Chapter 8

Effect of Local Delivery of GDNF Conjugated Iron Oxide Nanoparticles on Nerve Regeneration along Long Chitosan Nerve Guide

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Abstract

Local delivery of neurotrophic factors is a pillar of neural repair strategies in the peripheral nervous system. The main disadvantage of the free growth factors is their short half-life of few minutes. In previous studies, it was demonstrated that conjugation of various neurotrophic factors to iron oxide nanoparticles (IONP) led to stabilization of the growth factors and to the extension of their biological activity compared to the free factors. In vitro studies performed on organotypic dorsal root ganglion (DRG) cultures seeded in NVR gel (composed mainly of hyaluronic acid and laminin) revealed that the glial cell–derived neurotrophic factor (GDNF) conjugated to IONP-enhanced early nerve fiber sprouting and accelerated the onset and progression of myelin significantly earlier than the free GDNF and other free and conjugated factors. The present article summarizes results of in vivo study, aimed to test the effect of free versus conjugated GDNF on regeneration of the rat sciatic nerve after a severe segment loss. We confirmed that nerve device enriched with a matrix with GDNF gives more successful results in term of regeneration and functional recovery in respect to the hollow tube; moreover, there are no detectable differences between free versus conjugated GDNF.

Keywords: GDNF conjugated iron oxide nanoparticles, chitosan tube, peripheral nerve regeneration

1. Introduction

The incidence of nerve injury is quite high in the world, related also to the exposition of nerve tissue in the body. Despite the good regeneration ability retained by peripheral nerve system in adulthood, nerve injuries are associated with high morbidity and deep alteration of patient's life [1]. Severe transection injuries, in which nerve continuity is lost, require a surgical approach, specially where the distance between the proximal and the distal nerve stumps is extended. In order to avoid negative side effect of the autologous nerve graft, the current gold standard technique, and to improve the functional recovery and reduce neuropathic pain, various biosynthetic nerve grafts have been developed to bridge the two nerve stumps [2–4]. Some of the artificial nerve guides have been approved for clinical use, and indeed, their use in reconstructive surgery is restricted because they still show limitations, principally for long-distance repair [5]. One category of these nerve conduits is defined absorbable conduit, according to their characteristic to be degraded in the host body; among these, the chitosan tube has been widely used in pre-clinical studies obtaining promising results [4, 6, 7]. Chitosan is a polysaccharide derived from chitin, it is biocompatible, and it can promote glial cells survival and neurite outgrowth [8–10]. It has been shown that hollow chitosan tube can be effective as autologous nerve graft for bringing 10-mm gap in rat sciatic nerve model [7, 11, 12]. In order to increase the performance of chitosan tube for long defect repair; here, we filled the tube with a hydrogel, called NVR gel, composed mainly by hyaluronic acid and laminin. Previous works demonstrated that NVR gel is a promising hydrogel that allow neurite outgrowth and cell survival in vitro [13]. Furthermore, we used NVR gel as a carrier for neurotrophic factors. In particular, we focus our attention on glial cell line–derived neurotrophic factor (GDNF), which is mainly involved in motor neuron regrowth and remyelination [14–16]. After nerve injury, endogenous growth factors are released by neuronal and glial cells from the distal nerve stump, and they can stimulate and guide axon regeneration; yet, this support is ineffective in regeneration over long distance and extended in time due to the short life of neurotrophic factors and the decline of their production [17, 18]. The implementation of the nerve conduit with neurotrophic factors has the aim to maintain elevated the concentration of these growth factors and to extend their action for the prolonged time needed for axons to reach the target. For this purpose, we use the NVR gel as a scaffold for the release of GDNF conjugated to iron oxide nanoparticles (GDNF-IONP). We have previously demonstrated that conjugation of various neurotrophic factors to iron oxide nanoparticles (IONP) led to stabilization of the growth factors and to prolonged biological activity respect to the non-conjugated factors [19]. IONP are considered biocompatible and biodegradable, they are actually used for various biomedical application [20–22], and thus, they were suggested for in vivo use for peripheral nerve regeneration [23]. Moreover, in vitro experiments showed that the combination of NVR gel and IONP represents a permissive environment to neurite outgrowth [19], and GDNF-IONP has been shown to accelerate the onset and progression of myelin in organotypic dorsal root ganglion (DRG) cultures seeded in NVR gel, significantly earlier than the free GDNF and other free and conjugated factors [24].

In the present study, we explored the efficacy of chitosan tube enriched with NVR gel and GDNF-IONP to the repair of critical length, 15 mm, sciatic nerve defect in rat model. We

evaluated the functional and morphological outcome of nerve regeneration at 5 months after nerve injury, analyzing the effects given by the presence of NVR gel alone, NVR gel with GDNF-IONP or free GDNF inside the chitosan tube respect to the empty nerve device.

2. Materials and methods

2.1. Synthesis of GDNF-IONP

Dextran-coated iron oxide magnetic nanoparticles of 10 nm diameter were prepared as described previously [3]. The dextran coating was used to covalently bind the protein GDNF using divinyl sulfone binding reagent [1].

2.2. NVR gel preparation

NVR gel is a matrix supporting cell growth and survival composed by high-molecular-weight hyaluronic acid (3×10^6 Da, BTG Polymers, Kiryat Malachi, Israel) and laminin (Sigma, Rehovot, Israel) [4]. For the in vivo application as a tube filler, the NVR gel was diluted in nutrient medium corresponding to Dulbecco's modified eagle medium-nutrient mixture F-12 (DMEM-F12), supplemented with 10% fetal calf serum (FCS), 2-nM glutamine, 6-g/L D-glucose, 25-μg/mL gentamycine and 50-ng/mL IGF-I. The final concentration of the solution is 0.5% to render more suitable gel manipulation. NVR gel was filled into the chitosan tubes during the surgical implantation using a syringe.

2.3. Preparation of chitosan conduit

Chitosan tubes were manufactured by Medovent GmbH (Mainz, Germany) under ISO 13485 conditions from chitin tubes made following three main procedures: the extrusion process, distinctive washing, and hydrolysis steps to reach the required medium degree of acetylation (DAII). Tubes were finally cut into the length of 17 mm and treated with ethylene oxide for sterilization.

2.4. Animals

All animal experiments were approved by the Institutional Animal Care and Usage Committee (IACUC) and adhered strictly to the Animal Care guidelines. Female Wistar rats were brought to the vivarium 2 weeks prior to the surgery and housed two per cage with a 12-h light/dark cycle, with free access to food and water.

2.5. Experiment design and surgical technique

Fifty female Wistar rats, weighing 200–250 g each, were studied using an experimental model for producing a complete peripheral nerve injury with massive nerve defect that has recently been described [7]. During the 2 weeks before surgery and for the entire study, the rats were given Amitriptyline in their drinking water, in order to decrease autotomy—self-eating of

toes—in the operated limb. Before surgery, a general anesthesia was induced with intraperitoneal injection of xylazine (15 mg/kg) and ketamine (50 mg/kg).

During the surgical procedures, a high magnification microscope was used. The left sciatic nerve was uncovered and disconnected from biceps femoris and semimembranous muscles, beginning from the area of branches to the glutei and hamstring muscles and distally to the trifurcation into peroneal, tibial, and sural nerves. At the third femur level, the sciatic nerve was fully transected using microsurgical scissors, and a 15-mm nerve segment was removed.

Afterward, the rats were divided into five experimental groups according to the type of implant: empty tubes ($n = 10$); tubes filled with NVR gel ($n = 10$); tubes filled with NVR gel enriched with free GDNF ($n = 10$); tubes filled with NVR gel enriched with GDNF-IONP ($n = 10$); and autologous nerve grafts ($n = 10$).

For nerve reconstruction in treatment groups, a 17-mm chitosan empty tube was located between the proximal and the distal sides of the transected sciatic nerve. Both proximal and distal sides of the sciatic nerve were positioned 1 mm into the tube ends, providing a 15-mm nerve gap between the proximal and distal ends. Then, the tube ends were sutured to the epineurium at the proximal and distal nerve stumps using a 9-0 nonabsorbable suture.

In control group (autologous nerve graft reconstruction), after exposition the left sciatic nerve, a 15-mm nerve segment was severely cut, using micro scissors, at the femur level, below the superior gluteal nerve and above the dissection of the sciatic nerve into the tibial and peroneal nerves. Immediately thereafter, inverse end-to-end coaptation of the nerve segment was performed using 2–3 nonabsorbable 10-0 sutures. Coaptation of nerve fascicles was performed to preserve all the fascicles within the epineural sac. Then, the muscular, subcutaneous, and skin layers were closed.

2.6. End-point assessments

Assessments before and after surgery (30, 90, and 150 days postoperatively) were carried out in a blinded manner without disclosure of rat's affiliation to the evaluating team. The assessments consisted of functional motor assessment of the sciatic nerve utilizing sciatic function index (SFI), somatosensory evoked potentials (SSEP), ultrasound evaluation, and morphological analysis.

2.6.1. Electrophysiological evaluation

SSEPs were recorded in both the operated and intact limbs using a Dantec™ KEYPONT® PORTABLE. Conductivity of the sciatic nerve was measured by stimulating the sciatic nerve at the level of the tarsal joint with simultaneous recording from the skull over the somatosensory cortex in anesthetized rats. Two subcutaneous needle electrodes were placed under the skin of the skull, when the active electrode was placed above the somatosensory cortex along the midline, and the reference electrode was placed between the eyes. The ground electrode was placed subcutaneously on the dorsal neck. Stimulation of the sciatic nerve was conducted by a set of two polarized electrodes located on the lateral aspect of the tarsal joint. The sciatic

nerve was stimulated by 300 pulses of 0.2 ms in duration with a rate of 3 Hz. The intensity of the stimulus was set to 2–5 mA, causing a slight twitching of the limb under observation. A response to the stimulus was considered positive if an evoked potential appeared in at least two consecutive tests.

2.6.2. Ultrasound evaluation (US)

During the observational period, imaging studies employing ultrasonography were carried out, in anesthetized rats, for real-time evaluation of nerve regeneration inside the chitosan tubes. The lateral aspect of the right leg was shaved to improve transducer contact. The sonographic scanning technique included longitudinal and transverse sections. US examinations were performed using conventional US units equipped with color Doppler capabilities using 7–15 MHz linear transducer, yielding an axial resolution of 0.2–0.4 mm. The identification of the chitosan conduit on the US image was based on the recognition of a hyperechoic structure of a tubular shape in the longitudinal axis and a circular shape on the transverse section.

2.6.3. Sample resin embedding

All the nerve samples were harvested 5 months after the surgical implantation. The complete tube was collected taking care to preserve part of the proximal and the distal nerve and was fixed for 4–6 h at 4°C in 0.1-M phosphate buffer (pH 7.4) with 2.5% glutaraldehyde. After the fixation, all the tubes were opened using a scalpel and removed in order to free and clean the regenerated nerve inside the tube and prepared it to be processed for resin inclusion. The post-fixation of nerves regenerated inside tubes was done using 2% osmium tetroxide for 2 h, and then, the samples were dehydrated in ethanol from 30 to 100%. Two washings of 7 m using propylene oxide were performed, and then, samples were embedded in a mixture of propylene oxide and Glauerts' mixture of resins (50% Araldite HY964, 50% Araldite M and 0.5% dibutylphthalate) mixed in equal parts and left 1 h at room temperature. A second embedding with Glauerts' mixture of resins alone for an overnight was performed. The Glauerts' mixture of resins was changed, and samples were left 37°C for 1 h. Two following samples embedding were done using resin with 2% of accelerator 964. Samples were left for 3 days at 60°C.

For stereological analysis, resin-embedded nerve was cut from the distal stump using Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) to obtain transverse semi-thin sections (2.5 μm of thickness) for optical analysis.

2.6.4. Morphometrical analysis

Semi-thin transverse nerve sections were stained with 1% toluidine blue and analyzed in high-resolution light microscopy. The qualitative and quantitative morphological analysis was performed with DM4000B microscope and DFC320 digital camera, using IM50 image manager system (Leica Microsystems). One randomly selected semi-thin section was used to measure the total cross-section area of the nerve. Using a systematic protocol described in [25], 12–16 fields were selected, and the following parameters were measured: mean fiber density (number of fiber/field area), total number of myelinated fibers (mean fiber density ×

area of the nerve section) fiber area, axon area, fiber diameter (D) and fiber axon (d), myelin thickness [($D - d$)/2], and g-ratio (d/D).

2.7. Statistical analysis

Functional and electrophysiological analysis and calculations were done using MatLab software (Ver. 2008b, The MathWorks, Inc.). Nonparametric statistics were used in this study. Hence, all figures are presented with median ± mad. Significance levels were calculated using a Mann-Whitney U test and a Wilcoxon signed rank test. SSEP responses were analyzed as categorical parameters using χ^2 test.

For the stereological analysis, more than five sciatic nerves were analyzed for each single groups (autograft, $n = 5$; tube with NVR gel, $n = 8$; tube with NVR gel enriched with GDNF-IONP, $n = 8$; tube with NVR gel enriched with free GDNF, $n = 7$). The ANOVA one way followed by Bonferroni post hoc test was performed using SPSS Statistic Program. Results are reported as mean +SD.

3. Results

All the five defined animal groups (autograft, DAII empty tube, DAII tube with NVR gel, DAII tube with NVR gel and GDNF-IONP, DAII tube with NVR gel with GDNF) were followed-up until 5 months after nerve injury. During the follow-up period, the regeneration and the position of the implanted tubes were evaluated using ultrasound observation that enabled to observe in vivo the condition of the tube without scarifying the rats. All implants were found to be complete and in correct position (**Figure 1**).

Five months after nerve guide implantation, animals were sacrificed, and all the chitosan tubes were removed for nerve regeneration evaluation. For morphological and morphometrical analysis, the autograft group was considered as the control group, since in the comparison with healthy nerve already revealed the best regenerative aspect [26, 27].

A nerve regeneration was observed in all of the investigated conditions: a higher percentage of regenerated nerves was detected for tubes containing NVR gel with GDNF-IONP (100%), while a lower percentage of regenerated nerves (56%) was observed for tubes containing NVR

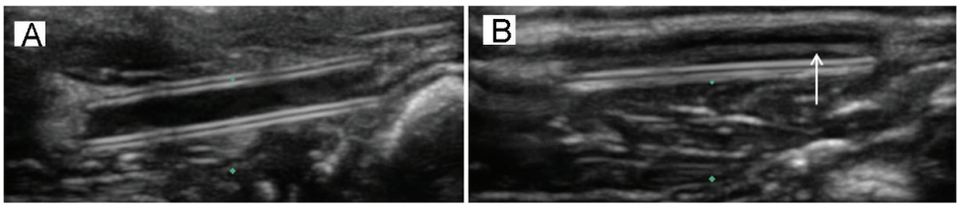

Figure 1. Ultrasound evaluation of implanted chitosan tube. (A) 20-day post-tube implantation—no regeneration is visible inside the tube and (B) 90 days post-tube implantation—nerve filaments are present inside the tube reconnecting nerve stumps (white arrows).

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gel with GDNF; the lowest percentage of regenerated nerves was found for empty tubes (11%) or tubes filled with NVR gel alone (30%).

3.1. Functional analysis

In order to assess the functional recovery, we used the electrophysiological evaluation. We recorded SSEP at different time points (0, 30, 90 and 150 days after injury) calculating SSEP peak to peak (P2P) amplitude (NR type) in which the operated limb was compared to the intact limb (**Figure 2**). Regarding the operated limb, all groups showed a decrease at the first follow-up (30 days), followed by an increase, except for the tube filled with NVR gel and NVR gel enriched with GDNF-IONP, which recovered only after 90 days. Interestingly, for what concerns the intact limb, the animal group treated with tube filled with NVR gel enriched with GDNF-IONP also exhibited a decrease in amplitude following the operation and then recovered, while both the autologous nerve graft reconstruction and the tube filled with NVR gel enriched with free GDNF groups demonstrated an increase in P2P.

It was interesting to observe the comparison of the P2P among the different treatments in both the operated and intact limbs (**Figure 3**). At 30 days after injury, P2P amplitude is significantly higher in the group of animals in which truncated nerves were repaired with tubes filled with NVR gel and GDNF-IONP respect to those repaired with autologous nerve graft ($p < 0.05$).

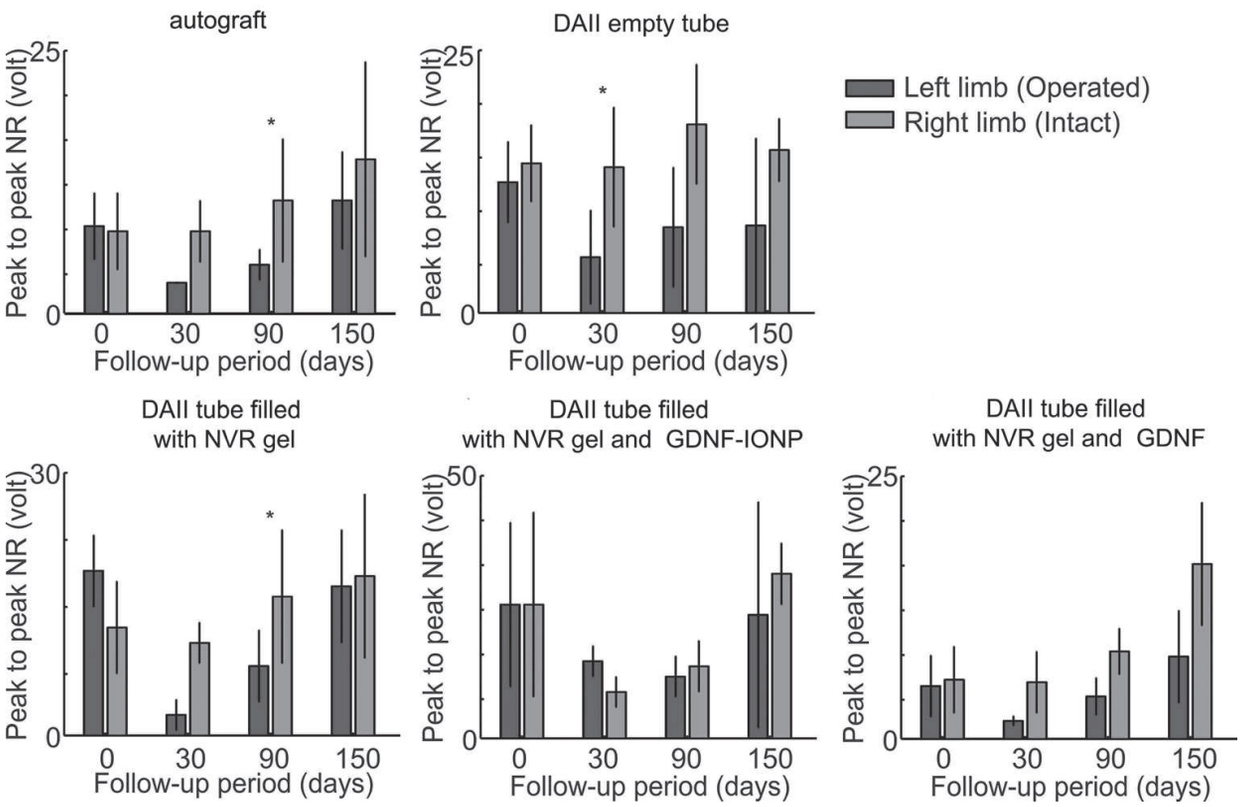

Figure 2. Comparison of somatosensory evoked potentials (SSEP) peak-to-peak (P2P) amplitude among various treatments. Data were gathered from each limb separately (operated and intact) at four follow-up periods: 0 (pre-operatively), 30, 90, and 150 days. Values are presented as median ± mad. Statistical analysis: Wilcoxon signed rank test. *$p < 0.05$.

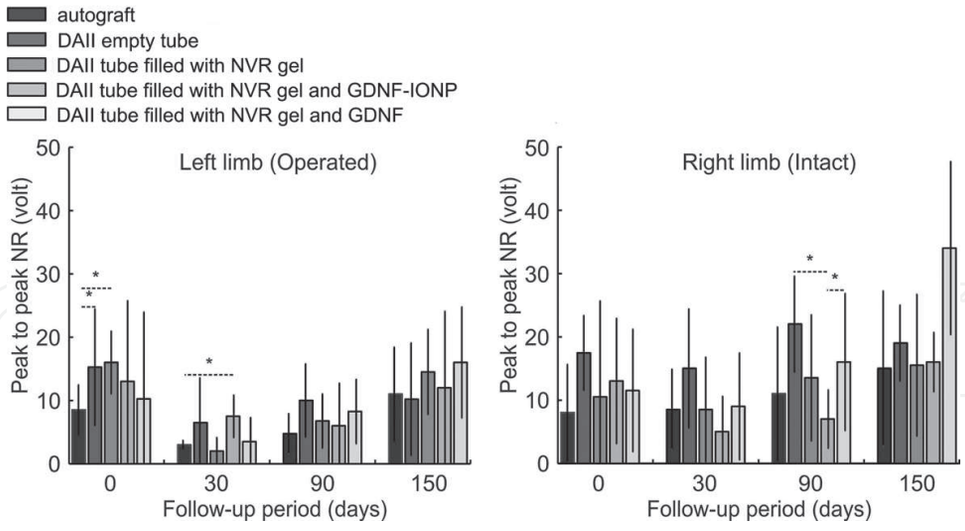

Figure 3. Comparison among the various treatments. Data were gathered from each limb separately (operated and intact) at four follow-up periods: 0 (pre-operatively), 30, 90, and 150 days. Values are presented as median ± mad statistical analysis: Mann-Whitney rank-sum test. *$p < 0.05$.

The same treatment seemed to provoke significant decrease in P2P amplitude at the intact limb but only during the first time point investigated. The difference was observed in relation to the empty tube group ($p < 0.05$), and the tube filled with NVR gel enriched with free GDNF ($p < 0.05$).

In order to normalize the results, we decided to subtract P2P measurement in the operated limb with the P2P measurement in the intact limb (**Figure 4**) in each of the four follow-up time points (0, 30, 90, and 150 days). The "0" value indicates similar amplitude in both intact and operated limbs. Since a dramatic decrease in amplitude is an indicator of neurological dysfunction, we assume that a large shift down from "0" is a marker for this pathology. As expected, at time point 0 (pre-operation), all groups exhibited an amplitude difference very close to "0." During the follow-up, most treatments exhibited a decrease at the amplitude. The most robust and sustained decrease was found at the empty tube group. Surprisingly, the rat group that was treated with tube filled with NVR gel enriched with GDNF-IONP had no decrease.

3.2. Morphological and morphometrical analysis

To determine how structural aspect of regenerated nerves is influenced by the choice of the dispositive used to repair the long gap, we performed morphological and morphometrical analysis on nerve regenerated inside the chitosan tube 5 months after the surgery. Beside only two animals showed regeneration in empty tube group, this group was excluded from morphometrical analysis. The morphometric quantification was carried out in the distal part of the regenerated nerve inside the tube (**Figure 5A**). Semi-thin section of regenerate nerves was used for the analysis and compared to the distal part of regenerated nerves of autograft group, our positive control (**Figure 5B–E**).

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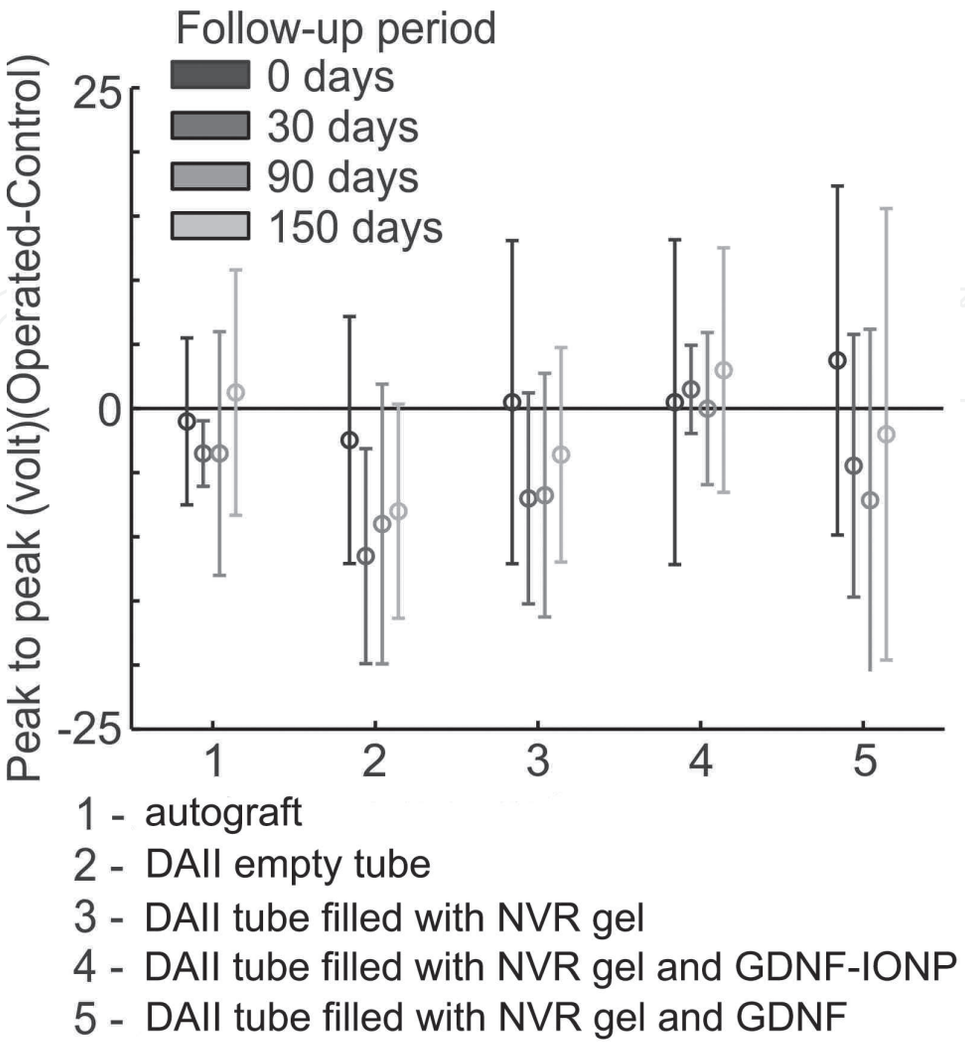

Figure 4. P2P measurement normalized to the intact limb. The graph shows the data obtained through the subtraction of P2P measurement in the operated limb with P2P measurement in the intact limb. The "0" value indicates similarity between the two limbs. Values are presented as median ± mad.

It is possible to notice the similarity among experimental groups in which nerve cross sections are organized in fascicles with small fibers.

The number of myelinated fibers is significantly higher in autograft group ($p \leq 0.001$) that remains the gold standard of regeneration (**Figure 6**); nevertheless, it is important to notice that in the experimental group repaired with the tube enriched with NVR gel and GDNF-IONP, the number of myelinated fibers is statistically significant higher ($p \leq 0.05$) respect to the group repaired with the conduit functionalized with NVR gel alone (**Figure 6**).

The myelin thickness parameter, recorded for the group repaired using a tube with NVR gel alone, shows a statistically relevant reduction compared to autograft group ($p \leq 0.05$). As regard the other parameters considered, it is interesting to note that there are no differences among the experimental groups.

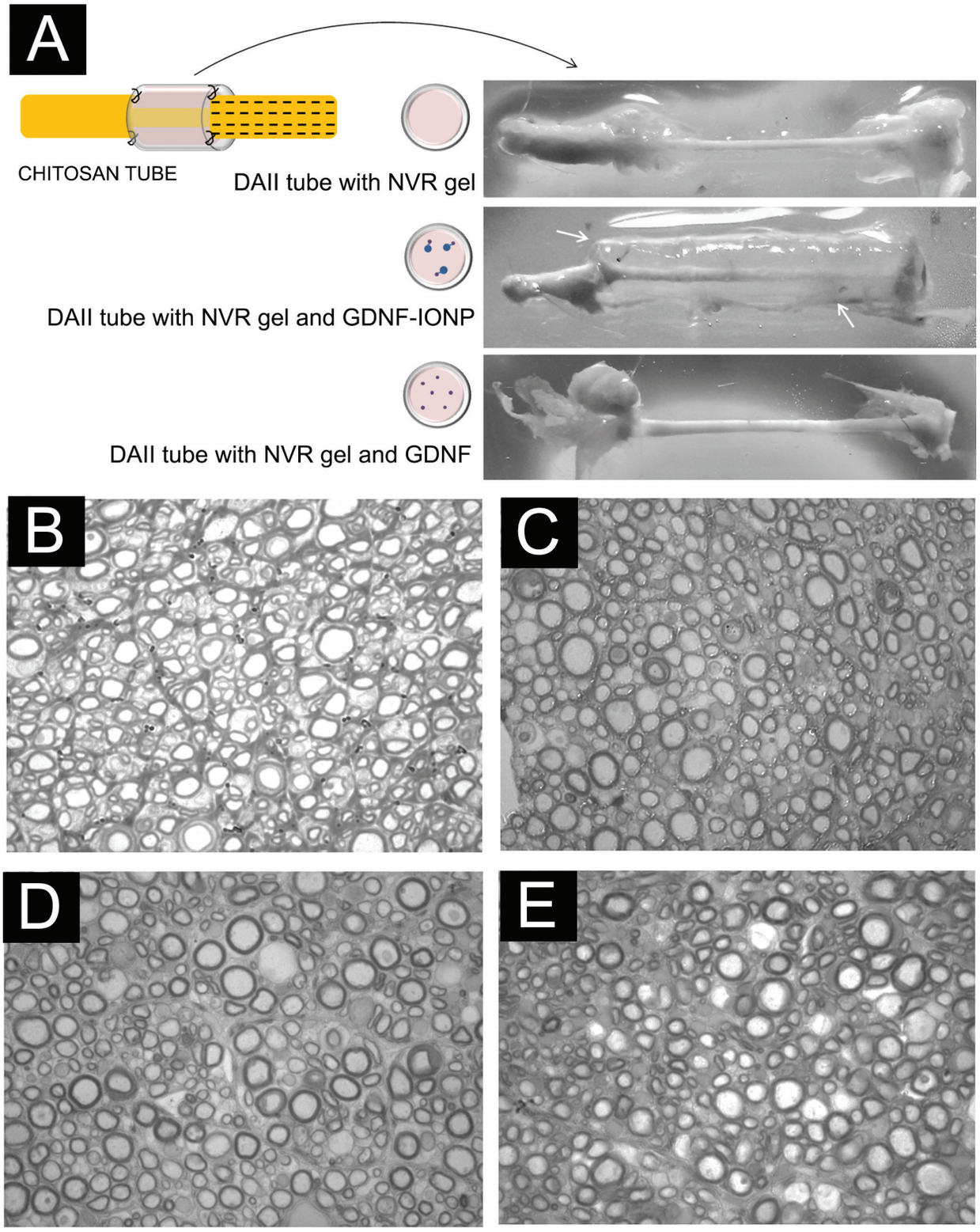

Figure 5. Morphological analysis of regenerated nerves inside chitosan tube. (A) Representative photos of sciatic rat regenerated nerves harvested inside chitosan tube 5 months after the implantation. (B–E) The figure shows representative semi-thin transversal nerve sections of regenerated sciatic nerves used for morphometrical analysis. For autograft group (B), the distal nerve is represented; for tube with NVR gel alone (C), tube with NVR gel enriched with GDNF-IONP (D) and tube with NVR gel enriched with free GDNF (E); the pictures refer to the nerve found inside the chitosan tube. For all the groups, a good regeneration is visible.

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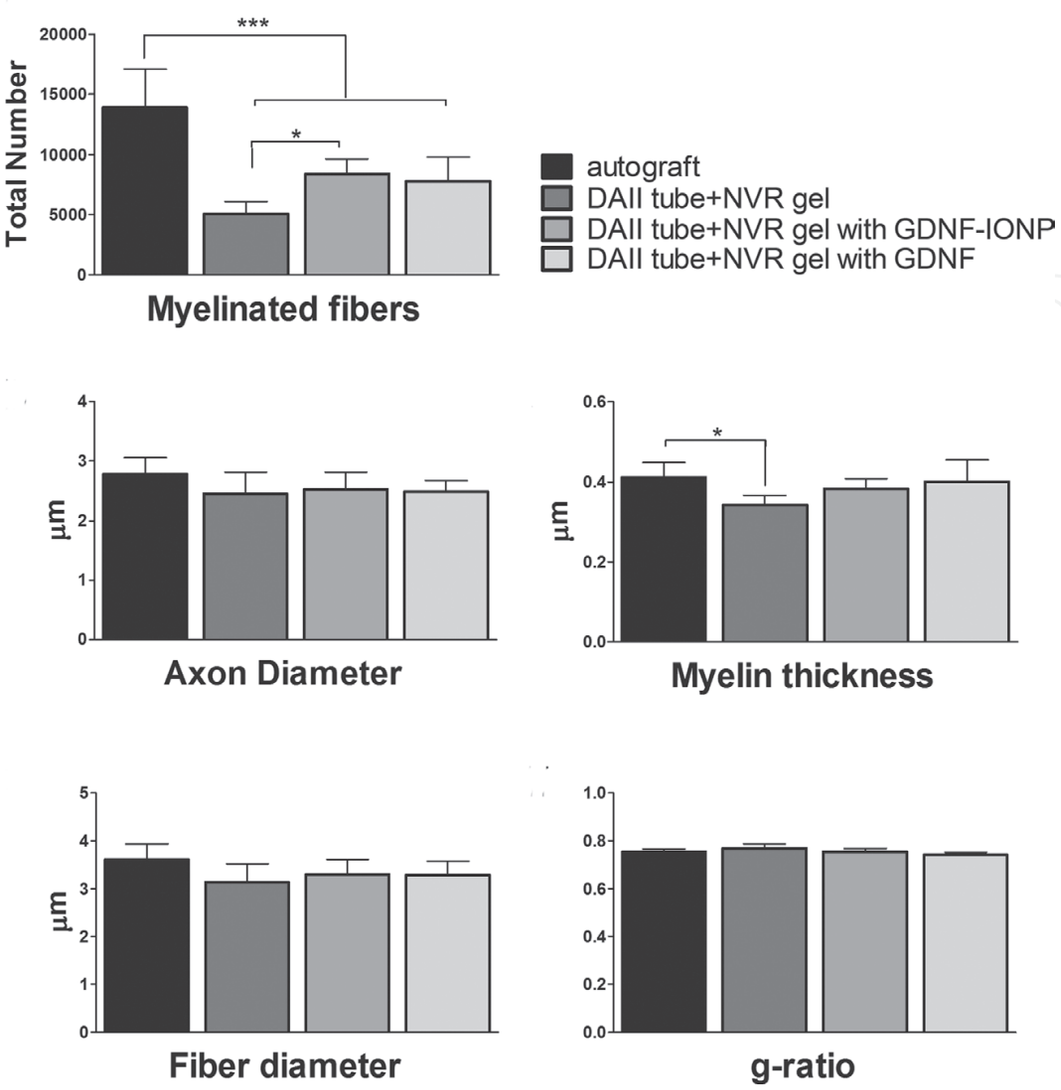

Figure 6. Morphometrical analysis of regenerated nerve inside the chitosan tube. The analysis was performed on semi-thin transverse sections of the regenerated nerves 5 months after implantation. For autograft group (used as control), the distal part of the nerve was analyzed; for all the other groups, the values in the graphs are referred to the distal regenerated nerve found inside the chitosan tube. The graphs show the total number of number of myelinated fibers, the axon diameter, the fiber diameter, the myelin thickness, and the g-ratio. In the filled tubes, there is less myelinated fibers respect to the autograft, but all the other parameters are comparable with the autograft. The two groups with GDNF factors, free or conjugated to iron-oxide nanoparticles, are similar. All data are presented as mean + SD. Statistical analysis: One-way ANOVA with post hoc Bonferroni Test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4. Discussion

A wide range of conduits with different internal design have been tested for reconstruction of peripheral nerves in animal models [28]. The efforts concern the attempt to replace the current gold standard, the autologous nerve graft, above all in the case of very serious injury and to avoid negative side-effects [2, 4]. Chitosan conduits, among the absorbable forms, have been shown to be able of supporting the peripheral nerve regeneration with values of recovery

fully comparable to the surgical gold standard technique, with regard to sciatic nerve lesions of 10 mm in the rat animal model [7, 11]. Despite this, in case of nerve injuries with a considerable loss of substance, it is necessary to functionalize the conduit, to provide a filler, in order to prevent the collapse of the walls, and to provide a releasing system of neurotrophic molecules able to accelerate the regenerative processes [26]. In this study, we used the NVR gel, made of hyaluronic acid and laminin [13], as internal filler and for the release of the neurotrophic factor GDNF, which has been further enhanced by stabilizing its duration through a covalent conjugation with IONP [19].

We have previously demonstrated that the administration of this factor induces an early myelination in organotypic cultures of neonatal DRG [24]. The whole device has been proposed for the repair of a rat sciatic nerve lesion with severe loss of substance (15 mm). In this study, the nerve regeneration and the correct position of the device have been constantly monitored. Ultrasound observations and functional analysis have allowed us to carry out regular follow-up (30, 90, and 150 days after surgery).

The ultrasound analysis carried out on the implants revealed that none of these have undergone a shift from the correct position by the moment of the surgery. The two representative figures show the implanted tube at 20 and 90 days after surgery; at the experimental time 90 days, nerve regeneration appears through nerve fascicles.

Electrophysiological analysis on the five experimental groups demonstrates a nerve conductivity recovery (P2P amplitude) by the end of the follow-up period (150 days), when the tube enriched either with NVR gel or NVR gel and GDNF-IONP treatments displayed a complete recovery, as time 0, whether other treatments showed only a partial recovery. Normalizing the SSEP results of the operated limb (left) to the intact one (right), all groups showed a decrease after surgery followed by an increase, as expected, except for the group treated using a tube enriched with NVR gel and GDNF-IONP that did not showed any decrease during the follow-up period after surgery. These electrophysiological findings suggest that the treatment, in which a tube is enriched with NVR gel, is comparable to the autologous nerve graft treatment.

The morphological aspect of regenerated nerves when, after 5 months, rats were sacrificed and tubes explanted revealed for all the cases analyzed a good nerve regeneration.

The morphometric quantification was referred on the distal part of the regenerated nerve inside the tube. Semi-thin sections of nerves were used for the analysis and compared to the ones from the distal part of regenerated nerves of autograft group, our positive control. All the nerves revealed the same cross section structure: fascicles with small fibers, a typical nerve regeneration framework.

It was interesting to observe similar values for the measured parameters, such as axon diameter, myelin thickness, fiber diameter, and g-ratio, demonstrating an equal good regeneration, because compared with the gold standard, the autograft. The only parameter in which the superiority of our positive control was found was the total number of the fibers, although the group represented by the tube enriched with NVR gel and GDNF-IONP had a statistically significant greater value compared to the tubes enriched with only NVR gel.

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5. Conclusions

In this work, we investigated the efficacy, in the repair of a nerve injury with a considerable loss of substance, of a conduit functionalized (i) with a factor that has been demonstrated to accelerate nerve regeneration, (ii) with a hydrogel, which has proven to be a good substrate for neurite outgrowth, (iii) and with the system of the IONP, able to stabilize the signaling of the factors. The analysis carried out has not shown a great improvement in nerve regeneration in animals treated with this functionalized device compared to the other devices investigated. IONPs are potentially a good candidate for nerve device enrichment; however, the best way to administer neurotrophic factors IONP in the tube needs further investigation, as well as the effects of their long time exposition.

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