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Local Administration of Methylprednisolone Laden Hydrogel Enhances Functional Recovery of Transected Sciatic Nerve in Rat

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ABSTRACT

Objective: To determine the effects of methylprednisolone-laden hydrogel loaded into a chitosan conduit on the functional recovery of peripheral nerve using a rat sciatic nerve regeneration model was assessed.
Methods10- **:**mm sciatic nerve defect was bridged using a chitosan conduit (CHIT/CGP-Hydrogel) filled with CGP-hydrogel. In authograft group (AUTO) a segment of sciatic nerve was transected and reimplanted reversely. In methylprednisolone treated group (CHIT/MP) the conduit was filled with methylprednisolone-laden CGP-hydrogel. The regenerated fibers were studied within 16 weeks after surgery.
Results: The behavioral, functional and electrophysiological studies confirmed faster recovery of the regenerated axons in methylprednisolone treated group compared to CHIT/Hydrogel group ($p<0.05$). The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of CHIT/MP and CHIT/Hydrogel groups ($p<0.05$). Morphometric indices of regenerated fibers showed number and diameter of the myelinated fibers were significantly higher in CHIT/MP than in CHIT/Hydrogel group.
Conclusion: Methylprednisolone-laden hydrogel when loaded in a chitosan conduit resulted in improvement of functional recovery and quantitative morphometric indices of sciatic nerve.

Keywords: Peripheral nerve repair; Sciatic; Methylprednisolone; Hydrogel; Local.

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Introduction

The repair of peripheral nerve injuries is still one of the most challenging tasks and concerns in neurosurgery. 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 [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 [2,3].

Biodegradable nerve guides as a temporary scaffold are better than non-degradable biomaterials because the latter remain *in situ* as a foreign body and ultimately result in limiting recovery of nerve function [4]. Nevertheless, the resistance to biodegradation can be a cause of chronic nerve compression in the long run and a second surgery may therefore be required for its removal.

Beneficial effects of chitosan as a conduit in promoting nerve regeneration have already been documented and it seems chitosan as a natural polymer has excellent properties including biocompatibility, biodegradability, non-toxicity and adsorption properties, and might be a suitable functional material for peripheral nerve regeneration [5,6]. The effects of glucocorticoids are mainly anti-inflammatory and anti-edema following injury [7]. Glucocorticoids enhance the rate of myelin formation by binding to glucocorticoid receptors that are expressed on Schwann cells [8]. Glucocorticoids also reduce injury-associated tissue and improve nerve recovery [9,10]. Recently, Yao and Kiyama have demonstrated that glucocorticoids can up-regulate the expression of growth associated protein after peripheral nerve injury which is associated with axon regeneration and function reconstruction [11,12]. Systemic glucocorticoid administration is used to treat facial nerve injury, however, in order to meet an important pharmacological principal high-doses of glucocorticoids are administrated after acute injures of the nerve system. Therefore, systemic toxicities and adverse reactions such as Cushing's syndrome, infections, ulcer, hypertension, arteriosclerosis and osteoporosis may ensue [13].

To enhance nerve regeneration and avoid or reduce adverse reactions, a chitosan conduit loaded with methylprednisolone-laden hydrogel was designed for the local drug delivery to injured nerves, which help increase local drug concentration while decrease systemic toxicities.

The present study was conducted to evaluate effects of methylprednisolone-laden hydrogel loaded chitosan conduit as an *in situ* delivery system of methylprednisolone in bridging the defects in rat sciatic nerve transection model.

Materials and Methods

Experimental Design

One hundred male White Wistar rats weighing approximately 290 g were divided into four experimental groups (n=20), randomly: Authograft group (AUTO), transected control group (TC), sham-surgery group (SHAM), chitosan control group (CHIT/CGP-Hydrogel) and methylprednisolone-laden CGP-hydrogel group (CHIT/MP). Each group was again subdivided into four subgroups of five animals each and surveyed in four time points of 4, 8, 12 and 16 weeks. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages with an ambient temperature of 23±3º 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 50ºC) for 3hour. 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 [14]. Chitosan conduit was made according to the method described by others by gentle injection of the prepared solution into a home-made mold [14]. 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.

Biodegradable Hydrogel and Methylprednisolone Solution Preparation

A methylprednisolone-laden hydrogel and a methylprednisolone aqueous solution were used in this experiment. These materials were freshly made on the day of usage. To make a blank control hydrogel (CGP-hydrogel), chitosan (CH02, Biosyntech, Québec) was dissolved in 0.2 mol/L acetic acid (0.01%) to yield a 3.85% (wt/wt) chitosan solution, and then a 55% (wt/wt) glycerophosphate (GP) salt (Sigma, St. Louis MO) aqueous solution was added drop-wise to the chitosan solution with agitation. To make the hydrogel loaded with methylprednisolone, the methylprednisolone powder (Sigma-Aldrich Chemie GmbH, Germany) and 55% (wt/wt) GP was added to the 3.85% chitosan solution with agitation. The final solution (MP-CGP-Hydrogel) contained 39% (wt/wt) MP.

Grafting Procedure

Animals were anesthetized by intraperitoneal administration of ketamine-xylazine (ketamine hydrochloride 5%, 90mg/kg and xylazine hydrochloride 2%, 5mg/kg). The procedures were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain [15]. The University Research Council approved all experiments.

Following surgical preparation 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 resorbable 4/0 sutures, and the skin with 3/0 nylon. In authograft group (AUTO) a segment of sciatic nerve was transected and reimplanted reversely.

In the CHIT/Hydrogel group the left sciatic nerve was exposed through a gluteal muscle incision and transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a gap about 10 mm due to retraction of nerve ends. Proximal and distal stumps were each inserted 2 mm into a chitosan tube and two 10/0 nylon sutures were placed at each end of the cuff to fix the tube in place and to leave a 10-mm gap between the stumps. The conduit was filled with CGP-Hydrogel and sterile Vaseline was used to seal the ends of the tubes to avoid leakage. In the CHIT/MP group the conduit was filled with methylprednisolone-laden hydrogel. The animals were anesthetized (see above) and euthanized with transcardial perfusion of a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH 7.4) 4, 8, 12 and 16 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 [16]. 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 [17]. 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 16 weeks.

Functional Assessment of Reinnervation

Sciatic Functional Index (SFI)

Walking track analysis was performed 4, 8, 12 and 16 weeks after surgery based on Bain *et al*., 1989 [18]. 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) in 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

Static Sciatic Index (SSI)

SSI is a time-saving digitized static footprint analysis described by others [19]. 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 [19]. 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:

SSI = [(108.44 × TSF) + (31.85 × ITSF)] − 5.49

Where:

TSF = (ETS-NTS)/NTS

ITSF = (EIT-NIT)/NIT

Biomechanical Testing

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. Samples were kept wet moist during testing using a drop of normal saline solution to the nerve segments.

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

Histological Preparation and Quantitative Morphometric Studies

Operated nerve was dissected from surrounding tissues and a segment including several millimeters proximal and distal to the graft was harvested. Graft middle cable of SHAM, TC, CHIT/CGP-Hydrogel and CHIT/MP groups were fixed in 2.5 percent glutaraldehyde. The grafts were postfixed in OsO4 (2%, 2 h), dehydrated through an ethanol series and embedded in Epon. Samples were cut in 5 µm, stained with toluidine blue and examined under light microscopy. Morphometrical 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 [20].

Statistical Analysis

Experimental 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. The differences were

considered significant when $P< 0.05$.

Results

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 methylprednisolone treated group showed significant improvement in locomotion of the operated limb compared to the CHIT/CGP-Hydrogel group during the study period ($p< 0.05$).

Recovery of Sciatic Nerve Function and Reinnervation

SFI Outcome

Figure 2 shows sciatic function index (SFI) values in 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. Four weeks after surgery had performed mean SFI was -69.5±-2.39 in methylprednisolone treated group, compared to -91.67±-3.22 in CHIT/CGP-Hydrogel group. Eight weeks after surgery had carried out the improvement in SFI was observed in animals of methylprednisolone treated group (-55.3±-2.25) that was significantly higher than CHIT/CGP-Hydrogel (-75.2±-3.39) animals ($p<0.05$). After 12 weeks, animals of group CHIT/MP achieved a mean value for SFI of -38.4±-3.57, whereas in group CHIT/CGP-Hydrogel, a mean value of -57.1±-4.10 was found. At the end of the study period, 16 weeks after surgery, animals of group CHIT/MP achieved a mean value of -29.5±-3.75 for SFI of, whereas in group CHIT/CGP-Hydrogel, a mean value of -46.3±-3.43 was found.

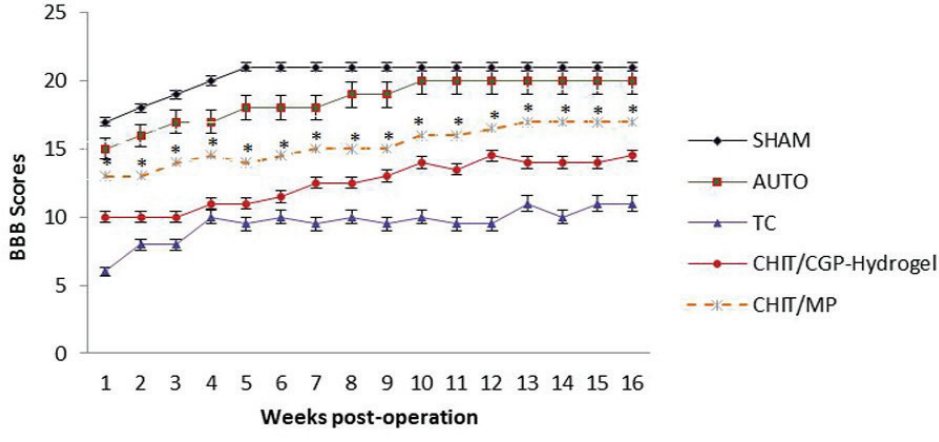

Fig. 1. BBB score for all experimental groups. Local administration of methylprednisolone with chitosan grafting gave better scores than in CHIT/CGP-Hydrogel group. Standard error at each data point is shown with bars.

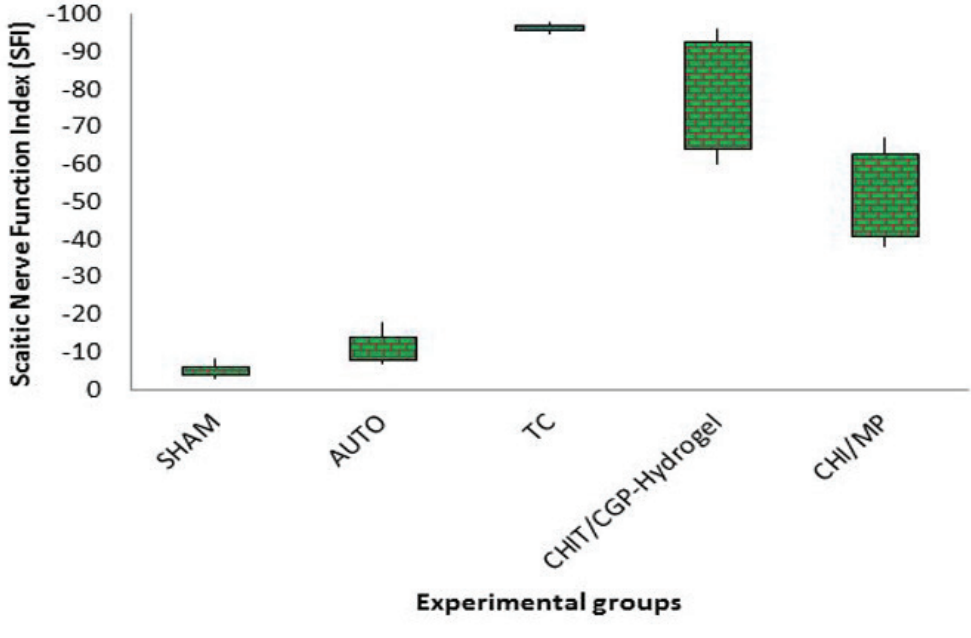

Fig. 2. Diagrammatic representation of effects on the sciatic nerve function index (SFI). Local administration of methylprednisolone with chitosan grafting resulted in improved functional recovery of the sciatic nerve than in CHIT/CGP-Hydrogel groups. Data are presented as mean±SD. * $p<0.05$ *vs* CHIT/CGP-Hydrogel group.

The statistical analyses revealed that the recovery of nerve function was significantly faster in CHIT/MP than in CHIT/CGP-Hydrogel group ($p<0.05$) and locally administered methylprednisolone promoted functional recovery.

SSI Outcome

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

Biomechanical Measurements

Maximum pull force (F_{max}) of normal sciatic nerve was found to be 5.49±0.40. F_{max} of nerve samples in experimental groups are shown in Table 1. F_{max} in CHIT/MP group was significantly higher than that in CHIT/CGP-Hydrogel group ($p<0.05$). 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. A 16-week assessment revealed tensile strength of regenerated nerves treated with ibuprofen was higher than those in CHIT/CGP-Hydrogel group ($p<0.05$). Ultimate strain, the amount of elongation divided by the initial specimen length achieved at the point of tensile failure, in CHIT/MP group was significantly higher than that in CHIT/CGP-Hydrogel group ($p<0.05$). 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/MP group was significantly higher than that in CHIT/CGP-Hydrogel group ($p<0.05$).

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 CHIT/MP and CHIT/CGP-Hydrogel groups ($p<0.05$). The results showed that in CHIT/MP group muscle weight ratio was bigger than in CHIT/CGP-Hydrogel group and weight loss of the gastrocnemius muscle was ameliorated by local administration of methylprednisolone (Figure 4).

Morphological Findings

Table 2 shows quantitative morphometric analyses of regenerated nerves for each of the experimental groups. Four weeks after surgery, CHIT/MP group presented significantly greater nerve fiber, axon diameter and myelin sheath thickness compared to CHIT/CGP-Hydrogel animals ($p<0.05$). Although CHIT/CGP-Hydrogel presented regeneration patterns, the morphometric indices in CHIT/MP group both after 8, 12 and 16 weeks were better than in CHIT/CGP-Hydrogel.

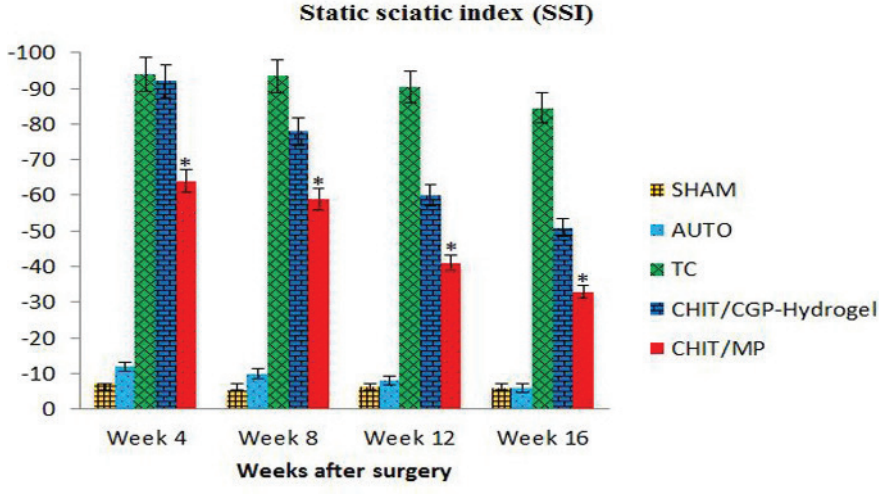

Fig. 3. Bar graph indicating static sciatic index (SSI) values in each experimental group during the study period. Local administration of methylprednisolone with chitosan grafting gave better results in functional recovery of the sciatic nerve than in CHIT/CGP-Hydrogel group. Data are presented as mean±SD.

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

Groups	Maximum Pull Force (N)	Tensile Strength (MPa)	Ultimate Strain	Toughness (N/mm)
SHAM	5.48±0.43	7.33±1.22	0.56±0.03	1.27±0.52
AUTO	5.02± 0.23	7.02±0.62	0.43±0.02	1.12±0.34
CHIT/CGP-Hydrogel	3.20±0.33	3.21±0.12	0.19±0.04	0.41±0.22
CHIT/MP	3.38±0.22[a]	3.30±0.15[a]	0.26±0.05[a]	0.55±0.12[a]

[a]The mean difference is significant at the .05 level *vs.* CHIT/CGP-Hydrogel group.

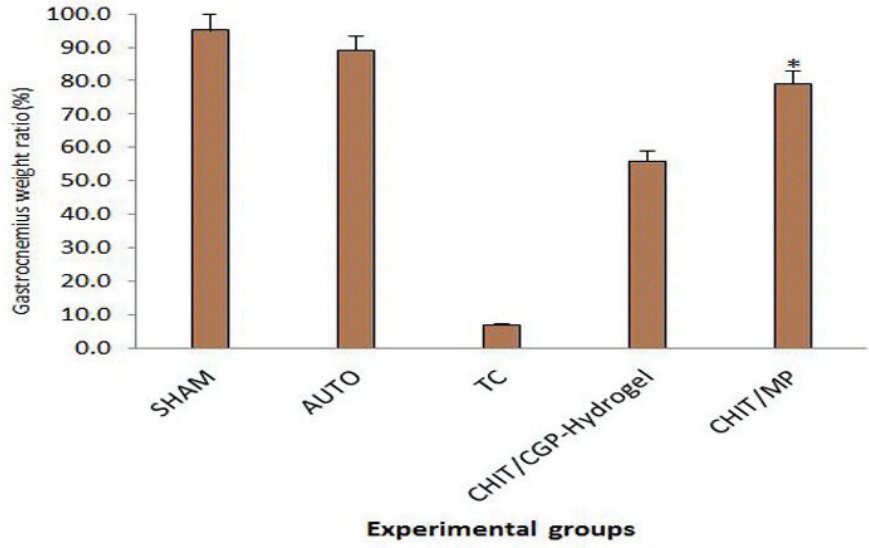

Fig. 4. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (operated left and unoperated right) were excised and weighed in the experimental groups at 16 weeks after surgery. Data are presented as mean±SD. *p<0.05 vs CHIT/CGP-Hydrogel group.

Table 2. Morphometric analyses of sciatic nerve in each of the experimental groups: Values are given as mean±SD.

Groups	Axon counts fb/mm²	Axon diameter (µm)	Myelin sheath thickness(µm)
SHAM	29534±2039	11.32±0.15	2.61±0.03
AUTO	27163±2234	9.32±0.14	1.63±0.02
CHIT/CGP-Hydrogel	21654±2204	3.73± 0.12	1.02±0.03
CHIT/MP	25634±2232 [a]	6.42±0.14 [a]	1.04±0.04

[a]The mean difference is significant at the .05 level *vs.* CHIT/CGP-Hydrogel group.

Using Factorial ANOVA analysis with two between-subjects factors (Group×time); in the CHIT/MP group number of nerve fibers and myelin thickness did not show significant difference between 8 ,12 and 16 weeks (p>0.05). Increase in mean thickness of myelin sheath did not show statistical difference between 8, 12 and 16 weeks inside each group (p>0.05). Mean thickness of myelin sheath from week 8 onward did not show significant difference between CHIT/MP and SHAM group (p>0.05).

Discussion

Peripheral nerve lesions represent a common problem for the reconstructive surgeon [21]. After transection of a peripheral nerve, Wallerian degeneration occurs distally to the lesion site, whereas proximally regenerating axons sprout into the surrounding tissue and may grow aberrantly forming dense nerve tangles called neuromas, which are often painful and become a source of functional and psychological disability for the patient. Therefore, one of the main challenges in treating damaged nerves is to prevent the patho-physiological response of transected nerves which leads to neuroma formation [22]. Restoration of normal neurological function of transected peripheral nerve remains a great challenge in regenerative medicine and surgery. Entubulation neurorrhaphy is an excellent alternative to short interposition nerve grafts [23]. Selection of an appropriate method to evaluate functional recovery of nerve regeneration is extremely influential. Walking is a coordinated activity involving sensory input, motor response and cortical integration [24]. Therefore, walking track analysis (sciatic function index) is a comprehensive test. The results of the present study showed that methylprednisolone-laden hydrogel when loaded in a chitosan tube ended up a faster and significant improvement of functional recovery of the sciatic nerve throughout the study period.

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. Walking track analysis has frequently been used to reliably determine functional recovery following nerve repair in rat models [26, 27]. Our results showed that moephometric indices were not significantly different between CHIT/MP and CHIT/CGP-Hydrogel groups after 8 weeks. In contrary, functional recovery occurred from week 8 to week 16 in CHIT/MP supporting again this idea that selection of an appropriate method to evaluate functional recovery is crucial. This study also supported the idea that the walking track analysis (SFI) is more comprehensive and reliable than histomorphometric methods in peripheral nerve repair studies [25, 28].

Uniaxial tensile tests are generally conduced to quantify the physical properties of a nerve. Tensile

force is applied to a fixed length of nerve and the elongation of nerve is measured. The force and elongation are recorded simultaneously until the nerve ultimately fails [29]. Tensile stretch is a major cause of nerve injury resulting in sensory and motor nerve impairment. It has also been indicated that the perineurium is primarily responsible for most of the tensile strength and the endometrium also makes some contribution. At failure, the perineurium develops longitudinal tears with fibrils bulging but the nerve looks grossly intact [30, 31]. 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 [32]. The connective tissue from the epineurium forms a layer of fiber membrane at the 3rd day postoperatively and then forms collagen 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 [33]. Methylprednisolone treatment in the present study resulted in the enhanced biomechanical indices that were in agreement with functional and morphometric findings

Recording wet muscle weight is a previously utilized alternative for motor target organ reinnervation [34-37]. *In vitro* evidence suggests that methylprednisolone treatment improves the motor neuron activity, possibly acting as a neurotrophic factor [38]. 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 [39, 40]. In the present study 16 weeks after surgery the muscle mass was found in both experimental groups. However, CHIT/MP group showed significantly greater ratios of the mean gastrocnemius muscle weight than CHIT/CGP-Hydrogel group indicating indirect evidence of successful end organ reinnervation. However, it should be taken into consideration that some increase on muscle mass could be possibly because of direct effect of steroid on muscle.

In the histological studies, quantitative morphometrical indices of regenerated nerve fibers showed significant difference between CHIT/CGP-Hydrogel and CHIT/MP groups indicating beneficial effect of local methylprednisolone on the nerve regeneration.

Earlier studies on the effects of methylprednisolon hormone on peripheral nerve regeneration have provided conflicting results [41-45]. In the present study, first of all, it was important to know whether administration of methylprednisolone in biodegradable chitosan tubes is 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 and electrophysiological results revealed that the chitosan biodegradable guides allow methylprednisolone 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 rats entubulized by chitosan conduits achieved a faster rate. The functional and morphometric results indicated that the degradation of the chitosan nerve guides did not prevent the stimulating action of methylprednisolone.

Even though our study showed the neuroprotective action of local methylprednisolone in peripheral nerve injuries, data regarding the molecular mechanisms leading to the neuroprotective action remain to be investigated in depth. We have not given the histological and molecular evidence for neuroprotective action of methylprednisolone. 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 methylprednisolone-laden hydrogel on nerve regeneration. The results of the present study indicated that a single local administration of methylprednisolone at the site of transected nerve could be of benefit after chitosan graft tubulization. Local formulation would be ideal because of no long term systemic complications and time consuming limitations. Entubulation neurorrhaphy using methylprednisolone loaded chitosan tube as an *in situ* delivery system of methylprednisolone in bridging the defects could be considered as an alternative to short interposition nerve grafts. The use of a chitosan tube seems to have several distinct advantages for the treatment of transected peripheral nerves because it is inert, does not induce extensive scarring or degeneration after implantation, available and easily performed.

In conclusion, this study demonstrated that methylprednisolone-laden hydrogel when loaded in a chitosan tube improved functional recovery of transected sciatic nerve in rat. Supported by previous findings, the results from our present study would imply that the final outcome of both motor and sensory regeneration and reinnervation following repair of a peripheral nerve and immediate local application of methylprednisolone may be of clinical benefit. There are reasonable grounds to believe that this approach could deliver a superior quality of reinnervation in a shorter period of time, compared to repair without immediate local adjuvant treatment.

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Conflicts of Interest: None declared.

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