THE USE OF GLYCEROL-TREATED VENOUS GRAFT IN DAMAGED NERVES REPAIR: AN EXPERIMENTAL STUDY IN RATS

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SUMMARY

Autografting is the treatment of choice for cases of major nervous tissue loss where the ruptured nerve ends cannot be reduced. The use of a venous autograft previously treated with glycerol may be an alternative treatment, as it reduces surgery time duration and level of morbidity. Blood vessel explants, used in vascular microsurgery, kept in glycerol maintain their original biological structure, and when used in autografting, present reduced levels of patient's immune response. The aim of this study was to compare the level of nervous tissue regeneration by using histological analysis, regenerated myelinized axons counts, and functional analysis, obtained with the interposition of autologous graft (group A) and glycerol-treated vein tube (group B) in 5-mm defects on Wistar rats' fibular nerves. Neuroma was observed in animals of group A only. Both groups presented histological pattern consistent with reduced number of regrown axons with myelin sheath, although the number of such neurons was smaller in animals in group B as compared with those in group A. Regarding the functional after-healing assessment, both groups presented no statistically significant differences.

Keywords: *Peripheral nerves/surgery; Nerve regeneration; Glycerol; Veins.*

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INTRODUCTION

Possible causes for partial destruction of nervous fibers are associated to traumas, neoplasias, and iatrogenesis[1]. The immediate result is functional deficit, which, according to severity and extension, can render repair by primary reduction impossible.

Among all techniques available for neural repair, primary reduction is outlined as one providing the best histological and functional results, followed by autografting technique[2,3]. This technique can guide axonal growth and join distal and proximal stump ends, reducing tension on suture line, which would inhibit neural repair[1].

With the use of autografting, some factors must be considered, namely: 1) it always causes morbidity to donator area; 2) extensive neural tissues loss demands large amounts of autologous tissue, sometimes insufficient; 3) the use of tubular replacements reduced surgery time[2,3].

Studies addressing extensive neural tissue losses and the need of bridges connecting proximal and distal ends have been performed as early as the second half of 19th Century[4].

One feasible alternative presenting low morbidity is the use of tubing chambers[5]. In this method, proximal and distal stumps are introduced at each end of a tube and anchored with epineural sutures. Axons regenerate from the proximal stump, penetrating into tube light to reach distal stump[5]. Tubes provide a mechanical support, reduce tension between stumps and the amount of material on suture, thereby reducing cicatricial response[5,6]. Tubing enables experimental manipulation of the neural micro-environment such as administration of drugs stimulating regeneration[7].

A number of materials have been studied as tubing forms, including materials such as decalcified bone, fascia tube, synthetic tubes, and re-absorbable tubes[8].

Among tubing forms, neural reconstruction using vessels has developed well. Foramitti[9], in 1904, reported successful neural regeneration using arterial graft. In 1909, Wrede[10] performed it by means of venous graft. In 1941, Swan[11] used a venous graft for fixing ulnar nerve injury in human beings, and, in 1943, Weiss[12] used a tube from dissected arteries and preserved in ice to guide neural regeneration.

In 1982, Brunelli et al.[13] demonstrated the possibility of full neural regeneration within autologous vein grafts in defects as small as 1 cm, and, later on, in 1987, they demonstrated the selective chemotactic attraction to sensitive and motor fibers using that same kind of graft. Walton et al.[14] in 1989, described the use of vein grafts in digital nerve injuries' repair comparable to controls (nerve grafts), showing superior outcomes compared to control subjects in 10-mm defects. However, this technique showed a disadvantage of being more susceptible to collapse.

Study conducted at the Microsurgery Laboratory, discipline of Plastic Surgery, Medical College - USP.

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The concept of venous grafting for neural reconstruction brought an alternative to reduce sequels on neural donator area, yet with limitations: 1. its biggest benefits are seen in injuries as small as 10 mm; 2. inverted venous graft seems to provide better results; 3. surgical time remains extensive, because it requires vessels dissection.

In order to reduce surgical time and morbidity, a venous graft collected from a cadaver donator could be used. As shown by Wolff et al.[15] vessels can be preserved on glycerol preventing its structure to be destructed, which enables its use in vascular microsurgery, having the additional advantage of reducing graft immunogenicity.

The objective of this study is to assess the degree of nervous repair by means of quantitative histology and of a functional study, after repair with autografting and glycerol-treated veins in injuries showing 5mm fibular nerve loss in Wistar rats.

MATERIALS AND METHODS

Ten 8-week old Wistar rates weighting 200g - 300g have been used in this study. For surgical procedure, the animals were anesthetized with sodium pentobarbital (5m/kg) intraperitoneally injected. With the microsurgical technique, 5-mm long defects were created on the fibular nerve of rats' right paws at 5 mm away from its sciatic nerve origin. The animals were divided into 2 groups of five animals according to the kind of repair provided.

In group A animals (control), defect correction was provided by suturing the removed nerve segment, keeping the original direction with the use of separated epineural stitches with Mononylon 10-0 (Ethicon) (acting as an autogenous graft).

In group B animals, an autogenous segment of the right internal jugular vein was interposed, which was prepared with trichotomy of the cervical anterior portion, incision at cervical paramedian portion and segregation of internal jugular vein with 10-mm resection of its extension, with distal and proximal stumps being connected. This vein graft was collected 7 days previously, and remained stored in individual and labeled tubes, with 20ml of 98% glycerol solution and refrigerated at a temperature of 4°C[16-18]. On graft placement day, the veins were kept into saline solution for 30 minutes previously to its use.

Jugular vein was anchored in position by means of "U" stitches with Mononylon 10-0 (Ethicon), passing on each end as follows: from outside in the vein, crossing the epineurium on nerve's stump and going back into the vein from inside out, so that the vein could cover 2.5 mm of the nervous stump. The second end was fixated using the same technique (Figure 2). Closing was made by muscle and skin planes with non-absorbable surgical suture.

The animals received water and food ad libitum and were sacrificed 6 weeks after surgery to enable histological analysis and counting of the number of regenerated myelinized axons. Functional recovery assessment was made according to the technique analyzing lower paws prints at walk (walking track analysis) pre- and postoperatively, on the third week and at the moment of sacrifice (6 weeks).

HISTOLOGICAL ASSESSMENT

Histological assessment was performed with a medial portion fragment of the interposed segment. The material was analyzed after fixation into 2% glutaraldehyde and 1% osmium tetroxide solution, included into pure 1% benzoyl peroxide and hydroxyethylmetacrilate resin. Two-microns thick cross-sections were made, which were stained with 1% toluidine blue.

On slices, the overall architecture of the regenerated nerve was analyzed, pursuing to identify an organization pattern of the neural tissue inside neural and venous grafts, the myelinization and axonal reorganization degree, fibroblasts and epiperineural connective tissue arrangement, the presence of axonal fibers escaping to beyond the limits of the epineurium, and the tissue response analysis[16-18].

FUNCTIONAL ASSESSMENT

The degree of functional recovery associated to neural regeneration was assessed by studying rats' ambulation patterns at baseline and postoperatively (early, 3 weeks, and at the moment of sacrifice - 6 weeks), by analyzing footprints of animals' posterior paws (walking track analysis), according to the methods previously described by De Medinaceli et al.[19] and modified by Bain et al.[20].

Footprint distances between first and fifth fingers (fingers extension - FE) and footprint length (FL) were collected. (Figure 1)

Those data were collected for calculating fibular function index (FFI) of each animal, using the formula proposed by Bain et al.[20]:

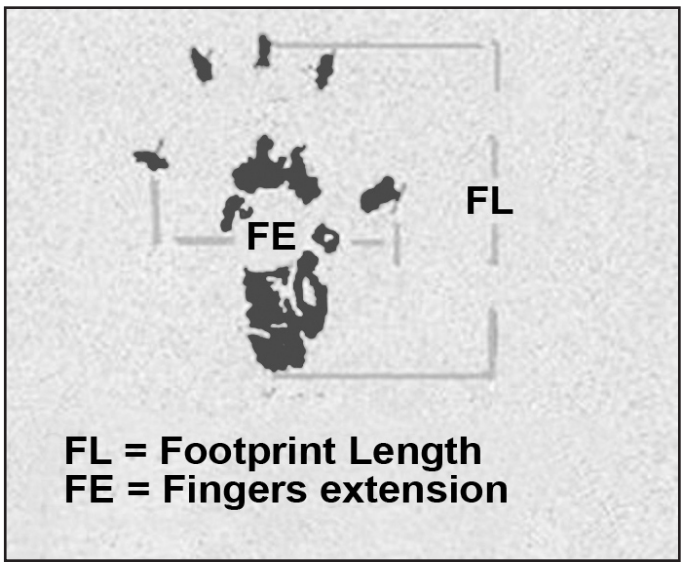

Figure 1 - *Schematic illustration of the measures used at sciatic function index calculations*

$$FFI = 174.9 \times [(FLO - FLN) \div FEN] + 80.3x [(FEO - FEN) \div FEN] - 13.4$$

where:
FLO = footprint length of the operated paw
FLN = footprint length of the normal paw
FEO = fingers extension of the operated paw
FEN = fingers extension of the normal paw

being:
FFI = near to zero® normal fibular nerve motor function
FFI = near to -100® full dysfunction.

RESULTS

During the six weeks of the study, all animals showed good health status, with no infection on surgical wound or neurodystrophic plantar ulcers being seen.
At the moment of sacrifice, group A (autograft), under gross analysis, showed that the examined grafts were intact, with no visible neuromas.
In animals from group B (autogenous vein + glycerol), a thin layer of fibrous tissue covering veins was seen, which were shown to be intact. Macroscopically, no neuroma was seen, as well as adherence cases.

Histological Assessment:

In Group A (autograft), the microscopic analysis of slides evidenced the presence of a large amount of fibers with variable diameters, reasonably myelinized, spread all over the neural stroma, sometimes grouped as small fascicles. Tissue response around the graft was greater when compared to group B. Regenerated fibers escape was detected going beyond the limits of the epineurium in all animals (Figure 2).
In group B (autogenous vein), fibrosis response around veins was clearly lower when compared to group A (autograft). Neural escapes were less frequently seen (Figure 3). Nervous fibers presented a moderate width, myelinized at median portion, and presented an arrangement as mini-fascicles with areas of low axonal density at the newly-formed stroma, containing only connective tissue. However, they presented strong neoangiogenesis. Overall, the area occupied by regenerated fibers in this group was smaller than that seen in group A (control).

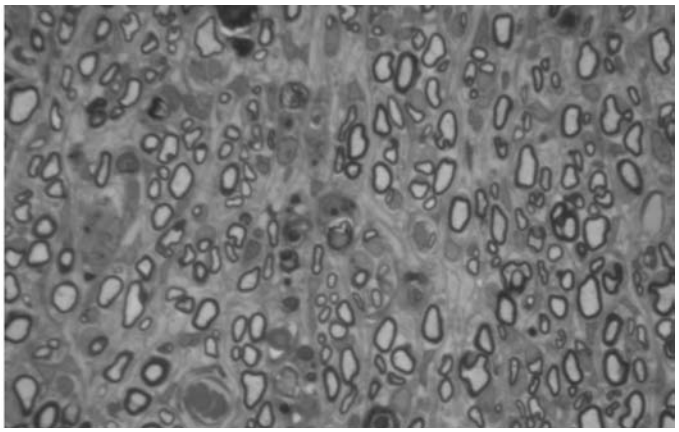

Figure 2 - *Group A: autograft*

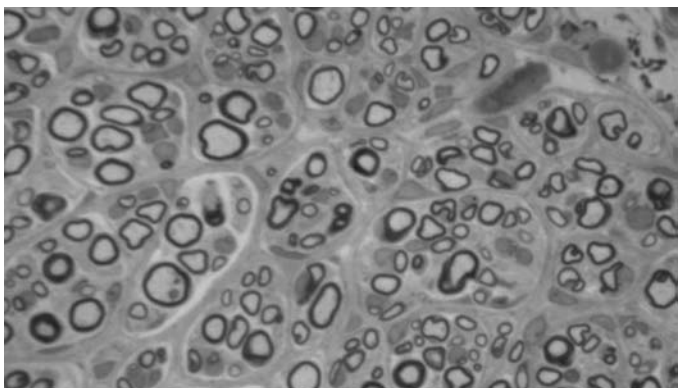

Figure 3 - *Group B: glycerol-treated vein*

Functional study:

The degree of functional recovery was measured by studying pre-and postoperative (early, 3 weeks, and 6 weeks) rats' ambulation patterns through the analysis of footprints of animals' posterior paws (walking track analysis), providing FFI average values, calculated for each group (Figure 4).

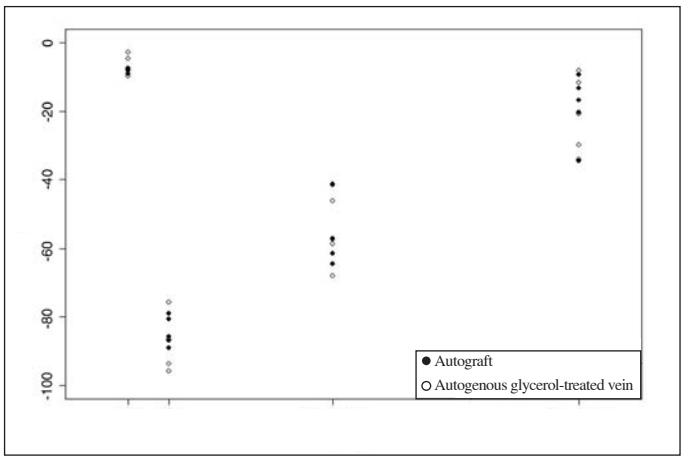

Figure 4 - *FFI for Group A (autograft) and Group B (autogenous glycerol-treated vein)*

As shown by Figure 4, FFI values for early postoperative period, 3 and 6 weeks were shown to be not statistically significantly different between both studied groups, but indeed a linear growth of the FFI was seen in both groups. These data were obtained from statistical analysis by Bonferroni's multiple comparisons method.

DISCUSSION

In cases of peripheral nerves injuries with substance loss, where the extension of the defect precludes direct re-approximation of the stumps, the best repair method seems to be autografting[2].
Nevertheless, some factors lead us to pursue a new conduit for axonal growth: 1. the removal of autologous material for grafting always produce morbidity to donator area; 2. large defects demand the removal of extensive portions of autogenous tissue, which are, many times, unavailable; 3. the use of artificial materials or collected from tissue libraries saves the time to remove autologous material[2]; 4. the results are not fully satisfactory with the use of autografts[20].
The interposition of tubular conduits as a bridge between sectioned nerve's stumps has presented excitingly good experimental and clinical results. For fixing small defects, when the distance between stumps is not extensive enough to disturb chemotactic and chemotrophic attraction played by distal stump over axonal growth cone, the results are comparable to those obtained with autografts[2].
The tubing technique also provides some additional theoretical advantages over traditional methods of grafting: it provides good coaptation of both stumps, causing less manipulation trauma; it enables a better confinement of the growing fibers inside the tube, isolating the repair site from surrounding inflammatory response; it guides fibers growth towards distal stump, enabling local neurotrophic factors to concentrate around; it reduces neuroma formation and fibers escape out of the conduit; it allows for the circulation of regeneration-enhancing substances[5].

Several materials have been used for building tubes, which can be absorbable or non-absorbable[5].
An optimal tube would have to be inert, flexible, absorbable, and able to inhibit cicatricial process and to facilitate healing and neural regeneration process.
Tube flexibility is necessary for protecting a regenerating nerve when mobilizing the injured portion. Although re-absorbable, this tube should remain "*in situ*", with no degradation, for the period required for axons to grow and reach distal stump[5].
Several materials have been used for building absorbable tubes[5]. Clinically, a lower inflammatory response is desirable in order to prevent adhesions to surrounding structures, especially tendons in hand repair surgeries.
Venous graft can be employed as tubing chamber; however, little is known about histological changes both clinically and in experimental models[15].
Weis used venous grafts in experimental models, stating that there should be tension between neural suture, because this would favor longitudinal direction for axonal fibers to regenerate[12]. Some decades later, tension over neural suture was shown to cause a deleterious effect[6]. Thus, venous grafts should serve as a guide between neural stumps, keeping the region free from tension.
The use of autologous vein graft as neural tube or as suture wires coating is associated to connective tissue formation and axonal regeneration reduction[6]. The contact of endothelial cells with neural tissue is believed to produce this cicatricial tissue before neuroregeneration can occur.
Recently, some authors showed in experiments some advantages with the use of inverted (from inside out) vein over the traditional vein graft, and also when compared to traditional nerve grafts. Collagen and laminin were shown to promote neural regeneration[6]. The adventitious layer of the veins is a rich collagen source, and the median muscular layer of the veins is laminin-rich. Inverting the normal vein direction provides a better axons exposure to the collagen existing on adventitious layer.
Brunelli et al.[2], conducted a study with venous conduit filled with autogenous fresh muscle and showed the superior quality of the results for distances twice as longer as the conduits filled with axons in empty tubes. The presence of muscle cells within it is believed to reduce the incidence of vessel adherence.
The use of a glycerol-preserved vessel has been described in vascular microsurgery, but had never been described in neural reconstruction. Rejection response after allogenous vein transplant is histologically detected by an infiltrate predominantly with mononuclear cells[17]. That response depends on the degree of histocompatibility and of the thickness of the median layer containing immunogenic muscle cells[15-17]. Preservation with glycerol leads to a loss of the intimae layer and median layer's muscle cells atrophy. However, elastic fibers remain intact on a preserved vein[17].
Concerning histology, we could see that a greater neural escape and a stronger inflammatory response around the graft occurred in group A (autograft) compared to group B, where rats were submitted to tubing technique. Neural regeneration showed similar patterns between groups A and B regarding distribution pattern of axonal fibers and their myelinization degree.

CONCLUSIONS

Histological analysis and functional assessment of nervous repair obtained after 6 weeks by means of nerve autografting and autogenous glycerol-preserved vein for fixing 5-mm defects in rats' fibular nerves allow us to conclude that control group A (autograft) showed a greater tissue response when compared to group B, as well as a higher amount of axonal fibers escaping. The use of non-preserved autogenous vein showed histological pattern of areas with lower axonal density, presence of connective tissue within neoformed stroma, and stronger neoangiogenesis. Nevertheless, the histological pattern was similar compared to control group (autograft).
In this study, objective functional data (FFI) were verified which evidence a similar evolution of animals submitted to treatment compared to control animals (autograft), and group B (autogenous glycerol-preserved vein) presented a functional response (FFI) statistically similar to that of control group throughout postoperative period (early, 3 and 6 weeks).
The use of autogenous glycerol-preserved vein seems to be an alternative for neural reconstruction. This material can be kept in tissue libraries, having no need of postoperative immunosuppressants and, like any other tubing method, allows for administering neurotrophic substances within it.

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