GLYCEROL-PRESERVED ALLOGENOUS NERVE: AN EXPERIMENTAL STUDY WITH RATS

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SUMMARY

The use of glycerol-preserved nerve allograft is an alternative to autografting in cases of peripheral nerve injury with loss of substance, which decreases surgical morbidity and provides sufficient material for neural repair. The objective of this study was to compare the degree of nervous repair, through interposition of autogenous graft (Group A), of glycerol-preserved vein tube (Group B), and interposition of glycerol-preserved allogenic nerve (Group C) in 5-mm defects of Wistar rats' fibular nerve, using histological and functional analyses. In group A (autograft) a perineural tissue reaction and myelinated axonal fibers escape out of the epineurium boundaries were greater when compared to those observed in Group B (autogenous vein + glycerol) and Group C (nerve allograft). The functional evaluation was made by analysis of the patterns of rats' posterior footprints (Walking Track Analysis) in preoperative, early postoperative period, week 3 and week 6. Regarding functional recovery, in none of the evaluated periods was there a statistically significant difference between the three groups.

Keywords: *Transplant; Nervous regeneration; Glycerol; Fibular nerve; Wistar rats; Surgery/methods*

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INTRODUCTION

The surgical treatment most frequently employed today for repairing peripheral nerves injuries with substance loss is the nerve autografting. This technique is based on the use of a donor nerve segment removed from the own individual, which is interposed between proximal and distal stumps of the injured nerve, and aims to fill the gap between stumps, fostering tension reduction on suture lines and the guidance for the advancement of neural growth cones[1,2].

The need to compromise another body region's nerve, most frequently the sural nerve, is correlated to some disadvantages that must be considered:

Presence of sequels at the sural nerve removal site. The following were noted: donor area infection in 10% of the patients; delayed healing in 12%, and; chronic pain in 5%[3]. Sensitivity loss was also noticed on lateral portions of the foot and ankle in 44% of the patients, while 42% presented with calf paresthesia, as well as calf pain in 16% of the patients[4].

Another disadvantage is the limited number or portions of nerves to be reconstructed due to the lack of sufficient donor material, as well as a relative incompatibility between injured and donor nerves´ width. This requires synthesis to be performed with fascicles of the injured nerve with many donor nerve segments, thus increasing the number of site sutures and creating a negative influence due to the resultant enhancement of the perineural inflammatory process[5,6].

Several alternatives to autografting have been studied, including tubing using cryopreserved or glycerol-preserved vessels, which have gained attention for being feasible options[7-13]. In literature, references to the use of allogenous nerve grafts are scarce[14], not listing so far any reference to glycerol-preserved nerve allografts.

The purpose of this study was to compare, in rats, the degree of neural regeneration using histological analysis and functional analysis by interposing glycerol-preserved nerve autografts, autogenous vein, and allogenous grafts, when repairing a 5-mm fibular nerve defect.

MATERIALS AND METHODS

Fifteen male Wistar rats, with weights ranging from 200 to 300g, and about 8 weeks old, were divided into three groups composed of 5 animals. For surgical procedure, the animals were submitted to anesthesia with sodium pentobarbital (at a dosage of 5mg/ kg) injected intraperitoneally. With a microsurgery

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technique a 5-mm gap was created on the fibular nerve on their right rear paws. The groups were divided accorded to the treatment employed for repairing the gaps, as follows:
Group A: control group; immediate interposition of fibular nerve autograft, repositioning of the autogenous nerve fragment removed, and re-suturing in the original direction with mononylon 10.0 (Figure 1).

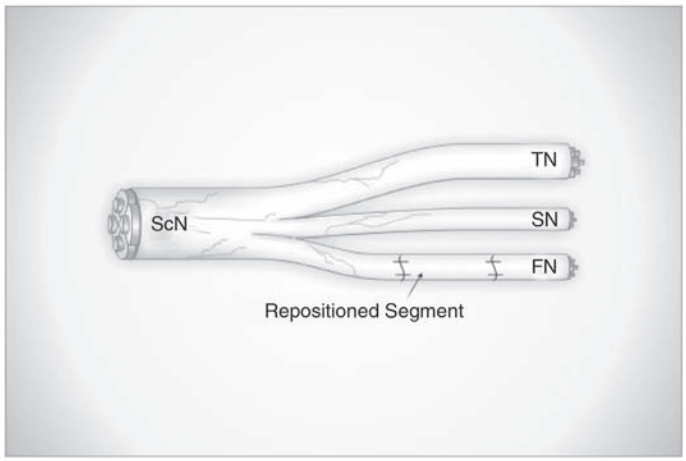


Figure 1 – *Repositioning of the autogenous nerve segment previously removed by suture with simple stitches using mononylon 10.0. ScN: sciatic nerve, FN: fibular nerve, SN: sural nerve, and TN: tibial posterior nerve.*

Group B: Glycerol-preserved autogenous vein tube. From each animal, a 10-mm segment of right internal jugular vein preserved with glycerol 98% for seven consecutive days in refrigerator at 4ºc.
After that period, each vein was hydrated for approximately 30 minutes into saline solution at room temperature and used for nerve tubing. Positioning was made at 2.5 mm from proximal and distal stump, with a 5-mm interval between each other.
For fixing the vein tube on proximal and distal stumps of the nerve, mononylon 10.0 "u"-shaped stitches were used between the epineurium and the jugular vein (Figure 2).

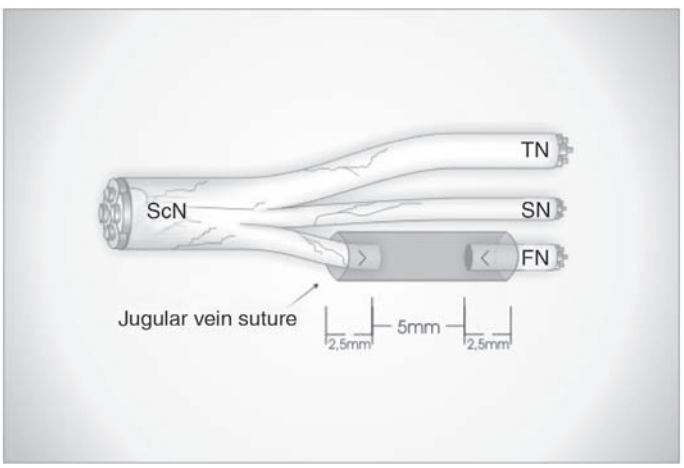


Figure 2 – *Interposition of the glycerol-preserved vein (group B) for a 5-mm defect tubing in a fibular nerve. Note that vein length is 10 mm and this covers 2.5 mm of the proximal stump and 2.5 mm of the distal stump, additionally to the fixation on each stump with "u"-shaped stitches.*

Group C: Interposition of allogenous nerve previously preserved with glycerol 98% for seven consecutive days at 4ºC. These nerves are originated from the right rear paws of Sprague-Dawley rats. These rats had the same weight, gender and ages as the Wistar rats.
The interposition of the allogenous nerve graft was made using a similar microsurgery technique as the one employed in autografting (Group A) and with hydration for about 30 minutes into saline solution at room temperature. The positioning of the allogenous nerve was made similarly to the autografting method performed on group A (Figure 3).

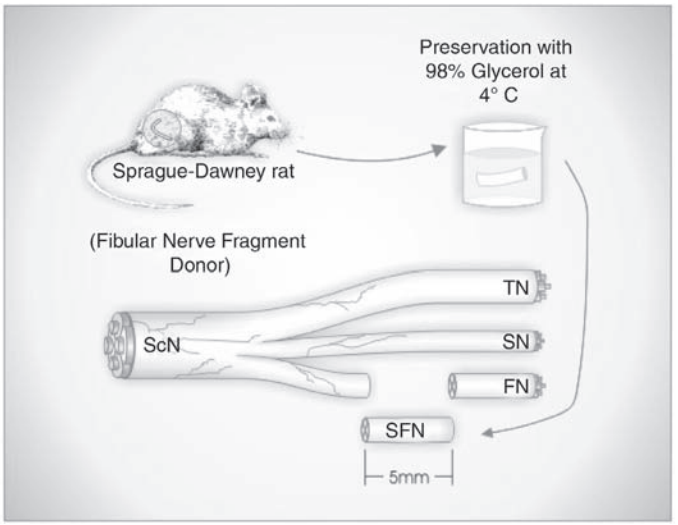


Figure 3 – *Interposition of glycerol-preserved allogenous nerve (group C), for treating a 5-mm defect in a Wistar rat's fibular nerve, after being removed from a different-specimen of donor rat (Sprague-Dawley) and preservation with 98% glycerol for seven days at 40C. ScN: sciatic nerve, SFN: donator rat's fibular nerve, SN: sural nerve and TN: tibial posterior nerve.*

Functional assessment

All animals were submitted to funcional assessment by the method analyzing rats' posterior paws print patterns ("Walking Track Analysis")[16,17], at the following times: early postoperatively, at the third postoperative week, and at the moment of sacrifice (six weeks). The animals' paws were soaked into blue stain and they were allowed to walk on a white paper track to provide footprints.
The footprints distances between the first and fifth toes (toes extension – TE) and the footprint length (FL) were measured (Figure 4).
These data were collected for the purposes of calculating each animal's fibular function rate (FFR) using the formula proposed by Bain et al[16].

$$FFR = 174.9 \times [(OFL - NFL) \div NFL] + 80.3x [(OTE - NTE) \div NTE] - 13.4$$

where:
OFL = operated footprint length
NFL = normal footprint length
OTE = operated paw´s toes extension
NTE = normal paw´s toes extension

with:
FFR = near zero → normal fibular nerve motor function
FFR = near -100 → total dysfunction.

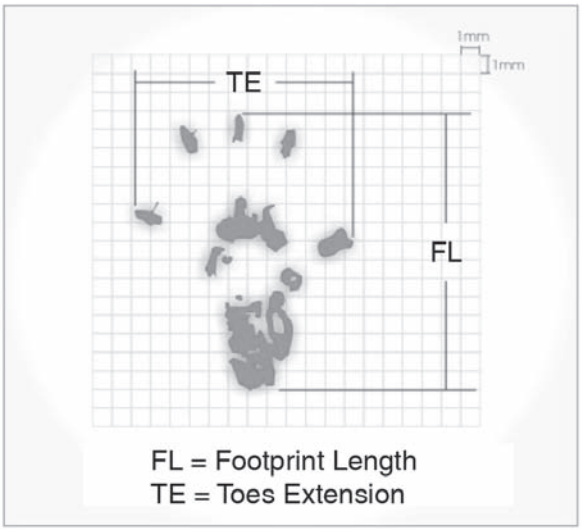


Figure 4 – *On rats' footprints, the required measures for quantifying the fibular nerve function rate are shown (16,17).*

Using data presented by the groups for postoperative FFR and submitting them to statistical analysis by the variance method with repeated measures and one factor (ANOVA), followed by multiple comparisons by Bonferroni method, the FFR range concerning postoperative time was shown[15]. Then, the animals were sacrificed at week six after fibular nerve repair surgery for performing qualitative histology tests.

Qualitative histology

For the histological analysis, the collection of fragments from the medium portion of the interposed segment was standardized, thus avoiding the suture area on both groups.

The material was fixated into 2% glutaraldehyde and into 1% osmium tetroxide included in pure 1% benzoyl peroxide resin and hydroxyethylmethacrylate. 2-micron thick cross sections were provided and stained with 1% toluidine blue.

RESULTS

During the six weeks of the study, all animals remained healthy, being kept in separated cages and with individual IDs and according to the group assigned. They were kept under heating and with water and ration "ad libitum" until full recovery of their vital functions, and neither surgical wound infections nor neuro-dystrophic plantar ulcers being found any time until the day of sacrifice.

At the moment of sacrifice, Group A (autografting) showed, macroscopically, intact grafts, with no visible neuromas on suture lines and little adherence to surrounding tissues.

Group B (autogenous vein + glycerol) showed little adherence between veins and surrounding tissues, with a thin fibrous tissue layer externally surrounding the veins. No neuroma or vein collapses were found.

On Group C (allogenous nerve + glycerol) as well, no neuromas were seen, and little adherence to surrounding tissues, a finding much similar to Group A (autografting).

Histological Analysis

In the microscope analysis of the Group A (autografting) slides we can see that the autograft preserved the epineurium, which is characterized by the presence of spindle-like cells and a thin fibrous sheath. Inside this wrapping, outlining the presence of a small amount of blood vessels is possible.

Small fascicles were found containing myelinated axons of different sizes. Wallerian degeneration is present, but in a small number of axons. Myelinated axonal fibers escape is also evident out of the epineurium boundaries in all five animals of the group (Figure 5).

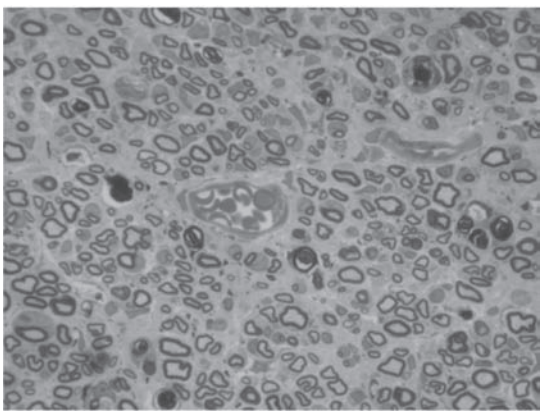


Figure 5 – *GROUP A (autografting). Minifascicles of axons, most of them myelinated and with different sizes (400x).*

On the five animals on Group B (autogenous vein + glycerol), a lower perineural tissue response and axonal escape amount was found when compared to Group A (autografting). Glycerol-preserved veins showed intact and easily distinguishable structure from neural tissue. A large amount of newly-formed vessels are noted permeating the fascicles formed by neural axons, most of these myelinated but with a relatively smaller diameter and containing a smaller number of axons compared to Group A fascicles (autografting) (Figure 06).

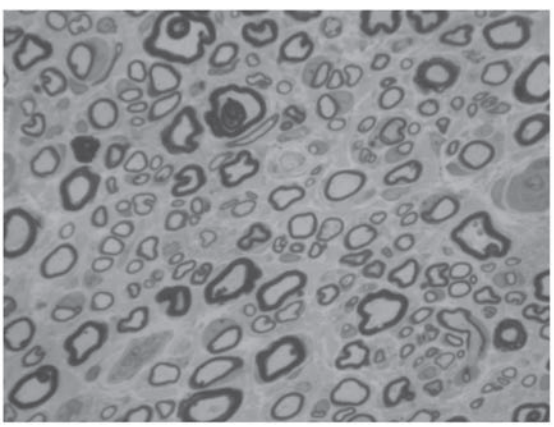


Figure 6 – *Group B (Autogenous vein + Glycerol): Minifascicles of axons, most of them myelinated and with different sizes are shown. (400x).*

On Group C (allogenous nerve + glycerol), the histological findings were similar to those found on group B (autogenous vein + glycerol). The allograft preserved its epineurium, and a fibrous sheath could be seen locally. Within this wrap the presence of neoangiogenesis can be seen in a small amount.

Fascicles formed by axons bundles were small, with few axonal fibers, most of these myelinated and with different sizes. Wallerian degeneration of axonal fibers was present, but in a small amount.

Myelinated axonal fibers escape out of the boundaries of the epineurium and the perineural tissue response were mild and inferior compared to group A (autografting) (Figure 7).

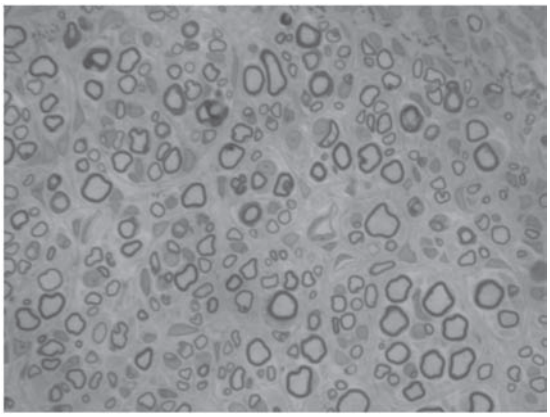
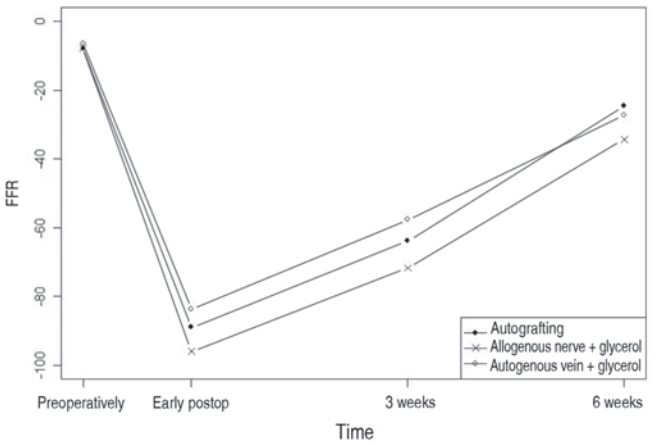


Figure 7 – *Group C (allogenous nerve + glycerol): Arrangement of axons in minifascicles, most of them myelinated and with different sizes (400x).*

FFR (fibular nerve function rate) mean values at preoperative and postoperative (early, 3 and 6 weeks) periods measured for each group are shown on Table 1 and depicted on Graph 1.

Group		Preop	Early Postop	3 weeks	6 weeks
Autografting	**Average**	-7.79	-88.96	-63.79	-24.51
	SD	0.23	8.01	6.59	10.79
	Minimum	-8.10	-102.23	-74.60	-37.40
	Maximum	-7.57	-80.74	-57.13	-13.38
Autogenous vein + glycerol	**Average**	-6.30	-83.57	57.68	-27.36
	SD	2.42	9.08	12.75	11.43
	Minimum	-8.26	-95.79	-68.06	-40.73
	Maximum	-2.80	-73.50	-41.61	-11.57
Allogenous nerve + glycerol	**Average**	-7.67	-96.04	-71.76	-34.36
	SD	2.87	7.50	7.24	14.48
	Minimum	-11.41	-107.87	-80.24	-53.44
	Maximum	-3.34	-87.76	-64.36	-17.03

Table 1 – *Descriptive FFR measures for each group over time.*



Graph 1 – *Mean profiles of the FFR variable for each group throughout the study period, starting from preoperative through early, 3- and 6-week postoperative periods.*

Statistical Analysis

The statistical analysis of the functional recovery as determined by FFR (fibular nerve function rate) was performed by using the variance analysis model with repeated measures (ANOVA) and by the multiple comparisons method by Bonferroni ($p<0.05$)[15].

The statistical analysis didn´t show significant differences between the three groups for preoperative, early postoperative, 3- and 6-week postoperative FFR.

DISCUSSION

Nerve autografting is the surgical treatment of choice for nerve injury cases affecting a larger segment than that where simple reapproximation and raphy are possible, with microsurgical wire[18].

However, the search for new techniques replacing nerve grafting for treating large portions of neural tissue loss, avoiding the damages caused to donor area, targeting the shortening of surgical time and functional outcomes improvement increasingly become a trend in neural regeneration literature. One of these possibilities describes the use of glycerol-preserved allogenous nerves, as employed in this study.

It is important to highlight that the tubing method on groups B (glycerol-preserved autogenous vein)[9] and C (glycerol-preserved nerve allograft) could show some additional advantages over autografting: absence of neural sequels on the donor area, shorter surgical times, and the potential to use large donor areas when repairing large neural tissue losses.

Also, the use of allografts could be regarded as a repair alternative when there is, in particular, a significant neural tissue loss[18]

The key reason for an unsuccessful procedure would be the high degree of antigenicity produced, being required the use of immunosuppressant agents, with the risk of strong local rejection with their early suspension[12,18]

In the evaluation of histological results, group A (autografting) showed the presence of axonal escapes, demonstrating that,

despite the presence of the epineurium and the local neurotrophic factors, these were unable to drive all growing axonal fibers to the target organ.

In the histological analysis of Group B (glycerol-preserved autogenous vein), a milder perineural tissue response was seen compared to Group A (autografting). This is probably related to preservation with glycerol, which collapsed the cells of the inner layer of the vein, as well as the muscle cells of the medium layer, making it a low antigenic structure [9].

Group B (glycerol-preserved autogenous vein) also showed less axon escape out of the boundaries of the vein, probably because the vein frame, collagen- and laminin-rich, better drives axonal growth to the injured nerve´s distal end[9].Another aspect that could have contributed with this is the fact that tubing eliminates the need to suture nerve stumps, thus determining a milder local inflammatory response[1,2,18] .

On group B (glycerol-preserved autogenous vein), the presence of neural mini-fascicles was found, most of these myelinated, as well as wallerian degeneration in small amounts, consistently to previous reports on scientific literature[9,13].

Group C (glycerol-preserved allogenous nerve) showed mild inflammatory response, characterized by a mild and poorer perineural response when compared to group A (autografting). It also showed axons, most of them myelinated and arranged as mini-fascicles, neural fibers in a wide range of diameters permeated by blood vessels and wallerian degeneration.

A smaller number of escapes to out of the boundaries of the nerve were found when compared to Group A (autografting) and similarly to group B (glycerol-preserved autogenous vein). These facts can be explained by the same reasons previously discussed on Group B.

Data for the mean early postoperative FFR of the three groups were submitted to statistical analysis, and no significant difference was found between groups. This lack of difference indicated that, similarly, all rats had the same kind of neural injury (by the resection of a 5-mm nerve segment) allowing for comparing the functional recovery process between groups.

At 3 and 6 weeks postoperatively, no statistically significant difference was found for FFR among the three studied groups. So, regardless of the method employed for treating a nerve defect, no statistical difference was found for animals' functional recovery in none of the assessed time points.

It is important to emphasize that, with these data, we can reasonably state that glycerol produced such a significant reduction of the allograft antigenicity that it enabled similar results to those found with the use of autogenous veins and with the autografting itself.

CONCLUSIONS

Based on the histological analysis and on the functional evaluation of the neural regeneration achieved for repairing 5-mm defects on fibular nerves of Wistar rats, in each of the groups studied: Group A (nerve autografting), Group B (glycerol-preserved autogenous vein) and Group C (glycerol-preserved allogenous nerve), we can conclude from the histological analysis that, in all groups, small fascicles were found containing myelinated axons of different sizes, and wallerian degeneration in a small number of axons.

On groups B (glycerol-preserved autogenous vein) and C (glycerol-preserved allogenous nerve), the escape of myelinated axonal fibers out of the boundaries of the epineurium was inferior to that seen on group A (autografting). On groups B (glycerol-preserved autogenous vein) and C (glycerol-preserved allogenous nerve) a milder inflammatory process was found when compared to group A (autografting).

Concerning functional assessment, we can conclude that there was no statistically significant difference between functional recoveries of the fibular nerve, regardless of the kind of repair employed: autografting, glycerol-preserved autogenous vein, and glycerol-preserved allogenous nerve.

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