COMPARATIVE STUDY BETWEEN AUTOGENOUS GRAFT AND MUSCULAR GRAFT COVERED WITH AUTOGENOUS VEIN TUBE IN WISTAR RATS' TIBIAL NERVES USING THE FLUORO-GOLD® AS A NEURONAL MARKER

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SUMMARY

The purpose of this work was to study nervous regeneration through neurons counts by comparing two surgical techniques for addressing nervous gaps on 15 rats' lower limbs. Initially, a 12-mm long vein tube from the left outer jugular was obtained, and then both lower limbs are operated, exposing the tibial nerve at each side and performing a resection of an 8-mm nerve segment, at the same time simulating a gap and an autogenous nerve graft. Left gap repair consisted of a usual conventional graft for nervous injury repair by means of microsurgical suture. The gap repair on right lower limbs was made through quadriceps muscle, treated with liquid nitrogen, covered with an 8-mm tube of jugular vein. After four months, the animals were submitted to a new surgery for exposing tibial nerves to the Fluoro-Gold® neuronal marker. After 48 hours, the rats were perfused and medullar segment between L3 and S1 was removed and subsequently cut into 40μm sections. Neurons on all sections were counted, and no statistical differences were found between both surgical techniques.

Keywords: autologous graft; Peripheral nerves; Cell count; Wistar rats.

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INTRODUCTION

In peripheral nerve injury, nervous fibers originate and grow from the proximal stump [1]. In digital nerves injuries, the best outcomes after repair were found in children, showing the need of further nervous regeneration studies in adult populations[2].

Using a tube for repairing an injured nerve is not new [3]. Tubes seem to be important for regeneration because they comprise neurotrophic substances that help on guiding axons towards their distal target [4]. The great driver towards researches using tubes with veins emerged after experiments in rats showing that the light of veins allows for nerve regeneration[5].

In large nervous substance losses, when a vein is used for filling the gap, adherences may occur. However, when a vein is filled with muscles, these adherences are prevented, and there is no fiber dispersion from proximal and distal stumps, also enabling the avoidance of fibrosis and blood intake improvement[6].

Based on these data, the present study intended to compare the effectiveness of a vein-covered denaturized muscle graft, in Wistar rats' tibial nerves, when compared to traditional autogenous graft, using counts of neurons marked with Fluoro-Gold®.

MATERIAL AND METHOD

The experimental protocols used in this study have been approved by the Committee on Medical Ethics in Research, Hospital São Paulo / Federal University of São Paulo, according to the International Ethical Guidelines[7].

Wistar race rats were used, which weighted 155 g in average, and kept under controlled conditions with light/dark cycle (12/12 h), at a temperature of 21 ± 2°C, with water and food ad libitum, throughout the experiment period.

In total, 29 rats were operated, but only 15 could be included in the study group, because the others presented complications during the performance of the various experimental procedures, making data quantification difficult.

The animals were anesthetized with a solution constituted of chloral hydrate, magnesium sulfate, thionembutal, propylene glycol, ethylene alcohol and 4ml/Kg distilled water.

Surgery constituted of removing external jugular vein (Figure 1)

Study conducted at the Department of Orthopaedics and Traumatology, UNIFESP.

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connecting tributaries and isolating a 12-mm long segment (Figure 2). The venous graft was kept soaked into 0.9% saline solution until its use.

The sciatic nerve and its branches (sural, peroneal, and tibial) were exposed. The right tibial nerve was dissected and manipulated as minimally as possible (Figure 3). An 8-mm segment was sectioned, leaving a proximal stump of approximately 3 mm (Figure 4). The nerve segment was maintained in 0.9% saline solution.

The injury provoked to the left tibial nerve was adjusted by interposing the nerve graft removed from the contralateral limb and sutured with two epineural nylon 10-0 monofilament stitches.

At the right side, 1 cm of muscle was removed, which was kept in liquid nitrogen for performing denaturing[8]. The denatured muscle was sutured to proximal stump and then covered with a vein tube (Figure 5). Once the vein was accommodated on the whole muscle segment, suturing was provided with the proximal and distal epineurium so that the vein could not be displaced from muscular graft. Muscle and skin planes were sutured with 4-0 nylon monofilament.

After 120 postoperative days, the animals were submitted to a new surgery for exposing right and left tibial nerves (Figure 6) for performing the neuronal marking with 3% Fluoro-Gold® (Fluorochrome Inc.) for subsequent neuron count at the anterior spinal cord horn.

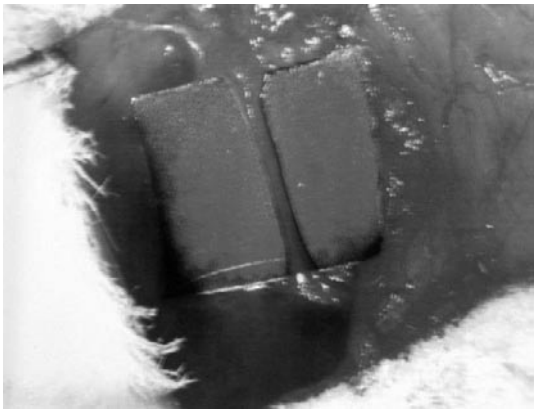

Figure 1 – *Identification of the external jugular vein.*

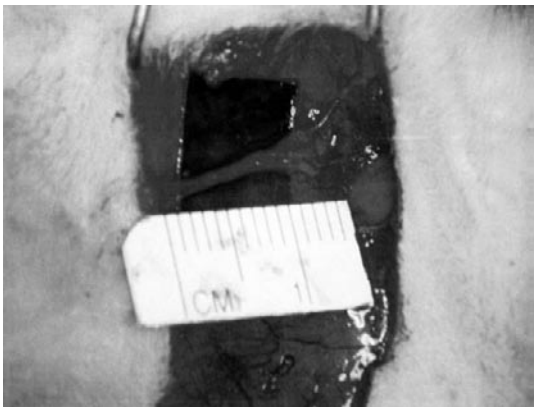

Figure 2 – *Measurement of the 12-mm segment to be removed from external jugular vein.*

Figure 3 – *– Exposure of a sciatic nerve and its branches.*

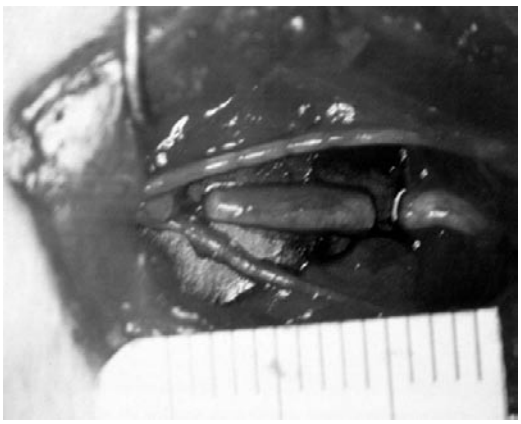

Figure 4 – *8- mm section of the right tibial nerve.*

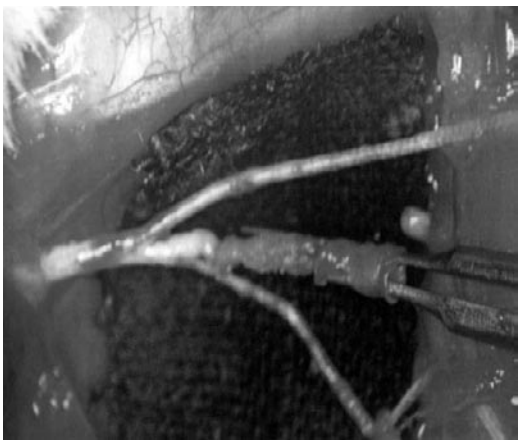

Figure 5 – *Jugular vein accommodation on muscle graft.*

The nerve was exposed to dye for 90 minutes. After that time, muscle and skin planes were sutured.

The animals, 48 hours after dye exposure, were anesthetized again and then submitted to a transcardiac perfusion by means of a port on thoracic box and heart exposure. The left ventricle was punctured with a needle and the infused solutions were drained by sectioning the right atrium.

During perfusion, the buffered solutions at physiological pH phosphate 0.2M and 10% sucrose 10% were used.

Finished the perfusion, the marrow was removed with the aid of a microscope. Laminectomy was initiated at S2 level.

Dorsal roots of L3, L4, L5, L6 and S1 were identified, and the L3 and S1 roots' path was followed up to its entry into the marrow, which was cross-sectionally sectioned in order to preserve L4, L5 and L6 segment.

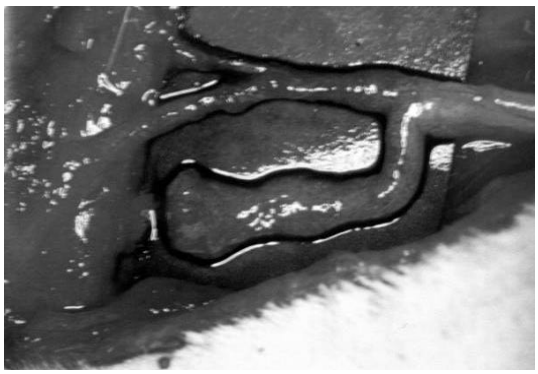

Figure 6 – *Section of the tibial nerve distal to muscle graft.*

The marrow segment was submitted to a cryoprotection process with 20% sucrose and subsequent cutting with a freezing microtome, into 40μm sections.
The slides were assembled and analyzed under fluorescent microscope. The anterior horn of the spinal cord was assessed and motor neurons were identified (Figure 7). Only the cells typically marked with Fluoro-Gold were assessed in order to avoid any misinterpretation. From the total of cells, the Abercrombie's[9] adjusting criteria were used for serial cuts to microtome.
As statistical evaluation methods, the following tests were used: the paired "t" test, the signaled Wilcoxon's test and the Mann-Whitney's test.

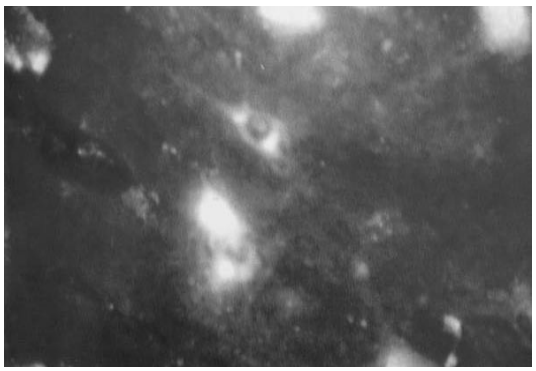

Figura 7 – *Cells stained with Fluoro-Gold®. Magnification: 20x.*

RESULTS

After neuron counting, results in absolute values were adjusted by using the Abercrombie's criteria[9] (Table 1).
Motor neurons counting at the anterior spinal cord horn
For statistical analysis, the paired "t" test was used, where it is performed by subtracting both sides of each rat and removing its individual effect. Following, the average of differences reaching zero was tested, showing no difference between groups.
The Wilcoxon's test was also employed, taking the differences between each rat's posts, and the Mann-Whitney's test, working with the averages of each group's posts, not removing rats' individual effects. In these two tests, no statistical difference was found as well.

RATS	RIGHT SIDE Muscle graft denatured, covered with vein	LEFT SIDE Conventional graft
1	257	250
2	705	657
3	1054	916
4	1331	1358
5	438	465
6	268	239
7	594	697
8	540	564
9	523	466
10	437	393
11	385	442
12	840	767
13	690	677
14	1258	1301
15	811	744

Table 1 - *Cell counts on right and left sides of the marrow, adjusted by the criteria by Abercrombie, for 40 µm sections.*

DISCUSSION

We used young (60 days old) Wistar rats because they are biologically well known and widely employed in experimental assays. That age was selected in order to avoid losing them by age-related diseases[10].
Fifteen out of the 29 operated rats were used at the end of the study, because during the experiment, some technical problems such as animals' deaths by cardiac-respiratory failure during anesthesia, postoperative complications, poor fixation or cryoprotection of the tissue, among others, made us unable to use the remaining animals. However, as a large amount of cells stained in each slide were analyzed, this number of animals was enough to find similar data to literature [11,12].
The selection of the external jugular vein is because of the advantages it presents when compared to the femoral vein. Those advantages include: a) a wider diameter - which allows a better quality suture and covers stumps' ends, avoiding the development of neuromas at the suture, b) it does not have valves - thus avoiding hurdles for axonal growth[13], c) vessel length is enough for tubing, and d) it avoids any significant tissue damage to rats' legs - by a double incision when addressing the nerve laterally and the venous graft medially.
The tibial nerve was used for two reasons: it reduces cell death and mimics peripheral injuries usually found in clinical practice. If the sciatic nerve was employed in this study, we would be at risk of finding some problems to the rats, typical of lower limbs injuries, such as: insensitive parts autophagy [10], skin ulcers[11] and difficult upright position stabilization allowing the animal to eat. The use of both limbs of the same animal was due to individual organic variations of each rat, which could compromise results reliability when comparing the proposed surgical procedures [10,12].
The selection of an 8-mm size for substance loss was made by complying with the limits described in literature, which reports that this measure should not exceed 10 mm [14].

Inside the vein graft, a muscle denaturized with liquid nitrogen was used[15], because it presents a lower size reduction rate, which is 42%, while microwave denaturizing is approximately 89%[16]. Denaturing rate in the muscles employed in this study was approximately 20%. This data was obtained by measuring values previously and subsequently to denaturizing process.

The established interval time of 4 months between the first and the second surgery is due to the fact that this time is enough for nerve regeneration[4], making the study to be more reliable, because by doing so, the nerve should not go through age-related biological changes, thus keeping its original structure. Some authors, however, consider 5 months as an optimal time interval between procedures [17].

After tibial nerve section, during the first surgery, the rats showed limping on both rear legs, while in the second procedure, they showed clinically well and without any atrophies. The dissection of the surgical plane was easier on the leg grafted with vein-covered muscle, which is consistent to literature findings[18]. It is worthy to highlight that this is an interesting data, enabling clinical application in cases where cicatricial response is important, such as for the treatment of compressive syndromes recurrences [19].

For nervous regeneration analysis, we used the Fluoro-Gold® as a retrograde neuronal marker at 3% strength, but this may range from 2 to 4%. This fluorescent marker enables a direct visualization under a microscope, not requiring chromogens use, which facilitates experiment reproduction[12]. This direct visualization is based on the presence of a marker, retrogradely transported to cell body[20] thus allowing the nerve to be represented on marrow.

The negative aspect of using Fluoro-Gold® as a neuronal marker lies on the fact that by being illuminated during the analysis, fluorescence is progressively reduced, making a reassessment of the same slide impossible.

During transcardiac perfusion, a sucrose solution was used for protecting the tissue against freezing aggressions, thus allowing a better visualization and analysis of the tissue to be assessed. This important step was reported by other authors[10,12].

In order to avoid counting the same cell more than once at different sections, the adjustment factor established by Abercrombie[9] was employed for 40 μm sections. The results were presented as absolute and adjusted values (Table 1), but the statistical analysis was made on the basis of adjusted values.

Three statistical tests were used for determining whether a significant difference existed or not between employed techniques.

There was no statistically significant difference between the methods; therefore, the denatured muscle graft covered with vein shows a similar number of marked cells when compared to the simple-graft method.

We have not been able to compare the results of this study to literature data, because no correlation was found between muscle-and-vein graft in reviewed studies.

Oliveira[14], using a denatured muscle graft compared to a conventional nervous graft, found no statistical difference between both methods as well.

Two of the animals employed presented a lower number cell count, but the differences between both sides remained reasonably proportional, which did not change the statistical analysis.

A combination of a muscle and vain graft was used, based on the principle that an injured nerve produces a high amount of axon-regenerating substances [11] and the vein, for being a tube system with selective permeability, would avoid these substances to be spread, facilitating nervous regeneration. The denatured muscle would maintain basal membrane, acting as a support system for regenerating neurons.

CONCLUSION

We did not detect statistical differences between the groups treated with conventional nervous graft and muscle graft denatured with liquid nitrogen covered with autogenous vein.

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REFERENCES

1. Ramon y Canal S. Degeneration and regeneration of the nervous system. London: Oxford University Press, 1928.
2. Altissimi M, Mancini GB, Azarrà A. Results of primary repair of digital nerves. J. Hand. Surg., 1991, 16B: 546-547.
3. Glück T. Ueber neuroplastic auf dem wege der transplation. Archs Klin Chir, 1880, 25: 606-616.
4. Politis MJ, Ederle k, Spencer PS. Tropism in nerve regeneration in vivo. Attraction of regenerating axons by diffusible factors derives from cells in distal nerve stumps of transected peripheral nerves. Brain Res. 1982, 253: 1-12.
5. Chiu DTW, Janecka I, Krizek TJ, Wolff M, Lovelace RE. Autogenous vein graft as a conduit for nerve regeneration. Surgery 1982, 91(2): 226-233.
6. Weiss p. The technology of nerve regeneration a review. Sjutureless tabulation and related methods of nerve repair. J Neurosurg 1944, 1: 400-449.
7. CIOMS/OMS. Council for International Organizations of Medical Services. WHO Distribution and sales service, 1211 Geneva 27, Switzerland. International Guiding Principles for Biomedical Research Involving Animals.
8. Brunelli GA, Vigasio A, Brunelli GR. Different conduits in peripheral nerve surgery. Microsurgery 1994; 15: 176-8.
9. Abercombrie M. Estimation of nuclear population from microtome sections. Anat Rec. 1946, 94: 239-247.
10. Leite VM. Estudo do comportamento das lesões altas do nervo ciático através da utilização de Fluoro-Gold® e horseradish peroxidase como marcador neuronais (tese). São Paulo: Escola Paulista de Medicina, 1994.
11. Smahel J, Jentsch B. Stimulation of peripheral nerve regeneration by na isolation nerve segment. Annals of Plastic Surgery 1986, 16(6): 494-500.
12. Moscalcoff MCS. Estudo comparative de neurorrafia término-terminal com neurorrafia termo-lateral utilizando o Fluoro-Gold® como marcador neuronal (tese). São Paulo: Universidade Federal de São Paulo, 1997.
13. Wang KK, Costas PD, Jones DS. Sleeve insertion and collagen coating improve nerve regeneration through vein conduits. J Reconstrutive Microsurgery 1993(b), 9(1): 39-48.
14. Oliveira EF. Enxerto de músculo-esquelético nas lesões de nervo ciático de ratos (tese). Universidade de São Paulo, Ribeirão Preto; 2000.
15. Whitworth H, Doré C, Hall S, Green CJ, Erengghi, GT. Different muscle graft denaturing methods and their use for nerve repair. Br J Plast Surg 1995, 48(7): 492-9.
16. Leniham DV, Carter AJ, Glasby MA. An electrophysiological and morphological comparison of the microwave muscle graft and the freeze-thawed muscle graft. Br J Plast Surg 1998, 51(4): 300-6,.
17. Aldskogius H, Thomander L. Selective reinnervation of somatotopically appropriate muscles after facial nerve transaction and regeneration in the neonatal rat. Brain Res. 1986, 375: 126-134,.
18. Lundborg G, Rosén B, Danielsen N. Tubular versus conventional repair of median and ulnar nerves in the human forearm. Early results from a prospective, randomized and clinical study. J Hand Surgery 1997, 22A: 99-106,.
19. Varitimidis SE, Riano F, Vardakas DG. Recurrent compressive neuropathy of the median nerve at the wrist: treatment with autogenous saphenous vein wrapping. J Hand Surgery 2000, 25B(3): 271-5.
20. Taylor DCM, Pierav, K, Schimid H. The use of fluorescent tracers in the peripheral sensory nervous system. J Neurosc Meth 1983, 8: 211-224.