



Local Effect of Heparin Binding Neurotrophic Factor Combined with Chitosan Entubulization on Sciatic Nerve Repair in Rats

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ABSTRACT

Objective: To assess the effect of heparin binding neurotrophic factor (HBNF) on sciatic nerve regeneration in animal model of rat.

Methods: Seventy-five male Wistar rats were divided into five experimental groups randomly (each group containing 15 animals): Sham operation group (SHAM), autograft group (AUTO), transected control (TC), chitosan conduit (CHIT) and heparin binding neurotrophic factor treated group (CHIT/HBNF). In AUTO group a segment of sciatic nerve was transected and reimplanted reversely. In SHAM group sciatic nerve was exposed and manipulated. In transected group left sciatic nerve was transected and stumps were fixed in adjacent muscle (TC). In treatment group defect was bridged using a chitosan conduit (CHIT) filled with 10 μ L (0.1 mg/mL) HBNF (CHIT/HBNF). Each group was subdivided into four subgroups of five animals each and nerve fibers were studied in a 12-week period.

Results: Behavioral, functional, biomechanical, electrophysiological and gastrocnemius muscle mass findings and morphometric indices confirmed faster recovery of regenerated axons in treatment group than in CHIT group ($p=0.001$). Immunohistochemical reactions to S-100 in treatment group were more positive than that in CHIT group.

Conclusion: Local administration of HBNF improved functional recovery and morphometric indices of sciatic nerve. It could be considered as an effective treatment for peripheral nerve repair in practice.

Keywords: Nerve regeneration; Sciatic; Heparin binding neurotrophic factor.

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Introduction

The ideal surgical technique should accomplish good wound healing with minimal scar formation and direct the nerve sprouts into their

correct targets [1]. Different graft equivalents have also been applied to bridge the nerve stump and regulated through the interaction of a variety of protein and cell signals [2]. 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 [3]. Neurotrophic factors support the survival of axotomized neurons and enhance the intrinsic regenerative capacity after retrograde uptake and induction of specific signaling cascades [3].

Heparin binding neurotrophic factor is a soluble secretory cytokine with mitogenic, angiogenic, and neurotropic activities, whose biological role should be made only after its binding with heparan sulfate proteoglycan [4,5]. Heparin binding neurotrophic factor has been reported to be highly expressed in central nervous system in the development or after injury and exhibits a strong neurite outgrowth activity as well as induction of neural progenitor cells to differentiate [6]. Heparin binding neurotrophic factor has been suggested as a growth factor that is upregulated in denervated distal nerve segments and as a neurotrophic factor for spinal motor neurons [7].

Aimed to study local effects of heparin binding neurotrophic factor on peripheral nerve regeneration, a study was designed to determine if it local could in fact reduce dysfunction after nerve injury in the rat sciatic nerve transection model.

Materials and Methods

Study Design and Animals

Seventy-five male Wistar rats weighing approximately 290g were divided into five experimental groups (n=15), randomly: Sham operation group (SHAM), autograft group (AUTO), transected control (TC), chitosan conduit (CHIT) and heparin binding neurotrophic factor treated group (CHIT/HBNF). Each group was further subdivided into three subgroups of five animals each and surveyed 4, 8 and 12 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.

Preparation of Chitosan Conduit

Chitosan solution was prepared by dissolving medium molecular weight, crab shell chitosan (~400kDa, 85% deacetylated) (Fluka, Sigma-Aldrich St. Louis, MO, USA) in an aqueous solution (1% v/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50°C) for 3 hours. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper then vacuum filtration to remove any un-dissolved particles. To overcome the fragility of chitosan, glycerol (Sigma Chemical Co., St. Louis, MO, USA) was added as 30% (w/w) of the total solid weight in solution [8,9]. Chitosan conduit was made by gentle injection of the prepared solution into a home-made mold. The prepared conduit was 2 mm

in external diameter, 1.8 mm in internal diameter and 10 mm in length.

Surgical Procedure

Animals were anesthetized by intraperitoneal administration of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain [10]. The University Research Council approved all experiments.

Through a muscle splitting approach, the plane between gluteus maximus and biceps femoris was developed and the right sciatic nerve was clearly visible on the underlying hamstrings muscles. Sutures were passed through the nerve epineurium (one on each side), 3 mm apart at a level of 1 cm above the trifurcation of the nerve. Sutures had the same circumferential orientation on the nerve to restore spatial longitudinal nerve continuity. Before transection, both needles were driven through chitosan conduit at each side 2 mm from the edge of the conduit. This facilitated proper and prompt insertion before endoneurial edema obscured the cut ends. Afterward, a complete transection between the sutures was undertaken and the cut ends of the nerve were driven carefully with the aid of the sutures inside the chitosan conduit and held in place.

A second epineurial suture was placed, at each side and through the conduit. After placement, the chambers were filled with 10 μL a neutral pH sterile solution of heparin binding neurotrophic factor (0.1 mg/mL) (Sigma-Aldrich Chemie, Munich, Germany). In the sham-operation group (SHAM), the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the muscle was sutured with Vicryl (Ethicon, Norderstedt) 4/0 sutures, and the skin with 3/0 nylon (Dafilon, B/Braun, Germany). In AUTO group the transected nerve segments were reimplanted reversely.

The animals of each group were anesthetized by intraperitoneal administration of ketamine-xylazine (see above) and were perfused via left cardiac ventricle with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) at 4, 8 and 12 weeks after surgery.

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 [11]. 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 [12]. 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-minute 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 12 weeks.

Functional Assessment of Reinnervation *Sciatic functional index (SFI)*

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on the method of others [13]. 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:

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

Biomechanical Testing

Following electrophysiological assessments the regenerated nerves were harvested and placed in a normal saline bath at room temperature. The samples were then fixed between frozen fixtures in a mechanical apparatus. The TA.XTPlus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). After 5 minutes, the frozen fixtures were tightened to ensure that no slippage occurred during testing. The initial length was set to 10 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure.

Muscle Mass

Recovery assessment was also indexed using the weight ratio of the gastrocnemius muscles 12 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.

Histological Preparation and Morphometric Studies

Nerve mid-substance in CHIT group, nerve mid-substance in CHIT/HBNF 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 μm 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 dissector rules were followed in order to cope with sampling-related, fiber-location-related and fiber-size related biases [14].

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 2h 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 minutes. 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 1h at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for 1h. Horseradish peroxidase-labelled 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.

Statistical Analysis

The results were expressed as means±SD. Statistical analyses were performed using Predictive Analytics SoftWare (PASW) version 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 $p < 0.05$.

Results

Behavioral Testing

BBB Recovery

In order to assess hind limb recovery the open field locomotor was used. Figure 1 shows BBB scores compared to the baseline. All experimental groups, except for SHAM, showed the greatest degree of functional deficit one week after surgery. The heparin binding neurotrophic factor treated group showed significant improvement in locomotion of the operated limb compared to the CHIT group during the study period ($p = 0.001$).

Recovery of Sciatic Nerve Function

SFI Outcome

Figure 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 CHIT/HBNF group achieved a mean value for SFI of -31.6 ± 18 whereas in group CHIT a mean value of $-50.3 \pm$

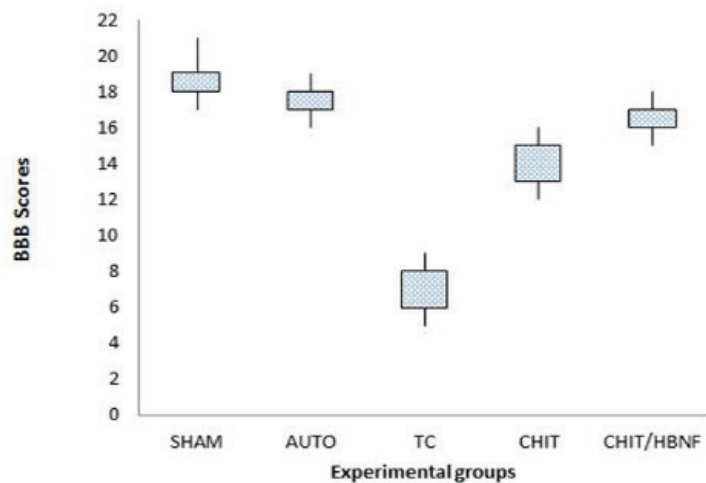


Fig. 1. Box-and-whisker plots of sciatic nerve BBB scores in each experimental group during the study period. Local administration of heparin binding neurotrophic factor with chitosan conduit gave better scores than in CHIT group. Standard error at each data point is shown in the bars.

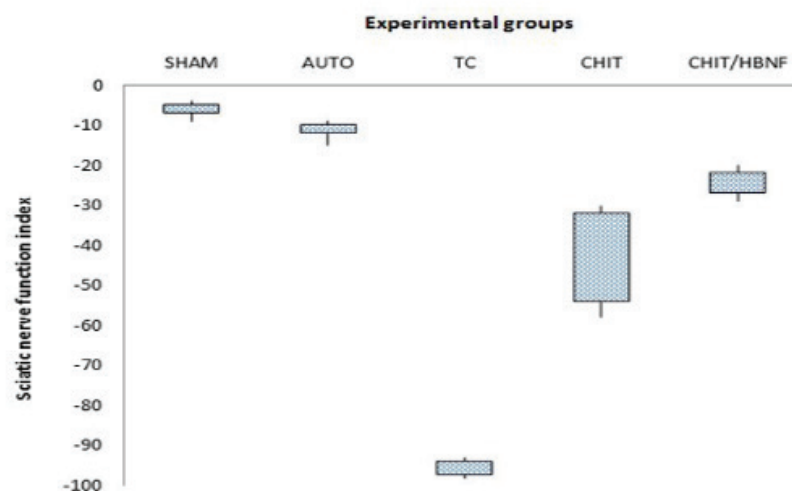


Fig. 2. Box-and-whisker plots of sciatic nerve function index values in each experimental group during the study period. Local administration of heparin binding neurotrophic factor with chitosan conduit gave better results in functional recovery of the sciatic nerve than in CHIT group.

4.39 was found. The statistical analyses revealed that the recovery of nerve function was significantly ($p=0.001$) different between CHIT/HBNF and CHIT groups and application of the heparin binding neurotrophic factor in chitosan conduit significantly accelerated functional recovery in the course of time.

Biomechanical Measurements

Maximum pull force (F_{\max}) of normal sciatic nerve was found to be 5.56 ± 0.43 . F_{\max} of nerve samples in experimental groups are shown in Figure 3. F_{\max} in CHIT/HBNF group was significantly higher than that in CHIT group ($p=0.001$). Tensile strength, the amount of force per unit of initial cross-sectional area at tensile failure, was measured based on F_{\max} and nerve cross sectional area. Assessment on week 12 revealed that tensile strength of regenerated nerves in CHIT/HBNF group was higher than those in CHIT group ($p=0.001$). Ultimate strain, the amount of elongation divided by the initial specimen length

achieved at the point of tensile failure, in CHIT/HBNF group was significantly higher than that in CHIT group ($p=0.001$). Toughness, reflecting the properties of anti-deformation and anti-fracture of nerve, was determined by the nerve itself and could reflect “looseness” or “toughness” of nerve. Toughness in CHIT/HBNF group was significantly higher than that in CHIT group ($p=0.001$).

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 CHIT/HBNF and CHIT groups ($p=0.001$). The results showed that in CHIT/HBNF the group, the muscle weight ratio was larger than in the CHIT group, and weight loss in the gastrocnemius muscle was ameliorated by local administration of heparin binding neurotrophic factor (Figure 4).

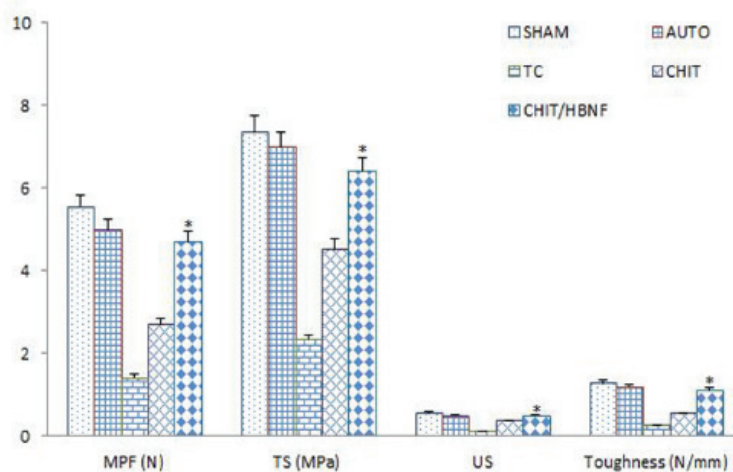


Fig. 3. Bar graph indicating biomechanical analyses of regenerated nerves for each of the experimental groups: Data are presented as mean±SD. * $p=0.001$ vs. CHIT group; MPF: Maximum Pull Force; TS: Tensile Strength; US: Ultimate Strain

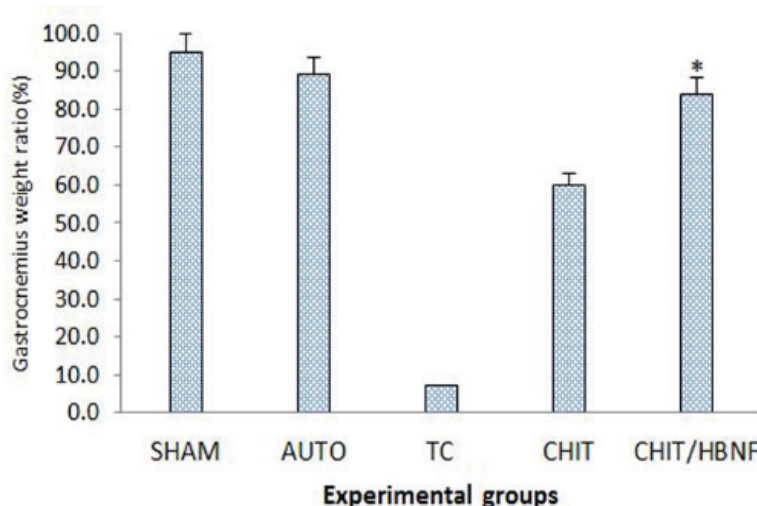


Fig. 4. Bar graph indicating percentage of gastrocnemius muscle weight ratio. The gastrocnemius muscles of both sides were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean±SD; * $p=0.001$ vs. CHIT group.

Histological and Morphometric Findings

The animals of CHIT/HBNF group presented significantly greater nerve fiber, axon diameter, and myelin sheath thickness during study period, compared to CHIT animals ($p=0.001$). Normal control group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to CHIT/HBNF and CHIT groups animals (Table 1). In case of myelin thickness there was no significant difference between CHIT/HBNF and CHIT groups, morphometrically ($p=0.065$).

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 (Figure 5). In both CHIT/HBNF and CHIT groups, the expression of S-100 and the findings resembled those of the histological evaluations.

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 [15]. Quantitatively, our results are consistent with these findings.

The results of the present study showed that application of HBNF in a chitosan conduit resulted in faster functional recovery of the sciatic nerve during the study period. Left gastrocnemius muscle weight was significantly greater in the CHIT/HBNF group than in the CHIT group, indicating indirect evidence of successful end organ reinnervation in the HBNF treated animals. The strongest connective tissue layers in peripheral nerves are the perineurium and, to a lesser extent, the epineurium. Changes in the epineurium and perineurium extracellular matrix composition are likely to have significant effects on the biomechanical properties of acellular nerve [16]. The connective tissue from the epineurium forms a layer of fiber membrane at the 3rd day postoperatively and then forms collagen

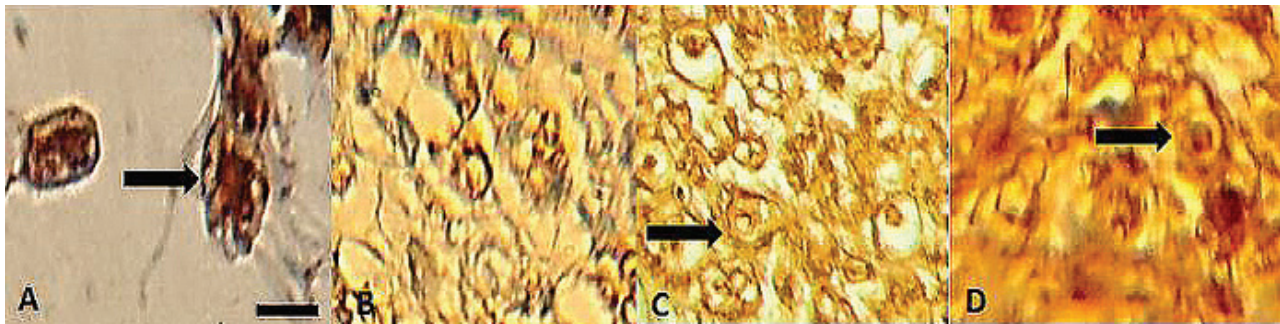


Fig. 5. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) SHAM, (B) TC, (C) CHIT and (D) CHIT/HBNF. There is clearly more positive staining of the myelin sheath-associated protein S-100 (arrows) within the periphery of nerve, indicating well organized structural nerve reconstruction in HBNF treated nerve compared to that of the CHIT. Scale bar: 10 μ m

Table 1. Morphometric analyses of sciatic nerve in each of the experimental groups.

Groups	Axon counts fb/mm ²	Axon diameter (μ m)	Myelin sheath thickness(μ m)
SHAM	29478 \pm 2312	11.36 \pm 0.18	2.63 \pm 0.02
TC ^d	4096 \pm 2005	3.30 \pm 0.15	1.00 \pm 0.02
AUTO ^a	27171 \pm 2201	9.24 \pm 0.12	1.60 \pm 0.03
CHIT ^b	20571 \pm 2156	6.24 \pm 0.14	1.39 \pm 0.04
CHIT/HBNF ^c	25004 \pm 2214 ^e	7.81 \pm 0.12 ^e	1.40 \pm 0.03

^aAUTO: Autograft; ^bCHIT: Chitosan conduit; ^cCHIT/HBNF: Chitosan conduit and heparin binding neurotrophic factor treated; ^dTC: Transected control; ^eThe mean difference is significant at the 0.05 level vs. CHIT group.

at the 8th day. The key point influencing functional recovery is the number of axons throughout the suture that enhances the anti-tension capacity of the nerve [17]. HBNF local administration in the present study resulted in the enhanced biomechanical indices that were in agreement with morphometric findings.

In immunohistochemistry the expression of myelin sheath special proteins was evident in both groups which indicate the normal histological structure. The location of reactions to S-100 in the CHIT/HBNF group was clearly more marked than in the SIL group implying that both regenerated axon and Schwann cell-like cells existed and were accompanied by the process of remyelination and the structural recovery of regenerated nerve fibers. It has been demonstrated that morphometric indices are measures of regenerated nerve maturity and quality of regeneration [18]. Larger diameters of axons and thicker myelination give rise to improved nerve function compared to smaller and thinner myelinated fibers [19].

Loading of HBNF into CHIT conduit at the nerve repair site increased fiber maturity. At week 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the CHIT and CHIT/HBNF groups, indicating a beneficial effect of local application of HBNF 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 [20-22]. Classical and newly developed methods of assessing nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling do not necessarily

predict the reestablishment of motor and sensory functions [21,23-26]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [21]. Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment. It has been suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function [27]. Information taken from BBB scale may be invaluable in evaluation of peripheral nerve process. Results of the present study showed that the HBNF treated animals had been improved in locomotion of the operated limb compared to the CHIT group during the study period. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [25-28]. Nerve conduction measurement is a direct evidence for the study of nerve transmission [29].

To achieve maximal efficacy in nerve transection models a dose-response studies remains to be conducted for HBNF to determine the combination of the conduit and the compound. Several nerve guidance conduits and nerve protectant wraps are approved by the US Food and Drug Administration (FDA) 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 [30]. Surgeons are often not aware of the different (bio) materials of these conduits when performing nerve repair [31].

The neurotrophin 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 [3]. Neurotrophins are unique among neurotrophic factors in their ability to act as guidance molecules for growth cones [32]. They can be synthesized by the target tissue, surrounding glial cells or the neuron itself [33]. Therefore, these molecules mediate their effects via receptor-mediated uptake and specific retrograde transport or via an autocrine or paracrine mechanism. Neurotrophic factors are acting via high-affinity receptors that in many cases these are tyrosine transmembrane receptors [34]. In addition to the neurotrophins, other neurotrophic factors, like glial cell line-derived neurotrophic factor and interleukin-6 are characterized also with regard to their expression and/or function in the peripheral nervous system [35]. In response to nerve injury, Schwann cells divide and form long chains of cells, known as bands of Büngner, which provide a substrate for axonal regeneration [36]. It is well known that denervated Schwann cells increase their production of several neurotrophic molecules, including nerve growth factor or brain-derived neurotrophic factor [37,38]. Axon–Schwann cell interaction mediated by neurotrophic factors may play a pivotal role in peripheral nerve regeneration [39]. Increased production of trophic factors by Schwann cells and fibroblasts may substitute for the usual target-derived trophic factors. The signals that induce these complex changes are not known and the interactions among various nonneural cells are incompletely understood, but the data presented in this study suggest a contribution of heparin binding neurotrophic factor to these mechanisms. After nerve injury, the damaged nerve tissue must be eliminated. In particular, myelin debris inhibits axon growth and must therefore be removed. Active Schwann cell division occurs in the distal stump following nerve injury, where Schwann cells act as phagocytes to digest axons and their myelin sheaths [40]. Several non-neurotrophin growth factors may potentially participate in the proliferative phase of peripheral nerve repair including fibroblast growth factor II, vascular endothelial growth factor or insulin-

like growth factor [41]. Some of these molecules, together with heparin binding neurotrophic factor, may act as autocrine mitogens for Schwann cells or as chemoattractants for macrophages and contribute to the neovascularization that is key to successful nerve regeneration [42].

In the present study, first of all it was important to know whether local exogenous administration of HBNF in biodegradable chitosan tubes was able to stimulate the regeneration of the transected rat sciatic nerve. With this aim, we compared the regeneration of the transected sciatic nerve within biodegradable guides. Our functional results revealed that the chitosan biodegradable guides allow HBNF to exert the stimulation of nerve regeneration. In addition, gasterocnemius muscle mass, obtained from muscles of operated and unoperated limbs, indicated that motor functional recovery in transected sciatic nerve bridged by chitosan conduits achieved a faster rate. The functional, morphometric and immunohistochemical results indicated that the degradation of the chitosan nerve guides did not prevent the stimulating action of HBNF. Even though our study shows the regenerative action of local HBNF in peripheral nerve injuries, data regarding the molecular mechanisms leading to the action remain to be investigated in depth. We have not given the histological and molecular evidence for regenerative action of HBNF. 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 HBNF on nerve regeneration.

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Conflict of Interest: None declared.

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