

Effect of the laser therapy in association with swimming for a morphological nerve repair and functional recovery in rats submitted to sciatic axonotmesis

Efeito da associação da laserterapia com a natação no reparo morfológico do nervo isquiático e na recuperação funcional de ratos submetidos à axonotmese

Efecto de la asociación entre la laserterapia y la natación en la recuperación morfológica del nervio isquiático y en la recuperación funcional de ratas sometidas a axonotmesis

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ABSTRACT | The peripheral nerve injuries occur frequently and generally cause functional loss impacting negatively on patient's life. The objective this study was to verify the efficiency of the combination of laser therapy and swimming in rats affected by axonotmesis. The sample was comprised of 50 Wistar rats and it was divided into 05 groups: Control Group; Surgical Control Group; Laser Experimental Group; Swimming Experimental Group and Laser Experimental combined with Swimming Group. The nerve was crushed into a 5 mm-long segment next to the sciatic nerve trifurcation with a pair of forceps for 60 seconds. The GaAs infrared laser (904nm) was used with energy radiated 0,4J the first week, the second week 0,8J and 1,2J in the third and fourth week. For functional (FCI) evaluation, the animals were immobilized and the plantar region of their paws were painted with ink stamp. The procedure was repeated twice to each animal. The nerve morphometry (areas, diameters and thicknesses of the fibers, axons and myelin sheath) was performed with the measurement of 220 fibers per animal in each group. We can see that the GEL and GEN groups, obtained the best results when compared with the other groups (GC, GCC and GELAN) in all morphometric variables studied, but no statistically significant difference

was found between them. In functional analysis, it was observed that the gelan group obtained the best results when compared with the other groups (GCC, GEN and GEL) and when the GEL and GEN groups were compared, there was no statistically significant difference between them. Was concluded the GEL and GEN groups had the best morphometric results, while the GELAN showed the best functional outcome. Therefore, it can be concluded that the combination of these features favoured the functional recovery of the animals

Keywords | Swimming; Laser Therapy; Rats, Wistar; Regeneration; Physiotherapy.

RESUMO | As lesões de nervos periféricos ocorrem frequentemente e, de modo geral, causam perda funcional impactando de forma negativa na vida do paciente. O objetivo do estudo foi verificar a eficiência da associação da laserterapia e natação em ratos acometidos por axonotmeses. A amostra foi composta por 50 ratos da linhagem Wistar. Foram divididos em 5 grupos, sendo: grupo controle (GC); grupo controle cirúrgico (GCC); grupo experimental laser (GEL); grupo experimental natação (GEN) e grupo experimental laser associado à natação (GELAN). O nervo foi esmagado em um segmento de 5

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mm de comprimento próximo a trifurcação do nervo isquiático, feito com uma pinça durante 60 segundos. Foi utilizado o laser infravermelho AsGa (904nm) com energia irradiada de 0,4 J na primeira semana, 0,8 J na segunda semana e 1,2 J na terceira e quarta semana. Para avaliação funcional (IFC), os animais foram imobilizados, e a região plantar das patas foram pintadas com tinta de carimbo. Esse procedimento foi repetido duas vezes com cada animal. Foi realizada a morfometria (áreas, diâmetros e espessuras das fibras, axônios e bainha de mielina) dos nervos com mensuração de 220 fibras por animal de cada grupo. Pudemos observar que os grupos GEL e GEN, em todas as variáveis morfométricas estudadas, obtiveram os melhores resultados, quando comparados com os outros grupos (GC, GCC e GELAN), mas não apresentou diferença estatisticamente significativa entre eles. Na análise funcional observou-se que o grupo GELAN obteve o melhor resultado quando comparado com os outros grupos (GCC, GEL e GEN) e quando comparados os grupos GEL e GEN entre eles não houve diferença estatisticamente significativa. A conclusão foi que os grupos GEL e GEN obtiveram os melhores resultados morfométricos, enquanto o GELAN apresentou o melhor resultado funcional. Portanto, pode-se concluir que a associação destes recursos favoreceu a recuperação funcional desses animais.

Descritores | Natação; Terapia a Laser; Ratos Wistar; Regeneração; Fisioterapia.

RESUMEN | Las lesiones de los nervios periféricos frecuentemente ocurren, y generalmente ocasionan pérdida funcional, lo que les causa daño a la vida de los pacientes. En este artículo se propone a verificar la eficacia de la asociación de la laserterapia

y de la natación en ratas sometidas a axonotmesis. El muestro se compuso de 50 ratas Wistar. Se las dividieron en 5 grupos: grupo control (GC); grupo control quirúrgico (GCQ); grupo experimental láser (GEL); grupo experimental natación (GEN) y grupo experimental láser asociado con la natación (GELAN). Se aplastó con una pinza durante 60 segundos el nervio en un segmento de 5 mm de extensión cerca de la trifurcación del nervio isquiático. Se empleó el láser infrarrojo AsGa (904nm) con energía irradiada de 0,4 J en la primera semana, 0,8 J en la segunda y 1,2 J en la tercera y cuarta semanas. Para la evaluación funcional (IFC), se los inmovilizaron los animales y se los pintó con tinta estampilla la región plantar de las patas. Se repitió dicho procedimiento dos veces en cada animal. Se realizó la morfometría (áreas, diámetros y espesuras de las fibras, axônios y vaina de mielina) de los nervios con mensuración de 220 fibras por cada animal de cada grupo. Se notó que los grupos GEL y GEN, en todas las variables morfométricas estudiadas, presentaron los mejores resultados, en comparación con los otros grupos (GC, GCQ y GELAN), sin embargo no presentó diferencia estadísticamente significativa entre ellos. En el análisis funcional se observó que el grupo GELAN tuvo el mejor resultado en comparación con otros grupos (GCQ, GEL y GEN), y al comparar los grupos GEL y GEN no presentaron diferencia estadísticamente significativa. Se concluyó que los grupos GEL y GEN tuvieron mejores resultados morfométricos, mientras que el GELAN presentó el mejor resultado funcional. Por lo que se concluye que la asociación de dichos recursos les favoreció la recuperación funcional de dichos animales.

Palabras clave | Natación; Terapia por Láser; Ratas Wistar; Regeneración; Fisioterapia.

INTRODUCTION

Peripheral nerve injuries are a common occurrence and generally cause the patient to suffer functional loss¹ that consequently has a negative effect on his/her life². The most common causes of peripheral nerve injury are firearm projectiles, falls and penetrative or contusion trauma, however, the main causative factor are automobile accidents³.

Peripheral nerve injury is more frequent in individuals aged between 25 and 40 years. This relatively young age may cause significant economic and social consequences due to functional disability occurring in early life, as most individuals are at the prime of their professional capacity during this

period. Thus, following an injury, any treatment that leads to a more accelerated functional recovery of the peripheral nerves is extremely valuable to society⁴. The degenerative process in the muscle is known to start shortly after nerve injury, therefore, intervention to re-establish myoneural interaction must be swift⁵.

There are several surgical techniques and treatments that have been developed⁶, these help to regenerate the peripheral nerves with the aim of morphological and functional improvement in less time^{7,8}.

Exercise is the preferred method out of the rehabilitation strategies that are currently used, mainly due to it being characterized as a non-invasive therapeutic resource⁹ and that it represents an important

mechanism for releasing neurotrophic factors, especially the mainly Brain-Derived Neurotrophic Factor (BDNF), which is considered paramount in terms of mediating neuroplasticity^{10,11}.

Several authors have shown beneficial results by using exercise, namely through greater budding and extension of the axons¹², increased number of myelinated nerve fibers,^{13,14} and improved functional recovery of the injured member¹⁵⁻¹⁷. There have been extensive discussions in the scientific community¹⁸ regarding recommendations as per the type of exercise as well as its duration and intensity.

Another feature used with the objective of promoting functional improvement for the injured person is phototherapy. Studies from the late 80s saw the beginning of thorough investigatory efforts whose purpose was to evaluate the protocols used as a therapeutic resource for regenerating peripheral nerves, these studies used crush injury¹⁹ as an experimental model. In modern times these protocols are still used as a great therapeutic resource for peripheral nerve regeneration²⁰.

Laser therapy, as an effective complementary therapeutic resource for treating nerve injuries, has gained prominence in physical therapy intervention protocols, which is mainly due to it being a non-invasive treatment that provides positive results for both regeneration and functional recovery. Among these benefits are: an anti-inflammatory and anti-oedematous effect, potential for wound healing, pain relief, increased mitochondrial respiration, ATP synthesis and proliferation of fibroblasts, stimulating the proliferation of Schwann cells that secrete neurotrophic factors for nerve regeneration, among other factors²¹⁻²⁴.

Based on the belief in the beneficial effects of laser therapy and physical exercise, while considering the importance of functional recovery and returning to daily activities, the objective of this research was to investigate the effectiveness of laser therapy and swimming regarding the morphofunctional characteristics of rats that have been subjected to axonotmesis. It is our belief that the association of these protocols, in addition to the laser energy being gradually increased over the time of the injury, makes it possible to trigger a more efficient regenerative process that can provide the individual with more effective functional improvement.

METHODOLOGY

This study was approved by the Research Ethics Committee under the protocol number 034/2012.

50 male, 80-day old, Wistar rats (*Rattus norvegicus albinus*) from the central bioterium were used. The animals were kept in cages and given ad libitum access to food and water in a controlled environment (temperature between 21 to 25°C with 12 hours in the light and 12 hours the dark) with no restrictions on movement.

The 50 animals were randomly divided in five groups (n=10, for each group) for axonotmesis, these being:

Control group (CG), where the animals were not subject to any intervention and observed for 30 days.

Surgical Control Group (SCG), where the animals were put through the surgical intervention, with the induction of nerve injury through crushing, however, they were not subjected to any treatment protocol following the surgery.

Experimental Laser Group (ELG), in which the animals were put through the surgical intervention, with the induction of nerve injury through crushing, and, subsequent to surgery, were treated with the laser therapy protocol.

Experimental Swimming Group (ESG), in which the animals were put through the surgical intervention, with the induction of nerve injury through crushing, and, subsequent to surgery, were subjected to the swimming exercise protocol.

Experimental Laser together with Swimming Group (ELSG), in which the animals were put through the surgical intervention, with the induction of nerve injury through crushing, and, subsequent to surgery, were treated with laser therapy and subjected to the swimming exercise protocol.

In order to perform the surgical procedure on the nerve, the animals were anesthetized with a drug combination of Ketamine Hydrochloride (80 mg/kg) and Xylazine Hydrochloride (15 mg/kg). The surgical procedure involved a 5cm incision on the dorsolateral region of the left pelvic limb of the animal. Following the incision, the adjacent musculature was separated in order to expose the right sciatic nerve.

The nerve crushing was performed on a 5mm long segment of the sciatic nerve, close to the trifurcation where the fibular nerve, tibial nerve and sural nerve originate. The procedure was performed using a hemostat for a period of 60 seconds, comprising

of three 20-second stages. The musculature and skin were sutured with 4-0 thread following the procedure.

The laser instrument used in the laser therapy groups was an AsGa-904nm (Arseneto de Gálio, ENDOPHOTON - KLD). The irradiation was performed on the skin of the animal's right pelvic limb, on the injured segment of the nerve. The procedures used by previously published studies with the same pattern were used^{25,26}. The instrument was properly calibrated before the applications were performed.

The laser was applied, three times a week, for exactly 8 seconds in the first week, 16 seconds in the second week and 24 seconds in the third and fourth for a total treatment time of 4 weeks. The animals were held by hand during the application of the laser.

The following parameters used were: 904nm wavelength, 50mw power, 40J/cm² energy fluency or density in the first week, 80J/cm² in the second week and 120J/cm² in the third and fourth week, the radiated energy was 0.4J in the first week, 0.8J in the second week and 1.2J in the third and fourth week. The purpose of increasing the dose was to advance the stages of peripheral nerve injury from the acute to subacute phase, and later to the chronic phase.

For the groups being submitted to the swimming exercise, the animals were placed in a glass tank with warm water (32±2°C), filled to a 40cm depth. The swimming exercises were performed five times a week, with Saturdays and Sundays being rest days. During the first week, the animals swam exclusively for 20 minutes on the first day, with 10 extra minutes being added each day up to a 40 minute duration, a time that was kept constant until the experiment's conclusion.

In order to evaluate the animals' functional capability, they were immobilized and placed in an acrylic pathway to walk on. The animals were filmed during their walk so that the footprints from their tracks could be recorded. This procedure was repeated with each animal twice. The distance between the footprints made by the hind limbs were evaluated according to the equation as described by Bain²⁷, based on studies by De Medinacelli²⁸. The videos were converted into sequential photos while following a calibration pattern for each image. The Sciatic Functional Index was used to evaluate functional recovery. The measurements were taken using 4.6.2 Image Pro plus software, and the data

were subjected to statistical analysis according to the P<0.05 index for all samples.

In order to collect the samples, the animals were anesthetized with a drug combination of Ketamine Hydrochloride (80 mg/kg) and Xylazine Hydrochloride (15 mg/kg), which was applied via intramuscular injection into the dorsolateral region of the left pelvic limb of the animal. Following the surgical sample collections, the animals were given a lethal dose of 150 mg/kg sodium pentobarbital and 2% lidocaine (20 mg/mL), which was administered intraperitoneally.

During the treatment, the histological samples from the sciatic nerve were fixed in Karnovsky solution, included in histo-resin and stained with osmium tetroxide. The samples were processed on a microtome in order to obtain the 5µm thick histological cuts.

The morphometry of the nerves was performed by measuring 220 fibers per animal in each group, which was done using a microcomputer with image capturing and analysis software linked to an optical microscope. The morphometric variables that were studied in the nerves are as follows: area of the fibers, area of the axons, minimum diameter of the fibers, minimum diameter of the axons, area of the myelin sheath and thickness of the myelin sheath.

The analysis of variance test (ANOVA) was used to compare the groups, followed by the TUKEY test when there was a significant difference detected. A paired T-test was used to make comparisons between the experimental and normal sciatic nerves. A significance level of p <0.05 was adopted for all analyses.

RESULTS

Upon comparing the area and the diameter of the nerve fiber, the ELG and ESG groups presented the best results (area = 38.42 µm² and 37.56 µm², respectively, and diameter = 4.02 µm and 3.96 µm, respectively) compared with the SCG and ELSG groups, which had 21.47 µm² and 28.76 µm² for area and 2.87 µm and 3.35 µm for diameter, respectively. The CG presented the highest mean for area with 50.12 µm² and 8.95 µm for diameter.

The data for the area and diameter of the nerve fibers can be seen in Table 1.

Table 1. Mean values and standard deviation for the area of the nerve fibers (m²) and mean values and standard deviation of the smaller diameter of the nerve fibers (μm)

	Area of the Nerve Fiber		Diameter of the Nerve Fiber	
	Mean	SD	Mean	SD
CG	50.12 ^a	4.77	8.95 ^a	0.22
SCG	21.47 ^b	3.18	2.87 ^b	0.14
ELG	38.42 ^c	5.82	4.02 ^c	0.20
ESG	37.56 ^c	4.52	3.96 ^c	0.21
ELSG	28.76 ^d	3.27	3.35 ^d	0.45

Different letters indicate a statistical difference (p <0.05)

Based on the results from Table 2, it is possible to see that the area and the diameter of the axon from the ELG and ESG groups had the best results in the two variables analyzed (area = 11.17μm² and 12.46μm², and diameter = 2.98μm and 3.06μm, respectively) when compared with the SCG and ELSG groups, which had 4.75μm² and 9.06μm² for the area of the axon and 2.05 μm and 2.57μm for the diameter of the axon. The CG showed the largest values with 15.05μm² of area and 5.1 μm of diameter.

Table 2. Mean values and standard deviation of the area of the axons (m²) and mean values and standard deviation of the smallest diameter of the axons (μm)

	Area of the Axon		Diameter of the Axon	
	Mean	SD	Mean	SD
CG	15.05 ^a	0.80	5.13 ^a	0.18
SCG	4.75 ^b	1.28	2.05 ^b	0.22
ELG	11.17 ^c	0.95	2.98 ^c	0.31
ESG	12.46 ^c	0.87	3.06 ^c	0.32
ELSG	9.06 ^d	0.94	2.57 ^d	0.56

Different letters indicate a statistical difference (p <0.05)

According to the results from Table 3, it is possible to see that the area and the thickness of the sheath from the ELG and ESG groups showed the best results (area = 25.25μm² and 25.10 μm², respectively; thickness = 2.86μm and 2.72μm, respectively) when compared with the SCG and ELSG groups, which showed 7.72m² and 21.70μm² in the area of the sheath, and 1.82μm and 2.28μm in the thickness of the sheath, respectively. The CG presented the highest values with 43.07μm² in the

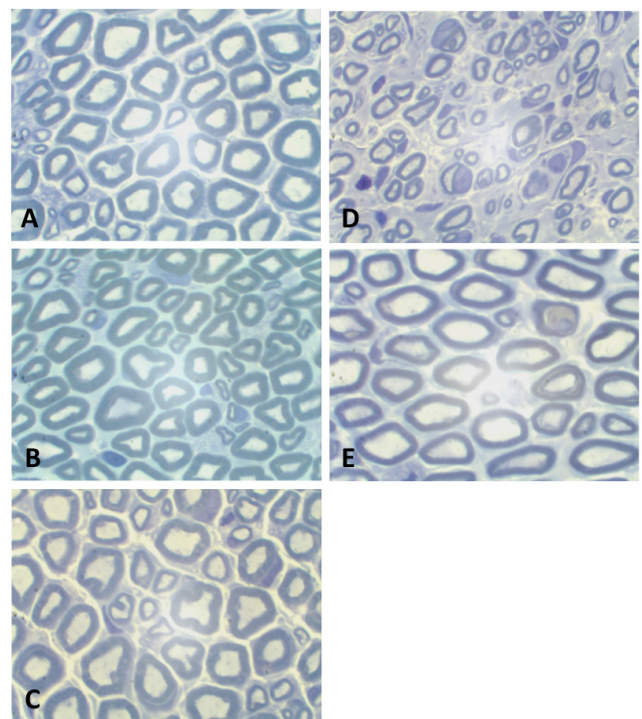
area of the sheath and 3.82μm in the thickness of the sheath.

Table 3. Mean values and standard deviation of the areas of myelin sheaths (m²) and mean values and standard deviation of the thicknesses of the myelin sheaths (μm)

	Area of Myelin Sheath		Thickness of Myelin Sheath	
	Mean	SD	Mean	SD
CG	43.07 ^a	1.80	3.82 ^a	0.33
SCG	7.72 ^b	4.56	1.82 ^b	0.25
ELG	25.25 ^c	1.74	2.86 ^c	0.37
ESG	25.10 ^c	2.46	2.72 ^c	0.45
ELSG	21.70 ^d	2.93	2.28 ^d	0.82

Different letters indicate a statistical difference (p <0.05)

Figure 1 shows the photomicrographs from the study groups and the morphological differences presented by these groups.

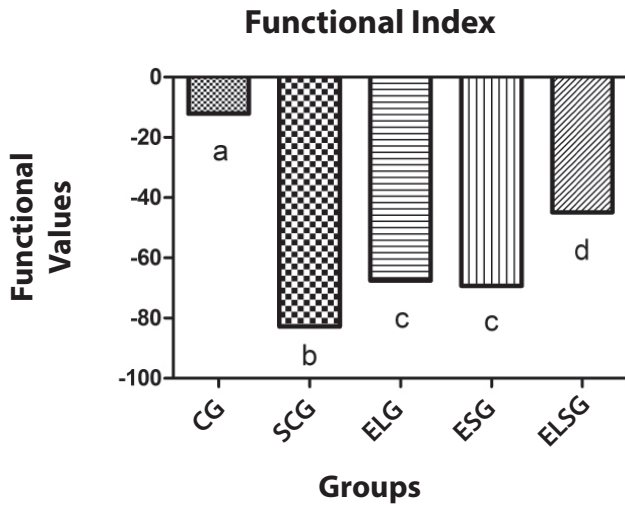


(A) ELG; (B) ESG; (C) ELSG; (D) SCG and (E) CG

Figure 1. Plate of the micrographs from the groups showing the morphology of the nerve (1000x)

Graphic 1 shows that the functional analysis results from the ELSG group were better than the other groups (SCG, ELG and ESG), with there being a statistically significant difference between them. The best functional

analysis result is the one closest to the control group. Different letters indicate statistical difference ($p < 0.05$).



Graphic 1. Graph showing the functional result of the groups. The ELSG Group showed a result closest to the CG

The ELSG groups had a result of -44.90, which was the best due to it being closest to the CG result (-12.11). The ESG and ELG groups did not show any statistical difference, reaching -69.32 and -67.51, respectively. As a result of being denervated, the SCG group had the worst result (-82.81).

DISCUSSION

Several authors have chosen to work experimentally using crushing²⁹ when studying peripheral nerve injuries, which are classified by Seddon³⁰ as an axonotmesis. This method partly preserves important support structures, such as: the endoneurium, the perineurium and the tubules from the Schwann cells, which function to guide the new axon in regenerating up to the target organ.

Several experiments involving denervated animals¹⁸ have described exercise as the factor that provides the most budding and extension of the axons,¹² the greatest increase in the number of myelinated fibers^{13,14} and the best improvement in functional recovery for the injured member¹⁵. The results from this study are similar to those found in the literature regarding evaluating functional recovery, due to the fact that the groups that performed exercise showed better results compared to groups who did not go through the swimming protocol. Other authors refer to the harmless effects of physical exercise on the peripheral nerve regeneration process^{31,32}.

Despite being somewhat controversial, some authors have suggested exercise as a complementary therapeutic resource³³ and, when combined with other therapeutic practices such as electrotherapy³⁴ or phototherapy^{6,19}, can result in a better recovery prognosis for peripheral nerve injuries. The ELSG group in this research, which combined laser therapy with swimming, presented a better functional result when compared to the ELG and ESG group, respectively.

Based on clinical and experimental research, there is evidence that some of the effects of laser therapy are an improvement in nerve function, increased metabolism of the neurons and greater production capacity of the myelin sheath. Due to laser therapy not being invasive, the ability to radiate injured nerves without intervention is useful³⁵.

Generally speaking, publications whose treatments involve a continuous laser output produced positive findings regarding peripheral nerve regeneration, however, the energy density used varies greatly, presenting data from $1.2\text{J}/\text{cm}^2$ to $140\text{J}/\text{cm}^2$. Thus, a laser energy density that became gradually greater over time, during the course of the injury was chosen for this study, which ranged between $40\text{J}/\text{cm}^2$, $80\text{J}/\text{cm}^2$ and $120\text{J}/\text{cm}^2$ week by week in order to find the ideal laser dosage (PISTARINI *et al.*, 2015; SOUSA *et al.*, 2013; LINS *et al.*, 2010; NORONHA *et al.*, 2004; LUCAS *et al.*, 2003)^{25,26,36-41}.

Very little is known regarding the role of laser irradiation in terms of rehabilitating tissues of the locomotor system. However, laser irradiation is widely used to treat a variety of pathological conditions of the musculoskeletal system, including the peripheral nerves²⁵.

Light energy absorption by nerve tissue increases ATP synthesis and cell proliferation, which increases axonal energy metabolism, improving scarring in the regenerative process, and thereby, the expression of neurotrophic factors, such as: GAP-43 protein, TGF-1, expression of the GCRP gene, which increase the regeneration rate and directs the axon to the target organ. The increase of axonal budding is also described as a result of the laser irradiation^{42,43}.

During research performed by Reis³⁵, the mean SFI (sciatic functional index) in the control group was -96.3, which is close to the figure found during this study, whose mean was of -82.81. The experimental laser group from the Reis research was different, with an average of -88.20 (-67.51 in our study).

According to Camargo²⁶, who also used the AsGa laser on peripheral nerve regeneration, the mean SFI was -47.71, which represents an even better result compared with the data from this study.

During a study conducted by Endo²⁵, who used low-level laser therapy to accelerate the regeneration of peripheral nerves, there was a progressive improvement of the SFI, both in irradiated and control nerves (69% and 45%, respectively). According to Endo²⁵, the fiber density increased for the irradiated nerves and decreased for the control nerves, showing a significant difference between the two ($p=0.001$). The authors concluded that low-level laser therapy effectively accelerates sciatic nerve regeneration in rats. This study also presented an increase in the area of the fibers, with the experimental laser group and the surgical control group obtaining values of $38.42\mu\text{m}^2$ and $21.47\mu\text{m}^2$, respectively.

According to a study performed by Oliveira³¹, which involved using electrical stimulation and swimming for nerve regeneration and functional recovery during the acute phase of a axonotmesis in mice, the diameter of the axon was lower in the denervated groups, and that, when compared together, the group for which the best results were observed was the swimming group with the following values: 6.32 ± 0.36 in the control group; 3.45 ± 0.64 in the denervated group; 3.67 ± 0.41 in the denervated + electro stimulation group; 4.34 ± 0.69 in the denervated + swimming group; 4.04 ± 0.38 in the denervated + swimming + electro stimulation group. Our study observed the same, the best result in relation to the diameter of the axon was in the swimming group.

During this study, the group in which there was an association between therapy and exercise, despite not showing good morphological results, presented an improvement in the functional analysis. We associate this improved functional result to the exercise, which requires the animal to release neurotrophic factors, and also to effect from the laser, which provides an anti-inflammatory, anti-oedematous and analgesic effect.

We believe that further research should be conducted in order to identify the expression of proteins involved in the peripheral nerve regenerative process, as well as to verify the muscular response to newly received innervation, which establishes a correlation between the mioneural interaction and possible readaptation by the motor plates

CONCLUSION

Based on the results presented here, the laser and isolated swimming were observed to promote a morphological improvement during the evaluation of nerve regenerative process, however, no statistically significant difference between them was found. However, the association of laser therapy and swimming was beneficial for functional recovery following peripheral nerve injury.

Therefore, the conclusion is that laser therapy and swimming can promote the efficient morphological recovery of rats with peripheral nerve injury, and that associating these resources demonstrated a tendency for functional recovery. New swimming protocols should be therefore investigated with a view to establish a direct relationship between exercise intensity and functional recovery.

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