecutive applications, with the help of a load cell. After the ten minutes of application of the forceps, the fibular nerve was carefully detached and put back in its bed, then the surgical wound was closed by planes, with separate sutures of 5/0 nylon (Mononylon®, Ethicon).

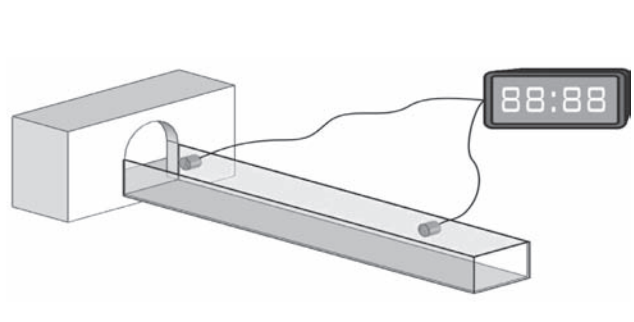

Figure 1 – *Diagram of the gait analysis track, with the track time recording sensors.*

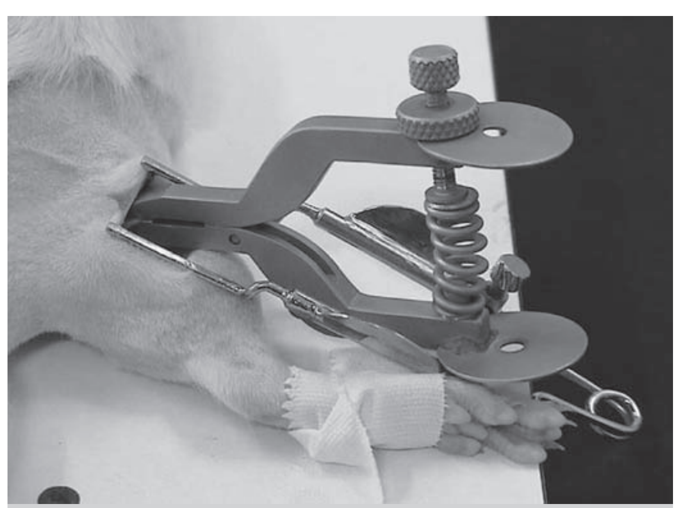

Figure 2 – *Representation of the nerve injury by crushing of the common fibular nerve.*

Laser irradiation

Laser irradiation (Figure 3) was performed with low power gallium-aluminum arsenide (GaAsAL)[1*] diode laser equipment, with the following features: Wavelength of 830 nm, emitter power of 100 mW, continuous mode, and dose of 140 J/cm^2. The irradiation was performed for 21 consecutive days, starting on the first postoperative day, by the punctual transcutaneous method, by static contact, at five points in the lesion region. Each irradiation point was at a distance of 1 cm from the other, with a time of 40 seconds per point, and a total time of 2 minutes of irradiation per animal.

Figure 3 – *Portable laser.*

Functional gait evaluation (PFI)

Sixty (2 x 30) footprints were analyzed, totaling 60 evaluations. The record of the images captured was analyzed by means of the FAPN - Functional Analysis of Peripheral Nerve computer program, developed in the Bioengineering Laboratory[2*3*], which calculated the parameters predetermined for the functional gait evaluation. (Figure 4) The program allows the analysis of all the functional indexes, but in this investigation only the functional fibular index (PFI, of peroneal), was used.

The following parameters were measured in the footprints: print length (PL), toe spread (TS) and intermediate toes (IT), in footprints considered normal (N) and experimental (E). These data were launched in the formula of Bain, Mackinnon, and Hunter (10) specifically for the functional fibular index (PFI):

$$PFI = 174.9 \times \left(\frac{EPL - NPL}{NPL}\right) + 80.3 \times \left(\frac{ETS - NTS}{NTS}\right) - 13.4$$

Statistical analysis

The linear regression model was used in this study, adopting the increase/decrease obtained after the intervention as variable-response for each one of the variables. This increase/decrease is achieved by the ratio of the respective values of each animal between the post- and preoperative periods (Post/Pre). The group to which the animal belonged was considered independent variable: groups 1 to 3 and control group. The analyses were conducted in the SAS 9.0 software through the PROC REG procedure, considering the significance level of 5% ($p<0.05$).

Figure 4 – *Visualization of the monitor screen, with two footprints, showing the key points to be clicked, with use of the program modified by Yamasita, Mazzer and Barbieri (2008).*

RESULTS

All the animals from Groups 1 to 3 presented moderate deformity of the right hind paw, with contracture in flexion of the paw and flexion-adduction of the toes, resulting from the paralysis of the extensor/eversor musculature of the paw and extensor of the toes. They were capable of resting their weight on the operated paw, but the gait was of the steppage kind, up to the end of the first postoperative week, when support became more complete. Appearance and support improved slowly on the rear hind paw in the next two weeks, which was reflected in the improvement of the PFI, but did not go back entirely to normal until the 21st day, end of the experimental period. Yet it was possible to distinguish that the gait was better in Group 3 (injury + effective irradiation) than in Groups 1 (injury, without irradiation) and 2 (injury + simulated irradiation).

The mean value of the preoperative PFI was -12.19, in the control group, and -5.61, -8.37 and -3.56, in Groups 1, 2 and 3, respectively, without statistical difference among the groups (Table 1). The time required to complete the track in this phase was 68.4 ms in the control group, and 69.6 ms, 62.4 ms and 62.7 ms, in Groups 1, 2 and 3, respectively, without statistical difference among groups (Table 2). The mean weight of the animals in the preoperative phase was 224 g in the control group, 225 g in Group 1, 226 in Group 2 and 219.5 in Group 3, with no statistical difference among groups. (Table 3)

On the 21st postoperative day, the mean value of PFI was -3.93 in the control group and -37.46 in Group 1, -26.59 in Group 2 and -4.16 in Group 3 (Table 1), with significant differences in the compa-

1* DMC, São Carlos, Brazil.
2,3* Yamasita, Mazzer, Barbieri, 2008.

Table 1 – *Description of the variable PFI, according to the groups and the period, and of the difference obtained before and after the intervention (21-0), in each one of the groups.*

Group	Period	N	Mean	SD	Minimum	Median	Maximum
PFI							
Control	Pre	5	-12.19	7.3	-24.23	-9.38	-6.03
	21 d	5	-3.93	1.32	-5.48	-4.14	-1.93
1	Pre	10	-5.61	1.88	-8.74	-5.01	-3.4
	21 d	10	-37.46	25.97	-81.99	-26.67	-13.54
2	Pre	10	-8.37	3.42	-15.19	-9.27	-3.18
	21 d	10	-26.59	20.63	-64.53	-17.35	-11.92
3	Pre	10	-3.56	2.6	-8.01	-2.84	-0.78
	21 d	10	-4.16	1.71	-6.39	-4.59	-1.37
Ratio							
Control		5	0.4	0.21	0.14	0.44	0.59
1		10	7.21	5.17	2.48	6.5	18.45
2		10	3.75	3.81	1.4	2.08	13.65
3		10	2.2	2	0.37	1.06	5.05

Table 2 - *Description of the variable TIME, according to the groups and the period, and of the difference obtained before and after the intervention (21-0), in each one of the groups.*

Group	Period	N	Mean	SD	Minimum	Median	Maximum
Time							
Control	Pre	5	68.04	2.41	65	69	71
	21 d	5	64.6	3.65	61	63	69
1	Pre	10	69.6	4.03	62	69.5	76
	21 d	10	79.5	1.65	77	79.5	82
2	Pre	10	62.4	7.12	55	60.5	75
	21 d	10	78.4	4.77	72	77	86
3	Pre	10	62.7	1.49	60	63	65
	21 d	10	52.9	1.79	50	53	56
Ratio							
Control		5	0.94	0.04	0.9	0.9	0.97
1		10	1.15	0.06	1.05	1.13	1.29
2		10	1.27	0.12	1.09	1.27	1.47
3		10	0.84	0.04	0.78	0.85	0.92

risons between the control group and Groups 1, 2 and 3 ($p<0.05$), and between Group 1 and Group 3, but not obtaining statistically significant difference between Groups 1 and 2, and Groups 2 and 3. Time spent on the walking track in the postoperative period was 64.6 ms in the control group and 79.5 ms, 78.4 ms and 52.9 ms in Groups 1, 2 and 3, respectively (Table 2), with no statistically significant differences among the groups in this period. The mean weight of the animals was 417 g in the control group and 415.5 g, 384.5 g and 389.5 g, in Groups 1, 2 and 3, respectively. (Table 3) The differences among groups in this period were not significant ($p<0.05$).

Table 3 – *Description of the variable WEIGHT, according to the groups and the period, and of the difference obtained before and after the intervention (21-0), in each one of the groups.*

Group	Period	N	Mean	SD	Minimum	Median	Maximum
Weight							
Control	Pre	5	224	11.94	210	225	240
	21 d	5	417	38.83	390	405	485
1	Pre	10	225	7.45	215	225	235
	21 d	10	415.5	41.19	375	405	495
2	Pre	10	226	7.38	215	227.5	235
	21 d	10	384.5	22.66	345	387.5	425
3	Pre	10	219.5	5.99	210	220	230
	21 d	10	389.5	8.96	380	387.5	405
Ratio							
Control		5	1.87	0.26	1.7	1.76	2.31
1		10	1.85	0.22	1.6	1.81	2.3
2		10	1.7	0.13	1.53	1.69	1.93
3		10	1.78	0.08	1.65	1.77	1.93

There were significant differences in the comparisons between pre- and postoperative values of weight of the animals for all the groups ($p<0.01$). For the time spent on walking track, the differences were significant for Groups 1, 2 and 3 ($p<0.01$), but not for the control group ($p=0.06$). For the PFI, the differences were significant for the control group and for Groups 1 and 2 ($p<0.01$), but not for Group 3 ($p=0.19$). (Table 4)

There were significant differences in the ratio between the values observed in the pre- and postoperative periods (increase/decrease) for the variable PFI in the comparison between the control group and Groups 1, 2 and 3 ($p<0.01$) and between Groups 1 and 3 ($p<0.01$), but not between Groups 1 and 2 ($p=0.06$) and

Table 4 – *Pre- and postoperative mean values for the three variables and increase/decrease ratio.*

Variable	Groups	Pre		Post		Increase/ Decrease (Ratio)		P
		Mean	SD	Mean	SD	Mean	SD	
PFI	Control	-12.19	7.3	-3.93	1.32	0.4	0.21	**< 0.01**
	Group 1	-5.61	1.88	-37.46	25.97	7.21	5.17	**< 0.01**
	Group 2	-8.37	3.42	-26.59	20.63	3.75	3.81	**< 0.01**
	Group 3	-3.56	2.6	-4.16	1.71	2.2	2	**0.19**
Time	Control	68.4	2.41	64.6	3.65	0.94	0.04	**0.06**
	Group 1	69.6	4.03	79.5	1.65	1.15	0.06	**< 0.01**
	Group 2	62.4	7.12	78.4	4.77	1.27	0.12	**< 0.03**
	Group 3	62.7	1.49	52.9	1.79	0.84	0.04	**< 0.01**
Weight	Control	224	11.94	417	38.83	1.87	0.26	**< 0.01**
	Group 1	225	7.45	415.5	41.19	1.85	0.22	**< 0.01**
	Group 2	226	7.38	384.5	22.66	1.7	0.13	**< 0.01**
	Group 3	219.5	5.99	389.5	8.96	1.78	0.08	**< 0.01**

between Groups 2 and 3 (p=0.07). For the variable time spent on walking track, the differences were significant in all the comparisons (p<0.01), while for the variable weight, there was no significant difference for any comparison. (Table 5)

Table 5 – *Comparisons of increase/decrease obtained among the groups, in the pre- and postoperative periods, for each variable*

Variable	Groups	Increase/Decrease (Ratio) Mean	SD	Group	Increase/Decrease (Ratio) Mean	SD	P
PFI	Control	0.4	0.21	Group 1	7.21	5.17	**< 0.01**
	Control	0.4	0.21	Group 2	3.75	3.81	**< 0.01**
	Control	0.4	0.21	Group 3	2.2	2	**< 0.01**
	Group 1	7.21	5.17	Group 2	3.75	3.81	**0.06**
	Group 2	7.21	5.17	Group 3	2.2	2	**< 0.01**
	Group 3	3.75	3.81	Group 3	2.2	2	**0.07**
TIME	Control	0.94	0.04	Group 1	1.15	0.06	**< 0.01**
	Control	0.94	0.04	Group 2	1.27	0.12	**< 0.01**
	Control	0.94	0.04	Group 3	0.84	0.04	**< 0.01**
	Group 1	1.15	0.06	Group 2	1.27	0.12	**< 0.01**
	Group 2	1.15	0.06	Group 3	0.84	0.04	**< 0.01**
	Group 3	1.27	0.12	Group 3	0.84	0.04	**< 0.03**
WEIGHT	Control	1.87	0.26	Group 1	1.85	0.22	**0.84**
	Control	1.87	0.26	Group 2	1.7	0.13	**0.08**
	Control	1.87	0.26	Group 3	1.78	0.08	**0.36**
	Group 1	1.85	0.22	Group 2	1.7	0.13	**0.06**
	Group 2	1.85	0.22	Group 3	1.78	0.08	**0.37**
	Group 3	1.7	0.13	Group 3	1.78	0.08	**0.29**

DISCUSSION

Despite its routine use for the treatment of musculoskeletal tissue lesions, laser is still a physical resource that is not well known, as refers both to the parameters of use, and its effects. There is a great deal of evidence that laser interferes with the function of the peripheral nerves, and might decrease the latency time and increase the speed of conduction of a normal nerve[1,5,8] besides accelerating the morphological and functional regeneration of an injured nerve.[7,8,20-22] According to the observation of these authors, low power laser increases the quantity of connective tissue (collagen) and accelerates the healing of the injury,[7,15] increases the number of fibroblasts and of mielinized axons at the site of the injury, accelerates nerve conduction,[23] induces faster recovery of muscular trophism[7] and promotes significant functional recovery,[7,22,23] and it acts both in regeneration and in the prevention of degeneration of the nerve fibers.16 Nevertheless, the conclusions of the various studies show an unacceptable degree of controversy, demonstrating that there is not yet a consensus about the true effects of laser irradiation in peripheral nerve regeneration.

Indeed, on one hand Rochkind et al.[16], Khullar et al.[6] and Gigo-Benato et al.[7] encountered positive effects for nerve regeneration. But, Bagis et al.[5] concluded that laser is inefficient in the repair of nerve injuries, as they did not find any evidence of improvement in nerve regeneration, which can be attributed, however, to the short time of treatment (7 consecutive days) and to the type of emission employed (pulsed and of low intensity). In a previous study carried out in our laboratory, but with the sciatic nerve of the rat, it was demonstrated that the morphological and functional recovery were more complete in the animals submitted to laser irradiation, although in a much lower dose than in this investigation.[8] These results were the starting point for this investigation, now using the common fibular nerve of rats and focused only on functional recovery, which is ultimately what really matters.

Until recently, it was believed that cells need prolonged applications of laser to reach a response threshold and that as well, high power therapeutic equipment could be delivering energy very rapidly and preventing adequate cellular absorption. However, some authors started to work with higher powers, initially with 35 to 50 mW and currently, between 100 and 300 mW, thus delivering greater energy densities.[19,24] They reached the conclusion that when dealing with high energy density, faster application results in greater cellular absorption, which induces more satisfactory responses. For this reason a dose of 140 J/cm^2 was employed in this investigation, much higher than in other investigations.

The experimental model employed was that of the controlled crush injury, now making use of forceps especially developed for this purpose, which can be calibrated with the same loads used in the universal testing machine[12] or in the deadweight machine,[25,26] with the advantage that it is much easier to use and does not submit the studied nerve to traction tensions, as is the case during adaptation to the aforesaid machines. The load employed to produce the lesion, however, was 5,000 g, lower than in the previous studies, in which a load of up to 15,000 g was employed, as the goal of the investigation was to produce a lesion that was not very severe, and that could benefit more quickly from laser irradiation, since the study period would be only 21 days. In actual fact, it was demonstrated that, starting from 5,000 g and up to 10,000 g, the type (axonotmesis) and the degree of injury do not change significantly with the increase of the load applied.[25,26] With loads of 10,000 g to 15,000 g, the injury is still of the axonotmesis type, without destruction of the supporting framework of the nerve, but much more severe, as there is preservation only of fibers of very small diameter.

In this stage of the investigation, the participants opted to perform just the functional gait evaluation, not reporting yet the results of other analyses to be performed on the nerve. Indeed, it was demonstrated that the functional evaluation is closely related to the morphometrical analysis, and may, in certain situations, be carried out and analyzed separately.[1,2,12] The method employed for the functional gait evaluation of rats is of routine use in our laboratory, particularly for investigations of the sciatic nerve, and is based on the method initially introduced by De Medinaceli et al.[9,10] and improved by Bain et al.[11] and by Lowdon et al.[18] The rat footprints were scanned and stored and analyzed in a computer, with the use of a specific program for this purpose, developed in our laboratory and employed routinely for this type of study. We must take into consideration the fact that the pathological gait pattern varies with the injured nerve; in the case of the fibular nerve, there is paralysis of the extensor/eversor musculature of the paw and extensor muscles of the toes, resulting in plantar flexion/inversion of the paw and flexion/adduction of the toes, with gait becoming very difficult, particularly in the first week after the injury, when the weight was borne practically on the dorsolateral surface of the paw and dorsal surface of the toes. With functional recovery, weight is gradually borne by the distal extremity of the paw and the plantar surface of the toes, resulting in a plantar print that is shorter and narrower than usual, as there is considerable reduction of total toe spread, despite the integrity of the intrinsic paw musculature.[10] In a previous investigation of our group, it was demonstrated that the obtainment of prints of good quality and easier interpretation is only possible from the third week, for sciatic nerve injuries,[21] and such a strategy was adopted in this investigation, only performing the functional evaluation on the 21st day and not weekly.

The results showed that, as expected, the animals gained significant weight between the preoperative period and the 21st postoperative day, but this apparently did not influence the speed at which they walked on the track. Indeed, on the 21st day, gait was slower in Groups 1 (injury, without irradiation) and 2 (injury + sham irradiation), in which the PFI did not present significant improvement, having been faster in Group 3 (injury + effective irradiation), in which the PFI improved practically to normal, since there was no significant difference ($p=0.19$) between the values obtained in the pre- and postoperative evaluations. As observed in previous investigations.1 the preoperative PFI preoperative, as well as the SFI (sciatic) and TFI (tibial), was never equal to zero, ranging between -3.56, in Group 3, and -12.19, in the control group, which can be considered a deficiency of the mathematical formula employed for its calculation, or a fault in the method used to measure the parameters, which might also be present in the calculation of the postoperative PFI's, but attaining all groups and certainly not to the extent of producing such a significant and elevated difference as that observed between Group 3 (-4.16) and Groups 1 (-37.46) and 2 (-26.59). Is worth remembering that the higher PFI in Group 2 than in Group 3 might mean that simulated irradiation (placebo) had a positive effect on regeneration, which, however, is not likely.

CONCLUSION

Low power GaAsAl laser irradiation accelerated, and probably potentialized the regeneration process of the fibular nerve of rats on the 21st day after the controlled crush injury and as verified by the functional gait evaluation.

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