STUDY OF TIBIAL NERVE REGENERATION IN WISTAR RATS IN PRIMARY NEURORRHAPHY WITH AND WITHOUT GAP, WRAPPED IN VEIN SEGMENTS

EWERTON BASTOS DOS SANTOS, MARCELA FERNANDES, JOÃO BAPTISTA GOMES DOS SANTOS, VILNEI MATTIOLI LEITE, SANDRA GOMES VALENTE, FLÁVIO FALOPPA

ABSTRACT

Objective: This study compared nerve regeneration in Wistar rats, using epineural neurorrhaphy with a gap of 1.0 mm and without a gap, both wrapped with jugular vein tubes. Motor neurons in the spinal cord between L3 and S1 were used for the count, marked by exposure of the tibial nerve to Fluoro-Gold (FG). Method: The tibial nerves on both sides were cut and sutured, with a gap on one side and no gap in the other. The sutures were wrapped with a jugular vein. Four months after surgery the tibial nerves were exposed to Fluoro-Gold and the motor neuron count performed in the spinal cord. Results: The results were statistically analyzed by the paired Wilcoxon test. There was a statistical difference between the groups with and without gap in relation to the motor neuron count ($p=0.013$). Conclusion: The epineural neurorraphy without gap wrapped with jugular vein showed better results for nerve regeneration than the same procedure with gap. ***Level of Evidence: Experimental Study.***

Keywords: Fluorescent dyes. Nerve regeneration. Tibial nerve. Wistar rats. Suture techniques.

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INTRODUCTION

The treatment of peripheral nerve lesions requires detailed knowledge of their anatomy and physiology and is achieved through clinical experiences and experimental studies. The objective of the nerve injury repair is to restore normal sensitivity and muscular function. Function restoration after a nerve injury requires the growth of the affected axons over the distance between the lesion and the target organ. In this situation, when we conduct experimental trials with animals (rats), we will encounter a difference in the study time since regeneration requires relatively short distances.

There are reports of the use of arterial and venous grafts in animal experiments, with the advantage of being autogenous.[1] It has been demonstrated, experimentally, that the vein lumen has the ability to regenerate nerve, serving as a conduit for axonal growth. This study was conducted through the clinical and electrophysiological observation of sciatic nerve regeneration in rats, using different autogenous vein graft models,[2] agreeing with other authors who claimed that the veins offer biological substrates for axon regeneration.[3] The improvement of the motor neuron count was demonstrated using vein tubes in national studies.[4,5]

Several researchers have defended neurorrhaphy with short gaps, which varied, in literature, between 0.5 and 5.0mm,[6-9] while in one of the studies, using injury and repair in the femoral nerve of rats with a gap of 0.5mm between the stumps, they observed disorientation in the motor neuron axons in the first two weeks, with improvement in the following weeks due to interaction of trophic factors between and among the axons.[6] Valero-Cabré et al.,[10] when comparing neurorrhaphy techniques, using silicone tubes, noted that when applied to the sutures with a gap, there is no improvement in the reinnervation process. Since good results were found in the literature with techniques using vein wrappings,[3,4,8] our goal in this study was to compare neurorrhaphy with and without gap wrapped in vein, through quantitative analysis of nerve regeneration, by means of the motor neuron count in the spinal cord.

OBJECTIVES

Compare, using counts of neurons marked with Fluoro-Gold®, the results of the regeneration of the tibial nerves of Wistar rats, through end-to-end neurorrhaphy, with and without gap, wrapped in vein graft.

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

Universidade Federal de São Paulo (UNIFESP) – São Paulo, SP, Brazil

Study conducted at the Department of Orthopedics and Rheumatology of Escola Paulista de Medicina da UNIFESP.
Mailing address: Marcela Fernandes.Rua Borges Lagoa, 786. Vila Clementino - 04038-001 - São Paulo – SP Brazil.
email: fernandesmarcela@hotmail.com

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MATERIAL AND METHODS

The experimental protocols used in this study were approved by the Research Ethics Committee of UNIFESP/EPM. Every effort was made to minimize the animal's suffering, according to the International Ethical Guidelines.[11]

The study subjects were 20 male Wistar rats, with an average weight of 250g and average age of eight weeks, kept under controlled conditions with light/dark cycle (12/12 hs.), temperature 21± 2°C, with unimpeded access to water and feed.

Assuming a difference of at least 10 motor neurons, on average, between the suture with a gap and that without a gap, with confidence of 95% and sample power of 80%, the sample necessary in this study would be 16 animals all told. Expecting a loss of animals during the study, 20 animals underwent surgery. At the end of the experiments, we arrived at a total of 11 animals, due to the losses during the course of the stages of the study.

Each animal received intraperitoneal anesthesia with an anesthetic solution composed of chloral hydrate (4.25g), magnesium sulfate (2.25g), propylene glycol (4.28ml), absolute ethyl alcohol (11.5ml), distilled water (45.7ml) and 3% thionembutal.[4,5] With the animal positioned in supination, using microscope and microsurgical instruments, we made an anterior-lateral cervical incision for the removal of a segment measuring 12mm in length of external jugular vein. (Figure 1A) The animal was then positioned in pronation and we made a posterior-lateral incision in both thighs, to dissect the sciatic nerve and its ramifications: sural, peroneal and tibial. We sectioned both tibial nerves and performed the epineural neurorrhaphy with a gap in the right paw and without a gap in the contralateral paw, with the sutures wrapped in the vein segment.[2] (Figure 1B, C, D) After four months, the animals were submitted to the new surgery for exposure of the tibial nerves to the FG® neuronal marker.[12] (Figure 2A) After 48 hours, the animals were anesthetized with thionembutal (50 mg/Kg, IP) and sacrificed via transcardiac perfusion[13] with 50ml of intracardiac isotonic saline solution for vascular system cleansing, 200ml of 4% paraformaldehyde, quickly for 10 minutes, 300ml slowly, for 20 minutes, 200ml of 10% sucrose buffer quickly for 10 minutes, 300ml slowly, for 20 minutes.[4,5] (Figure 2B) Dorsal and lumbar laminectomy was performed at the end of this procedure, with the animal in pronation.[4,5] (Figure 3) After spinal cord exposure, we resected the corresponding segment from L3 to S1.[4,5] We then marked a groove in the dorsal region of this segment to indicate the right side.

The spinal cords were sectioned in 40μm slices and mounted on glass slides. The slides were examined under a Zeiss-Axiolab fluorescence microscope to evidence the FG® (Figure 4) The slices were examined under magnification of 25 to 100 and the marked motor neurons were counted.[12] The number of motor neurons obtained was corrected by the criterion of Abercrombie and Johnson[14] for 40X increase, aiming to eliminate counts of the same motor neuron in different sections, since it is possible, with this thickness, for the cell body to appear in more than one serial cut.

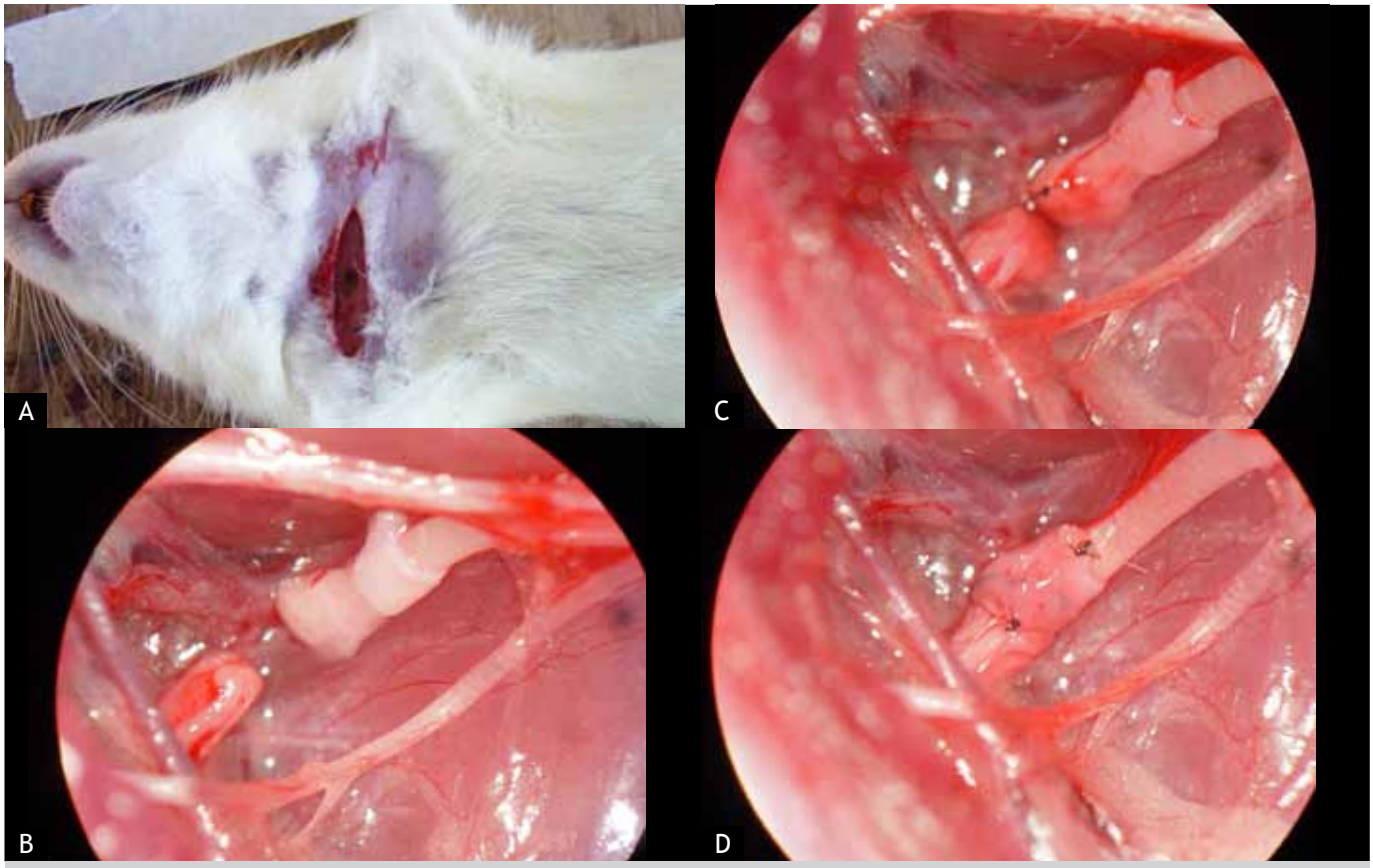

Figure 1. *Approach for removal of the external jugular vein (A). Tibial nerve sectioned with vein tube collected in the proximal stump (B). Neurorrhaphy with 1.0mm gap between the stumps (C). Final aspect of the neurorrhaphy with vein wrapping repaired by two stitches (D).*

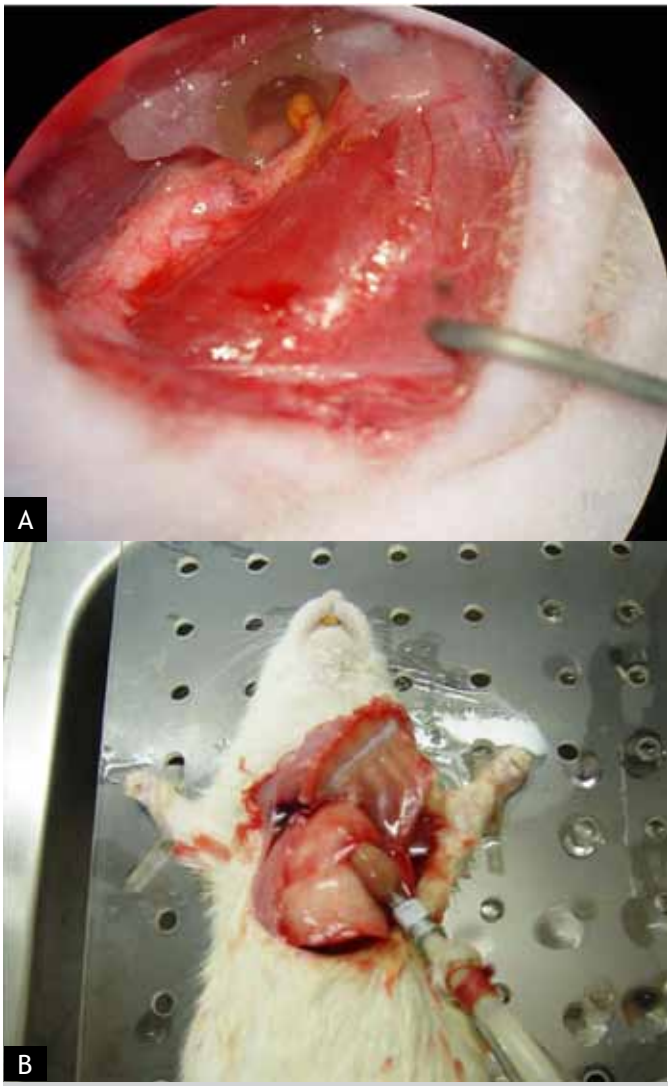

Figure 2. *Neuronal marking with FG® of the tibial nerve sectioned distally to the neurorrhaphy (A), Intracardiac profusion of the rat with catheter in the aorta 48 hours after neuronal marking (B).*

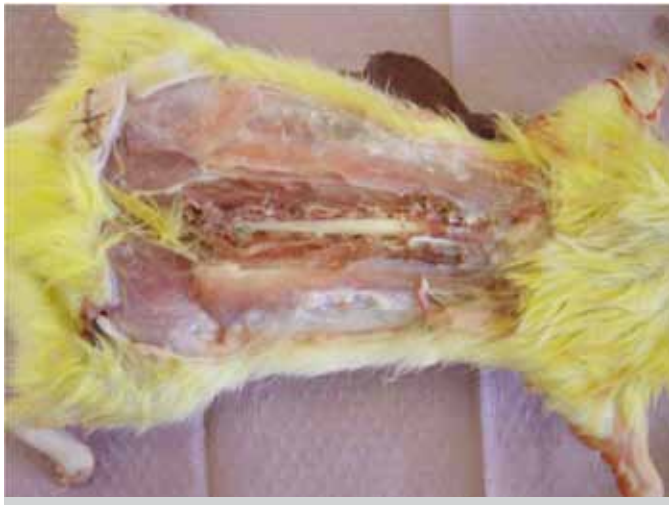

Figure 3. *Laminectomy with exposure of the spinal cord from L3 to S1 and identification of the spinal roots and medullary segment to be removed for analysis.*

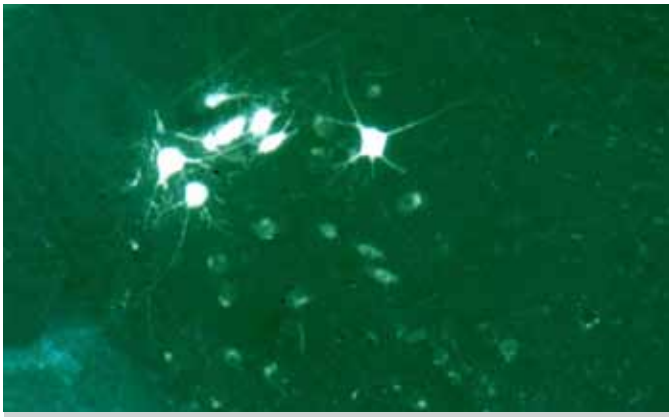

Figure 4. *Visualization of the motor neurons stained with Fluoro-Gold® under a 40X fluorescence microscope.*

RESULTS

After counting the motor neurons in the spinal cord of the 11 rats, we presented their results in value corrected by the criterion of Abercrombie and Johnson.[14] (Table 1, Figure 5)

Table 1. *Number of motor neurons, stained with Fluoro-Gold® in the spinal cord of Wistar rats, corrected by the Abercrombie criterion for 40µm section.*

Rats	Neurorrhaphy with GAP	Neurorrhaphy without GAP
1	233	293
2	228	289
3	223	263
4	489	546
5	226	256
6	359	324
7	178	153
8	310	393
9	510	653
10	169	222
11	259	285
MEAN	**289.4**	**334.3***

*Neurorrhaphy without gap presents better results than neurorrhaphy with gap (*p= 0.013).*

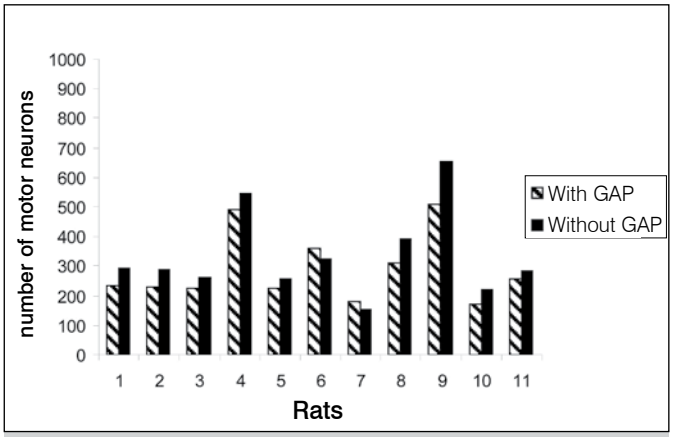

Figure 5. *Representation of the numbers of motor neurons in the spinal cord of the animals that were operated with gap and without gap for the 11 rats.*

The Wilcoxon test for paired samples was used to compare the number of motor neurons between the groups of neurorrhaphy with gap and without gap on the left and right sides, respectively, in the same rate. The test showed that the number of motor neurons with a gap is statistically lower than that without a gap ($p=0.013$).

DISCUSSION

Several methods for repair of injured peripheral nerves have been proposed in the last few decades.

Tubulization, which consists of the introduction of the distal and proximal extremities of a nerve inside a tubular structure that may or may not contain substances that promote axon regeneration, was first tested with decalcified bone, between the two ends of the peripheral nerve.[15]

The ideal material for tubulization would be: low-cost, inert (biocompatible), thin, flexible, bioabsorbable, inhibiter of inflammatory processes (fibrosis, glioma, neuroma, edema, ischemia and adherence) and which benefits the processes that contribute to regeneration (accumulators of the factors that promote nerve growth).[16,17] The autogenous vein tube is a material option that satisfies many of these criteria.

Studies concluded that the nerves regenerate through the vein light[2] and were demonstrated in electronic microscopy.[18]

Observations comparing veins demonstrated that the jugular vein as a neurotube, presents superior quality in nerve regeneration when compared with the femoral vein.[19] Several researchers have obtained better results when they used vein tubes.[3,5,19]

We started our study with 20 operated rats, and lost 9 during different stages: anesthesia, perfusion or poor quality of the histological sections observed in the microscopy.

We determined an interval of four months between the first and the second surgery, since this time is sufficient for nerve regeneration.[20] Accordingly, after this period, the neuronal regeneration study becomes more reliable as it is known that the nerve will not suffer major biological changes, approaching its original structure.[19]

To analyze nerve regeneration we used retrograde neuronal marking with FG®, in the concentration 3%. FG® was chosen to evaluate the result of the neurorrhaphy due to its low short--term toxicity and ease of use, and as it is a fast, reproducible and non-subjective method.[21] This marker is fluorescent and allows its direct observation under the microscope with the use of filters, without chemical reaction with color developer, permitting easier reproduction in other experiences.[1,22] Moreover, it is a quantitative study method that is just as important as the other equally quantitative methods, as in the case of electrophysiology, in which its results are objective and clearly defined.[2] It is based on direct observation of the presence of markers transported retrogradedly to the cell body,[1,21] allowing the representation of the nerve at the spinal cord level.

As the study aims to demonstrate the reconnection of the peripheral nerves with the CNS, we used retrograde axonal transport with FG to visualize the motor neurons in the anterior horn of the spinal cord. In a previous study[5] we used the histomorphometric analysis and attempted to correlate with the findings of retrograde axonal marking with FG, concluding that the motor neuron presents an axon and this, when injured, can generate several axonal sprouts in the attempt to reach the target organ. Although the density of fibers is increased, showing axonal sprouting, this sprouting does not always reach the target organ effectively. Consequently, not all the motor neurons of the spinal cord present marking with FG, even though the number of fibers is increased. The number of fibers counted is not related to the number of motor neurons in the spinal cord. As the main objective was to evaluate the reconnection of the peripheral nerves with the CNS, we only used the FG® methodology.

During dissection for neuronal marking with FG®, we observed the presence of a more intense fibrosis on the side with the gap, when compared to the side without a gap, in 9 rats. In the literature we found that the vein tube forms a more intense fibrosis, when used.[7]

Considering the possibility of the same cell being counted more than once in different histological sections, we used the correction factor established by Abercrombie and Johnson[14] for 40μm sections, in which the result is multiplied by 0.65, in an attempt to eliminate the counting error of the same cell body in different sections.

Studies suggest that axons are attracted by chemical influences and that this can be improved by leaving a gap between the nerve stumps instead of suturing without a gap.[8,23]

During our bibliographical review we did not find any study showing negative evidence against neurorrhaphy with a gap; we were in doubt about how to choose the ideal gap. The short gaps cited in the literature range from 0.5 to 5.0mm[6-9] though we found 0.5mm a very short distance since when we sectioned the nerve and repaired it, the actual stump edema would cause us to lose this distance. We opted for the gap of 1.0mm, but do not find any studies conducted with this spacing in literature.

Studies comparing neurorrhaphy with and without a gap, both tubularized, in rabbits and monkeys, concluded that the use of a small gap improved nerve regeneration specificity.[8,24]

The advocates of neurorrhaphy with tubularized gap believe that this procedure has the following advantages: less surgical trauma to the nerve because its stumps located inside a guidewire would decrease the quantity of stitches in the neurorrhaphy.[9] The presence of the proximal stump in a guidewire would lead to axonal distal migration without interposition of other tissues and without deviation in alignment, improving regeneration due to the accumulation of neurotrophic factors in the tube lumen.[2,23,25]

At the end of the study, the loss of animals was greater than foreseen, with a total of 11 animals remaining. Assuming the same difference proposed of at least 10 motor neurons, on average, between neurorrhaphy with and without gap and with the same confidence, the study power came to 66%. After the statistical study we found, in this experimental trial, that the best primary neurorrhaphy method is that performed without a gap, since this presents better results when compared with neurorrhaphy with gap.

CONCLUSION

Primary neurorrhaphy wrapped with a vein segment, in the tibial nerves of Wistar rats, presents better results in suturing without a gap, when compared with suturing with a gap.

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