



Original research

Functional effect of local administration of glial derived neurotrophic factor combined with inside-out artery graft on sciatic nerve regeneration in rat



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ABSTRACT

Effect of local glial derived neurotrophic factor (GDNF) on nerve regeneration was assessed. Eighty male Wistar rats were divided into four experimental groups ($n = 20$), randomly: In transected group left sciatic nerve was transected and stumps were fixed in adjacent muscle. In treatment group defect was bridged using an artery graft filled with 10 μ L GDNF. In artery group graft was filled with phosphate-buffered saline. In normal control group sciatic nerve was exposed and manipulated. Each group was subdivided into four subgroups of five animals each and nerve fibers were studied in a 16-week period. Behavioral, functional, electrophysiological and gastrocnemius muscle mass findings and morphometric indices confirmed faster recovery of regenerated axons in IOAG/GDNF than in IOAG group ($p < 0.05$). Immunohistochemical reactions to S-100 in IOAG/GDNF were more positive than that in IOAG group. GDNF improved functional recovery and morphometric indices of sciatic nerve. It could be considered as an effective treatment for peripheral nerve repair in practice.

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1. Introduction

Despite variability and unpredictability of functional outcome after nerve repair, technological advances in diagnostic imaging, neurosurgical instrumentation and the use of a surgical microscope have resulted in pronounced improvements in the diagnosis and repair of transected peripheral nerves [1]. Nerve autograft remains the gold standard, however, there are several drawbacks such as sacrifice of functioning nerves, loss of sensation and mismatch between nerve and graft [2]. The ideal surgical repair technique should accomplish good wound healing with minimal scar formation and direct the nerve sprouts into their correct targets [3]. The conduits act to guide axons sprouting from the regenerating nerve end, provide a microenvironment for diffusion of neurotrophic and neurotropic factors secreted by the injured nerve stump, as well as help protect from infiltration of fibrous tissue [4]. An artery graft presents large quantities of laminin and some collagen. These

substances are also found in Schwann-cell basal membrane and are reported as axonal outgrowth factors [5,6]. Laminin, one of the main basal membrane components, stimulates neurite outgrowth, induces Schwann-cell mitosis, and plays a fundamental role in peripheral nerve regeneration [7,8]. As well as requiring nerve fiber contact, normal Schwann-cell differentiation requires contact with a connective tissue matrix or some associated material such as collagen [9]. Standard artery graft basal membrane tube diameter is large, and the contact surface for migrating Schwann cell or axonal outgrowth cone adhesion becomes very small [10]. Nevertheless, in inside-out artery grafts, this negative effect might be diminished once the adventitia provides a permissive matrix which increases the contact surface for axons [11].

Neurotrophic factors have been extensively investigated in animal models of nerve injury to further enhance and accelerate the process of nerve regeneration and functional recovery [12]. Neurotrophic factors support the survival of axotomized neurons and enhance the intrinsic regenerative capacity after retrograde uptake and induction of specific signaling cascades. One member of the transforming growth factor superfamily, glial cell line-derived neurotrophic factor (GDNF), has been shown to promote the survival and function of several neuronal populations in the peripheral

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nervous system [13,14]. Many neurotrophic factors support the survival of neurons in culture and *in vivo* after peripheral nerve injury. GDNF is a potent motoneuron factor which prevents motoneuron degeneration in mice and rats after axotomy [15,16].

Aimed to study local effects of GDNF on peripheral nerve regeneration, a study was designed to determine if local GDNF could in fact reduce dysfunction after nerve injury in the rat sciatic nerve transection model. Assessment of the nerve regeneration was based on behavioral, functional, histomorphometric and immunohistochemical (Schwann cell detection by S-100 expression) criteria 4, 8, 12 and 16 weeks after surgery.

2. Materials and methods

2.1. Study design and animals

Eighty male Wistar rats weighing approximately 290 g were divided into four experimental groups ($n = 20$), randomly: normal control group as normal control (NC), transected control (TC), inside–out artery graft (IOAG) and GDNF treated group (IOAG/GDNF). Twenty rats were used as artery graft donors. Each group was further subdivided into four subgroups of five animals each and surveyed 4, 8, 12 and 16 weeks after surgery. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of $(23 \pm 3) ^\circ\text{C}$, stable air humidity and a natural day/night cycle. The rats had free access to standard rodent laboratory food and tap water. All measurements were made by two blinded observers unaware of the analyzed groups.

2.2. Surgical procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain [17]. The University Research Council approved all experiments.

Following surgical preparation in the normal control group, the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with resorbable 4/0 sutures, and the skin with 3/0 nylon. In TC group, the left sciatic nerve was transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a 10 mm gap due to retraction of nerve ends. Proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture. No graft was interposed between the stumps. In the IOAG group, a 7-mm nerve segment was resected to produce a 10 mm nerve gap after retraction of the nerve transected ends. The gap was bridged using an artery graft, entubulating 2 mm of the nerve stump at each end. The artery graft was harvested from abdominal aorta of donor animals. The abdominal aorta artery was exposed through a midline abdominal incision and cannulated. Then, a 15 mm segment was harvested on the cannula. Donor animals were sacrificed after graft harvest using a high-dose anesthetic. Harvested grafts were washed in physiological solution and left at room temperature for 40 min. A subtle retraction of 1 mm was already expected. Each graft was inverted inside-out by pulling it down the cannula with microsurgery tweezers. Allografts did not receive preliminary treatment to reduce their antigenicity. Two 10/0 nylon sutures were used to anchor the graft to the epineurium at each end. In GDNF treated group (IOAG/GDNF) the graft was filled with 10 μl GDNF (100 ng/kg). The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2%

paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) 4, 8, 12 and 16 weeks after surgery.

2.3. Behavioral testing

Functional recovery of the nerve was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale for rat hind limb motor function [18]. Although BBB is widely used to assess functional recovery in spinal cord injured animals, however, it has been demonstrated that it could be most useful in assessment of never repair processes in peripheral nerve injuries [19]. Scores of 0 and 21 were given when there were no spontaneous movement and normal movement, respectively. A score of 14 shows full weight support and complete limbs coordination. BBB recordings were performed by a trained observer who was blinded to the experimental design. The testing was performed in a serene environment. The animals were observed and assessed within a course of a 4-min exposure to an open area of a mental circular enclosure. BBB scores were recorded once before surgery in order to establish a baseline control and again weekly thereafter to assess functional recovery during 16 weeks.

3. Functional assessment of reinnervation

3.1. Sciatic functional index (SFI)

Walking track analysis was performed 4, 8, 12 and 16 weeks after surgery based on the method of others [20]. The lengths of the third toe to its heel (PL), the first to the fifth toe (TS), and the second toe to the fourth toe (IT) were measured on the experimental side (E) and the contralateral normal side (N) in each rat. The sciatic function index (SFI) of each animal was calculated by the following formula:

$$\text{SFI} = -38.3 \times (\text{EPL} - \text{NPL})/\text{NPL} + 109.5 \times (\text{ETS} - \text{NTS})/\text{NTS} + 13.3 \times (\text{EIT} - \text{NIT})/\text{NIT} - 8.8$$

In general, SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. SFI was assessed in the NC group and the normal level was considered as 0. SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

3.2. Static sciatic index (SSI)

SSI is a time-saving digitized static footprint analysis described by others [21]. A good correlation between the traditional SFI and the newly developed static sciatic index (SSI) and static toe spread factor (TSF), respectively, has been reported by others [21]. The SSI is a time-saving and easy technique for accurate functional assessment of peripheral nerve regeneration in rats and is calculated using the static factors, not considering the print length factor (PL), according to the equation:

$$\text{SSI} = [(108.44 \times \text{TSF}) + (31.85 \times \text{ITSF})] - 5.49$$

Where:

$$\text{TSF} = (\text{ETS} - \text{NTS})/\text{NTS}$$

$$\text{ITSF} = (\text{EIT} - \text{NIT})/\text{NIT}$$

Like SFI, an index score of 0 was considered normal and an index of -100 indicated total impairment. When no footprints were measurable, the index score of -100 was given.

3.3. Electrophysiological assessment

At the end of the study period, following walking track, all animals were subjected to electrophysiological studies using Nacro bio system 320-3760 A trace 80 (USA). Under general anesthesia, the left sciatic nerve was re-exposed by incision of the skin at the previous surgical site. Single electrical pulses at supramaximal intensity were delivered via bipolar electrodes placed in turn at the proximal and distal trunk of the grafted nerve and EMG was recorded by inserting an electrode into the belly of gastrocnemius muscle.

The difference in latency of EMG was measured, and the distance between the proximal and distal sites of stimulation was measured to calculate the conduction velocity across the regenerated tissue cable. On the contralateral side of each animal similar measurement was made for determination of conduction velocity. The conduction velocity of the bridged nerve was expressed as a percentage of that on the intact side of each animal to cancel off variations between animals (% CVR) [22].

3.4. Muscle mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 16 weeks after surgery. Immediately after sacrificing of animals, gastrocnemius muscles were dissected and harvested carefully from intact and injured sides and weighed while still wet, using an electronic balance.

3.5. Histological preparation and morphometric studies

Nerve mid-substance in IOAG group, nerve mid-substance in IOAG/GDNF treated group, midpoint of normal sciatic nerve (NC) and regenerated mid substance of TC group were harvested and fixed with glutaraldehyde 2.5%. They were post fixed in OsO₄ (2%, 2 h), dehydrated through an ethanol series and embedded in paraffin. The nerves were cut in 5 μ m in the middle, stained with toluidine blue and examined under light microscopy. Morphometric analysis was carried out using an image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). Equal opportunity, systematic random sampling and two-dimensional disector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases [23].

3.6. Immunohistochemical analysis

In this study, anti-S-100 (1:200, DAKO, USA) was used as marker for myelin sheath. Specimens were post fixed with 4% paraformaldehyde for 2 h and embedded in paraffin. Prior to immunohistochemistry nerve sections were dewaxed and rehydrated in PBS (pH 7.4). Then the nerve sections were incubated with 0.6% hydrogen peroxide for 30 min. To block non-specific immunoreactions the sections were incubated with normal swine serum (1:50, DAKO, USA). Sections were then incubated in S-100 protein antibody solution for 1 h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1 h. Horseradish peroxidase-labeled secondary antibody was applied for 1 h. After that all sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride chromogene substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

3.7. Statistical analysis

The results were expressed as means \pm SD. Statistical analyses were performed using PASW 18.0 (SPSS Inc., Chicago, IL, USA). Model assumptions were evaluated by examining the residual plot. Results were analyzed using a factorial ANOVA with two between-subjects factors. Bonferroni test for pairwise comparisons was used to examine the effect of time and treatments. The differences were set at $P < 0.05$.

4. Results

4.1. Behavioral testing

4.1.1. BBB recovery

In order to assess hind limb recovery the open field locomotor was used. Fig. 1 shows BBB scores compared to the baseline. All experimental groups, except for NC, showed the greatest degree of functional deficit one week after surgery. The GDNF treated group showed significant improvement in locomotion of the operated limb compared to the IOAG group during the study period ($P < 0.05$).

4.2. Recovery of sciatic nerve function

4.2.1. SFI outcome

Fig. 2 shows sciatic function index (SFI) values in all four experimental groups. Prior to surgery, SFI values in all groups were near zero. After the nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. At the end of the study period, animals of GDNF group achieved a mean value for SFI of -31.2 ± 4.19 whereas in group IOAG a mean value of -52.3 ± 4.12 was found. The statistical analyses revealed that the recovery of nerve function was significantly ($P < 0.05$) different between IOAG/GDNF and IOAG groups and application of the GDNF in artery graft significantly accelerated functional recovery in the course of time.

4.2.2. SSI outcome

Changes in SSI were similar to those observed in SFI, indicating significant deficit following the sciatic nerve transection (Fig. 3). Changes in SSI were significant at weeks 4, 8, 12 and 16 of recovery ($P < 0.05$). The contrasts indicate SSI values in group IOAG/GDNF at week 16 to differ significantly from those obtained from IOAG, a trend also noticed for SFI ($P < 0.05$).

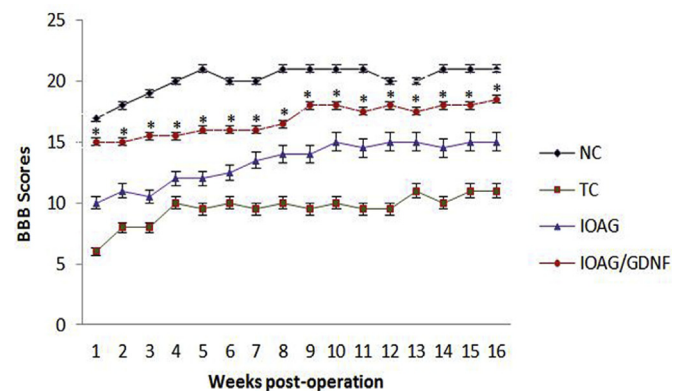


Fig. 1. BBB score for all experimental groups. Topical administration of GDNF I with artery grafting gave better scores than in IOAG group. Standard error at each data point is shown with bars.

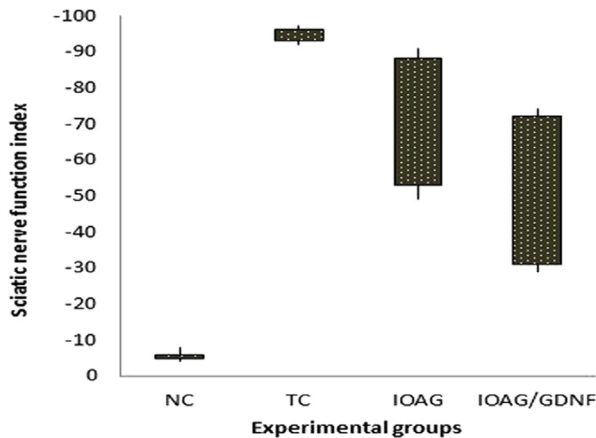


Fig. 2. Box-and-whisker plots of sciatic nerve function index values in each experimental group during the study period. Topical administration of GDNF I with artery grafting gave better results in functional recovery of the sciatic nerve than in IOAG group.

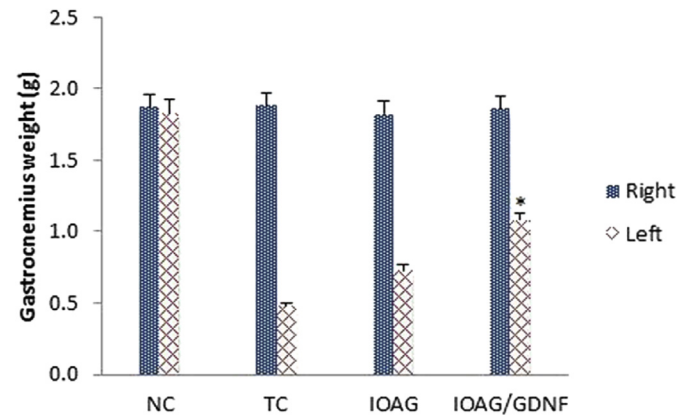


Fig. 4. Percentage recovery of conduction velocity in experimental groups. Data are presented as mean \pm SD. * $P < 0.005$ vs IOAG group.

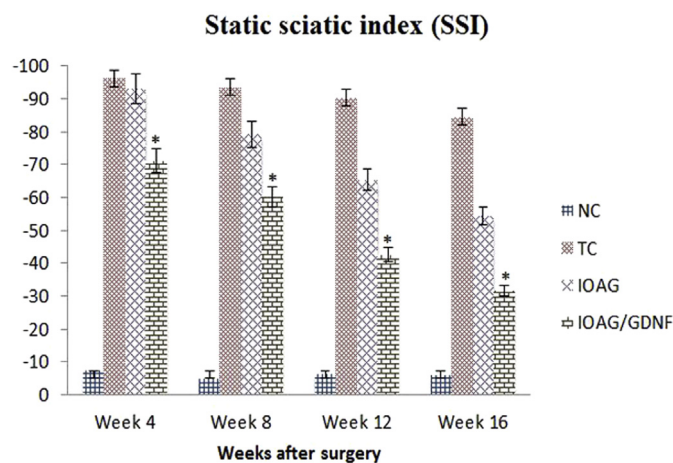


Fig. 3. Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Topical administration of GDNF I with inside-out artery grafting gave better results in functional recovery of the sciatic nerve than in IOAG group. Data are presented as mean \pm SD. * $P < 0.05$ vs IOAG group.

4.2.3. Electrophysiological measurement

Fig. 4 shows nerve conduction velocity (NCV) along regenerated sciatic nerves in experimental groups. NCV in GDNF treated animals was significantly higher than that in IOAG group ($P < 0.05$).

4.2.4. Muscle mass measurement

The mean ratios of gastrocnemius muscle weight were measured at the end of the study period. There was a statistically significant difference between the muscle weight ratios of the IOAG/GDNF and IOAG groups ($P < 0.05$). The results showed that in the GDNF treated group, the muscle weight ratio was larger than in the IOAG group, and weight loss in the gastrocnemius muscle was ameliorated by GDNF local administration (Fig. 5).

4.2.5. Histological and morphometric findings

The GDNF treated group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness during study period, compared to IOAG animals ($P < 0.05$). Normal control group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to IOAG/GDNF and IOAG groups animals (Figs. 6–9). In case of myelin thickness there was no

significant difference between IOAG/GDNF and IOAG groups, morphometrically ($P > 0.05$).

4.2.6. Immunohistochemistry

Immunoreactivity to S-100 protein was extensively observed in the cross sections of regenerated nerve segments. The expression of S-100 protein signal was located mainly in the myelin sheath. The axon also showed a weak expression indicating that Schwann cell-like phenotype existed around the myelinated axons (Fig. 10). In both IOAG/GDNF and IOAG groups, the expression of S-100 and the findings resembled those of the histological evaluations.

5. Discussion

It is known from previous studies that regeneration process in rats would not have been completed by 12 weeks, a phenomenon which has been reported in a variety of experimental models [24]. Quantitatively, our results are consistent with these findings. However, a 12-week experimental period is sufficient for evaluation of regeneration process because in rats functional recovery after repair of a transected peripheral nerve occurs during this timeline [25].

The results of the present study showed that application of GDNF in an artery graft resulted in faster functional recovery of the sciatic nerve during the study period. Left gastrocnemius muscle weight was significantly greater in the IOAG/GDNF group than in the IOAG group, indicating indirect evidence of successful end organ reinnervation in the GDNF treated animals. It has been demonstrated that morphometric indices are measures of regenerated nerve maturity and quality of regeneration [26,27]. Larger diameters of axons and thicker myelination give rise to improved nerve function compared to smaller and thinner myelinated fibers [28]. Loading of GDNF into IOAG conduit at the nerve repair site increased fiber maturity.

At week 16 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the IOAG and IOAG/GDNF groups, indicating a beneficial effect of local application of GDNF on the nerve regeneration.

Although both morphological and functional data have been used to assess neural regeneration after induced crush injuries, the correlation between these two types of assessment is usually poor [29–31]. Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling [32] do not necessarily predict the reestablishment of motor and sensory

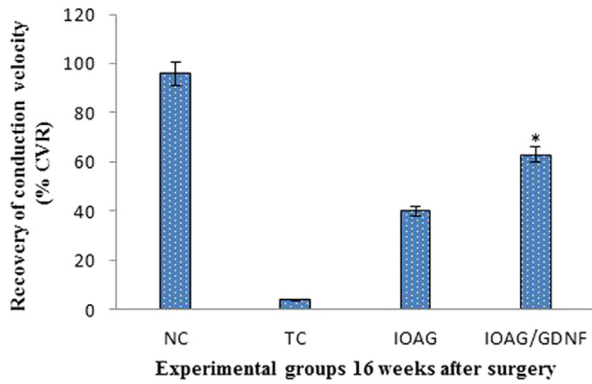


Fig. 5. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 16 weeks after surgery. Data are presented as mean \pm SD. * $P < 0.05$ vs IOAG group.

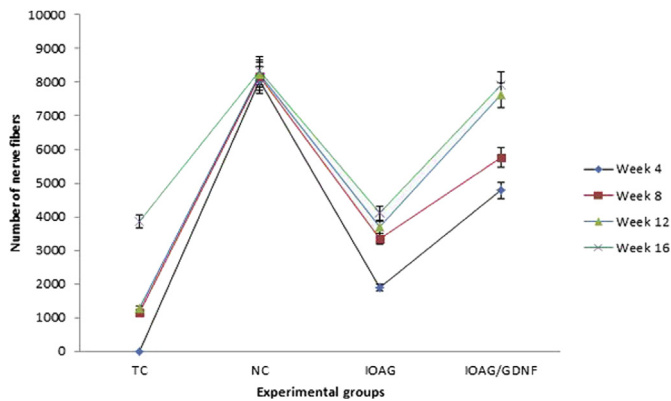


Fig. 6. The graph shows the quantitative results of fiber counting. The mean number of nerve fibers in NC group was nearly 8326 ± 211 (mean \pm SD). Both groups of IOAG and IOAG/GDNF showed the lower number of fibers than the NC group even at the end of the study period.

functions [30,33–35]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [30]. Therefore, research on peripheral nerve injury needs to combine both functional and

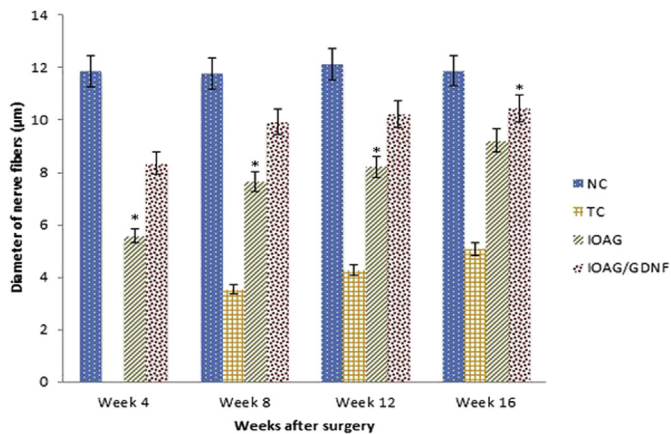


Fig. 7. The graph shows the quantitative results of mean diameter of nerves fibers. The mean diameter of nerve fibers in NC group was nearly 12.6 ± 0.11 (mean \pm SD). Both groups of IOAG and IOAG/GDNF showed the lower mean diameter of nerve fibers than the NC group even at the end of the study.

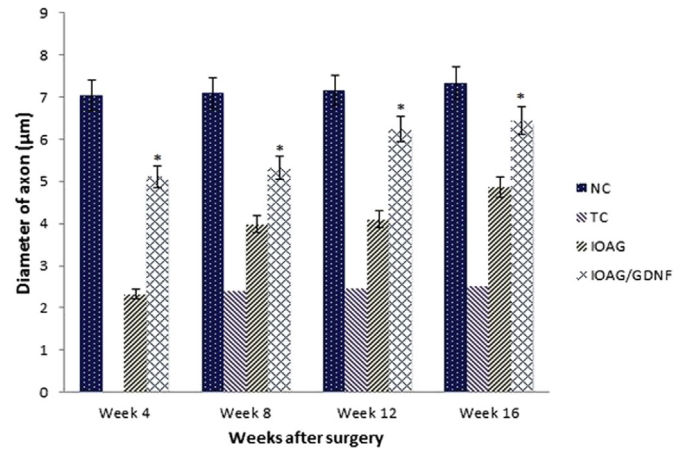


Fig. 8. The graph shows the quantitative results of mean diameter of axons. The mean diameter of axons in NC group was nearly 7.3 ± 0.09 (mean \pm SD). Both groups of IOAG and IOAG/GDNF showed the lower mean diameter of axons than the NC group even at the end of the study period.

morphological assessment. Castaneda et al., [25] suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that the GDNF treated animals had been improved in locomotion of the operated limb compared to the IOAG group during the study period. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [21,34]. Nerve conduction measurement is a direct evidence for the study of nerve transmission [36]. The conduction velocity depends on the diameter of axons and the thickness of myelin sheath [37]. The results of the present study showed significantly different conduction velocity between the GDNF treated animals and IOAG bridged regenerated sciatic nerves, therefore, the IOAG conduit in combination with GDNF could be assumed as a safe nerve guide with no nerve conduction interference. To achieve maximal efficacy in nerve transection models dose–response studies remain to be conducted for GDNF to determine the combination of the graft and the compound.

Several nerve guidance conduits (NGCs) and nerve protectant wraps are approved by the US Food and Drug Administration (FDA)

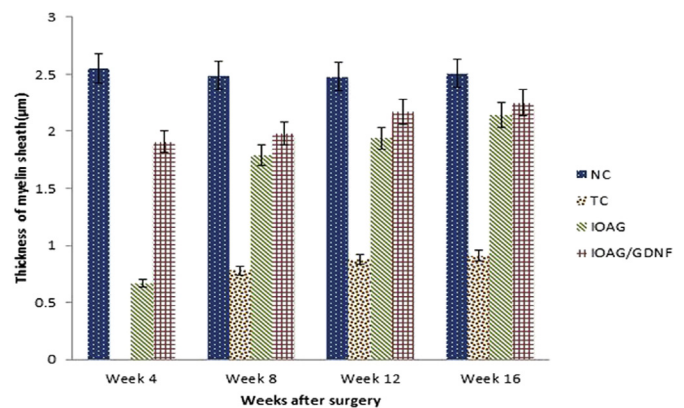


Fig. 9. The graph shows the quantitative results of mean thickness of myelin sheath. The mean thickness of myelin sheath in NC group was nearly 2.4 ± 0.04 (mean \pm SD). Both groups of IOAG and IOAG/GDNF showed the lower mean diameter of axons than the NC group even at the end of the study period.

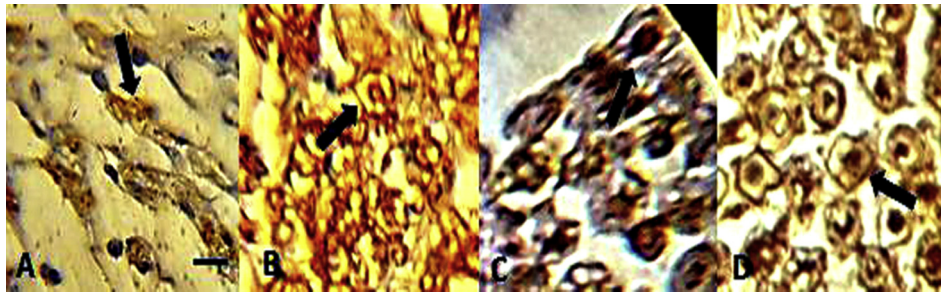


Fig. 10. Immunohistochemical analysis of the regenerated nerves 16 weeks after surgery from (A) middle cable TC, (B) IOAG, (C) IOAG/GDNF and (D) NC. There is clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in GDNF treated nerve compared to that of the IOAG. Scale bar:10 μ m.

for clinical use in peripheral nerve repair. These devices cover a wide range of natural and synthetic materials, which may or may not be resorbable [38]. Surgeons are often not aware of the different(bio) materials of these conduits when performing nerve repair [39].

Because of its inert and elastic properties, the silicon tube was one of the first and most frequently used to bridge the transected nerves [40]. Nevertheless, these non-biodegradable tubes induce fibrous capsule formation, leading to chronic nerve compression and an inflammatory response [6]. In order to avoid problems associated with non-degradable guides, recent research has been focused on the production of biodegradable nerve guides [41]. These guides provide a good tool to administrate factors which can improve the regeneration of injured peripheral nerves in human. However, such a biodegradable guides collapse easily because of their thin walls [42]. Arteries have been experimentally used as grafting tubules and promising results have been achieved [11].

The neurotrophin family of neurotrophic factors is a family of structurally and functionally related peptides which mediate potent survival and differentiation effects on a wide variety of neuronal populations in the central and peripheral nervous systems [12]. Neurotrophins are unique among neurotrophic factors in their ability to act as guidance molecules for growth cones [43,44].

GDNF, a member of the transforming growth factor-beta family of growth factor encoding genes, is initially identified as a survival factor for midbrain dopaminergic neurons [45]. It has been shown that it acts on a wide range of neuronal populations in the central and peripheral nervous system. GDNF has been shown to promote the survival of developing motoneurons *in vivo* and *in vitro*. It can also promote the survival of adult mouse spinal motoneurons after injury *in vivo* [46]. In studying the effects of GDNF on embryonic motoneuron differentiation neurite outgrowth is promoted in varying degrees [47]. GDNF exert its effects via a receptor complex which consists of a high affinity ligand-binding subunit and subsequent coupling to a common signal transduction subunit [48].

Reportedly, synthetic guidance channels continuously releasing GDNF can support sciatic nerve regeneration across long gaps [49]. Besides functional impairment, nerve injury can also cause debilitating neuropathic pain. Neurotrophic factors can also act as pain modulators [50]. GDNF has been described to have analgesic effects in animal models of neuropathic pain [51]. Apart from its better regenerative power, GDNF released by synthetic nerve guidance channels has been proposed to be an effective treatment for peripheral nerve repair in the clinic [49].

Even though our preliminary study shows the neuroprotective action of local GDNF in peripheral nerve injuries, determining the molecular mechanisms leading to the neuroprotective action remains needs to be investigated. We have not given the histological and molecular evidence for neuroprotective action of GDNF. This

may be considered as a limitation to our study. Therefore, the authors stress that the aim of the current investigation was to evaluate a single local dose and clinical treatment potential of GDNF on nerve regeneration including functional assessments of the nerve repair, a case not considered in previous studies. The results of the present study indicated that a single local administration of GDNF at the site of transected nerve could be of benefit after artery graft tubulization. Detailed mechanism of neuroprotective action remains to be investigated.

In Conclusion results of the present study demonstrated that a single local application of GDNF could accelerate functional recovery after transection of sciatic nerve.

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