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# In vivo study of ethyl-2-cyanoacrylate applied in direct contact with nerves regenerating in a novel nerve-guide

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**Abstract** Stitch suture is still the most recommended method to hold a nerve-guide in place but stitch suture is a well known cause of local inflammatory response. Glues of several kinds have been proposed as an alternative but they are not easy to apply in a real surgical setting. In 2006 authors developed a new concept of nerve-guide termed "NeuroBox" which is double-halved, not-degradable and rigid, and allows the use of cyanoacrylic glues. In this study, Authors analyzed histologically the nerve-glue interface. Wistar rats were used as animal model. In group 1, animals were implanted a NeuroBox to promote the regeneration of an experimentally produced 4 mm gap in the sciatic nerve. In group 2, the gap was left without repair ("sham-operated" group). Group 3 was assembled by harvesting 10 contralateral intact nerves to document the normal anatomy. Semi-thin sections for visible light microscopy and ultra-thin sections for Transmission Electron Microscopy were analyzed. Results showed that application of ethyl-2-cyanoacrylate directly to the epineurium produced no significative insult to the underlining nerve fibers nor impaired nerve regeneration. No regeneration occurred in the "sham-operated" group.

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## 1 Introduction

The gold standard in treating nerve gap-injuries is the autograft [1]. Unfortunately, there are several limitations and complications associated with autografts: (a) harvesting a donor nerve graft may have significant co-morbidity [2, 3]; (b) the donor nerve is, often, a smaller sensitive nerve which limits, from the beginning, a full recovery when a bigger and more important motor nerve requires the treatment; (c) there is an increasing difficulty in proposing an autograft to patients who neither accept the sacrifice of their nerves and its associated morbidity nor the lack of a guaranteed positive outcome (in the worst case, they will perceive two lesions instead of one); (d) the two stitch sutures securing the autograft (one proximal and one distal) may be the site of an unfavourable fibroblastic proliferation [4].

An alternative to autograft is the allograft but it may bring the even greater problem of a life-long immunosuppressive therapy [5]. A new class of commercial allografts are now commercially available as non-immunogenic to the host, since they are so highly processed that only the Laminin-laden structure of the original nerve fascicles remains [6]; this treatment assimilate them to multichanneled artificial nerve guides.

Artificial nerve guides (or conduits) have been introduced into clinical practice more than 20 years ago; they are cylindrical conduits inside which a regenerating nerve stump may find protection and guidance [7]. An overview of the clinical outcome showed that they perform at least as good as autografts in peripheral nerve injuries where gaps are not-longer-than 20 mm; in this situation they bring the advantage of avoiding donor site sacrifice and morbidity and provide an easier and quicker surgical technique [8, 9]. Nowadays there are several degradable nerve guides in clinical use [10] made of: poly-glycolic-acid ("Neurotube"

Synovis USA); poly-lactic-acid ("Neurolac" Ascension USA—Polyganics NL); treated bovine collagen ("Neuragen" Integra USA; "Neuroflex" and "Neuromatrix" Stryker—Collagen Matrix USA); a proprietary hydrogel non-degradable in vivo ("SaluBridge" SaluMedica USA). Several other experimental guides have been proposed and tested in vitro and in vivo [3, 11–39].

From the biomaterial point of view, there are three structural districts in a nerve guide, namely: (a) the outer structure, which is basically the tube inside which the nerve stumps are accommodated; (b) the inner structure, which is how the tube is filled; (c) the suture, which is the site where a mechanical force is being applied to the guide and where a mechanical and biological insult is being received by the nerve stump.

Stitch suture is still the most recommended method to hold the guide in place but stitch suture is a well known cause of local inflammatory response [4] (Fig. 1a). Glues of several kinds have been proposed as an alternative [40–47] but, unfortunately, they have proved not easy to apply in a real surgical setting, where the presence of blood and other fluids is highly variable and little manageable.

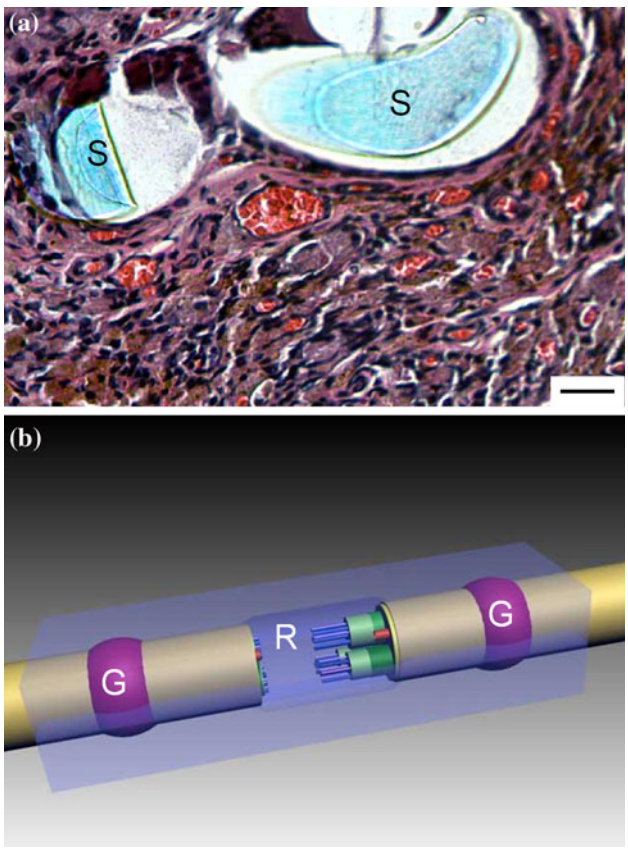


**Fig. 1** **a** A transverse section showing two heads of an epineural knot (S), where a great number of hyperhemic capillaries and hemosiderin deposits can be seen (H&E; bar = 10 μm). **b** Schematic drawing of a NeuroBox device: the volume for the acrylic glue (G) and the flat "regeneration chamber" (R) are labelled

In 2006 authors developed, and tested in vivo, a new concept of nerve-guide termed "NeuroBox" (patent WO/2008/029373) which is double-halved, not-degradable and rigid, and does not require the use of any stitch to be sutured to the nerve stump, allowing the use of cyanoacrylic glues instead. The device proved to allow a successful nerve regeneration in vivo [26].

In this study, Authors analyzed histologically the nerve-glue interface in vivo. Their hypothesis is that the little invasiveness in applying ethyl-2-cyanoacrylate directly to the epineurium (which is allowed by the peculiar design of the device) is instrumental to the absence of any significative insult to the underlining nerve fibers (which was observed) and this contributed significantly to the successful nerve regeneration that had taken place.

## 2 Materials and methods

Twenty-two male Wistar rats, weighing about 300 g, were used as animal model. Three groups of samples were studied. In group 1 (G1), 15 animals were implanted a NeuroBox double-halved stitch-less nerve-guide to promote the regeneration of an experimentally produced 4 mm gap in the sciatic nerve. In group 2 (G2), 7 animals had the 4 mm gap left without repair ("sham-operated" group). Group 3 (G3) was assembled by harvesting 10 contralateral intact nerves (6 from G1 and 4 from G2) and this group of samples documented the normal anatomy of the sciatic nerve in the experimental model.

The NeuroBox was micro-machined from a solid block of poly-methyl-methacrylate (PMMA) (Repsol, Madrid, Spain) using computer-aided manufacturing (CAM) techniques, at the Institute of Bioengineering of Catalunia. Devices were degreased by sonication in a mix of distilled water and ethyl alcohol (50–50%) and sterilized by low-temperature (55°C) hydrogen peroxide gas plasma (STERRAD Sterilization Systems, Johnson & Johnson, USA). In the NeuroBox the traditional cylindrical nerve guide is replaced by a box of two-halves into which three main compartments are recognizable: (1) a lodgement for the neural stump (one proximal and one distal); (2) a compartment for the acrylic glue (one proximal and one distal); (3) a flat "regeneration chamber", where elongating axons from the proximal stump are invited to spread (Fig. 1b).

Ethyl-2-cyanoacrylate (Loctite, Henkel, Germany) was employed as cyanoacrylic glue. The dedicated glue-compartment of the NeuroBox promotes the polymerization of the glue with just the minimum amount which is needed for its wetting. Neural stumps are gently accommodated

within their compartments, in the bottom half of the guide, just prior the end of the polymerization process. The acrylic glue conforms to the stumps. The top-half was gently positioned so to close the device symmetrically (by wetting, with the glue, the dedicated top-half glue-compartments). However, the NeuroBox guide cannot be considered completely sealed but, on the contrary, some empty space remains at both entrances and fluids and cells may access the regeneration chamber.

Adequate measures were taken to minimize pain or discomfort to the animals and experiments were conducted in accordance with ECC D86/609 and with the approval of the National Committee for Animal Experimentation. Anaesthesia was induced by 75 mg/kg ketamine chlorhydrate (KETAVET 100, Farmaceutici Gellini, Aprilia, Italy) and 0.5 mg/kg medetomidine chlorhydrate (DOMITOR, Farmos Orion Corporation, Espoo, Finland) with intramuscular injection on the right tight. A 30 mg/kg methylpredinisolone (SOLU-MEDROL, Pharmacia & Up John NV/SA, Puurs, Belgium) was administered prior to surgery.

A curvilinear transverse incision with superior convexity was performed to gain a smooth access to the intramuscular interstice to expose the sciatic nerve, in the left tight. The sciatic nerve was cut proximally to its trifurcation and a gap of 4 mm in length ensued. Surgical operation required the assistance of optical magnification (Zeiss OP MI 1, Carl Zeiss, Jena, Germany).

**Table 1** Implant and retrieval scheme

| | NeuroBox | Gap | Controlateral |
|---|---|---|---|
| 3 Days | 2 | | |
| 1 Week | 2 | | |
| 2 Weeks | 2 | | |
| 1 Month | 3 | 3 | 3 |
| 2 Months | 3 | 4 | 4 |
| 3 Months | 3 | | 3 |

Implantation scheme for G1 (NeuroBox), G2 (sham operated group) and G3 (intact contralateral nerve)

Animals were sacrificed under deep anaesthesia, in an atmosphere saturated with $CO_2$; retrievals followed the timing shown in Table 1. At retrieval, the nerve is simply dislodged from the guide. Macroscopic examination of the operated site was performed. Retrieved nerves were fixed in buffered formaline; post-fixed in osmium tetroxide and

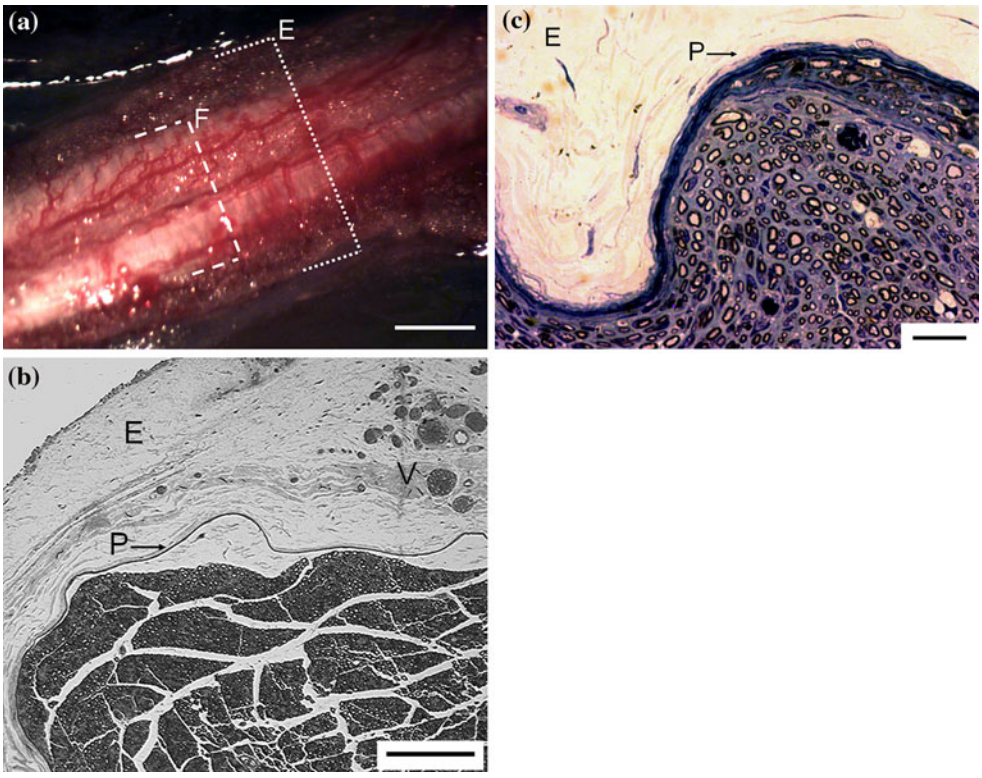


**Fig. 2** **a** Intact sciatic nerve of a Wistar rat shows that the epineurium (E) is a wide bumping protective coat wrapped around the nerve fascicles (F), with main vascular trunks and their collateral sinusoidal branches embedded in (bar = 0.5 mm). **b** Transverse section of an intact sciatic nerve: the epineurium (E) is made of loose areolar connective tissue and adipose tissue, with embedded epineural vessels (V); a distinct perineurium (P) surrounds the nerve fascicle (toluidine blue; bar = 100 μm). **c** Transverse section of an intact sciatic nerve: the perineurium (P), with its peculiar lamellar arrangement of roughly 7–8 concentric layers, surrounds the nerve fascicle (epineurium is labelled with E) (H&E; bar = 10 μm)

dehydrated in serial passages of acetone, then embedded in araldite. Semi-thin sections were stained for visible light microscopy (Nikon SMZ 800); ultra-thin sections were cut for Transmission Electron Microscopy (TEM) (Zeiss EM 109T). Images were processed by a commercial software ("PhotoDeLuxe", Adobe).

Five zones were mapped in a retrieved nerve. (1) The Proximal End (PE), 5 mm proximal to the surgical transection. (2) The Proximal Glueing Region (PGR), 1 mm in length and about 1 mm proximal to the surgical transection (this is the proximal area embraced and locked by the polymerized acrylate glue). (3) The Regenerate (R). (4) The Distal Glueing Region (DGR), 1 mm in length and about 1 mm distal to the surgical transection (this is the distal area embraced and locked by the polymerized acrylate glue). (5) The Distal End (DE), 5 mm distal to the surgical transection.

## 3 Results

Ethyl-2-cyanoacrylate was applied in direct contact with the epineural sheath. In the rat, as controls show, Epineurium is a wide bumping protective coat wrapped around the nerve fascicle(s) (Fig. 2a), made of loose areolar connective tissue and adipose tissue, with embedded main vascular trunks and their collateral sinusoidal branches (epineural vessels) (Fig. 2b). A distinct perineurium, with a peculiar lamellar arrangement of roughly 7–8 concentric layers, surrounds the nerve fibers (Fig. 2c).

Authors did not find any image of adverse early inflammatory response or tissue necrosis. No glue appeared inside the epineurium or inside the nerve bundles, in sections taken at the glueing regions or anywhere else, at any time.

When 6 animals in G1 were sacrificed in an early stage (2 after 3 days; 2 after 1 week; 2 after 2 weeks; as shown in Table 1) no finding was suggestive of any impairment of nerve fibers at the level of the glueing regions (Fig. 3). In this stage, the regenerate consists mostly of an assembled jelly structure of fibrin, blood cells, Schwann's cells and neurites [26, 48].

In G1 nerve regeneration occurred after 1 months and it was observed in 9/9 cases (3 after 1 month; 3 after 2 months; 3 after 3 months; as shown in Table 1). The retrieved nerve was about 4 mm longer than the contralateral intact nerve. There were no signs of any massive and adverse intraneural fibroblastic proliferation; large and small myelinated fibers were identified and also several non-myelinated axons; fine blood vessels were well represented (confirming what already reported [26]). A fibrous capsule was found around the guide but not inside the regeneration chamber (Fig. 4).

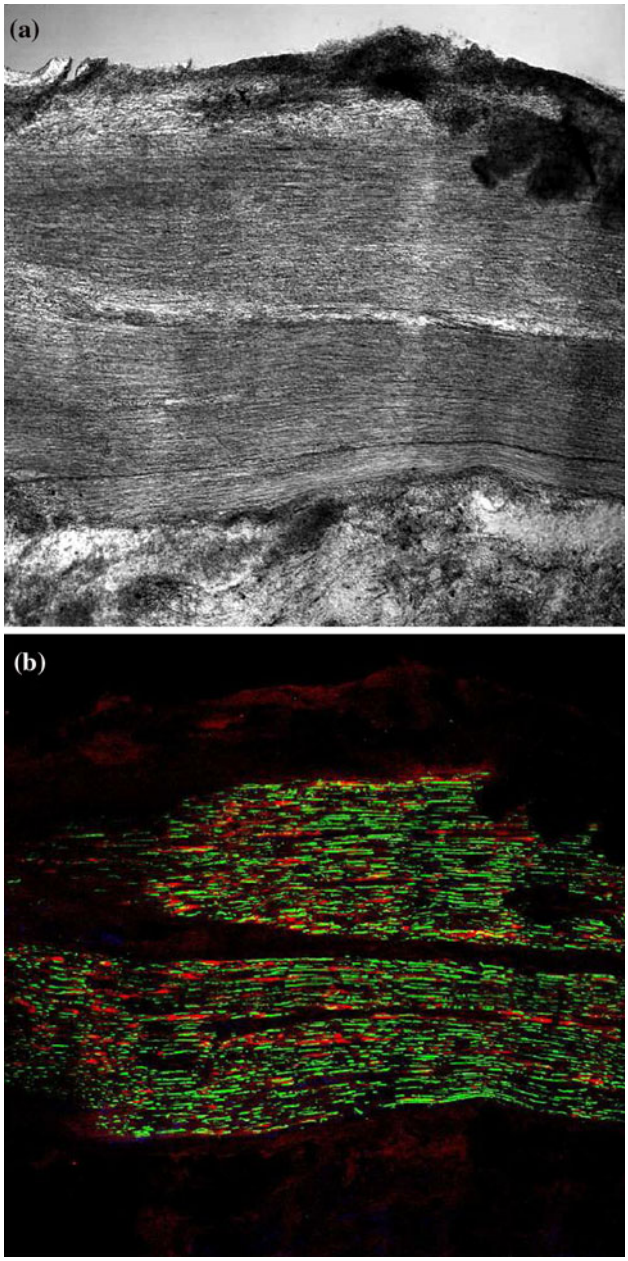


**Fig. 3** After 3 days, no morphological alteration of nerve fibers was found at the level of the glueing regions, both in unstained sections (**a**) and in immuno-stained sections (**b**; *green* = axons; *red* = Schwann's cells) (courtesy of the Institute of Bioengineering of Catalunia)

Epineural sheath which was in direct contact with ethyl-2-cyanoacrylate in the proximal and distal glueing regions (PGR and DGR) showed no major microscopic alterations nor they were found in the underlining nerve fibers; a clear fascicular structure, well demarcated from the surrounding epineurium, was preserved (Fig. 5).

More in details, in the PGR the normal structure of the nerve was preserved, with myelinated large diameter fibers and smaller non-myelinated fibers and intraneural

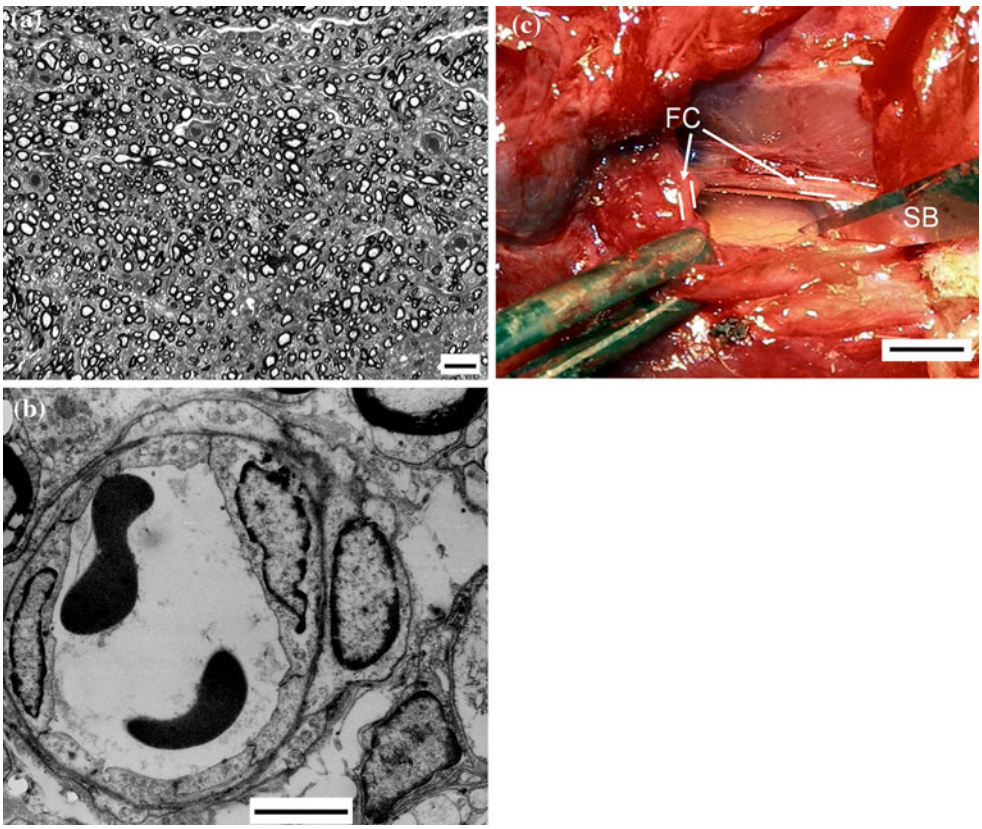


**Fig. 4** **a** A transverse section of the nerve regenerated inside the NeuroBox (toluidine blue; bar = 10 μm). **b** A tiny intraneural capillary in the regenerated nerve (TEM; bar = 1 μm). **c** A thick fibrous capsule (FC) was found around the nerve-guide; it was cut by a sharp blade (SB) and the transparent wall of the regeneration chamber showed tiny blood vessels in the regenerated nerve (bar = 1 mm)

vascularization. TEM showed smaller fibers, with thin myelin sheath, which represent newly regenerating axons; there are also larger fibers with disruption myelin figures, as expected in degenerating axons proximal to a lesion (Fig. 6a, b). In DGR, tiny myelinated and non myelinated fibers represent axons that entered the distal stump; there is, also, the presence of Schwann's cells digesting the myelin of axons which underwent Wallerian degeneration (Fig. 6c).

On the contrary, no regeneration was observed in G2, where the gap was left un-treated (3 after 1 month; 4 after 2 months; as shown in Table 1).

## 4 Discussion

There are several proposals in the literature about the best solution to adopt for each of the three districts which constitute an artificial nerve guide [49] but the suture district, in our opinion, has received minor attention until now. This district is the mechanical interface between the guide and the nerve and is a crucial point both biologically and surgically; established surgical treatment, in acute transection with negligible or absent gap, prescribes the joining of the two nerve-stumps by an end-to-end suture (neurorraphy) [50] and it is widely accepted that the stumps must not be sutured under tensional stress [51] otherwise the development of a fibroblastic and myofibroblastic proliferation will be greatly favoured. The latter phenomenon will impair and eventually stop any axonal regeneration [52]. However the use of stitches (both degradable or not degradable) represent a significative local inflammatory stimulus [4, 44] even without tensioning the stumps and is able to provoke enough fibrosis to impair nerve regeneration.

One should not assume the fibroblastic and myofibroblastic proliferation to be un-avoidable, just because of our present inability to abandon the use of stitches. Research on "stitch-less" techniques in any surgery associated with artificial nerve guides seems instrumental, in our opinion, to the successful development of the guides themselves and glues seem to most straightforward option.

The present knowledge on nerve-glues is, however, limited. In the past, cyano-acrylic glues, and ethyl-2-cyanoacrylate in particular, have been associated with Asthma [53–55] and Allergic Contact Dermatitis [56–58].

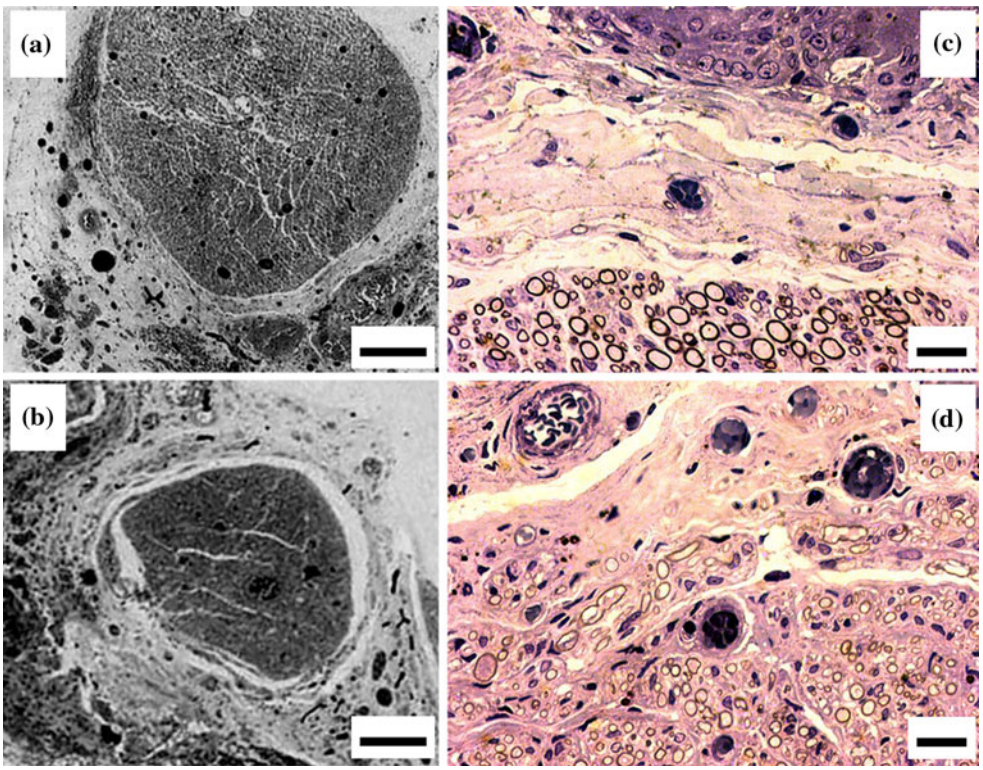


**Fig. 5** A clear fascicular structure of the nerve was preserved in the proximal glueing region (**a**, **c**) and in the distal glueing region (**b**, **d**), were well demarcated from the surrounding epineurium; no particles of glue were found (**a** and **b**: toluidine blue; bar = 0.1 mm) (**c** and **d**: H&E; bar = 10 μm)

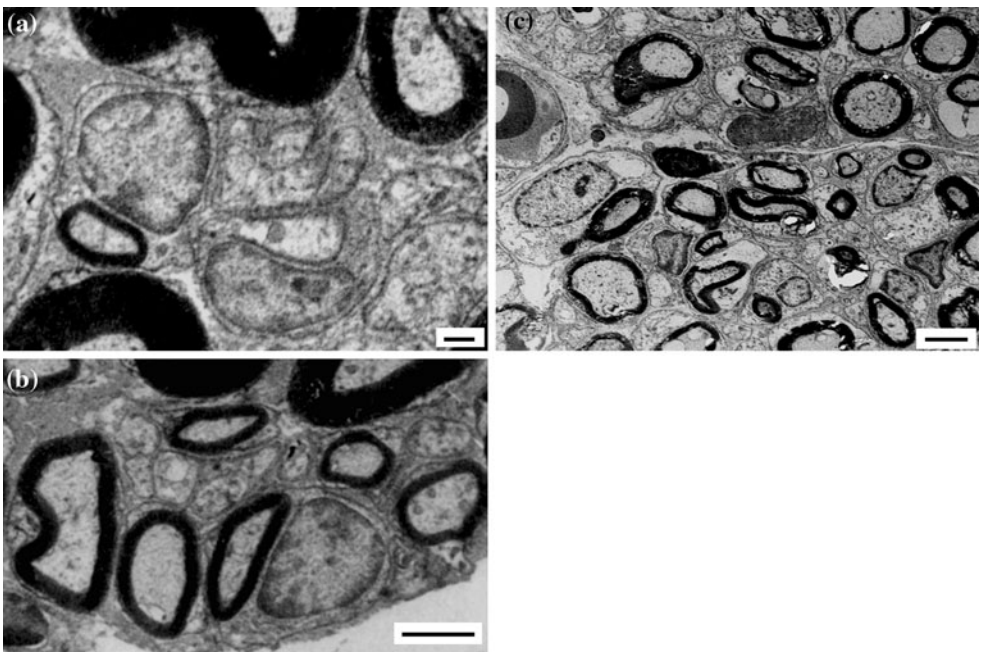


**Fig. 6** **a** Transmission electron microscopy of the proximal glueing region with myelinated and non-myelinated fibers (bar = 1 μm) representing newly regenerating axons; **b** there are also fibers with disruption myelin figures, as expected in degenerating axons proximal to a lesion (bar = 10 μm). **c** The distal glueing region shows tiny myelinated and non myelinated fibers as well; Schwann's cells are digesting the myelin of axons which underwent Wallerian degeneration (bar = 10 μm)

Furthermore, ethyl-2-cyanoacrylate has been correlated with neuropathy in sporadic cases in which, it must be noted, other factors were possibly involved and great quantity and/or prolonged exposure were reported [59–61].

As a group of rapidly polymerizing adhesives, cyanoacrylates have found surgical applications as skin-wound sutures as well as hemostatic and embolizing agents [62].

In more recent literature promising results have been reported with cyanoacrylate molecules in nerve surgery, in particular: ethyl-2-cyanoacrylate; *n*-butyl-2-cyanoacrylate; 2-octyl-cyanoacrylate. These papers show that cyanoacrylic glues can be used in direct contact with the nerve [41–47].

The actual use of cyanoacrylate glue in a true surgical setting may bear a lot of technical difficulties and this may explain, in part, early negative recommendations [40]. First: it is not easy to control the curing time of the glue, which should be not too fast, so giving the surgeon adequate time to accurately put the stumps in place, but (at the same time) should be not too slow, to avoid the accidental flow of part of the glue in front of the nerve stump (bringing the misfortunate consequence of sealing it and impairing the regeneration process). Second: to find a suitable method of delivering the glue in a real surgical environment may be an additional problem; for example, tiny quantities required by a digital nerve suture could polymerize early inside a microscopic delivery tube, before reaching the stump. Third: in a real surgical setting, an unpredictable bleeding may vanish the effectiveness of a glueing procedure; this could be a quite frequent occurrence if nerve surgery has to be performed with a limited (or absent) intraoperative ischemia (tourniquet), as advocated to better preserve the vitality of the nerve.

In the present work, Authors found that ethyl-2-cyanoacrylate was easily applied in the peculiar construct of the NeuroBox and nerve regeneration was not affected by the presence of the acrylic glue all around the epineural sheath of the glueing regions. In particular, no alterations were found in the morphology of axons and Schwann's cells and these results confirm that ethyl-2-cyanoacrylate can be used in direct contact with the nerve [47].

The peculiar geometry of the NeuroBox, in our opinion, helps greatly in the surgical application of the glue and greatly reduces the quantity which is needed; this may, in turn, minimize the fibroblastic response. However, the device may possibly have a further role in diverging the fibroblasts away from the Regeneration Chamber since it was observed that most of the fibroblasts in the area are engaged in the formation of an outside capsule around the PMMA-made NeuroBox. It may be speculated that the device acts as a decoy for the large number of fibroblasts that, instead of entangling the tiny regenerating axons inside the Regeneration Chamber, primarily attack the nerve-guide outer structure.

Discussing the experimental design, it must be said that the limited number of implanted guides was dictated mostly by the high costs associated with their production, while the short length of the treated gap was chosen to simplify the experiment aimed primarily at testing the adequacy of the new concept of the "stitch-less" guide. Despite these limitations, a safe use of ethyl-2-cyanoacrylate in direct contact with a nerve regenerating inside the NeuroBox was demonstrated and this maintains our commitment to refine a "stich-less" surgical technique for nerve repair. Any progress in nerve-guide surgery will lead, someday, to a significant reduction in nerve-autografts requirement; this will mean lesser associated morbidity, shorter surgical time, minor complexity. In brief, a real improvement for treating a large number of patients.

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