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PERIPHERAL NERVE REGENERATION THROUGH THE NERVE TUBULIZATION TECHNIQUE

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ABSTRACT

Transection of a peripheral nerve results in a loss of function at the target organ that can rarely be recovered without surgical repair. Such an intervention usually involves nerve autografting but is complicated by problems such as the need for secondary surgery, a limited donor nerve supply and loss of sensitivity in the donor nerve area. An alternative approach involving repair by nerve tubulization has been extensively used to study substances that may improve the regenerative process. An interesting feature of the tubulization technique is the possibility of filling the tube with substances that can enhance regeneration. Such substances include collagen, laminin, hyaluronic acid, fibronectin and, more recently, glycosaminoglycans alone or with collagen. Biopolymers, purified glial cells, and neurotrophic factors have also been tested. By using the tubulization technique, it has been possible to increase the number of regenerating fibers and the gap between the stumps. In this review, we discuss some of the basic concepts of this technique, as well as recent advances in this field.

Key words: Collagen, extracellular matrix, glycosaminoglycans, laminin, neurotrophic factors, proteoglycans, tubulization.

Peripheral nerve regeneration

The regeneration of a lesioned peripheral nerve is an intricate process that has been extensively studied in recent decades [23,57,73]. The obvious negative impact of such an injury on the life quality, has led to the development of a number of surgical approaches to reconnect the transected nerve stumps [23]. End-to-end anastomosis and autologous nerve grafting have been the most commonly used approaches [20,28,46,47,50,62]. However, the simple reconnection of the stumps is not sufficient for successful targeted reinnervation since it does not allow precise control of the orientation of the fascicles and their substructures [28,40]. Another critical issue is the level of the lesion, which is crucial for the outcome of regeneration, since the closer the lesion occurs to the neuron cell body, the smaller are the chances of success and the greater are the chances of neuronal death [15]. The intrinsic regenerative capacity of each neuron is also an important determinant in degeneration [22].

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The interaction between growing axons and non-neuronal cells present in the distal stump, and the rearrangement of the extracellular matrix are central phenomena for achieving guided, high quality regeneration [7,58,73].

Indeed, the fine changes in the extracellular matrix associated with the stages of regeneration have been widely studied (for review, see [58]). One experimental model that has proven to be useful for exploring this subject is the nerve tubulization technique. This method provides a unique microenvironment to which different substances and cells can be added in order to assess their role in regeneration [5,20,21,23,31,33,60,62]. In this review, we provide an overview of the knowledge on nerve regeneration that has been gained by using the tubulization technique.

The Tubulization technique: a micro chamber for studying peripheral nerve regeneration

Tubulization or entubulation is a surgical procedure in which the sectioned nerve stumps are introduced and fixed into a tubular prosthesis, with a gap left between the nerve ends (Fig. 1A). Regeneration within the tubular prosthesis is dependent on the early formation of a non-cellular

bridge to connect the two stumps [23,35]. This connection consists of a fibrin matrix that provides the initial substrate for the migration of non-neuronal cells (Fig. 1B). The fibrin matrix is then degraded and substituted by longitudinally oriented collagen fibrils that will serve as the substrate for the regenerating sprouts of the proximal stump (Fig. 2).

The occurrence of the events described above depends on the length of the gap between the stumps and on the cross-sectional dimensions of the tube. Butí *et al.* [9] examined the influence of these two parameters on the regeneration of mouse sciatic nerve and found that although tubulization considerably enhanced the recovery for 2-4 mm gaps, it was less efficient with larger gaps. In addition, the best functional recovery was obtained with tubes having an inner cross-sectional area 2.5 times greater than that of the nerve. Thinner tube walls also improved the axonal regeneration [9,23].

The tube may be constructed of different materials and the stumps may be glued or sutured to its extremities [23]. Tubes made of silicone are the most commonly used and, along with polyglycolic acid, are systematically used in clinical practice to repair median, ulnar and digital nerves [41,42,44,64].

Ideally, the material used as conduits, should satisfy a variety of criteria. First, the substance must be biocompatible, which means that it should not elicit any vigorous immune response that would interfere with the regeneration [1,14,30]. Second, the material should last for a minimum critical period after implantation in order to ensure an appropriate path for the growing axons [20,67,68]. Third, the material should stimulate cell migration as well as axonal growth, thereby speeding up the regeneration. Fourth, the material should stimulate vascularization and the accumulation of neurotrophic factors in the gap between the nerves ends [11,14,57]. From a surgical point of view, the material should also be flexible, and the tube wall should be as thin as possible and transparent in order to facilitate fixation and orientation of the stumps [23].

A potential problem with the tubulization technique is that the tube itself may compress the regenerated nerve cable and induce a late degenerative process that includes demyelination and axonal loss [11,16,45]. In such cases, large caliber fibers are the most affected and, a few months after surgery, there is a decrease in the amplitude of the compound action potential, with a direct impact on motor function [11,36].

Although this abnormalities caused by compression may be avoided by adjusting the internal diameter of the tube to accommodate the lesioned nerve [9, 23], the use of biological material or of synthetic bioresorbable materials represents a promising option. In the past, conduits of biological origin such as arteries, veins, decalcified bone, peritoneum and dura were used (reviewed in [23]). However, most of these failed to provide optimal mechanical support for guided axonal growth, and were also often difficult to obtain in the necessary dimensions and amounts without lesioning other body areas. As a result, the use of such biological conduits has become less attractive and has gradually been replaced by synthetic bioresorbable polymers.

Several types of biopolymers have been used as nerve conduits, with results comparable to autografts [5,11,21,24,29,30,47]. A number of variables are particularly important in order to achieve nerve regeneration, and include the method used to build the tube, the tube porosity, and the biocompatibility of the material. The time frame of degradation is also a relevant consideration. In this context, biodegradable materials provide better results compared with non-bioresorbable substances since one main disadvantage of the latter is the need for a second surgical intervention to remove the prosthesis.

Of the numerous biocompatible materials available, collagen, EVA (ethylene-vinyl acetate copolymer) [1], PLLA (poly(lactic acid))

[20,21], poly(glycolic acid) (PGA) [33], Poly(L-lactic acid co-glycolic acid) (PLGA) [8,30] and LA/CPL (a copolymer of lactic acid and caprolactone) [5,17] have yielded good results in nerve regeneration. More recently, Flynn *et al.* [24] developed a method to create longitudinally oriented channels within poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels for neural tissue engineering applications. Such technological advances, combined with the use of tubulization, growth factors and extracellular matrix components, has provided a promising alternative to autografts [48].

Resorbable collagen tubes have been the most successful and frequently used prosthesis for repairing peripheral nerves. These tubes have been used to repair monkey median nerve [3,4,39], with results comparable to those obtained by conventional graft repair. The physiological recovery was also similar to that seen with direct suture repair [3].

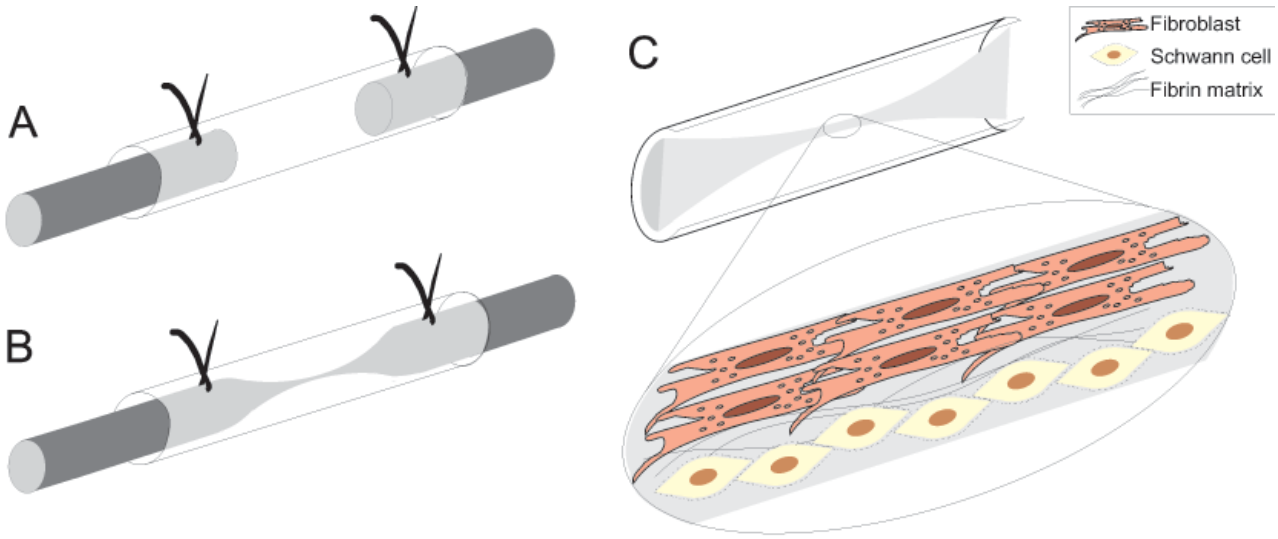

Figure 1A. Schematic representation of the tubulization technique. Both nerve ends are sutured to the tube walls, leaving a gap between the stumps. **B**. Formation of a bridge between the stumps. This bridge is initially acellular and has a fibrin matrix. **C**. Schematic representation of a longitudinal section through a regeneration cable already containing fibroblasts and Schwann cells that will support the ingrowing axons of the proximal stump.

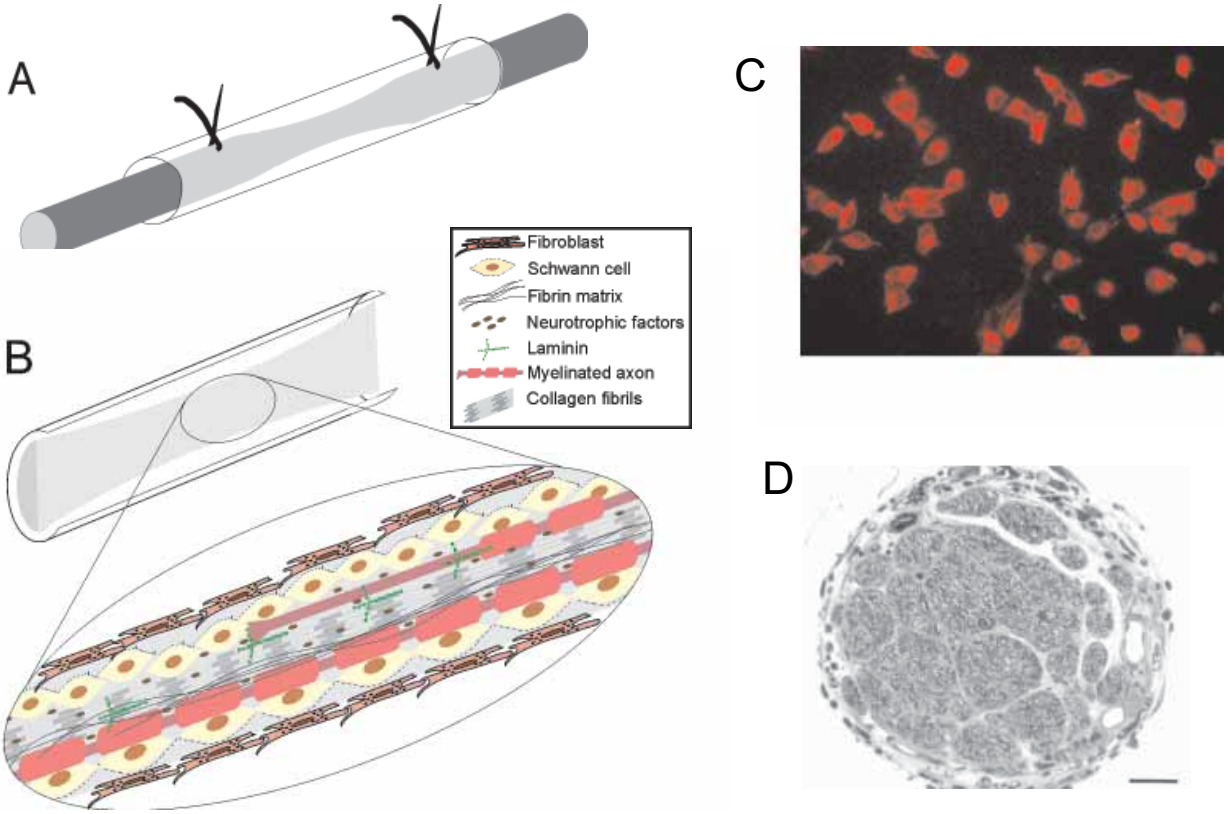

Figure 2A. Drawing of a regenerated nerve inside a tubular prosthesis. **B**. Schematic representation of a longitudinal section through A. Note the presence of a collagen matrix, containing neurotrophic molecules that provide support for axonal growth. The Schwann cells are organized to form bands of Büngner that guide the ingrowing axons. **C**. Micrograph of a pure Schwann cell culture immunolabeled with S100 antibody. **D**. Transverse section of a regenerated nerve at the midpoint of the tube, five weeks after tubulization. Scale bar = 50 µm. (Panel D was reproduced from Pierucci A. *et al.* Braz. J. morphol. Sci. 2004; 21:125-130; Reprinted with permission of the Braz. J. morphol. Sci.).

Role of purified extracellular matrix proteins and neurotrophic factors in peripheral nerve regeneration

An attractive feature of the tubulization technique is the possibility of filling the tube with different substances and cells in order to study their role in nerve regeneration [12,13,31,46,50,53,65]. As mentioned above, the first stage of nerve regeneration after tubulization is the formation of a longitudinally oriented matrix that provides a substrate for non-neuronal cell migration and axonal elongation. Hence, prefilling the tube with extracellular matrix components may improve the regenerative process [51]. Several molecules, including collagen, laminin, hyaluronic acid, fibronectin and, more recently, glycosaminoglycans alone or associated with collagen, are useful for this purpose [6,10,11,25,35,38,47,59,69,70]. The use of these substances, resulted in a greater number of regenerating fibers and allowed the gap between the stumps to be increased. However, although promising, neither collagen nor laminin gels have yielded a significantly greater number of myelinated axons when compared to saline-filled tubes. Indeed, as shown by Valentini *et al*. [61], collagen- and laminin-containing gels decreased the axonal elongation in semipermeable tubes. In these experiments, the density of the solutions used may have impeded the diffusion of neurotrophic factors, Schwann cell migration, and axonal elongation. In contrast, Labrador *et al*. [34] reported successful regeneration with more dilute collagen or laminin solutions: a low-concentratin collagen gel (1.28 mg/ml) provided the best recovery, whereas an intermediate concentration (1.92 mg/ml) gave the worst results. The findings show that a slight change in the extracellular composition can have a marked effect on regeneration.

This conclusion agrees with another study showing the dependence of nerve regeneration on the concentration of the agarose gel used in tubulization. In this case, the degree of reinnervation decreased progressively with increasing concentration (0.5, 1 and 2%) of the agarose gel used to fill a 4 mm gap in silicone tubes in mice, probably because higher gel concentrations produced a matrix with progressively narrower pores that obstructed axonal regeneration and molecular diffusion. Labrador *et al.* [35] also reported that the size of the regenerating cable within silicone tubes was inversely proportional to the concentration of the hyaluronate gels used.

Laminin is another reported well-characterized extracellular matrix component. Madison *et al.* [47] reported an increase in the number of mouse myelinated axons crossing a 4 mm gap filled with laminin, when compared to collagen and an empty chamber. However, Labrador *et al*. [35], observed no functional recovery under the same experimental conditions. Indeed, in this case, the less concentrated collagen solution provided better results than laminin, probably because the less dense matrix, allowed greater axonal ingrowth. With longer gaps (6 mm), there was improved target reinnervation with both laminin and collagen gels when compared with saline solution.

Another ECM component that has shown positive results is the fibronectin. It has been used to prefill tubes and to construct nerve guides (for details, see [2, 32, 66]). Indeed, fibronectin is one of the few natural extracellular matrix molecules that can provide the correct axonal growth in a permissive environment and also retain neurotrophic substances [26].

Other components of the basal lamina include heparan sulphate and chondroitin sulfate proteoglycans [52], which can interact with laminin via their glycosaminoglycan side-chains. Dow *et al.* [19] suggested that the glycosaminoglycan residues of heparan sulphate proteoglycans have neurite-promoting properties *in vitro*. These side chains are also able to bind to several growth factors, including acidic and basic fibroblast growth factors. Gorio *et al.* [25] reported that the treatment of rats with exogenous glycosaminoglycans stimulated peripheral nerve regeneration, and enhanced the expression of mRNA for myelin proteins. Chamberlain *et al.* [11] studied nerve regeneration across a 10 mm gap in rat sciatic nerve using porous collagen tubes prefilled with a collagen-glycosaminoglycan matrix. This matrix was designed to last for six weeks and had a surface with different degrees of porosity. Under these conditions, regenerated nerves contained axons with a significantly larger diameter and significantly higher A-fiber conduction velocities when compared to the controls.

Little is known about the role of high molecular mass proteoglycans in the regeneration of peripheral nerves *in situ*. Aggrecan is a large, complex proteoglycan with a protein backbone that has three globular domains (G1, G2 and G3). Interestingly, the G2 domain is capable of interacting with different extracellular molecules, and the G3 domain has a

sequence that is homologous to that of epidermal growth factor [63]. Since this proteoglycan contains hundreds of glycosaminoglycan side chains, it is able to aggregate and form a gel-like matrix that can retain water and soluble trophic factors [18,27]. Pierucci *et al.* [49] tested the ability of aggrecan, extracted from avian xyphoid process, to provide an adequate matrix for axonal sprouting and regeneration after sciatic nerve tubulization. The number of regenerated myelinated fibers increased significantly when compared to the control, and important Schwann cell activity was observed *in vivo* and *in vitro*.

Although extracellular matrix components have an important role in nerve regeneration, neurotrophic factors are also pivotal because of their ability to regulate the time course of the process and to provide support for neuronal survival, neurite guided outgrowth to the target organ, and the multiplication and migration of non-neuronal cells [12,13,37,53,71]. A number of studies have combined the use of matrix molecules with neurotrophic factors in order to improve the number of regenerating axons and functional recovery [29,58]. Zhang *et al.* [72] recently reported significant motor recovery after silicone tube implantation and treatment with ciliary neurotrophic factor (CNTF). Brain-derived neurotrophic factor (BDNF) associated with collagen also favored functional recovery in different repair techniques, including tubulization [60]. Additionally, fibroblast growth factor 1 (FGF-1) has been reported to improve the outcome of regeneration when incorporated into a synthetic polymer and administered locally [48]. Glial cell line-derived neurotrophic factor (GDNF) also improved nerve regeneration when associated with collagen gel [12]. This treatment also up-regulated GAP-43 protein expression, which could partially account for the increase in axonal growth rate. No long-lasting improvement in anatomical and functional recovery was observed 12 weeks after tubulization or after treatment with nerve growth factor (NGF) and neurotrophin-3 (NT-3) [71]. However, Terenghi *et al.* [58] reported that the addition of NT-3 and NT-4 to fibronectin guides did improve regeneration [54, 56].

The use of biomaterials, extracellular matrix molecules and neurotrophic factors in tissue engineering is one of the most promising approaches for the treatment of nerve defects after surgical ablation and injury. One strategy that has been widely studied in recent years is the association of biocompatible conduits and purified Schwann cells seeded inside the prosthesis. Schwann cells and their basal lamina are the primary support cells of the peripheral nervous system [55] (Figure 2C). After injury, these cells proliferate, synthesize trophic factors and help macrophages to phagocyte axonal and myelin debris. The bioactive substrates on which axons migrate best are produced mainly by Schwann cells and provide sustained and guided ingrowth. Rodríguez *et al.* [50] reported that Schwann cells from predegenerated sciatic nerves, enhanced peripheral nerve regeneration across a 6 mm gap in mice when transplanted into a poly(L-lactide-μ-caprolactone) copolymer (PLC) tube. Similarly, Evans *et al.* [20] obtained promising results by using poly(L-lactic acid) (PLLA) conduits filled with purified Schwann cells. However, there were no detectable functional differences between the polymer-mediated regenerated nerves and those repaired with allografts, indicating that methodological improvements in the former approach are still necessary. In addition, the survival of Schwann cells *in vivo* (30 days) may be too short for adequate regeneration [50]. Nevertheless, the number of regenerated axons per mm^2 at the tube mid-point was twice as much as that in a silicone tube, indicating much faster axonal growth in the presence of a large number of Schwann cells.

Concluding remarks

The use of bioresorbable and bioactive polymers associated with a defined matrix containing purified Schwann cells can significantly improve the axonal number and growth rate, and may restore motor and sensory functions to levels comparable to those seen with autografts. In the future, nerve tubulization may become a therapeutic option of choice for treating extensive nerve lesions, thereby avoiding the need for autografs.

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