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Effect of Local Administration of Verapamil Combined with Chitosan Based Hybrid Nanofiber Conduit on Transected Sciatic Nerve in Rat

***Mahan Alizadeh-Mohajer*[1, 2], *Abbas Raisi*[1*], *Ghasem Farjanikish*[3], *Rahim Mohammadi*[4]**

[1]*Department of Clinical Sciences, Lorestan University, Khorramabad, Iran*
[2]*Resident of Veterinary Surgery Islamic Azad University, Science and Research Branch, Tehran*
[3]*Department of Pathobiology, Lorestan University, Khorramabad, Iran*
[4]*Department of Surgery and Diagnostic Imaging, Urmia University, Urmia, Iran*

***Corresponding author:** *Abbas Raisi*
Address: Department of Clinical Sciences, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran.
Tel/Fax: +98-66-33120649
e-mail: raisi.a@lu.ac.ir

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ABSTRACT

Objective: To assess the effect of locally administered verapamil on transected peripheral nerve regeneration and functional recovery.
Methods: Sixty male healthy white Wistar rats were divided into four experimental groups (n=15), randomly: In transected group (TC), left sciatic nerve was transected and stumps were fixed in the adjacent muscle. In treatment group defect was bridged using chitosan tube (CHIT/Verapamil) filled with 10 µL verapamil (100ng/mL). In chitosan conduit group (CHIT), the tube was filled with phosphate-buffered saline alone. In sham-operated group (SHAM), sciatic nerve was exposed and manipulated. The repair trend was examined based on behavioral and performance tests as well as the variations of the gastrocnemius muscle, morphometric indices, and immunohistochemical indices.
Results: Sciatic nerve functional study, muscle mass and morphometric indices confirmed faster recovery of regenerated axons in CHIT/Verapamil than CHIT group (p0.001=). When loaded in a chitosan tube verapamil accelerated and improved functional recovery and morphometric indices of sciatic nerve. Immunohistochemical analysis revealed the S-100 protein was vastly present in the transverse nerve sections and the myelin sheath. In the treatment group (chit/verapamil), the immunohistochemical susceptibility of the axons being repaired and the axons in the myelin sheath to S-100 protein was higher than the other groups.
Conclusion: The present study demonstrated that a single local application of verapamil could accelerate functional recovery after transection of sciatic nerve.

Keywords: Peripheral nerve repair; Sciatic; Verapamil; Chitosan nanofiber.

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Introduction

Unlike other tissues in the body, peripheral nerve regeneration is slow and usually incomplete. Less than half of patients who undergo nerve repair after injury regain good to excellent motor or sensory function and current surgical techniques are similar to those described by Sunderland more than 60

years ago [1]. The conduits have been proposed 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 [2].Chitosan tubes have been shown to be proposed in bridging of nerve defects [3].Chitosan conduits are an attractive alternative to other standard grafts because of no donor morbidity, availability, affordability and no foreign reactions [4, 5].

All clinically available conduits are hollow tubes although extensive research continues to focus on adding internal structure, Schwann cells, and growth factors to support axonal regeneration. Therefore, all autologous nerve graft alternatives including decellularized nerve grafts and autogenous and non-autogenous conduits demonstrate similar efficacy but their use is limited to sensory nerves with small gaps <3 cm. Primary nerve repair or autogenous nerve grafts remain the mainstay of surgical nerve reconstruction for severe nerve injuries [6, 7].In patients with subarachnoid hemorrhage the calcium channel blockers have been used to reduce the morbidity and mortality associated with delayed ischemic deficits [8-10].

To the best knowledge of the authors, the literature is poor regarding the beneficial local effects of verapamil (a calcium channel blocker) on transected sciatic nerve. Aimed to study local effects of verapamil on sciatic nerve regeneration, a study was designed to determine if local verapamil could in fact reduce dysfunction after nerve injury in the rat sciatic nerve transection model. Assessment of the nerve regeneration was based on functional and histomorphometric criteria 4, 8 and 12 weeks after surgery.

Materials and Methods

Study Design and Animals

Sixty male Wistar rats weighing approximately 250g were divided into four experimental groups (n=15), randomly: sham-operation group as normal control (SHAM), transected control (TC), chitosan conduit (CHIT) and verapamil treated group (CHIT/Verapamil). Each group was further subdivided into three subgroups of five animals each and studied 4, 8 and12 weeks after surgery. Two weeks before and during the experiments, the animals were housed in individual plastic cages with an ambient temperature of (23±3) °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.

Preparation of Chitosan Based Hybrid Nanofiber Conduit

The conduit was prepared based on a methods described by others [11]. 5 wt% Chitosan in Trifluroacetic Acid (TFA) and 12 wt% polycrapolactone in TFE solutions were prepared separately. They were then combined in PCL/chitosan weight ratios varying from 40/60 to 80/20 2 grams of solution at a time. The resulting solutions were then vortexed for 1 min to ensure the complete mixture of polymers. A DC voltage of approximately 19 kV was applied (High DC power supply, Del Electronics Corp.) between the syringe tip and a cylindrical collector. The distance between the syringe tip and collector was approximately 29 cm with the syringe angled down approximately 30 degrees below the horizontal. The positive voltage was supplied to the solution via a platinum wire, connected to the positive electrode, inserted into the solution. The cylindrical collector was electrically grounded by attachment of the negative electrode. The mats were first affixed to 2 cm x 2 cm cover slides and then soaked in 14 % NH4OH for 5 min. They were then thoroughly rinsed in deionized (DI) water and soaked in 1 wt% genipin (aqueous) for 24 h. Finally, they were rinsed thoroughly in DI water for 5 min before further processing. Chitosan conduit was made by gentle injection of the prepared solution into a home-made mold [12]. The prepared conduit was 2 mm in external diameter, 1.8 mm in internal diameter and 10 mm in length. This internal diameter complies with optimal function in rat models.

Surgical Procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine 5%, 90mg/kg and xylazine 2%, 5mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain [13]. The University Research Council approved all experiments. Following surgical preparation in the sham-operation 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 7mm segment was excised, leaving a 10mm 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 CHIT 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 achitosan conduit, entubulating 2 mm of the nerve stump at each end. The chitosan conduit was 2 mm in diameter with 2 thickness in wall. Two 10/0 nylon sutures were used to anchor the conduit to the epineurium at each end. In verapamil treated group (CHIT/Verapamil) the conduit was filled with 10 µl verapamil(100ng/mL). The animals were anesthetized and euthanized with transcardiac perfusion of a fixative containing 2% paraformaldehyde and 1%glutaraldehyde buffer (pH

7.4) 4, 8 and 12 weeks after surgery.

Functional Assessment of Reinnervation

Sciatic Functional Index (SFI)

Walking track analysis was performed 4, 8 12 and 16 weeks after surgery based on the method of others [14, 15]. 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:

SFI = -38.3× (EPL-NPL)/NPL+109.5× (ETS-NTS)/NTS +13.3× (EIT-NIT)/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.

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. All measurements were made by two independent observers unaware of the analyzed group.

Histological Preparation and Morphometric Studies

Nerve mid-substance in CHIT group, nerve mid-substance in verapamil treated group, midpoint of normal sciatic nerve (SHAM) 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 Epon. 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 [16].

Immunohistochemisrty

Tissue specimens were 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 anti-S-100 antibody (1:200, DAKO, USA) 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 chromogen substrate solution (DAB, DAKO, USA) for 10 min. The results of immunohistochemistry were examined under a light microscope.

Statistical Analysis

The results were expressed as means±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. A two-sided p-value of less than 0.05 was considered statistically significant.

Results

Recovery of Sciatic Nerve Function

SFI Outcome

Figure 1 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. The statistical analyses revealed that the recovery of nerve function was significantly (P=0.001) different between CHIT/Verapamil and CHIT groups and application of the verapamil in chitosan conduit significantly accelerated functional recovery in the course of time.

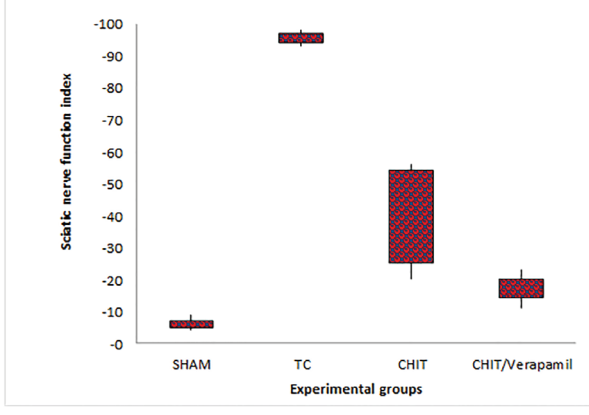

Fig. 1. Box-and-whisker plots of sciatic nerve function index values (SFI) in each experimental group during the study period. Local administration of Verapamil gave better results in functional recovery of the sciatic nerve than in CHIT group.

Muscle Mass Measurement

Gastrocnemius muscles weight of injured and uninjured sides were measured in each group. There was statistically significant difference between percentage of the mean muscle weight ratios of

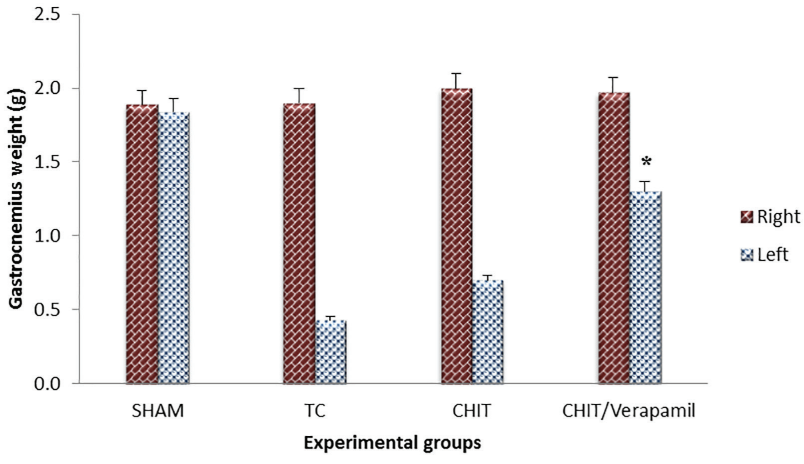

Fig. 2. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 12 weeks after surgery. Data are presented as mean±SD. * $p<0.05$ vs Chitosan group.

CHIT/Verapamil and Chitosan groups ($p=0.001$). The results showed that in CHIT/Verapamil group muscle weight ratio was bigger than in CHIT group and weight loss of the gastrocnemius muscle was ameliorated by local administration of Verapamil solution (Figure 2).

Histological and Morphometric Findings

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

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 3). In both CHIT/Verapamil and CHIT groups, the expression of S-100 and the findings resembled those of the histological evaluations.

Discussion

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 [14-16]. Classical and newly developed methods of assessing

Table 1. Morphometric analyses of sciatic nerve in each of the experimental groups: Values are given as mean±SD.

Groups	Axon counts fb/mm^2	Axon diameter (µm)	Myelin sheath thickness(µm)
SHAM[a]	29402±2265	11.30±0.11	2.60±0.12
TC[b]	4080±2015	3.37±0.12	1.06±0.08
CHIT[c]	20307±2112	6.19±0.14	1.18±0.07
CHIT/Verapamil	25104±2287[a]	7.78±0.13[a]	1.47± 0.09

[a]SHAM: Control group; [b]TC: Transected Control, [c]CHIT: Chitosan; The mean difference is significant at the 0.05 level *vs.* other groups ($p=0.001$)

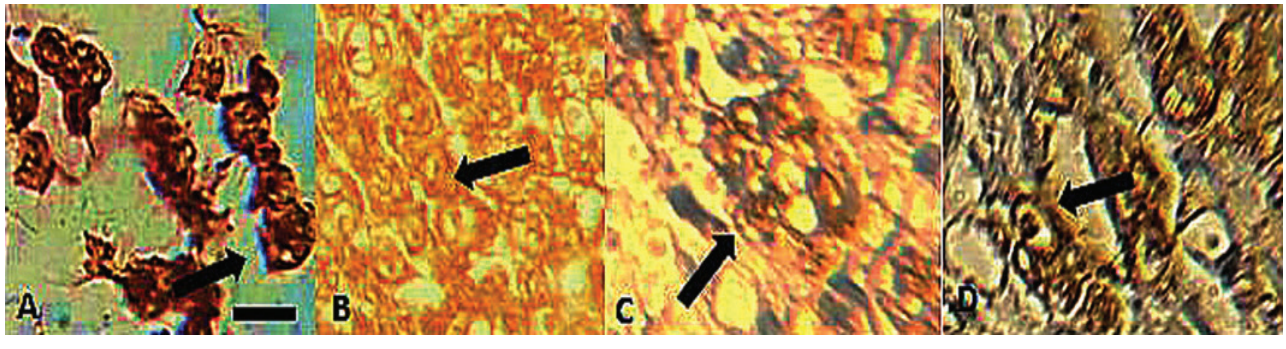

Fig. 3. Immunohistochemical analysis of the regenerated nerves 12 weeks after surgery from middle cable (A) SHAM, (B) TC, (C) CHIT and (D) CHIT/Verapamil. There was clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well organized structural nerve reconstruction in CHIT/Verapamil group. Scale bar: 10 µm

nerve recovery, including histomorphometry, retrograde transport of horseradish peroxidase and retrograde fluorescent labeling do not necessarily predict the reestablishment of motor and sensory functions [16-20]. Although such techniques are useful in studying the nerve regeneration process, they generally fail in assessing functional recovery [16]. Therefore, research on peripheral nerve injury needs to combine both functional and morphological assessment. Castaneda and Kinne (2002) suggested that arrival of sprouts from the proximal stump at the distal nerve stump does not necessarily imply recovery of nerve function [21]. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [18-22]. It has been demonstrated that conventional and nano-conduits accelerate nerve regeneration in animal models [23-26].

The results of the present study showed that administration of verapamil into 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/Verapamil group than in the CHIT group, indicating indirect evidence of successful end organ reinnervation in the verapamil treated animals. At week 12 quantitative morphometrical indices of regenerated nerve fibers showed significant differences between the CHIT and CHIT/Verapamil groups, indicating a beneficial effect of topical application of verapamil on the nerve regeneration.

As the posterior tibial branch of the sciatic nerve regenerates into the gastrocnemius muscle, it will regain its mass proportional to the amount of axonal reinnervation [27, 28]. In the present study 12 weeks after surgery the muscle mass was found in both experimental groups. However, CHIT/Verapamil group showed significantly greater ratios of the mean gastrocnemius muscle weight than Chitosan group indicating indirect evidence of successful end organ reinnervation.

In the histological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant difference between Chitosan and CHIT/Verapamil groups indicating beneficial effect of local verapamil on the nerve regeneration. 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 CHIT/Verapamil group was clearly more marked than in the CHIT 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.

Membranes of the proximal and distal portions of the transected axons are resealed 5-30 min after the transaction [29]. Thereafter, the proximal stump gradually regrows, fostered by the neural cell surface molecule of the transmembrane glycoprotein Ll, nerve cell adhesion molecule (N-CAM), myelin associated glycoprotein PO, and the extracellular matrix components laminin and tenascin. The regenerating axons are guided by the Schwann cell processes and their growth within the Schwann cell basal lamina tubes is synchronous with the withdrawal and degeneration of the axonal remnants of the distal stump [30, 31]. Calcium ions play a crucial role in depolarization, outgrowth, excitability, aging, learning, and cell proliferation-in short, neuronal plasticity [32]. It is well known that peripheral nerve injury disrupts the permeability barrier function of the plasma membrane, allowing an influx of Ca2+ down a steep electrochemical gradient between the outside and the inside of the cell [33]. The resultant intracellular free Ca2+overload triggers a wide array of chain reactions, which eventually may lead to cell death [34, 35]. Therefore,an agent preventing the excessive influx of Ca'+ might attenuate cellular damage caused by mechanical neuronal injury and thus improve neuronal recovery [36].By reducing the amount of calcium influx into the axoplasm of the resprouting nerve fiber, the treatment with verapamil may provide the necessary optimum level ("set-point") of Ca'+ influxthat promotes accelerated growth cone elongation[37-39]. However, it further reduces the buffering capacity of the terminals for Ca'+, which might render their responsiveness toCa2+ even stronger [40].

Even though our preliminary study shows the neuroprotective action of local verapamil 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 verapamil. 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 verapamil on transected sciatic 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 verapamil at the site of transected nerve could be of benefit after chitosan conduit tubulization. Detailed mechanism of neuroprotective action remains to be investigated.

In conclusion, the present study demonstrated that a single local application of verapamil could accelerate functional recovery after transection of sciatic nerve and may have clinical implications for the surgical management of patients after facial nerve transection. Thus, dose–response studies should be conducted for verapamil to determine the combination of the graft and the compound that achieve maximal efficacy in nerve transection models.

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Conflicts of Interest: None declared.

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