PERIPHERAL NERVE GAP REPAIR BY ENGINEERED TISSUE CONDUITS

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Bruno BATTISTON, Stefano ARTIACO, Paolo TITOLO, Stefano GEUNA*

Bruno BATTISTON, MD - PhD
Head of Traumatology - Hand Surgery - Microsurgery Unit
C.T.O. Hospital - Turin Italy

Stefano ARTIACO , MD
Hand Surgery - Microsurgery Unit
C.T.O. Hospital - Turin Italy

Paolo TITOLO, MD
Hand Surgery - Microsurgery Unit
C.T.O. Hospital - Turin Italy

* Stefano GEUNA, MD, Associate Professor
Department of Clinical and Biological Sciences, University of Turin, S. Luigi Hospital, Orbassano, Italy.

Corresponding Author: Bruno BATTISTON
C.T.O. Hospital
Via Zuretti 29, 10126, Turin, Italy
Phone: +39 3386464388
Fax: +39 0116963662
E-mail: bruno.battiston@virgilio.it

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SUMMARY

AIMS: If nerve continuity is lost, surgical reconstruction is required to reconnect nerve endings, and if substance loss occurs, the two stumps must be bridged. Thanks to the fundamental work by Millesi on nerve repair by means of interfascicular nerve grafting, and studies by Rita Levi Montalcini, Lundborg and other researchers on nerve growth factors, chemotropism and many other relevant fields, we are now able to better understand the rules of nerve regeneration. This has led to new reconstruction techniques, including biological and synthetic conduits-an approach to a nerve injury in which the role of the surgeon is limited, and special emphasis is placed on the intrinsic healing capacities of the nerve tissue itself inside a guide.

MATERIAL AND METHODS. In this paper we present the experimental and clinical experience published in the literature on the use of tissue engineering of synthetic and biological conduits and autologous tissues for repair of damaged peripheral nerves.

RESULTS AND DISCUSSION. Based on literature reports and personal experience, the use of tissue-engineered multiple-component conduits hold promise, since many experimental studies have shown that these types of nerve guides lead to better outcomes in comparison to conduits made from single components alone. More work, however, is required to shed light on the many unknown

mechanisms over which the surgeon has no control in the effort to obtain good nerve healing and better clinical results.

KEY WORDS: Nerve gap - Conduits - Tissue engineering

Introduction

Traumatic nerve lesions are increasing due to the growing incidence of road accidents and workplace trauma. If nerve continuity is lost, surgical reconstruction is required to reconnect nerve endings, and if substance loss occurs, the two stumps must be bridged. Nerve autografts, which still represent the gold standard [1], bridge such gaps, guide regeneration, and protect axons from the surrounding scar. Although autologous nerve grafts have been the most widely used for bridging nerve gaps, this technique has several disadvantages associated with withdrawal of a sensory nerve from healthy tissue, creating damage, skin scarring, and sensory loss in the donor area, and can increase the risk of neuroma formation; moreover, at times these autografts are not long enough to repair the nerve gap.

During the first three decades of the twentieth century, there was considerable interest in improving peripheral nerve repair and regeneration, with basic research and clinical scientists working in synergy. However, in the second half of that century, this trend decreased, probably because of the criticism arising about the real usefulness of nerve reconstruction techniques in promoting nerve regeneration. Although very important work was carried out by many surgeons worldwide (including very famous surgeons such as Herbert Seddon and Sydney Sunderland), research across most of the remaining years of the twentieth century was mainly dedicated to optimising of surgical techniques for nerve reconstruction.

However, the observation that peripheral nerve axons retain a capability for spontaneous regeneration after trauma led researchers to focus on how to repair nerves, rather than how to improve nerve regeneration. It was not until recent years that a new research trend bagun, uniting surgical science and molecular neurobiology in an effort to address this issue. Indeed, the increasing awareness that, although possible, peripheral regeneration is far from being optimal [2,3] led to the awareness among surgeons that advancements in peripheral nerve reconstruction would need a stronger biological basis; meanwhile, basic scientists' commitment to peripheral nerve regeneration research began to grow, as demonstrated by the dedication of special issues of important international neuroscience journals over recent years.

This new trend towards interdisciplinary and multitranslational research opens several new scientific fields, and makes it possible to foresee that the decades to come will see significant scientific advancements in nerve repair and regeneration. Indeed, to achieve good functional reconstruction, any alternative surgical treatment proposed for nerve lesions must take into consideration the various factors which condition nerve regeneration.

Factors influencing nerve regeneration

Six groups of factors that influence nerve regeneration, and consequently the final outcome of nerve repair, may be distinguished. i.e., general factors, lesion type and site, timing, technical factors, and biomolecolar factors.

General factors

The *age* of the patient and *associated diseases* may influence nerve regeneration and final recovery [4]. Indeed, it is well known that children generally experience better functional recovery than adults. This is due, in part, to more valid nerve regeneration, but overall to an easier recovery of the body scheme thanks to greater plasticity of the central nervous system.

Associated diseases such as diabetes, metabolic dysfunction, etc., may also influence nerve regeneration, as does the misuse of alcohol or drugs.

Type of lesion
Lesion margins, surrounding tissues and the *amount of nerve substance loss* are important factors that condition the possibility and quality of nerve reconstruction.
Lesion margins: a neat lesion leads to better results. In mangled hands, crushed stumps are almost always present. Even if a good repair is performed, it will lead to a fibrous reaction inside the nerve and hamper nerve regeneration. Contused nerve stumps must be excised, and good trimming is essential [1].
Surrounding tissues: the reconstructed nerve must be kept in a soft and well-vascularized bed. So, if a surgeon is unsure of the neighbouring tissues, delayed repair must be considered, and good soft tissue coverage must be prepared.
Defect length: the nerve gap influences the clinical oucomes, as longer gaps require longer grafts. In presence of a nerve gap over 10 cm, prognosis is poorer [5].

Site of lesion
The site of the lesion is very important due to both the *level* of the injury and the *anatomical district*. The more proximal the lesion, the more difficult it will be to obtain a good functional outcome, as fibre mixing increases at proximal levels, and there is a greater distance between the regenerating fibres and the final target. The anatomical site is important in as much as it influences the possibility of mobilizing the nerve stumps.

Timing
Several studies have shown that primary nerve repairs give better clinical results than delayed ones [6]. However, experimentally speaking, nerve regeneration is improved if the repair follows the lesion at 3 to 4 weeks. Therefore, some authors [7] prefer to wait 20 to 60 days before performing nerve reconstruction. Primary repair is, however, always to be preferred in neat, isolated and distal lesions, or in replantation surgery.

Technical factors
Tension is a critical factor in nerve repair. Millesi has well emphasized that, with tension, fibrous tissue develops and hinders axonal regeneration [8]. This prompted surgeons to use grafts systematically, even in small gaps, to prevent tension and fibrous reaction. Wrong orientation of fascicles could lead the regenerating axons to a mistaken final target. All methods facilitating spontaneous orientation of new sprouts could improve the results of surgery. Of course, the use of *magnification* and dedicated *suture materials* is important in nerve reconstruction.
Suture materials: traditional threads or alternative techniques such as fibrine glue or laser nerve-welding may be used. Generally, microsurgeons still prefer traditional monofilament threads (nylon, polypropylene) because of their biocompatibility and due to the absence of local inflammatory reaction. Furthermore, the use of single stitches gives the surgeon the possibility to adapt fascicle facing in every single case. In the case of interfascicular nerve grafting, we prefer to use epi-perineurial stitches (perineurium of the lesioned nerve fascicles and epineurium of the sural grafts), as suggested by Millesi. That being said, fibrin glue makes for an easier and faster suture. Some authors use it systematically for nerve grafting [9]. The glue assembles sural nerve cables, and is also used for the suture site, thereby saving time and providing similar clinical results to the ones reported with traditional sutures. Laser welding coaptation, on the other hand, lacks tensile strength, and is not to be recommended in severe injuries.

Biomolecular factors
Several morphological and biochemical changes occur in the nerve cell body following transection of a nerve trunk. This reflects the changes in the synthesis of the cytoskeletal elements required to replace the loss of axon substance. At the site of axonal injury, sprouts start to grow distally, and

several biomolecular factors are involved in supporting the outgrowth and direction of axonal growth.
These biomolecular factors can be subdivided into three major (simplified) groups: neurotrophic factors, neurotropic factors and neurite promoting factors (NPF).
Neurotrophic factors are endogenous soluble proteins that influence the survival, development and morphological plasticity of nerve cells ("neurotrophism"). These factors are synthesized in neurons, muscle and glands, and are classified on the basis of their receptors: *neurotrophins* (NGF, BDNF, NT-3, NT-4/5), *neuropoietic cytokines* (CNTF, IL-6), *fibroblast grow factors* (aFGF, bFGF,FGF-5, FGF-6), *insulin gene family* (ITF-I, IGF-II, insulin) and *other*s (LIF, EGF, TGFα, TGFβ, CDNF). The prototype for a trophic factor, the nerve growth factor (NGF) described by Rita Levi Montalcini [10], binds to its receptors, is internalized in vesicles, and then transported, by retrograde axonal transport, to the cell body, where it exerts its action.
Neurotropic factors, on the other hand, influence the axonal growth direction by exerting an attraction at a distance ("neurotropism"). These factors, delivered by the distal nerve segment, create a concentration gradient. It is not strictly correct to separate "trophic" and "tropic factors" completely, and it has been suggested that the terms "trophic" and "tropic influence" be used to describe those factors secreted after an injury by non-neuronal cells in a distal nerve segment that normally have a trophic influence and may act like tropic factors, thereby exerting an attraction at a distance, also influencing axonal growth direction.
Neurite promoting factors (NPF) are substances that axons like to grow on, and they promote growth cone formation. *Laminin* and *fibronectin* are examples of such substances within the extracellular matrix, while N-CAM and L1 are examples of cell surface molecules providing adequate adhesion for the advancing sprouts.

Better understanding of all these factors involved in nerve reconstruction has guided researchers in their efforts to improve nerve repair. Even though conventional autografts usually provide good functional results, they require an extra surgical procedure, which may lead to damage created by the withdrawal of a healthy nerve (surgical incisions in sound areas, sensory residual deficits), and graft material is limited (in terms of length), especially in cases requiring the repair of extensive lesions, such as brachial plexus lesions. Now, however, much has been done to overcome problems connected with the correct orientation of fascicles, not only in direct sutures, but also in nerve repair in the case of loss of nerve substance. These two problems have both been overcome by means of the so-called *tubulization techniques*, generally using tubes or conduits to repair nerve defects.
The tubulization principle represents a biological approach to a nerve injury in which the role of the surgeon is limited, and special emphasis is placed on the role of the intrinsic healing capacity of the nerve tissue itself. To solve the problem of *misdirection* of the regenerating fibres leading to inappropriate distal reinnervation, Lundborg has suggested encasing both ends of a transected nerve in a silicon tube, leaving a short gap in between (3-4 mm), and allowing the accumulation of biological factors inside the tube. Early results from a prospective randomised clinical trial of this technique show that tube repair provides at least as good prerequisites for the recovery of nerve function as conventional repair techniques [11, 12].
Many biological and synthetic materials have been tested to bridge a peripheral loss of substance, including mesothelial chambers [13], veins [14], predegenerated or fresh skeletal muscle [15], and empty artificial tubes [16]. Unfortunately, all these "tubes" or "conduits" are useful for short distances only, the main limiting factor being the absence of Schwann cells inside them. Another consideration is that, in spite of the high number of published articles on nerve repair by the use of conduits, applications in patients are still limited. This suggests that researchers need to optimize the strategy for tissue engineering of peripheral nerves, striving for a new level of innovation that will brings together, in a multitranslational approach, the main pillars of tissue engineering, namely microsurgery, cell and tissue transplantation, materials science and gene transfer.

A combined tissue autotransplantation approach: the combined muscle-vein technique

Although the efficacy of vein or muscle (single tissue conduits) has been proven both experimentally [17] and in patients [18], its effectiveness is usually limited to reconstruction of short nerve gaps. Indeed, the vein tends to collapse and axon dispersion occurs when muscle autografts alone are used to bridge long nerve gaps. For this reason, Brunelli, Battiston and coworkers [19] decided to investigate the possibility of engineering a combined conduit by enriching vein segments with fresh skeletal muscle fibres to improve effectiveness of tubulization nerve repair. The original rationale behind the combined muscle-vein approach was that muscle enrichment would prevent vein collapse, while the vein wall would prevent axon dispersion; the choice of fresh rather than predegenerated muscle was aimed at reducing surgical times, obviating the need for a predegeneration procedure. Indeed, basic morphological investigation using confocal and electron microscopy showed that basal lamina scaffolds of fresh muscle fibres are available to migratory Schwann cells, without the need for any preliminary degeneration of the skeletal muscle. However, it has also been shown that most muscle fibres degenerate during the first postoperative weeks, and only some of them remain alive over time [20] but play no further role in relation to nerve fibres because, at late postoperative times, nerve fascicles are always completely separated from muscle by clearly delineated perineurial tubes [21]. Nevertheless, results from confocal imaging of Schwann cells and regenerating axons showed that combined muscle-vein grafts were massively colonized by a number of actively proliferating Schwann cells from the two nerve stumps starting from the first postoperative days, while axon regeneration was clearly detected inside the graft at only week 2 postoperatively.

The molecular mechanisms behind the effectiveness of this tissue engineering approach for peripheral nerve reconstruction have also been explored, focusing in particular on the gliotrophic system based on NRG1/ErbB signalling. NRG1 is the consensus name for a group of molecules encoded by the NRG1 gene. From the single NRG1 gene, various isoforms of the protein are produced; mRNA that encodes the isoforms is transcribed by several promoters, and alternative splicing contributes to their hererogeneity [22]. Some of these isoforms are known to be closely involved in the regulation of myelination in the peripheral nervous system [23].

Due to the pivotal role exerted by NRG1, through its interaction with the ErbB tyrosine receptor family [22], in Schwann cell development and function, we have studied how it can affect Schwann cell behaviour during nerve regeneration. Results showed that, during early postoperative times, only the nonmitogenic isoform of NRG1 is overexpressed inside combined muscle-vein tubes [24]. Similar results have been obtained in denervated skeletal muscles, where expression of the NRG1/ErbB system is detectable at low levels in normal skeletal muscle and increases after muscle denervation [25], thereby suggesting that skeletal muscle fibres and Schwann cells share a common autocrine trophic loop that is overactivated in the case of loss of contact with axons. Interference between the two (muscle and glial) autotrophic loops could be one of the mechanisms for explaining the effectiveness of the combined muscle-vein technique for nerve tissue engineering. It should be noted that fresh muscle enrichment is particularly effective when vein conduits are used, while in combination with synthetic conduits its effectiveness is reduced [26]. Finally, some evidence has been obtained on the involvement of NRG1/ErbB system in different types of peripheral nerve injury. In fact, ErbB receptor mRNA expression is modulated in the early phases of nerve regeneration after end-to-end and end-to-side coaptation [27]. These results raise the possibility that manipulation of this gliotrophic system by means of gene transfer at the site of injury may be useful for improving nerve regeneration and functional recovery after nerve lesion both with and without substance loss.

Biomaterials for nerve reconstruction

Concurrent with the attempts to use autologous tissues for engineering damaged peripheral nerves, much effort has also been expended on exploring the use of biomaterials as substitutes for tissues.

The use of materials for nerve reconstruction has a lengthy history, and many attempts to use various nonbiological materials, such as metals, permeable cellulose esters, gelatine tubes, rubber, plastics, etc., have also been carried out [28].
While both absorbable and nonabsorbable synthetic materials have been investigated for the purposes of nerve regeneration, concerns about the occurrence of complications due to local fibrosis and nerve compression in the case of the latter approach [29] has focused much interest on bioabsorbable tubes; many experimental studies have shown that their effectiveness is similar to traditional nerve autografts, and sometimes tubes made of polyglycolic acid (PGA) even perform better [30]. Furthermore, some authors have found resorbable guides made of collagen or polylactate caprolactone (PLC) superior to nonresorbable guides such as silicone, Teflon or polysylfone [31]. We too have recently published results from a study in which we show that wrapping with type-III chitosan membranes, which are characterized by a highly porous microstructure, improves nerve regeneration after contusion injury to the sciatic nerve, also providing a good substrate for the local delivery of stem cell therapy [32].

Gene transfer

Biotechnological progress that today makes it possible to induce therapeutic changes through gene transfer represents one of the pillars of tissue engineering, and has engendered much excitement, opening new perspectives in many disciplines of biomedicine, including nerve regeneration improvement. Gene therapy may contribute to stimulating regeneration of the peripheral nerve by locally supplying several neurotrophic factors, the efficacy of which is limited in exogenous application due to their rapid degradation. Moreover, systemic application of trophic factors can have side effects that are reduced if they are produced locally.
The use of viral vectors provides a high rate of transduction and expression, and the recent development of nontoxic, nonimmunogenic viral vectors that drive long-term local transgene expression makes their use much safer. We have recently focused our attention on viral vectors based on the adenoassociated virus (AAV), a nonpathogenic and widespread parvovirus incapable of autonomous replication and able to transduce both dividing and nondividing cells, and show a specific tropism for postmitotic cells, including neurons [33]. Because these vectors do not contain any viral genes-which are transiently transfected in trans for the packaging process-they elicit virtually no inflammatory or immune response [34]. As a consequence, transgene expression from these vectors persists for several months in a variety of animal tissues in vivo. AAV-mediated gene transfer is a promising tool for the delivery of therapeutic genes into the central and peripheral nervous systems. Indeed, vectors based on AAV have recently been used in phase I clinical trials for the treatment of neurological disorders, such as Parkinson's and Canavan's diseases. In fact, it should just be noted that the great effectiveness of skeletal muscle infection by AAVs makes it possible to foresee that this tissue could be a vehicle for transferring genes that can improve nerve regeneration, either by infecting the muscles that surround the nerve lesion site, or even by creating combined muscle-vein scaffolds previously potentiated by AAV gene transfer.

Clinical experience

A. Synthetic conduits

Tubes consisting of various materials have been successfully used for bridging nerve defects in patients. Dahlin and Lundborg demonstrated that, in short gaps (less than 5 mm), the use of silicone tubes can lead to successful nerve regeneration [35]. However, a major concern with the clinical use of nonabsorbable synthetic materials in humans is the occurrence of complications due to local fibrosis, induced by the implanted material, and nerve compression.
As an alternative to nonabsorbable tubes, bioabsorbable tubes have been tested both experimentally and in clinical practice. For example, a randomized prospective multicentre trial on the use of PGA conduits for human digital nerve construction has been conducted by Weber et al. [36]. PGA

conduits were found to lead to similar functional outcomes to nerve grafts and end-to-end repair in nerve gaps of 4 mm or less. We had a similar experience when comparing PGA tubes with biological muscle-in-vein conduits. Results showed good mean recovery with no significant differences ($P > 0.05$) between the two groups in any of the assessment criteria. In the PGA group there was one case with poor outcome, but this was tentatively ascribed to the severity of the initial associated lesions [37].

In a similar vein, Lohmeyer and coworkers employed collagen I conduits (NeuraGen; Integra LifeSciences, Plainsboro, NJ) in a series of 12 patients, and reporting good results [38]. Future clinical trials need to demonstrate which of the more recently devised biodegradable materials (for example, chitosan) could be valid alternatives to polyglycolic acid for fashioning conduits for human nerve repair [32].

B. Biological conduits

Veins have been used successfully in patients for bridging nerve gaps less than 3 cm long [18]. Risitano reported a retrospective study on 22 sensory nerves repaired using vein grafts in emergency hand reconstruction cases in which primary suture was not feasible, with good results [17]. Unfortunately, the tendency to collapse is high. Moreover, scarring of the surrounding tissue might subsequently prevent the vein from expanding later on, when the nerve growth cone passes the nerve gap .

An interesting strategy that has been proposed for avoiding vein collapse is filling the vein lumen with small pieces of nerve tissue [39]. On the basis of previously reported experimental evidence, combined muscle-vein conduits should provide the advantages of multiple-component conduits by relying on tissue engineering concepts. Indeed, they have been used in clinical practice to fill gaps of up to 4 cm in sensory and 6 cm (mean 2.5) in mixed nerves [40]. Good results were reported in 85% of the published series, at a minimum follow-up of 14 months. These results seem to be superior to those reported with other kinds of artificial or biological conduits. Furthermore, such conduits are cost free and prepared in relation to reconstructive needs after consideration of nerve size and length defect.

However, in a more recent review of a greater number of patients with very restricted indications (treatment in emergency, especially in the case of crush lesions), good or very good recovery was obtained in 52% of mixed nerves, while 13% had unsatisfactory recovery. A partial recovery was obtained in 35% of patients with mixed nerve lesions; in these patients only the motor or the sensory nerve fibres displayed good recovery. Nevertheless, for pure motor or sensory nerves, only 10% of patients had an unsatisfactory outcome [41]. These results seems to be at least similar to those reported for autograft reconstruction and to those reported for the use of synthetic conduits [37].

Some authors propose different tissues using a hollow conduit made of human amniotic membrane combined with autologous skeletal muscle fragments [42]. In cases of extensive substance loss of up to 5 cm, this technique provided good sensory and motor recovery.

Finally, allografts also seem to be a promising solution. They have the same structure as peripheral nerves, so provide better adhesion and support for the regenerating axons. Although the first investigators describing the use of immunogenic grafts needing immunosuppression had poor results [43], more recently nonimmunogenic allografts have been developed and used with some encouraging initial results [44, 45]. These allografts may be employed in gaps of up to 7 cm in length as an alternative source of tissue to bolster the diameter of a cable graft, and for the management of neuromas in unreconstructable injuries. The neurotropic effect of these grafts may lead to better results than empty conduits.

Conclusions

Today, the use of artificial nerve conduits is limited to nerve gaps of up to 30 mm, as results deteriorate with extended gap length. Biological tubes, on the other hand are less expensive and

seem to give similar outcomes. The newly devised tissue-engineered conduits can yield similar, and sometimes better, histomorphometrical and functional results in comparison to autogenous nerve grafts, avoiding donor site morbidity. Although the success of tissue engineering approaches based on single-component strategies, such as single-tissue biological transplants with veins or skeletal muscles, is usually limited by gap length, this be overcome by using combined biological conduits. Our experience on the use of conduits made from a vein filled with fresh skeletal muscle exploits the advantages of both tissues: the vein guides regeneration and the muscle prevents vein collapse. Furthermore, the muscle provides adequate "adhesion" for the advancing sprouts by means of neurite promoting factors present in its basal lamina (laminin and fibronectin), mimicking the Schwann cell adhesion role. Other types of bioengineered conduits, based on the enrichment of tubes with laboratory-based elements such as nerve growth factor (NGF) [46], glial growth factor (GGF), denatured muscle [47] and Schwann cells [48], have been experimentally tested, but have not yet been tried in patients. The latter type of adjuvant seems to hold the most promise because of the central role played by Schwann cells in nerve restoration.

In conclusion, we are still looking for an "ideal" tissue-engineered peripheral nerve, i.e., one that is compatible with the surrounding tissues, of adequate size and length, and contains substances that enable and support axonal regeneration, as well as protecting regeneration of nerve fibres from scar invasion. Indeed, analysis of the literature published to date tells us that those ideal requirements are only partially met by the large variety of tissue-engineered constructs that have been devised so far, and further basic and applied research is therefore definitely needed in this field.

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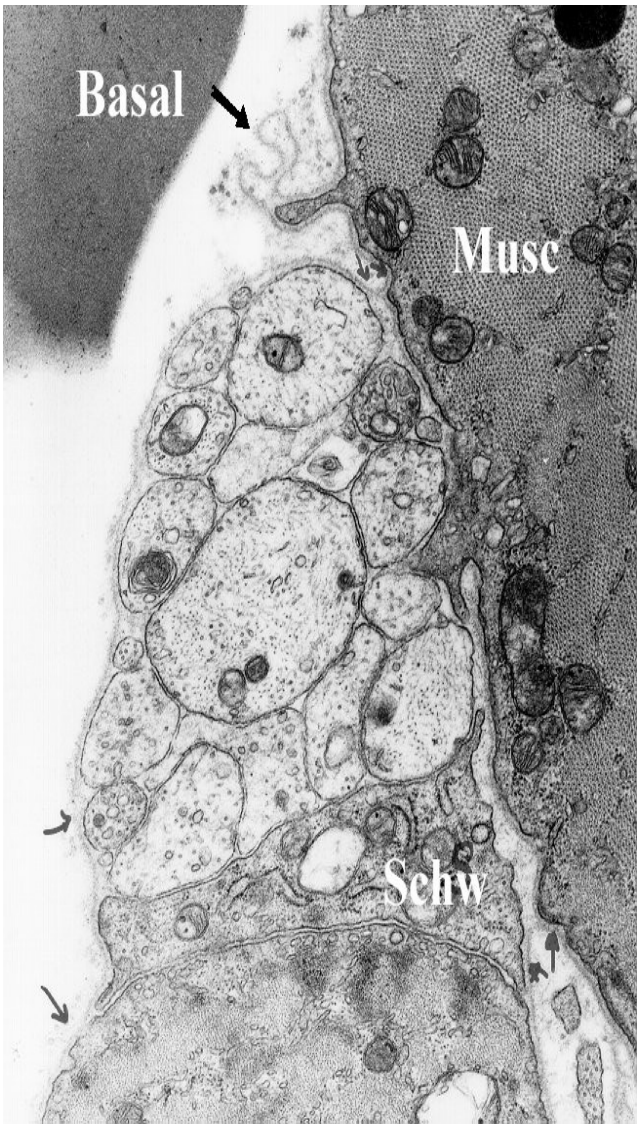

FIG. 1

Schwann cells and new axonal sprouts enter in the bio-engineered conduit made by muscle inside a vein gliding underneath the muscle basal membrane .

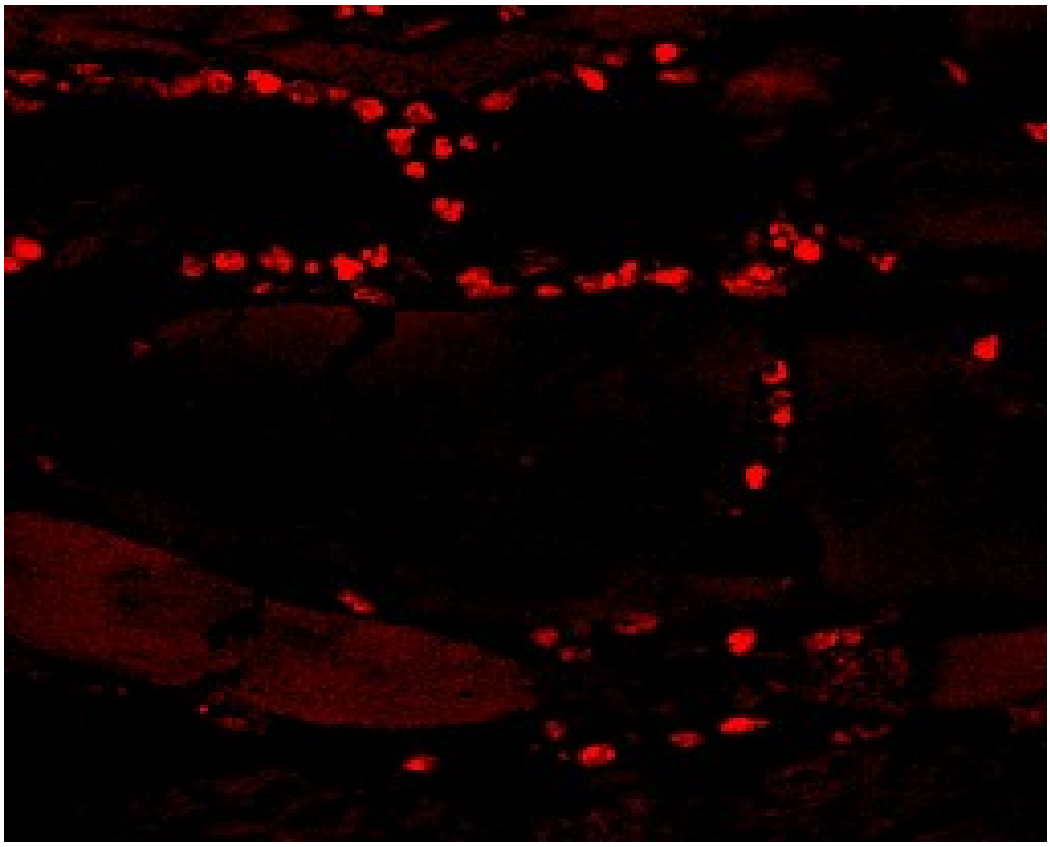

FIG. 2

Chains of migratory Schwann cells comes from the nerve stumps preparing the following nerve reconstruction