

Use of Chitosan Conduit for Bridging Small-Gap Peripheral Nerve Defect in Sciatic Nerve Transection Model of Rat

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Abstract

Objective- To evaluate effect of chitosan conduit for peripheral nerve regeneration using sciatic nerve transection model in rat

Design- Experimental *in vivo* study.

Animals- Sixty healthy male Wistar rats.

Procedures- The rats were divided into four experimental groups (n=15) randomly. In sham group the left sciatic nerve was exposed through a gluteal muscle incision and after careful homeostasis the wound was sutured. In transected control group the left sciatic nerve was exposed the same way, transected proximal to the tibio-peroneal bifurcation leaving a 10 mm gap and the nerve ends were sutured to the adjacent muscles. In silicone or chitosan groups the left sciatic nerve was transected the same way and proximal and distal stumps were each inserted into a silicone or chitosan tube. Each group was further subdivided into three subgroups of five animals each and were studied 4, 8, 12 weeks post operatively.

Results- Functional and electrophysiological analyses showed significant improvement of nerve function in chitosan than in silicone group ($P < 0.05$). Morphometric indices and immunohistochemistry indicated that there were significant differences ($P < 0.05$) between chitosan and silicone with transected control groups 12 weeks after surgery.

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Conclusion and Clinical Relevance- Chitosan conduit could be considered clinically as an effective biodegradable tube for peripheral nerve regeneration in the least harmful way that is available, easily performed and affordable. It also averts the need for foreign materials that are likely to provoke a foreign body reaction.

Key words-peripheral nerve regeneration, chitosan conduit, rat.

Introduction

Peripheral nerve injuries are common in clinical practice due to trauma or deliberate surgical resection.¹ Several methods as direct neuroorrhaphy, nerve grafting, neurotisation, end-to-side neuroorrhaphy and tissue engineering have been used in order to bridge peripheral nerve gaps. The gaps which are not suitable to the neuroorrhaphy can be bridged with autologous, heterologous (allografts) grafts or synthetic biomaterials. Autologous nerve graft is widely accepted, however, it has several disadvantages and donor graft nerves are limited.² Therefore, studies on nerve conduits as an alternative method in nerve regeneration have been focused on using a non-bioabsorbable material such as silicone or natural bioabsorbable grafts such as artery and vein.³⁻⁵

Biodegradable nerve guides as a temporary scaffold are better than non-degradable biomaterials because latter remain in situ as a foreign body and ultimately result in limiting recovery of nerve function.⁶ Silicone has been extensively tested in the form of a prototype nerve tube. Nevertheless, its resistance to biodegradation can be a cause of chronic nerve compression in the long run.⁷ A second surgery may therefore be required for its removal. Recent studies show a benefit of using chitosan as scaffold in promoting wound healing, cartilage repair and nerve regeneration.⁸⁻¹⁰ It seems chitosan as a natural polymer has excellent properties including biocompatibility, biodegradability, non-toxicity and adsorption properties might be a suitable functional material for peripheral nerve regeneration.^{10,11}

The objective of this study was to evaluate the efficacy of chitosan as a conduit on peripheral nerve regeneration using a rat sciatic nerve transection model. Assessment of the nerve regeneration was based on functional (walking track analysis), electrophysiological study, muscle mass measurement, histomorphometric, and immunohistochemical (Schwann cell detection by S-100 expression) criteria 4, 8, and 12 weeks after surgery.

Materials and Methods

Experimental Design

Sixty male Wistar rats weighing approximately 210 g were divided into four experimental groups (n = 15), randomly: Sham-operation (Sham), transected control (TC), silicone conduit positive control (SIL) and chitosan conduit (Chit). Each group was further subdivided into three subgroups of five animals each. Two weeks before and during the entire experiments, the animals were housed in individual plastic cages (50 × 40 × 20 cm) with an ambient temperature of 23 ± 3 °C, stable air humidity, and a natural day/night cycle. The animals were handled on a regular daily basis for 2 weeks prior to the study in order to acclimatize them with testing area and experiments. The rats had free access to standard rodent laboratory food and tap water. All procedures were carried out in accordance with the guidelines of the Ethics Committee and the University Research Council approved all experiments.

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 3hour. 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.¹² Chitozan conduit was made according to the method described by Qiang Ao et al.,¹¹ by gentle injection of the prepared solution into a home-made mold.

Grafting procedure

Animals were anesthetized by intraperitoneal administration of a combination of ketamine hydrochloride 5%, 90 mg/kg (Ketaset 5%; Alfasan, Woerden, The Netherlands) and xylazine hydrochloride 2%, 5 mg/kg (Rompun 2%, Bayer, Leverkusen, Germany). Following surgical preparation, in the sham-operation group the left sciatic nerve was exposed through a gluteal muscle incision and after sciatic nerve exposure the splitted muscle was sutured with 4/0 Vicryl (Ethicon, Norderstedt) and the skin with 3/0 nylon (Dafilon, B/Braun, Germany). In transected control group (TC) the left sciatic nerve was exposed the same way, transected proximal to the tibio-peroneal bifurcation where a 7 mm segment was excised, leaving a gap about 10 mm due to retraction of the nerve ends. The proximal and distal stumps were fixed in the adjacent muscle with 10/0 nylon epineurial suture and no conduit was placed between the stumps. In both silicone conduit, as positive control, and chitosan conduit, as treatment, the transected proximal and distal stumps were each inserted 2 mm into the conduit and two 10/0 nylon sutures were placed at each end of the cuff to fix the tube in place and leave a 10-mm gap between the stumps. The conduits were filled with 10-µL PBS solution, and sterile Vaseline was used to seal the ends of the tubes to avoid leakage. Then, the animals were housed in groups of five per cage under the same conditions mentioned above. 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 (n = 5), 8 (n = 5) and 12 weeks (n = 5) after surgery.

Functional assessment of nerve regeneration

Walking track analysis was performed 4, 8 and 12 weeks after surgery based on Bain et al.,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 contra lateral normal side (N) in each rat. The Sciatic Function Index (SFI) in 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, the SFI oscillates around 0 for normal nerve function, whereas around -100 SFI represents total dysfunction. The SFI was assessed based on the sham-operated group and the normal level was considered as 0. The SFI was a negative value and a higher SFI meant the better function of the sciatic nerve.

Electrophysiological measurement

After 12 weeks, following the track test, all animals were subjected to electrophysiological studies using Nacro bio system 320-3760 A trace 80 (USA). Under general anesthesia (see above), the left sciatic nerve (operated side) 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 contra lateral, intact side of each animal, similar measurements were made for the 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).¹⁴

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 blinded observers unaware of the analyzed group.

Histological preparation and morphometric studies

Graft middle cable (chit) and (SIL), midpoint of normal sciatic nerve (NC) and regenerated cable (TC) were harvested and fixed with glutaraldehyde 2.5%. The grafts were then embedded in paraplast paraffin, cut in 5 μ m and were next stained with methylene blue. 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.¹⁵

Immunohistochemical analysis

In this study, anti-S-100 (1:200, DAKO) was used as marker for myelin sheath. Specimens prior to immunohistochemistry were post fixed with 4% paraformaldehyde for two hours and embedded in paraffin. After non-specific immunoreactions were blocked, sections were incubated in S-100 protein antibody solution for one hour at room temperature. They were washed three times with PBS and incubated in biotinylated anti-mouse rabbit IgG solution for one hour. Horseradish peroxidase-labelled secondary antibody was developed by the diaminobenzidine method. The results of Immunohistochemistry were examined under a light microscope.

Statistical analysis

Experimental results were expressed as means \pm SD. All data were analyzed by one-way analysis of variance (ANOVA) to assess statistical significance between experimental groups (SPSS 17.0 for Windows). Dunnett's test for pair wise comparisons was used to examine the effect of time and treatments. The differences were considered significant when $P < 0.05$.

Results

Functional assessment of nerve regeneration

Table-1 shows sciatic function index (SFI) values in all four experimental groups. Prior to surgery, SFI values in all groups were near zero. After sciatic nerve transection, the mean SFI decreased to -100 due to the complete loss of sciatic nerve function in all animals. Four weeks after surgery, SFI improvement in Chit group was better than SIL group ($p < 0.05$). Eight weeks after surgery, the improvement in SFI was observed in both Chit and SIL groups, whereas in group TC, no comparable SFI value was obtained. After 12 weeks, animals of group Chit achieved a mean value for SFI of -23.7 ± 3.51 i.e. an approximate improvement of 72% and in SIL group a mean value of -57 ± 3.5 i.e. an approximate improvement of 52%, whereas in group TC, a mean value of -94 ± 2.8 i.e. an approximate improvement of 5%, was found. The statistical analysis revealed that the recovery of nerve function was significantly ($P < 0.05$) different between Chit and SIL with TC groups and interposition of the chitosan and silicone conduits significantly promoted functional recovery in the course of time.

Electrophysiological measurement

Conduction velocity along the chitosan-bridged and Silicone-bridged regenerated sciatic nerves was 28% CVR and 28.5% CVR of the intact right side, respectively. These were significantly higher than that of TC group that was 4% CVR ($P < 0.005$) (Fig.1).

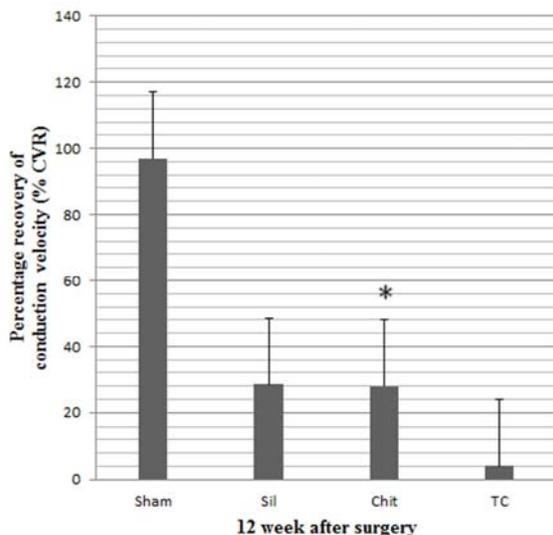


Figure 1. Percentage recovery of conduction velocity in experimental groups. Data are presented as mean \pm SD. * $P < 0.005$ vs TC group.

Muscle mass measurement

The mean ratios of gastrocnemius muscles weight were measured. There was statistically significant difference between the muscle weight ratios of Chit and SIL groups with TC group ($P < 0.05$). It showed that muscle weight ratios were bigger in Chit and SIL groups than of TC group and weight loss of the gastrocnemius muscle was ameliorated by chitosan and silicone entubulation (Fig 2).

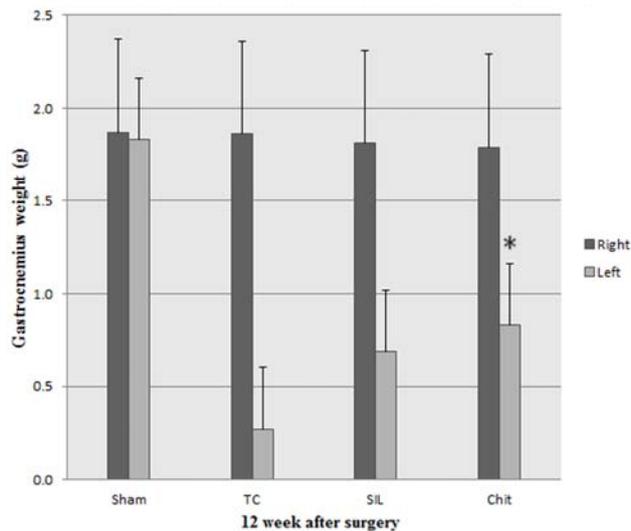


Figure 2. Gastrocnemius muscle weight measurement. The gastrocnemius muscles of both sides (injured left and uninjured right) were removed and weighed in the experimental groups at 12 weeks after surgery. * $P < 0.005$ vs TC group

Histological preparation and morphometric studies

All animals of the Chit group showed nerve fibers at distal stumps 4 weeks after surgery. In the early phases of regeneration (at four weeks post operation) regenerated nerve fibers were present within the chitosan guide and regenerated nerve fibers could be confirmed after 4 weeks without any foreign body reaction. In the SIL group, the finding was similar to Chit group. In TC group, three animals presented lower number of nerve fibers at distal stumps after 8 weeks. The other two showed degenerated distal stumps. Sham-operation group presented significantly greater nerve fiber and axon diameter, and myelin sheath thickness compared to Chit, SIL and TC animals.

Both of Chit and SIL animals presented regeneration patterns. The number of nerve fibers in both of them was significantly higher than TC group. The mean diameter of the nerve fibers in the Chit (4.86 ± 0.19) was significantly larger than that of TC (4.11 ± 0.22) ($P < 0.05$). The diameter of axon in Chit (3.77 ± 0.21) was significantly larger than in TC (2.44 ± 0.63) ($P < 0.05$) (Table 2). However, Morphometric indices in SIL group showed significantly greater values than those of Chit group ($P < 0.05$).

Immunohistochemical analysis

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 3). In the Chit and SIL groups, the structure and function of regenerated axons and myelin sheath were far more similar than TC group to those of normal nerve. In TC, the expression of S-100 was dispersed and the findings resembled those of the histological evaluations.

Table 1. Sciatic function index (SFI) 4, 8 and 12 weeks after surgery in experimental groups.

Rat No.	Sham			TC			SIL			Chit		
	4	8	12	4	8	12	4	8	12	4	8	12
1	-6.4	-3.7	-4.6	-97.4	-92.1	-95.3	-96.4	-75.8	-60.7	-54.5	-36.6	-23.4
2	-6.1	-2.1	-5.1	-96.7	-93.3	-96.4	-92.3	-76.6	-59.3	-57.8	-40.4	-23.5
3	-5.9	-3.9	-4.5	-98.1	-94.4	-95.8	-93.6	-80.2	-54.8	-53.6	-39.5	-26.3
4	-6.8	-2.6	-5.5	-96.4	-91.9	-93.6	-95.8	-77.4	-60.9	-57.4	-37.7	-25.9
5	-6.1	-3.8	-4.9	-97.1	-94.1	-94.7	-90.3	-79.8	-58.2	-59.3	-39.7	-21.5
(Mean ± SD)	-6 ± 0.8	-3 ± 0.7	-5 ± 0.8	-95.12 ± 2.4	-93 ± 3.6	-94 ± 2.8	-93 ± 3.5	-77 ± 3.2	-57 ± 3.5	-56 ± 3.7 ††	-38 ± 2.6 ††	-23 ± -3.3 ††

† P<0.05 versus TC group, ‡ P<0.05 versus SIL group

Table 2- Morphometric analyses of regenerated nerves for each of the experimental groups: values are given as mean ± SD

Group	Number of fibers			Diameters of fibers (µm)			Diameter of axon (µm)			Myelin thickness (µm)		
	4	8	12	4	8	12	4	8	12	4	8	12
Sham	802±404	8379 ± 446	8124 ± 385	12.01±0.01	11.93±0.17	12.06±0.23	7.03 ± 0.02	6.97 ± 0.39	7.06 ± 0.46	2.56 ± 0.01	2.48 ± 0.02	2.53 ± 0.01
TC	0	1003 ± 295	1131±219†	0	3.98 ± 0.55	4.11 ± 0.22	0	2.38 ± 0.36	2.44 ± 0.63	0	0.81 ± 0.13	0.83 ± 0.02
Chit	1378±176†	2673±215†	3024±198††	2.83±0.18††	4.51±0.24††	4.86±0.19††	2.68 ± 0.53	3.74±0.23†	3.77±0.21††	0.41±0.02††	0.43±0.03††	0.63±0.03††
SIL	1654 ± 301	3026 ± 285	3674 ± 272	3.57 ± 0.83	8.03 ± 0.21	8.45 ± 0.72	2.12±0.22†	4.21 ± 0.38	4.78 ± 0.34	0.52 ± 0.07	2.06 ± 0.41	2.21 ± 0.24

† P<0.05 versus TC group, ‡ P<0.05 versus SIL group.

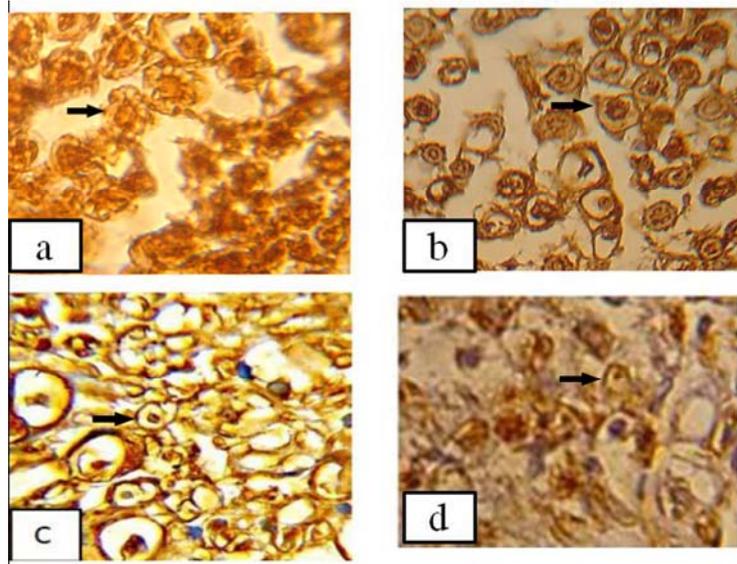


Figure 3. Immunohistochemical analysis of the regenerated nerve 12 weeks after surgery from (a) midpoint of Sham (Sham), (b) middle cable (SIL), (c) middle cable Chit and (d) regenerated cable (TC). There is clearly more positive staining of the myelin sheath-associated protein S- 100 (arrow) within the periphery of nerve, indicating well-organized structural nerve reconstruction in entubulated nerve compared to transected control. ($\times 1000$)

Discussion

The assessment of sciatic nerve regeneration based on walking track analysis, electrophysiological, muscle mass, histomorphometric and immunohistochemical studies showed that chitosan as well as silicone could be used as a conduit for entubulation of transected short-gap nerve ends to enhance rat sciatic nerve regeneration.

Walking track analysis as a reliable determining functional recovery test¹⁵⁻¹⁷ showed that improvement in recovery of sciatic nerve function in the course of time in Chit was significantly better than SIL group. Nerve conduction measurement is a direct evidence for the study of nerve transmission.¹⁸ The conduction velocity depends on the diameter of axons and the thickness of myelin sheath.¹⁹ The results of the present study showed similar conduction velocity along the chitosan-bridged and silicone-bridged regenerated sciatic nerves, therefore, the chitosan conduit could be assumed as a safe nerve guide with no nerve conduction interference.

In the present study, similar muscle mass results in both experimental groups showed again the chitosan could be as effective as silicone in entubulation of sciatic nerve defect. 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.^{20,21}

The results of present study indicated that number of nerve fiber and axon diameter in both experimental groups was similar during the entire study except for week 12. Diameter of fibers and myelin thickness values in SIL group were significantly higher than those of Chit group. Regenerating axonal sprouts tended to be smaller than those from uninjured axons. In contrary, in Chit group, functional recovery occurred from week 8 to week 12 and SFI values showed significant improvement 8 weeks after surgery. This again supports the idea that selection of an appropriate method to evaluate nerve repair process is crucial. Others have

suggested that walking track analysis is more comprehensive than histomorphometrical methods alone. This study also supports the idea that the walking track-analysis is more comprehensive and reliable than histomorphometric methods in peripheral nerve repair studies.²²⁻²³

In the present study as observed histologically, morphometrical values did not differ significantly between 8 and 12 weeks in entubulated defects by chitosan. Also, there were no significant differences in diameter of nerve fibers and myelin thickness between Chit and TC groups. This study again supports the idea that the walking track analysis (SFI) is more comprehensive than histomorphometrical methods.²²

We demonstrated in this proof of principle study that chitosan conduit can support axonal regrowth across a one cm gap in an adult rat sciatic nerve. We used well-established test systems because we aspired to examine comprehensive behavior of the conduit. Ideally, the scaffold material should last long enough for axonal regrowth and tissue repair. Previous studies shows chitosan is a biocompatible, timely biodegradable material, low toxic property, and low cost^{10,11} and also the capacity of adherence and survival of Schwann cells on its substratum in vitro has a benefit for nerve regeneration.^{24,25} This natural polysaccharide, has structural characteristics similar to glycosaminoglycans and it seems to mimic the functional behavior of glycosaminoglycans and enzymatically can degrade to absorbable oligosaccharides.¹ Some reports have also showed that chitosan has bacteriostatic^{26,27} and hemostatic²⁸ properties which reinforcing the suitability of as a scaffold biomaterial for neural tissue engineering.

Serum lysozyme degrades chitosan²⁹ and the degradation products are nontoxic, non-immunogenic and noncarcinogenic.³⁰ Chitosan has been shown to physiologically stimulate the tissue repair process and favors angiogenesis.³¹ Chitosan in the form of a scaffold has been shown to carry angiogenic activity when implanted.³² Chitosan solution can be easily formed due to its film-forming property and the tough and visco-elastic properties of gelled chitosan suitably provide mechanical support for nerve regeneration.³³ However, initial pilot of our study showed that when chitosan fabricated into conduits it would be firm, dry and fragile enough that make it not suitable for suturing and surgical handling. Therefore, it was blended with glycerol while preserving its biocompatibility.¹²

In conclusion, chitosan entubulation technique has offered the hope of providing a method for achieving the peripheral nerve regeneration in the least harmful way that is available, easily performed and affordable. Using chitosan tubes in bridging of nerve defects could be promising because it is inert and does not induce extensive scarring or degeneration after implantation.

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استفاده از کاندوئیت کیتوزانی در ترمیم نقیصه کوتاه عصب سیاتیک در مدل قطع عصب رت

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روش کار- رت‌ها بطور تصادفی به چهار گروه ۱۵ تایی تقسیم شدند. در گروه کنترل نرمال عصب سیاتیک چپ پس از برش پوست و عضله سرینی شناسایی شده و پس از خونبندی، محل بخیه گردید. در گروه کنترل منفی نقیصه‌ای به طول ۱۰ میلی متر بر روی عصب سیاتیک ایجاد گردید سپس انتهای بریده شده به عضلات ناحیه بخیه گردید. در گروه کنترل مثبت و درمان پس از ایجاد نقیصه ۱۰ میلی متری، انتهای قطع شده بالایی و پایینی عصب با استفاده از لوله سیلیکونی یا لوله کیتوزانی به هم مرتبط شدند. هر گروه سپس به سه زیرگروه ۵ تایی تقسیم گردیده و در مقاطع زمانی ۴، ۸ و ۱۲ هفته بعد از عمل مورد مطالعه قرار گرفتند.

نتایج- ارزیابی‌های عملکردی و الکتروفیزیولوژی عملکرد عصب سیاتیک در رت‌های دریافت‌کننده سیلیکون و کیتوزان بهبودی قابل ملاحظه‌ای را نشان داد ($P < 0.05$). شاخص‌های مورفومتریک و ارزیابی‌های ایمنوهیستوشیمیایی بیانگر ترمیم قابل ملاحظه در هر دو گروه در مقایسه با گروه کنترل منفی در پایان ۱۲ هفته بود ($P < 0.05$).

نتیجه‌گیری و کاربرد بالینی- از نظر بالینی لوله کیتوزانی را می‌توان به عنوان یک کاندوئیت تجزیه‌پذیر زیستی در ترمیم بهتر اعصاب محیطی در نظر گرفت که این روش نسبت به سایر روش‌های ترمیمی ساده‌تر، کم خطرتر، در دسترس و ارزان‌تر بوده و خطر ایجاد واکنش‌های اجسام خارجی را نیز ایجاد نمی‌کند.

کلید واژگان- ترمیم عصب محیطی، کاندوئیت کیتوزان، رت.