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Immediate *versus* later exercises for rat sciatic nerve regeneration after axonotmesis: histomorphometric and functional analyses

Exercício imediato *versus* tardio na regeneração do nervo isquiático de ratos após axoniotmese: análise histomorfométrica e funcional

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Abstract

Objective: Considering the controversies regarding the best period to begin physical exercise in relation to peripheral nerve regeneration, along with its influence on regeneration, this study accomplished a histomorphometric and functional analysis to evaluate the influence of physical exercise on a treadmill, applied to the immediate and late stages of sciatic nerve regeneration in rats following crushing injury. **Methods:** Twenty male Wistar rats (229.05±18.02g) were divided into the following groups: control (CON); denervated (D); denervated+exercise+cage (DEC) and denervated+cage+exercise (DCE). The DEC group started the exercise 24 hours after the nerve injury, while the DCE group started on the 14th day after the injury, with the following protocol: speed=8m/min, inclination=0%, 30min/day, for 14 days. The distal segment of the sciatic nerve was then removed for histomorphometric analysis. The gait was recorded before the operation and on the 7th, 14th, 21st and 28th days after the operation, using the sciatic functional index (SFI). **Results:** The number of regenerated axons in the D groups was greater than in the CON group ($p<0.05$), without differences between the D groups. The axon diameter in the DCE group was greater than the diameter in the D group, whereas the other morphometric parameters only showed significant differences with the CON group. There was no difference in SFI values between the groups, whereas within the groups, the 7th and 14th days differed from the values before the operation and on the 21st and 28th days after the operation. **Conclusions:** The treadmill exercise protocol that was applied to the immediate and late stages of nerve regeneration did not influence the axonal budding, the degree of maturation of the regenerated nerve fibers or the functional performance of the reinnervated muscles.

Key words: nerve regeneration; physical exercise; histomorphometry; sciatic functional index (SFI); neuromuscular plasticity.

Resumo

Objetivo: Devido à controvérsia sobre o melhor momento para iniciar o exercício físico, bem como sua influência sobre a regeneração nervosa periférica, este estudo realizou uma análise histomorfométrica e funcional para avaliar a influência do exercício físico em esteira, aplicado nas fases imediata e tardia da regeneração do nervo isquiático de ratos, após axoniotmese. **Métodos:** Vinte ratos Wistar machos (229,05±18,02g) foram divididos nos grupos: controle (CON); desnervado (D); desnervado+exercício+gaiola (DEG) e desnervado+ gaiola+exercício (DGE). Após 24 horas da axoniotmese, o grupo DEG iniciou o exercício, enquanto o grupo DGE iniciou no 14º dia, com o seguinte protocolo: velocidade=8m/min, inclinação=0%, 30min/dia, durante 14 dias. Em seguida, a porção distal do nervo isquiático foi retirada para análise histomorfométrica. Realizou-se o registro da marcha (pré-operatório e 7º, 14º, 21º, 28º dias pós-operatório (PO)), através do índice funcional do ciático (IFC). **Resultados:** O número de axônios regenerados nos grupos D foi maior que no CON ($p<0,05$), não havendo diferença intergrupos D. O diâmetro do axônio do grupo DGE foi maior que do grupo D, enquanto os demais parâmetros morfométricos apenas apresentaram diferença significativa com o grupo CON. Não houve diferença nos valores de IFC entre os grupos, enquanto na comparação intragrupos, o 7º e o 14º dias diferem do pré-operatório, 21º e o 28º dias PO. **Conclusões:** O protocolo de exercício em esteira aplicado nas fases imediata e tardia, não influenciou o brotamento axonal, o grau de maturação das fibras regeneradas e nem a funcionalidade dos músculos reinervados.

Palavras-chave: regeneração nervosa; exercício físico; histomorfometria; índice funcional do ciático (IFC); plasticidade neuromuscular.

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Introduction

Functional recovery following peripheral nerve injury is still a challenge for rehabilitation. Even with the efforts directed towards surgical repair of injuries in each nerve section, many factors limit the return of sensitivity and motor function in the involved region, such as the formation of scar tissue and axon route loss, among others[1,2].

When the injury occurs by crushing or compression and the continuity of the nerve is preserved, the prognosis is favorable[1]. However, due to Wallerian degeneration that begins immediately after the injury[3], nerve regeneration occurs slowly and complete maturation of the regenerated fibers is rarely achieved[4]. While the muscle remains denervated, the cross-sectional area of its fibers decreases, causing concomitant increases in conjunctive tissue, especially in the perimysium[5], which characterizes atrophy and loss of elasticity in the muscle.

Efforts to prevent muscle atrophy, in order to preserve the structural and metabolic conditions of the muscle while the nerve regeneration takes place, have been carried out in many experimental studies. For this, low-frequency phased electrical stimulation[5], chronic low-frequency electrostimulation[6] and physical exercise[7-12] have been used.

Kinesiotherapy is prominent among physical therapy methods for rehabilitation following peripheral nerve injury. In both humans and other animals, there is the capability of causing a set of functional and structural adaptations[7,8,12] that have the aim of preventing muscle atrophy and recovering the motor function and sensitivity of the affected areas.

However, there is a controversy in the literature regarding the best time for physical activity to be performed. Most studies discuss the effects of physical exercise during the reinnervation phase or at a later phase (approximately two weeks after the nerve injury). They state that there must be a resting period before exercise[7], because muscle atrophy and subsequent reinnervation after sciatic nerve compression in rats becomes evident only between the 14th and 21st day after the injury[13]. On the other hand, exercise done during the denervation or immediate phase (one to three days after the nerve injury) accelerates the return of motor-sensory function during the initial phase of recovery[12] and improves functional recovery in rats[9,11,12]. So far, there have been few studies on the influence of exercise on the regeneration process in the injured nerve[12].

Considering the controversy regarding the most appropriate time to begin exercising following denervation, the aim of this study was to investigate the influence of exercise starting during the immediate and late phases of nerve regeneration, on the histomorphometric and functional characteristics of the sciatic nerve regenerated following axonotmesis, with a view to provide support for future clinical investigations and discussion of the approaches used for rehabilitation.

Material and methods

This study was approved by the Ethics Committee for Animal Experimentation of the Federal University of São Carlos (CEEA /UFSCar), under protocol number 027/2006.

Twenty male Wistar rats (229.05±18.02gr) were obtained from the central vivarium of the Institution and were kept in polyethylene cages, with free access to water and commercially produced animal feed, under controlled temperature and 12-hour light/darkness cycles. They were divided into four groups (n=5):control(CON),denervated(D),denervated+exercise+cage (DEC) and denervated+cage+exercise (DCE). During this study, there were no sample losses.

Considering that rats are not born with the ability to run, unlike their ability to swim[14], the ten animals of the DEC and DCE groups were selected from a groups of 50 rats, using an electrical treadmill for rats containing eight lanes and a digital speed control. The selection criteria were their capacity to run for five to ten minutes a day at a speed of 17m/min, over a one-week period[15,16].

Before starting the experiment, the rats in the DEC and DCE groups were adapted to the treadmill, running at a speed of 17m/min, without inclination, over a one-week period respecting with the exception of weekends. They started with ten minutes on the first day, increasing five minutes each day until achieving 30 minutes, in accordance to Pills et al.[15] and Machado et al.[16]. This adaptation allowed the animals to become familiarized with the exercise protocol, thus reducing stress without provoking physical adaptations[10].

The animals in the D, DEC and DCE groups were anesthetized using a mixture of ketamine hydrochloride (50mg/mL) and xylazine hydrochloride (2g/100mL), in proportions of 1:1 (0.3mL/100g of body weight). An incision of approximately 1.5cm was made in the skin of the left gluteus region, allowing the sciatic nerve to be viewed. The nerve was nipped for 20 seconds, four times (with a one second interval between each one), using adapted hemostatic tweezers, in accordance with Fernandes et al[5].

The animals in the DEC group started exercising 24 hours after denervation (30min/day, five days/week, at a speed of 8m/min and an inclination of 0%), for 14 days. After this period, they remained in the cage up to the 30th day. The DCE group remained in the cage up to the 13th day and started exercises on the 14th post-operative day (POD), following the same protocol as used for the DEC group, for 14 days.

For functional analysis of walking, a runway of 8.2x42cm was used[17], with a dark shelter at the end. The runway was covered with white paper and the animals were then set to walk, with

their hind paws marked with fingerprinting ink. Thus, a record of normal and experimental paw impressions were obtained before the operation and on the 7th, 14th, 21st and 28th POD.

Using a digital pachymeter with an accuracy of 0.01mm, the following experimental (E) and normal (N) paw distances were determined: step length (SL), between the extremity of the third toe and the heel bone; step width (SW), between the first and fifth toe and intermediate step width (ISW), between the second and fourth toe[17,18].

The values found at all the analyses times were applied to the equation proposed by Bain, Mackinnon and Hunter[18]. The results obtained expressed the functional loss in percentages, such that the value of 0 represented normal function or absence of dysfunction and the value of -100 represented total loss of nerve function.

On the 31st day, the sciatic nerves for the animals in all groups were exposed, fixed *in situ* and removed. The distal portion was kept in a fixing solution (modified Karnovsky[19]) for 24 hours and then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer at pH 7.3 for two hours. It was then immersed in 5% uranyl (24 hours) and dehydrated in solutions of increasing acetone concentration (30 to 100%). Following this, the nerve fragment was embedded in Araldite resin (UEGAMA). After the removal of the nerve, the animals were sacrificed by breaking their necks.

Transversal sections of 1 μm were cut and stained with 1% toluidine blue in aqueous borax solution. They were examined under a Zeiss optical microscope (Standard 25) coupled to an image analyzing system using the Image Pro-Plus 4.0 software (Media Cybernetics®). Images were obtained using a 10x objective to determine the total nerve area of each animal. From these, five randomly defined fields were selected (corresponding to 5% of the sample) for analysis of the number of axons and the diameters of the nerve fibers and axons. The myelin sheath thickness and G ratio (corresponding to the division of the axon diameter by the nerve fiber diameter) were calculated from this data.

The Shapiro-Wilk test was used, since the analysis of data distribution among all the studied variables showed that the distribution was normal under the different conditions. For histomorphometric analysis, a one-way Anova-F test was used, followed by Tukey. For the functional gait analysis, the two-way Anova-F test was used to evaluate each group, at the different evaluation times (comparison within groups). For comparisons between groups, the one-way Anova-F test was used, followed by Tukey. The data were processed using the Biostat 4.0 software, with a significance level of 5% and the values were expressed as means and standard deviations.

Results

The number of axons regenerated in the D, DEC and DCE groups was greater than the CON ($p<0.05$). The axon diameter in the D was smaller than in the CON ($p<0.05$), but it was greater in the DCE group than in the D group ($p<0.05$). For the nerve fiber diameter and the myelin sheath thickness, the values were always lower in the D groups, in relation to the CON ($p<0.05$). The G ratio did not differ between the groups (Table 1).

In all the groups before surgery, the sciatic functional index (SFI) values reflected normal function. On the 7th and 14th POD, the values reflected significant functional loss, while between the 21st and 28th POD, these values showed functional recovery (Table 2).

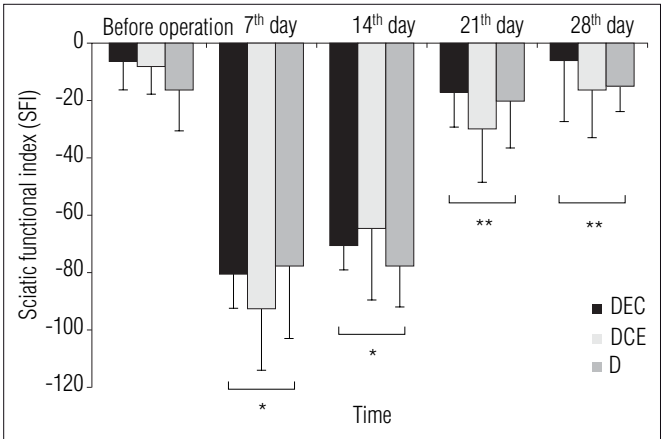
Table 1. Mean values±standard deviations (sd) of quantitative and histomorphometric analyses for the groups: control (CON); denervated (D); denervated+exercise+cage (DEC); denervated+cage+exercise (DCE); n=5.

	CON	D	DEC	DCE
Number of axons	11.976±1.439	21.345±2.372*	18.591±2.305*	18.436±2.520*
Axon diameter (μm)	5.62±0.19	3.60±0.23 *	3.77±0.18*	4.18±0.36*†
Fiber diameter (μm)	9.12±0.33	5.80±0.30*	6.06±0.41*	6.55±0.76*
Myelin thickness (μm)	1.75±0.16	1.10±0.05*	1.14±0.12*	1.18±0.20*
G-ratio	0.6±0.02	0.62±0.01	0.62±0.01	0.64±0.01

*$p<0.05$ *versus* CON; †$p< 0.05$ *versus* D.

Table 2. Mean values±standard deviations (sd) of sciatic functional index (SFI) for the groups: denervated (D); denervated+exercise+cage (DEC); denervated+cage+exercise (DCE), at the different analysis times (n=5).

	D	DEG	DGE
Before operation	-16.18±14.24	-6.44±9.46	-8.06±9.30
7th day	-77.62±25.17	-80.62±11.61	92.84±20.94
14th day	-78.00±13.66	-70.62±7.95	-64.93±24.38
21st day	-20.20±15.57	-17.01±11.85	-9.91±18.18
28th day	-14.66±8.70	-5.60±21.21	-16.47±16.25



Denervated (D), denervated+exercise+cage (DEC) and denervated+cage+exercise (DCE), at the different evaluation times (n=5); (*) *versus* before operation; (**) versus seventh and 14th postoperative day (p<0.05).

Figure 1. Sciatic functional index (SFI) within the groups.

Comparing the SFI values between the groups, at the different evaluation times (Table 2), it was found that the differences were not statistically significant (p<0.05). On the other hand, comparison within the groups (Figure 1) showed that the 7th and 14th POD differed from the period before the surgery, and the 21st and 28th POD differed from the 7th and 14th POD (p<0.05).

Discussion

Axonotmesis injury is a useful and reproducible type of injury for studying peripheral nerve regeneration. In this, the aggression to the nerve is sufficient to cause Wallerian degeneration in the distal portion and allow nerve regeneration, because the conjunctive coverings, basal membranes and local microcirculation are preserved[20].

Studying the muscle reinnervation process following axonotmesis in rats, Gorio et al.[13] observed that, ten days after the injury, no axons had established contact with the neuromuscular junction. This occurred from the second week of the injury onwards, and at this time it was found that 25% of the muscle fibers were reinnervated by more than one axon (multi-innervation). The multi-innervation peak occurred between 21 and 25 days after the injury and was followed by the synaptic elimination process. Sixty days after the injury, the fibers were mono-innervated.

Considering that, from the 14th day after axonotmesis, the regenerated axons would be recovering their contacts with muscle fibers, it was chosen to investigate whether this process would be modified by exercise applied before this time (during the immediate phase of regeneration) or after this time (later phase of regeneration).

In the D groups, it was found that exercise did not influence axon budding, in either the immediate or the late phase. This was shown by the similarity of the number of regenerated axons in the three D groups, in which the number of axons was seen to have practically doubled 30 days of the injury. This can be explained by the fact that each injured axon issues two or three buds directed towards in the muscle[21].

With regard to the maturation of the regenerated fibers, Verdú et al.4 stated that this reached approximately 75% of the control values after the injury, but rarely reached normal values. In the present study, the diameter of the fibers in the D group reached 63.59% of the control values, while they reached 71.82% in the DCE group and 66.44% in the DEC group. However, it is worth emphasizing that this analysis was obtained 31 days after the injury. An analysis after longer periods might show values closer to the ones found in the literature.

Thus, it can be stated that physical activity on the treadmill during the immediate or late phase of the injury did not influence the degree of maturation of the regenerated fibers, considering that only the axon diameter in the DCE group was higher than in the D group, whereas the other analyzed parameters did not differ between the D groups.

From the viewpoint of clinical practice, these histomorphometric results suggest that induction to physical exercise after denervation, with the aim of delaying the trophic alterations to the muscle, may not impair the nerve regeneration process.

However, there are reports that the increased neuromuscular activity provoked by the running exercise on the wheel for eight hours a day, during the acute phase of the injury, inhibits axon budding in the denervated muscle[22,23]. It is possible that, in those studies, the impairment of reinnervation was due to the duration of the exercise, thereby differing from the results of the present study, in which the exercise lasted for 30 minutes each day.

Electrophysiology, histomorphometry and functional tests have been greatly used to quantify nerve regeneration in experimental studies, because they make it possible to analyze the results from different types of intervention that might favor nerve regeneration and, consequently, functional recovery[24,25].

The SFI values close to -100 on the 7th day after the injury showed the consequences of denervation, with complete loss of function. From the 14th day onwards, the SFI values became progressively less negative, which coincided with the period during which the muscle reinnervation began. On the 21st day, the SFI values appeared close to zero, thus characterizing functional recovery relating to the multi-innervation peak[13], while on the 28th day they reflected a functional condition compatible with normal nerves[26].

Comparing the SFI results between the D groups, it was observed that the exercise on the treadmill applied during the immediate or late phases of nerve regeneration following

axonotmesis did not influence the degree of functional recovery. Thus, it was reported here the importance of early stimulation of denervated muscles. As observed in the present study, not only does this not damage nerve regeneration and functional recovery, but also it acts towards avoiding post-denervation atrophy[27], which may additionally favor functional recovery following nerve regeneration.

Comparing the SFI values within the groups, significant differences between the analysis times were found. This is in agreement with several investigators who affirmed that good functional recovery took place over the course of the evaluations, after crushing the sciatic nerve of rats[11,12,24,28].

Therefore, the benefits of physical activity for the denervated animal, such as the increased numbers and diameters of the axons[29], increased weight, muscle strength, and oxidative capacity of the muscle[10], rapid return of motor-sensory function during the initial and late phases (thereby accelerating functional recovery)[9] and increased myelinization of the fibers[12], among others, reaffirmed the importance of investigating and discussing clinical practice. It needs to be taken into account that the induction of neuromuscular activity (from the applied exercise protocol) in denervated muscles did not impair the process of axon regeneration. Thus, its benefits for the muscle may support its applicability, especially with regard to delaying atrophy, which may be reflected directly through functional recovery following nerve regeneration that is more effective.

Conclusions

The treadmill exercise protocol applied in the present study, during the immediate and late phases of nerve regeneration after crushing the sciatic nerve of rats, did not influence the axonal budding, degree of maturation of the regenerated fibers or the functionality of the reinnervated muscles.

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