


# Assessment of the Effects of Swimming as a Postoperative Rehabilitation on Nerve Regeneration of Wistar Rats Submitted to Grafting of Autologous Nerves after Injury to the Sciatic Nerve\*

## *Avaliação dos efeitos da natação como reabilitação pós-operatória na regeneração nervosa de ratos da linhagem Wistar submetidos a enxerto de nervos autólogos após lesão do nervo ciático*

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### Abstract

**Objective** To evaluate the effects of swimming on nerve regeneration after sciatic nerve injury in Wistar rats.

**Methods** A total of 30 Wistar rats was divided into 3 groups: Sham + Nat group animals that were not submitted to graft surgery and were submitted to swimming ( $n = 10$ ); Graft group: animals submitted to autologous sciatic nerve graft ( $n = 10$ ); and Graft + Nat group: animals submitted to autologous sciatic nerve graft surgery and to swimming ( $n = 10$ ). The results were analyzed on the software (GraphPad Software, San Diego, CA, USA).

**Results** In the first evaluation, all sciatic functional index (SFI) values were similar ( $p = 0.609$ ). Thirty days after the surgical procedure, we observed differences between all the comparisons: Sham + Nat ( $-34.64 \pm 13.89$ ) versus Graft ( $-145.9 \pm 26.06$ ); Sham + Nat versus Graft + Nat ( $-89.40 \pm 7.501$ ); Graft ( $-145.9 \pm 26.06$ ) versus Graft + Nat ( $-89.40 \pm 7.501$ ). In the measurements (60 and 90 days), there was no statistical difference between the Graft and Graft + Nat groups, with significantly lower values in relation to the control group ( $p < 0.001$ ). The number of motor neurons presented differences in the comparisons between the Sham + Nat and Graft groups ( $647.1 \pm 16.42$  versus  $563.4 \pm 8.07$ ;  $p < 0.05$ ), and between the Sham + Nat and Graft + Nat groups ( $647.1 \pm 16.42$  versus  $558.8 \pm 14.79$ ;  $p < 0.05$ ). There was no difference between the Graft and Graft + Nat groups.

### Keywords

- ▶ peripheral nerve injuries
- ▶ nervous Regeneration
- ▶ swimming
- ▶ sciatic nerve

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**Conclusion** Animals submitted to the swimming protocol after the sciatic nerve grafting procedure did not present differences in the SFI values and motor neuron numbers when compared to the control group. Therefore, this type of protocol is not efficient for the rehabilitation of peripheral nerve lesions that require grafting. Therefore, further studies are needed.

## Resumo

**Objetivo** Avaliar os efeitos da natação na regeneração nervosa após a lesão do nervo ciático em ratos Wistar.

**Métodos** Um total de 30 ratos Wistar foram divididos em 3 grupos: grupo Sham + Nat: animais que não foram submetidos à cirurgia de enxerto e foram submetidos à natação ( $n = 10$ ); grupo Enxerto: animais que foram submetidos à cirurgia de enxerto autólogo de nervo ciático ( $n = 10$ ); e grupo Enx + Nat: animais submetidos à cirurgia de enxerto autólogo de nervo ciático e à natação ( $n = 10$ ). Os resultados foram analisados pelo software GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, EUA).

**Resultados** Na primeira avaliação, todos os valores do índice funcional do ciático (IFC) foram semelhantes ( $p = 0.609$ ). Após 30 dias do procedimento cirúrgico, foram observadas diferenças entre todas as comparações: Sham + Nat ( $-34,64 \pm 13,89$ ) versus Enxerto ( $-145,9 \pm 26,06$ ), grupos Sham + Nat versus Enx + Nat ( $-89,40 \pm 7,501$ ), grupos Enxerto ( $-145,9 \pm 26,06$ ) versus Enx + Nat ( $-89,40 \pm 7,501$ ). Nas medidas (60 e 90 dias), não houve diferença estatística entre os grupos Enxerto e Enx + Nat, com valores significativamente menores em relação ao grupo controle ( $p < 0,001$ ). O número de motoneurônios apresentou diferenças nas comparações entre os grupos Sham + Nat e Enxerto ( $647,1 \pm 16,42$  versus  $563,4 \pm 8,07$ ;  $p < 0,05$ ) e Sham + Nat e Enx + Nat ( $647,1 \pm 16,42$  versus  $558,8 \pm 14,79$ ;  $p < 0,05$ ), não havendo diferença entre os grupos Enxerto e Enx + Nat.

**Conclusão** Os animais submetidos ao protocolo de natação após o procedimento de enxerto do nervo ciático não apresentaram diferenças nos valores de IFC e nos números de motoneurônios quando comparados com grupo controle. Portanto, este tipo de protocolo não é eficiente para reabilitação de lesões nervosas periféricas que necessitam de enxerto, sendo necessários novos estudos.

## Palavras-chave

- ▶ traumatismos dos nervos periféricos
- ▶ regeneração nervosa
- ▶ natação
- ▶ nervo ciático

## Introduction

Peripheral nerve lesions are frequent in the clinical practice and represent a health problem capable of generating disability in the population.<sup>1</sup> The etiological factors most associated with injuries include: motor vehicle collision, penetrating injury, and sports-related injuries.<sup>2</sup>

Morphologically, there is an increase in intramuscular connective tissue and muscular atrophy evidenced by the decrease in the cross-sectional area of muscle fibers.<sup>3</sup> These changes cause pain and tenderness along the nerve fiber,<sup>4</sup> which may result in limitations to the patients.

Although peripheral nerves have the capacity for regeneration,<sup>5</sup> recovery is critically dependent on postinjury treatment.<sup>1</sup> Nonsurgical treatments, such as physical exercise, may act on peripheral nerve regeneration.<sup>4</sup>

The physical exercise performed in the aquatic environment induces physiological effects that provide benefits to the cardiovascular, skeletal, muscular, and nervous systems that increase the process of tissue repair.<sup>6</sup> Swimming helps in the regeneration of the sciatic nerve in rabbits,<sup>7</sup> fish,<sup>8</sup> and

rats.<sup>9-11</sup> It seems to act in the removal of the degenerate myelin and in its synthesis in the regenerative phase,<sup>7</sup> accelerating the nervous recovery.<sup>10,11</sup>

Immersion in aquatic environments generates physiological effects, such as the general elevation in body temperature. As the skin becomes warm, the superficial blood vessels dilate, the peripheral blood supply is increased, and the heart rate rises. Heat from the water reduces the sensitivity of sensitive nerve endings, aiding in pain relief and reduced muscle spasm.<sup>12,13</sup> However, some studies have shown that swimming does not promote benefits in the sensorymotor recovery of rats after sciatic nerve injury.<sup>14,15</sup>

The effects of swimming on nerve regeneration are conflicting, as some authors have reported that forced exercise may have detrimental effects, especially in restoring muscle function.<sup>16,17</sup> Stress induced by physical training could prevent functional recovery after nerve injury.

Due to the scientific controversies of the benefits of swimming, studies are needed to show the effects of this physical activity on the regeneration of the sciatic nerve. In this context, the present study aims to evaluate the effects of

swimming on nerve regeneration after sciatic nerve injury in Wistar rats.

## Materials and Methods

### Casuistry

A total of 30 male Wistar rats from the Experimental Models Development Center, aged between 7 and 8 weeks, weighing on average 250 g, were used. The animals were kept under controlled lighting conditions (light/dark cycle of 12 hours), controlled temperature ( $21 \pm 2^\circ\text{C}$ ) and free access to water and feed. All of the animals were kept in a vivarium until euthanasia.

The present work followed the norms of the current legislation for animal experimentation, in accordance with national standards and with the international legislation (Guidelines of the Brazilian College of Animal Experimentation [COBEA, in the Portuguese acronym], and the National Institutes of Health [NIH] Guide for Care and Use of Laboratory Animals). The present work was approved by the Ethical Commission on the Use of Animals (CEUA, in the Portuguese acronym), under the number 5060051217.

### Definition of Study Groups

The animals were randomly divided into three groups:

- SHAM + SWIMMING (Sham + Swim) – Animals that underwent the surgical procedure, without graft removal, and were submitted to swimming ( $n = 10$ );
- GRAFT (Graft) – Animals that underwent autologous sciatic nerve graft surgery ( $n = 10$ );
- GRAFT + SWIMMING (Graft + Swim) – Animals submitted to autologous sciatic nerve graft surgery and to swimming ( $n = 10$ ).

### Surgical Procedure

In the Sham + Swim group, the right sciatic nerve was exposed for 10 minutes, and the muscle layer sutured with 4-0 monofilament nylon (Monofilamento preto NY44CT30, Tecnofil, Goiânia, GO, Brazil). In the other groups, the sciatic nerve was cut and an 8 mm segment was resected, leaving a distal stump  $\sim 3$  mm before the nerve branch, as described by Fernandes et al.<sup>18</sup> The nerve segment, now considered nerve graft, was inverted and sutured with 2 points of monofilament yarn 10-0 (Microcirurgia preto N-10005, Techsuture, Bauru, SP, Brazil).<sup>19</sup> The skin and muscle were sutured with nylon 4-0 monofilament yarn (Monofilamento preto NY44CT30, Tecnofil, Goiânia, GO, Brasil) and the operation was finished.

### Postoperative

After the surgery, the rats were kept in cages, submitted to the standard diet of the vivarium, water ad libitum, and to a light/dark cycle of 12 hours. Immediate treatment was given with the administration of tramadol hydrochloride (5mg/kg, intraperitoneal). Treatment with paracetamol and ketoprofen was then orally administered at the recommended dose for rats: 1.5 mg/ml and 5 mg/kg, respectively, every 24 hours for  $\geq 5$  days if there were signs of pain.

### Gait Evaluation

The gait evaluation was performed before the surgical procedure (time 0) and after 30, 60, and 90 days. The animals were evaluated using the CatWalk XT system (Noldus Information Technology Inc., Leesburg, VA, EUA), which analyzes footprints through a transparent walkway coupled to a computer system. These footprints were used to calculate the sciatic functional index (SFI).<sup>19</sup>

### Swimming Protocol

The rats of the Sham + Swim and Graft + Swim groups were placed in a pool with a height of 55 cm and filled with 150 liters of water at a temperature of  $30 \pm 2^\circ\text{C}$ , and were induced to swim. Before the surgical procedure, the animals underwent a period of progressive adaptation during 5 days, starting the training with 20 minutes until reaching 1 hour of swimming. The swimming exercise was started on the 14<sup>th</sup> day after the surgery, lasting for 2 weeks, when the animals swam 30 minutes 5 times a week. After this step, the animals performed the swimming activity for 9 weeks following the 30-minute schedule 3 times a week.<sup>9</sup>

### Surgery for Neural Tracing

Neuronal tracing was performed with the neuronal retrograde tracer Fluoro-Gold (Fluorochrome, LLC, Denver, CO, USA), in a concentration of 3%, in the same procedure, by exposing the proximal stump, distal to the second nerve suture for 90 minutes, for later counting of neurons in the anterior horn of the spinal cord.<sup>20</sup>

### Transcardiac Perfusion and Spinal Cord Extraction

Transcardiac perfusion was performed 48 hours after the neuronal tracing, through the opening of the rib cage and the exposure of the heart. The left ventricle was punctured with a needle and the infused solutions were drained through a section of the right atrium. During the infusion, the solutions used were 0.2M phosphate buffer, 4% paraformaldehyde, and 10% sucrose, at physiological pH.<sup>20</sup>

Immediately after the perfusion, a laminectomy was performed, and a segment of the spinal cord was removed using a Surgical microscope (MCT MU-M 19, DFVasconcelos, São Paulo, SP, Brasil). The roots of L3 and S1 were identified at the origin and sectioned transversely in order to keep the L4, L5 and L6 segments intact. The segments of the spinal cord were cryoprotected in 20% sucrose and sectioned in a cryostat at a thickness of 40  $\mu\text{m}$ . The slices were mounted on glass slides and analyzed by fluorescence microscopy.<sup>18</sup>

### Histological Analysis of the Bone Marrow Segment

For the histological analysis, a quantitative study of neurons marked by the neuronal retrograde tracer Fluoro-Gold in the anterior horn of the spinal cord was performed. Using the 25, 50, 100 and 400 fold magnifications of the ZEISS Axiolab fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) all of the sections were examined and the traced motor neurons were counted. All of the analyzes were performed by the same examiner. The histological analysis was performed randomly in the marrow of five animals from

each group. Among these, only cells strongly positive for Fluoro-Gold were considered, and the criterion of Abercrombie correction (1946) apud Fernandes et al.<sup>20</sup> was used.

### Statistical Analysis

The results were evaluated in the software GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk test was used to determine the normality of the data distribution. The parametric variables among the three groups were determined by the analysis of variance (ANOVA), with correction by the Bonferroni post-test. P-values  $p \leq 0.05$  were considered statistically significant differences.

## Results

### Sciatic Functional Index

In the first evaluation, all SFI values were similar between the Sham + Swim (-17.78), Graft (-20.64) and Graft + Swim (-14.184) groups, with  $p = 0.6099$ .

The Sham + Swim group obtained the indices at the limits of normality in all of the measurements, with their values remaining negative and close to zero. After the surgical procedure, the SFI values in the experimental groups (Graft and Graft + Swim) were significantly lower than those of the Sham + Swim group ( $p < 0.001$ , ► **Table 1**; ► **Fig. 1**). In the Graft and Graft + Swim groups, as expected, the 1<sup>st</sup> measurement after the grafting surgery showed a sharp fall in the SFI30 values (-145.9 ± 26.06 and -89.40 ± 7.501, respectively).

In the postoperative follow-up (60 and 90 days), the 2 grafted groups had a progressive functional improvement, although still lower in the Sham + Swim group ( $p < 0.0001$ ), with no differences between the Graft and Graft + Swim groups. In the SFI60, the Graft and Graft + Swim groups showed improvement in the SFI values (-77.52 ± 11.34 and -84.69 ± 21.65), respectively,

and these presented a similar value in the SFI90 (-72.14 ± 19.50 and -84.27 ± 5.78), respectively.

### Histology of the Medulla (Counting of Motoneurons)

According to the number of motoneurons, we can observe that there was a difference in the comparisons between the Sham + Swim and Graft groups (647.1 ± 16.42 versus 563.4 ± 8.07;  $p < 0.05$ ), and between the Sham + Swim and Graft + Swim groups (647.1 ± 16.42 versus 558.8 ± 14.79;  $p < 0.05$ ), but there was no difference between the Graft and Graft + Swim groups (► **Fig. 2**).

## Discussion

Swimming is a rehabilitation exercise to treat nerve damage. However, the level of resistance and the duration of effective exercise are still unclear.<sup>11</sup>

Swimming was applied to the rats 14 days after the surgical procedure, since the functional reinnervation of the muscles starts ~ 2 or 3 weeks after crushing of the sciatic nerve in rats. Excessive muscle work before this time may be detrimental to recovery.<sup>16,21,22</sup>

Studies that evaluate end-to-end neurorrhaphy without graft generally use rehabilitation protocols during 4 weeks of the postoperative period<sup>21,23,24</sup> or a shorter period.<sup>25</sup> Because the grafting application has a worse prognosis and a slower clinical recovery, we adopted an increase from the period of exposure of the animals to swimming.

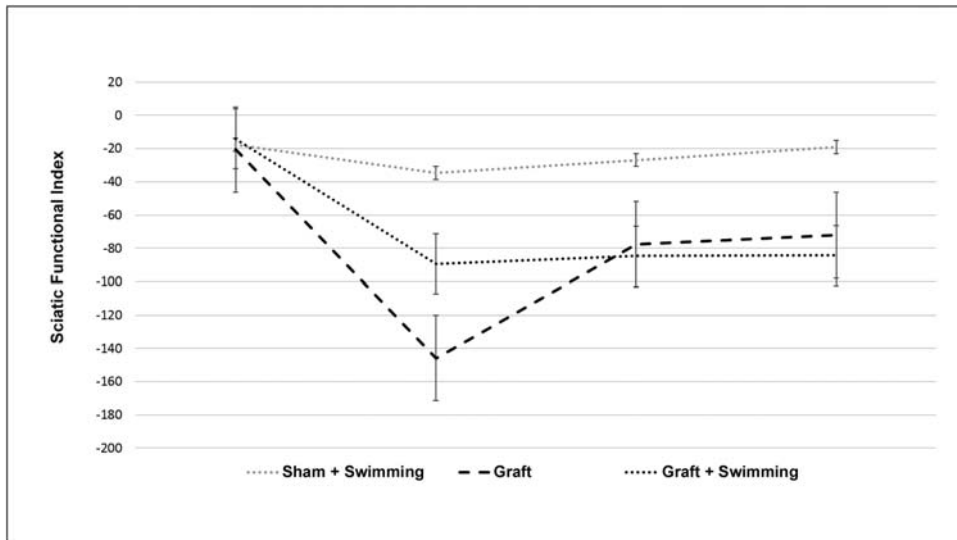
In our study, the IFC values close to -100 in the group Graft at 60 days showed complete nerve function impairment, suggesting the absence of innervation in this period. In the other evaluations, the values of Graft and Graft + Swim groups became less negative, that indicated a gradual return of function.<sup>15</sup> The Graft and Graft + Swim groups had a less

**Table 1** Sciatic functional index analysis between groups Sham + Swimming, Graft, and Graft + Swimming at times of 0, 30, 60, and 90 days

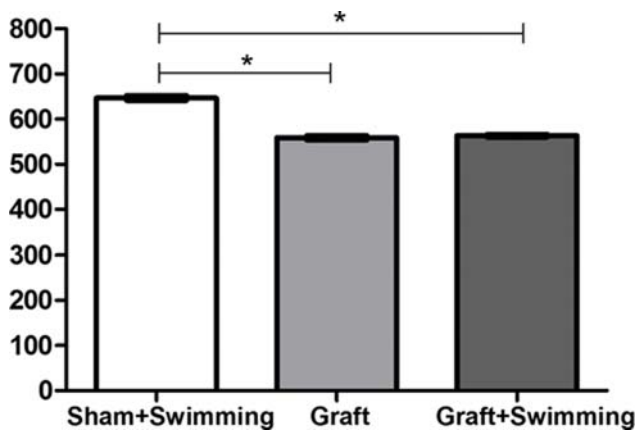
Time	Group	Average	SD	Median	Minimum	Maximum	n	p-value
0	Sham + Swimming	-17.78	17.27	-24.27	-25.74	24.82	8	0.6099
	Graft	-20.64	9.679	-18.82	-35.16	-8.360	9	
	Graft + Swimming	-14.18	13.46	-17.83	-25.57	-25.57	9	
30	Sham + Swimming	-34.64	13.89	-32.41	-50.20	-13.79	7	$p < 0.0001^*$
	Graft	-145.9	26.06	-143.3	-190.2	-100.4	10	
	Graft + Swimming	-89.40	7.501	-89.03	-100.2	-74.30	9	
60	Sham + Swimming	-26.97	13.28	-20.28	-50.12	-13.32	7	$p < 0.0001^{\#}$
	Graft	-77.52	11.34	-80.24	-91.15	-58.36	10	
	Graft + Swimming	-84.69	21.65	-91.44	-102.6	-27.64	10	
90	Sham + Swimming	-19.11	11.33	-21.26	-36.82	-2.870	8	$p < 0.0001^{\#}$
	Graft	-72.14	19.50	-73.37	-107.5	-41.16	10	
	Graft + Swimming	-84.27	5,782	-83,74	-92,28	-75,10	8	

Abbreviation: SD, standard deviation.

**Note:** n = sample number. Analysis of variance (ANOVA) with correction by the Bonferroni post-test. Statistically significant differences were considered with p-values  $\leq 0.05$ . \*; differences between all comparisons (Sham + Swimming versus Graft, Sham + Swimming versus Graft + Swimming, Graft versus Graft + Swimming); #; differences between groups: Sham + Swimming versus Graft, Sham + Swimming versus Graft + Swimming.



**Fig. 1** Sciatic functional index analysis between Sham + Swimming, Graft, and Graft + Swimming groups at 0, 30, 60, and 90 days.



**Fig. 2** Motoneuron count values after correction by the Abercrombie factor. Thirty animals were divided into the following groups: Sham + Swimming ( $n = 10$ ), Graft ( $n = 10$ ) and Graft + Swimming ( $n = 10$ ) at 0, 30, 60, and 90 days. Analysis of variance (ANOVA) with correction by the Bonferroni post-test. \* Differences were found between comparisons (Sham + Swimming versus Graft, Sham + Swimming versus Graft + Swimming); differences with  $p$ -values  $< 0.05$  were considered statistically significant.

negative SFI at 90 days after the postoperative period, but this group distanced itself of the values presented by the control group.

Ilha et al<sup>21</sup> observed an early improvement of the SFI values in animals submitted to physical exercise after sciatic nerve crush surgery when compared to sedentary rats.

Teodori et al<sup>9</sup> evaluated the morphological aspects and the functional characteristics of sciatic nerves of rats and reported that animals submitted to swimming immediately after nerve injury by crushing and animals submitted to swimming after 14 days of injury had fewer axons and a greater diameter of the axons and nerve fibers than control

animals, suggesting that the exercise can be initiated immediately after the injury or in the late phase of the nerve injury.

The number of motoneurons traced with Fluoro-Gold can be used to measure the reconnection of the peripheral nerves in the spinal cord.<sup>27</sup> When we evaluated the number of motoneurons, the values of the anterior horn of the marrow in the Graft and Graft+ Swim groups did not show any statistical difference, remaining below those of the control group.

It has been reported that animals submitted to swimming showed accelerated nervous regeneration compared with control animals with crushing-type nerve damage and increased nerve fiber diameter.<sup>8</sup>

Similar to the studies made by Teodori et al.<sup>9</sup> and Oliveira et al.<sup>15</sup>, the protocol adopted here established a phase of adaptation of the animals to the swimming activity, which starts with 20 minutes on the first day and increases 10 minutes every day until reaching 60 minutes on the fifth day. This adaptation period allows animals to become familiar with swimming, avoiding both physical accommodation and stress. On the other hand, there are some differences between the protocol used in the present study and the other data present in the literature.

The surgical method used here was autologous sciatic nerve graft, while other studies used sciatic nerve crushing<sup>9,16</sup> and sciatic nerve transection.<sup>11</sup> The time of the beginning of the applied intervention was on the 14<sup>th</sup> day after the surgical procedure, and this period was similar to the study by Teodori et al,<sup>9</sup> and superior to other protocols that started the activity after the 1<sup>st</sup> day<sup>9,15</sup> and the 7<sup>th</sup> day<sup>11</sup> of the surgical method. Besides, the duration of the swimming intervention was superior to studies that applied activity during 2,<sup>9</sup> 3,<sup>15</sup> and 4 weeks.<sup>11</sup> These variables may have affected the effectiveness of the swimming activity in sciatic nerve regeneration.

Thus, the comparison between the results obtained and the findings of the literature is a complex measure, since



there is no homogeneity regarding the methodology applied, the surgical technique, the starting time of the protocol, the intensity, the frequency, and the methods of measurement.

## Conclusions

Our study demonstrated that the animals submitted to the swimming protocol after the sciatic nerve grafting procedure did not present differences in the SFI values and in the numbers of motoneurons when compared with the control group. We conclude that the type of protocol described is not efficient for the rehabilitation of peripheral nerve lesions that require grafting. Further studies are needed to evaluate methods that accelerate and improve functional outcomes following the sciatic nerve grafting procedure.

Study conducted with animals. Approval number: CEUA 5060051217.

### Conflict of Interests

The authors have no conflict of interests to declare.

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