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Peripheral Nerve Regeneration Through Nerve Conduit Composed of Alginate-Collagen-Chitosan

Sang Wan Kim, Hong Ki Bae, Hye Sung Nam, and Dong June Chung*

Department of Polymer Science and Engineering, Sungkyunkwan University, Suwon 440-741, Korea
IBEC, Seoul National University, Seoul 110-768, Korea

Pill Hoon Choung

Department of Oral and Maxillofacial Surgery, Seoul National University, Seoul 110-768, Korea

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Abstract: Although the peripheral nerve system has a relatively good regenerating capacity compared to the central nerve system, peripheral nerve repair remains a clinical challenge as restoration of normal nerve function is highly variable. Synthetic tubular nerve conduits were designed as an alternative repair method in order to replace the need for an isograft. These nerve conduits guide regenerating axons from the proximal toward the distal end, maintain within growth-promoting molecules released by the nerve stumps, and protect regenerating axons from infiltrating scar tissue. In this work, we prepared cinnamoylated alginate (CA)-collagen-chitosan nerve conduit using the lyophilization method to generate a controllable parallel channel in the center and then investigated its influence on peripheral nerve regeneration in an animal study. At 12 weeks after implantation, histological study showed that tissue cable was continuously bridging the gap of the sciatic nerve in all rats. Our newly developed nerve conduit is a promising tool for use in peripheral nerve regeneration and provides a suitable experimental model for future clinical application.

Keywords: nerve conduit, implantation, peripheral nerve, regeneration, lyophilization.

Introduction

There are two kinds of nerve system including central and peripheral nerve for the control of external stimuli from human sensory system and responsive action from the signal transductions in brain tissue. Among the differences between the central and peripheral nerve system, the regeneration behaviors of nerve tissue after severe damage show different functions and characteristics. From many clinical reports, it was well accepted that central nerve system can not recover from clinically or externally injured tissue.[1]

Seddon *et al.* classified peripheral nerve damages as 3 types of neurapraxia, axonotmesis and neurotmesis.[2] Neurapraxia is temporal damage on neuron fiber and can be easily reconstructed in few hours or several weeks according to the degree of injury, because of keeping of distal conductivity and connection of axon. Axonotmesis is a complete severance of axon but conserve its connection of Schwann's cell sheath. After damage, movement including sensory and action was paralyzed completely, but axon was self-regenerated and recovered its original function through the conserved Schwann's cell sheath. Neurotmesis is a complete severance of axon and Schwann's cell sheath, therefore surgical treatment such as autologous nerve implantation or ostomy is good candidate for damaged nerve regeneration because of lack of possibility of self-healing (self-recovery).

In the case of nerve severance, surgical suturing on the damaged nerve site of distal and proximal can induce regeneration in the case of short nerve injury, but natural nerve implantation must be adopted as substitute materials for long or severe nerve injury. In this isograft implantation methods, it can not be avoidable to reduce pains of nerve donor and lack of original nerve tissue in donor's body.[3] Recently, to solve previously mentioned disadvantages, various polymeric artificial conduit including natural and synthetic materials was applied for nerve regeneration as shown in Figure 1.[4,5]

In polymeric materials, biodegradable polymers are very useful materials of conduit for its unique characteristics such as good mechanical strength and time dependent degradation control. Alginic acid from seaweeds origin is hydrophilic and anionic polysaccharides which consists of D-mannuronic

*Corresponding Author. E-mail: djchung@skku.edu

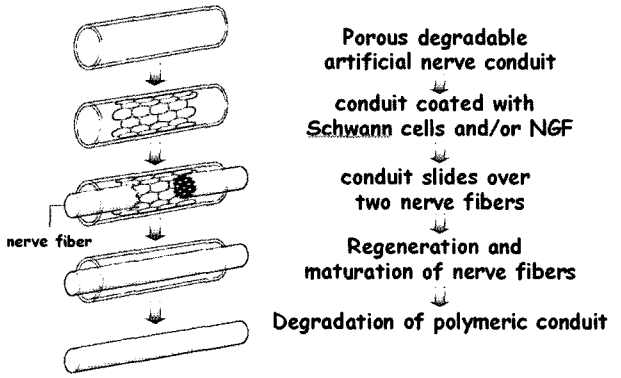

Figure 1. Concept of nerve regeneration through polymeric conduit.

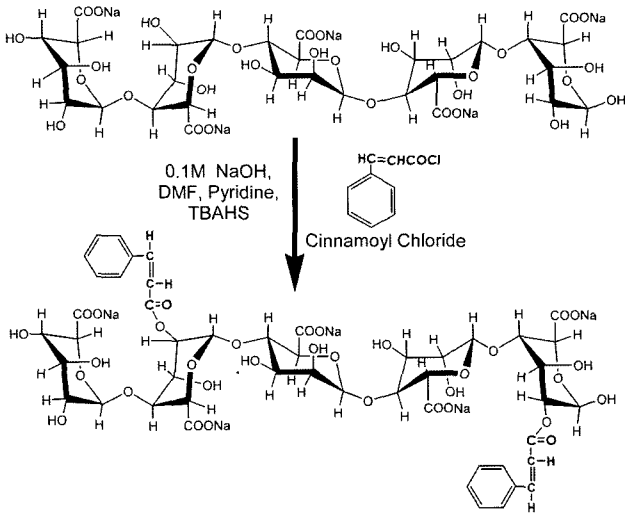

Figure 2. Reaction mechanism for the synthesis of CA.

acid and *L*-guluronic acid,[6,7] and sodium alginate (sodium salt of alginic acid) can be obtained with reaction of cations (Na^+, K^+, NH_4^+ etc) and shows good solubility to water and also make salt complex with Ca^{++} ion which turns to be hydrogel by crosslinking through ion interactions between added cation (Ca^{++}) and anions (OH^-) in sodium alginate.[8] According to the concentration of sodium alginate solution, we can get a very viscous solution and also applicable to make a synthetic matrices for drug containing, food modification.[9] In recent reports,[10] sodium alginate was used for the scaffolds of 3-dimensional cell culture in tissue engineering.

Sponge type porous collagen is suitable for the scaffolds of dermis including epithelium and cartilage cultivation for artificial skin and joint because of its good mechanical characteristics, biocompatibility and slow biodegradability.[11,12] Chitosan is abundant natural polysaccharide next to cellulose and also has biocompatibility, antibiocides, biodegradability and good metallic ion adsorption ability. So, this material was tried to apply for high-tech industry including new-fiber and biomedical industry.[13,14]

In this study, we used natural polymeric materials (sodium alginate, collagen, chitosan) for development of artificial nerve conduits and evaluate the characteristics of synthetic conduit, and finally also investigate the *in vivo* peripheral nerve regeneration through animal test using rat.

Experimental

Materials. Trans-cinnamoyl chloride and chitosan (Aldrich Chemical Co., Milwaukee, MO, USA), sodium alginate (Wako Pure Chemical Inc., Tokyo, Japan), type I collagen from bovine (Sigma Chemicals Inc., St. Louis, MO, USA) were purchased and used without further purification. NaOH, acetone, *N,N*-dimethylformamide (DMF), pyridine, citric acid, calcium chloride, EtOH were also purchased as reagent grade and used without further purification.

Synthesis of Cinnamoylated Alginate (CA). Porous structured nerve conduit was manufactured through photo-crosslinking method. Photo crosslinkable trans-cinnamoyl chloride was introduced to sodium alginate by the following reaction. 6.0 g of sodium alginate was solved in the 200 mL of 0.1 M NaOH solution containing 0.6 g of tetrabutylammonium hydrogensulphate (TBAHS) for 12 hrs at 40 °C with stirring. After reaction, reactor was located in ice bath and DMF/pyridine mixed solution (160 mL/60 mL) was slowly dropped to the reactor with stirring. And in dark place trans-cinnamoyl chloride and DMF mixture (25 mL each) was dropped for 8 hrs for the purpose of CA synthesis (Figure 2).

After completion of reaction in dark place, reaction products were precipitated in acetone and filterated, white powdered CA was obtained through vacuum drying. For the elimination of unreacted reactants, powdered CA was resolved in distilled water and dialyzed using dialysis tube (MW cut off; 3,500) for 3 days and fine products were obtained by lyophilization.

Manufacturing of Nerve Conduit. CA solution in distilled water and type I collagen solution in 5.0% citric acid (w/w, 2 : 1) were thoroughly mixed and irradiated by UV lamp (400 W) with UV filter (cut off wavelength, >260 nm) for 30 min for the purpose of photo dimerization of cinnamoyl group and photo-crosslinking of alginate (Figure 3).[15]

Concentrated photo-crosslinked mixture solution of collagen and CA was inserted to the PE tube (25 mm length and 3 mm diameter) and 14G needle was located at the center of the PE tube for the formation of internal rod like space. PE tube including mixture solution was deep freezed at -80 °C and after 2 days lyophilization, we can get a hollow tube type porous nerve conduit.

To strengthen mechanical properties of collagen/alginate conduit, lyophilized polymeric nerve conduit was immersed into the 10% calcium chloride solution for 1 day to introducing additional ionic bonding in alginate. And repeated

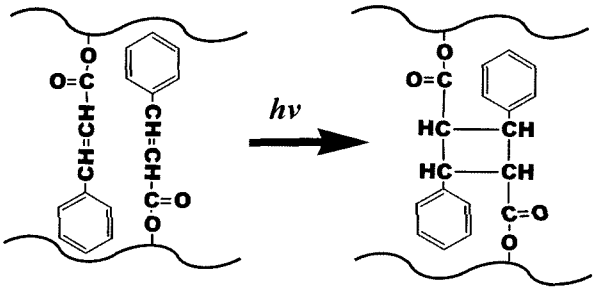

Figure 3. Potodimerization between cinnamoyl groups.

immersing collagen/alginate conduit into 1.0% aqueous chitosan solution also gives above conduit an additional enhancing of mechanical properties.

Porous surface morphologies of obtained nerve conduits were investigated with SEM observation (SEM, Hitachi S-2400, Tokyo, Japan) through pre-sample treatment method.

Characterization of Nerve Conduit. To confirm the mechanical characteristics of synthetic nerve guide, we measured the swelling degree of nerve guide using tube type conduit (10 mm in length, 3 mm in diameter). After vacuum drying and swelling in distilled water at 37 °C, dry and wet weight of nerve conduit were measured precisely using 5 samples.

Degree of swelling was defined the difference ratio between dry weight and wet weight after swelling as follows. W_d means the weight of nerve guide in dry state and W_s means the total weight of polymeric nerve guide and contained water after swelling.

$$\text{Swelling Degree (\%)} = (W_s - W_d)/W_d \times 100 \quad (1)$$

To investigate degradation behavior, 5 samples of nerve conduits were immersed in PBS solution (pH 7.4) at 37 °C for predetermined time intervals, and measured the weight difference of initial and time dependent degraded samples. Degradation ratio was determined by the ratio of initial weight and residual weight at predetermined time intervals.

$$\text{Degradation Ratio (\%)} = \text{(Residual Weight/Initial Weight)} \times 100 \quad (2)$$

Mechanical characteristics were measured through 3-point bending test by Instron (UTM-4467, Norwood, MA, USA) using tube type nerve conduits (20 mm in length, 3 mm in diameter). Measurements were performed at 25 °C with 10 mm/min head speed for compression.

***In vivo* Test.** 10 Sprague Dawley rats (250~300 g) (Samtaco Inc., Osan, Korea) were utilized in this study. Briefly the rats were anesthetized and maintained by 0.4 mL intramuscular injection of a premixed solution containing 64 mg/mL ketamine HCL (Keta-Sthetic™ Boehringer Ingelheim, St. Joseph, MO, USA), 3.6 mg/mL xylazine (Rompun™, Miles Inc., Scawnee Mission, KA, USA) and 0.07 mg/mL

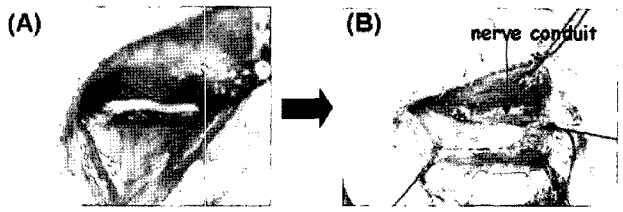

Figure 4. Intraoperative views. (A) Exposed left sciatic nerve and (B) after mplantation of the nerve conduit.

atropine sulfate (Elkins-Sinn Inc., Cherry Hill, NJ, USA). The skin from the clipped lateral thigh was scrubbed in a routine fashion with antiseptic solution. The incision extended from the greater trochanter to the midcalf distally. The sciatic and posterior tibial nerves were exposed by a muscle splitting incision; the sciatic nerve was divided near its origin to create an adequate distal segment. The 12 mm nerve conduits (CA-Col-Chi(insol)) were placed into this nerve gap (10 mm) using 10-0 nylon sutures under microsurgery technique (Figure 4). The nerve was sutured into the conduit such that approximately 1 mm of each nerve end remained within the tubular polymeric conduit.

Walking track analysis was performed 2 months later after surgery. Walking track analysis is an indirect method to measure functional muscle reinnervation following the correction of the hind paw prints after nerve regeneration comparing the control versus surgical limb. The sciatic functional index (SFI) was calculated as eq. (3) and were expressed as absolute values.[16,17] As the value tends toward 0, a complete functional recovery of nerve through conduit is noted and number of -100 means the overall damage originated from mismatching of synthetic conduit and natural nerve tissue.

$$\text{SFI} = -38.3\,(\text{EPL} - \text{NPL})/\text{NPL} + 109.5\,(\text{ETS} - \text{NTS})/\text{NTS} + 13.3\,(\text{EIT} - \text{NIT})/\text{NIT} \quad (3)$$

EPL ; experimental paw length
NPL ; non operated normal paw length
ETS ; distance between the first and fifth toes of experimental foot
NTS ; distance between the first and fifth toes of non operated (control) foot
EIT ; distance between the second and forth toes of experimental foot
NIT ; distance between the second and forth toes of non operated (control) foot

For histological investigation, following muscle harvest after 2 months, 4 and 8 mm from proximal conduit, and distal conduit were harvested and histomorphologically analyzed. The harvested conduit was fixed with 3% glutaraldehyde, embedded in epoxy resin and stained with hematoxylin-eosin for the histological section.

Results and Discussion

Synthesis of CA. Introduction of cinnamoyl group for photo-dimerization was identified by the UV absorption peak at 260 nm according to the carbon-carbon double bond, and also identified by IR absorption peak at 1730, 1640 cm^{-1} according to the elastic vibration molecular motion of C=O and C=C bond in cinnamoyl group.

After photo irradiation, decrease of UV absorbance at 260 nm in CA solution was observed, and this decrease was awing to [2+2] cyclo-addition of π bond in double bond of cinnamoly group,[18] finally this revealed the formation of intermolecular crosslinking structure of alginate through cyclobutane ring as shown in Figure 3.

Characterization of Nerve Conduit. Porous nerve conduit using CA and type I collagen was obtained by the photo crosslinking and freeze drying of mixture solution. From the SEM observation results of Figure 5, porous structured was confirmed and outer surface had more dense morphology than inner surface.

Nontoxic, biocompatible and anti-biocidic material, chitosan, was added to above mentioned CA and type I collagen mixed solution to improve mechanical strength through the ionic interaction[19] between anionic moieties in alginate and cationic glucosamine moieties in chitosan.[20]

In Table I, mechanical characteristics (degree of swelling, degradation rate and compression strength) were summarized. After 20 min swelling, degree of swelling showed maximum value and this value was decreased by the addition of chitosan. Nerve conduit containing chitosan showed reduced water content, therefore initial swelling behavior turned to be retarded and also reduced maximum swelling degree at equilibrium. Figure 6 showed degradation behaviors of nerve conduits *in vitro*. In case of CA-collagen nerve conduit, 70% of dry weight was diminished after 60 days

Table I. Physical and Mechanical Properties of Nerve Conduit

Groups	n	CA-Col	CA-Col-Chi
Max. swelling degree (%)	5	610.2±21.9	540.0±17.3
Degradation ratio(%) (after 60 days)	5	29.1±2.3	45.3±2.6
Comperesive strength (Kg_f)	10	181.5±7.8	209.7±6.9

Note. CA, CA; Col, collagen; Chi, chitosan;
n, number of tested sample. Values are means±SD.

and chitosan added CA-collagen nerve conduit showed 55% degradability in same time interval. Such differences were elucidated the addition effect of chitosan which caused structural stabilization owing to ionic interactions between them. As the same reason above mentioned, enhancement of compression strength could be explained in CA-Col-Chi conduit.

Macroscopic Evaluation and Walking Track Analysis Using Rat. 2 months passage after surgery, outside surface hind paw of rat was examined with the naked eye for the ascertaining of nerve regeneration. Mild automutilation was defined as surface damage limited on skin or claw of hind paw. Severe automutilation was defined as wide range damage including bone exposure or loss of a part of paw. Moderate automutilation was defined some damages located in the range between them. 8 of 10 experimental rats showed automutilation and half of them revealed severe automutilation. 2 of non-automutilated rats showed only trophic anomalies.

SFI values which were the evaluation of recovery for nerve and muscle after surgery was calculated using eq. (3) and summarized in Table II.

Histological Evaluation. Regenerated nerve tissue through implanted nerve conduit was illustrated in Figure 7. After 2 months, there was no significant difference in any of the

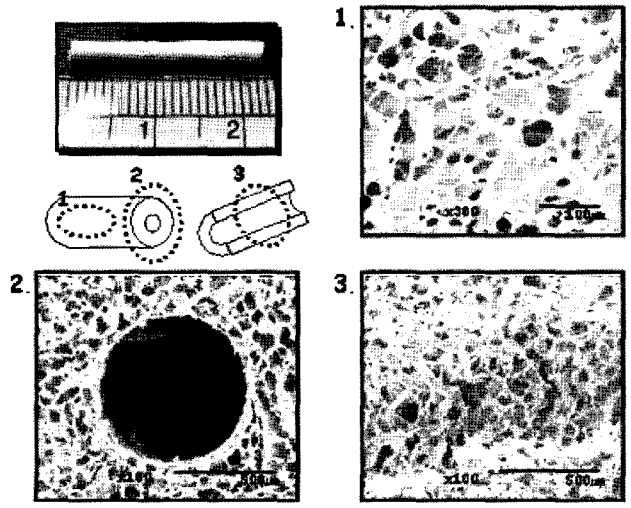

Figure 5. SEM micrograph of CA-Collagen nerve conduit.

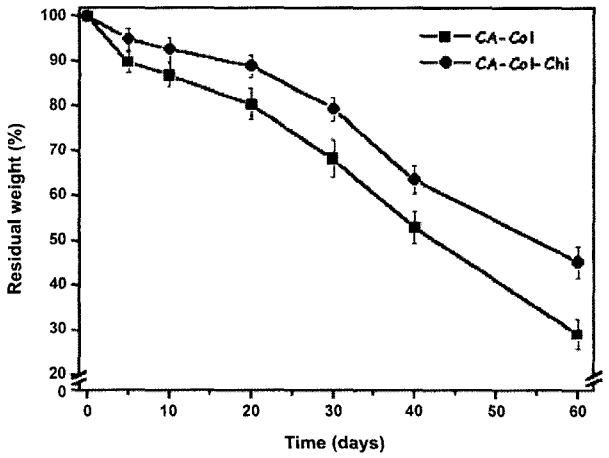

Figure 6. *In vitro* degradation behaviors of nerve conduits.

Table II. Macroscopic Evaluation and Sciatic Functional Index (SFI) of Experimental Groups Following Implantation

	No Automutilation	Mild Automutilation	Moderate Automutilation	Severe Automutilation	Total
No.	2	1	3	4	10
SFI	-65.2 -65.7	-75.8	-89.2 -82.1 -91.5	-100	-87.0±14.1

Note. SFI of Control(autograft); -63.5±18.1 (n=4). Values are means±SD.

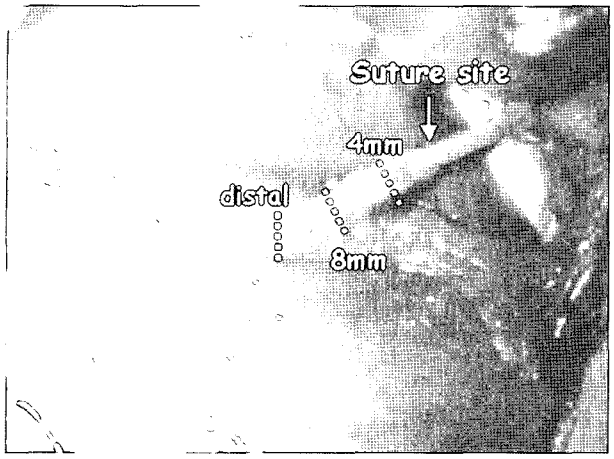

Figure 7. Intraoperative view of the regenerated sciatic nerve.

operated group but not equivalent to no automutilation when compared to control group. All cases of surgery, regenerated nerve tissue and newly connected sciatic nerve was observed at the incision of original nerve, and also observed that boundary muscle around incision area attached regenerating nerve.

For the histological investigation of nerve regeneration, harvested nerve tissue was sectioned and stained with hematoxylin-eosin and observed by optical microscopy.

In Figure 8, much fibrous myelinated nerve were observed and this result means that nerve connectivity was fully obtained. Unmyelinated axons do not have original functionality of nerve, so regeneration of fibrous myelinated nerve was very important factor for nerve regeneration of damaged nerve in living body.

For the evaluation of axon regeneration, area of fasciculus, density of axon and thickness of myelin sheath were measured using histological section of harvest regenerating nerve by optical microscopy (Figure 9).

In peripheral nerve, axons were sheathed with Schwann cell (neuroglial cell) and connective tissue sheath, and these cell wall structured membrane was called myelin sheath. All of axons do not sheathed by myelin sheath, myelinated nerve fiber is sheathed axon by myelin sheath. Unmyelinated nerve fiber does not have myelin sheath and does not reveal original nerve function. Therefore number, density of myelinated nerve fiber and adequate thickness of myelin sheath

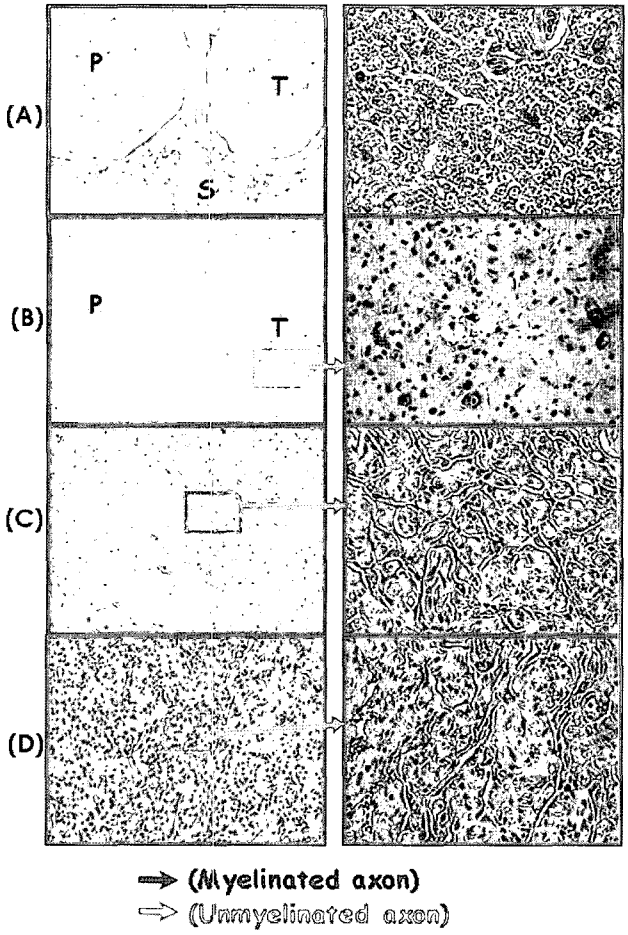

Figure 8. Representative transverse sections of (A) normal sciatic nerve and regenerated nerve at the (B) 4 mm, (C) 8 mm, (D) distal from the suture site. P: peroneal nerve, T: tibial nerve, S: sural nerve.

were also very important factors in nerve regeneration.

In this research work, the area of fasciculus, density of axon and thickness of myelin sheath of harvest nerve were not sufficient to those of control (autograft). The area of fasciculus and thickness of myelin sheath of distal section was reduced comparing to midpoint section, and same tendency was observed in control group. Such tendency is deeply related to the directional nerve regeneration characteristics from proximal to distal. But in case of the density of axon

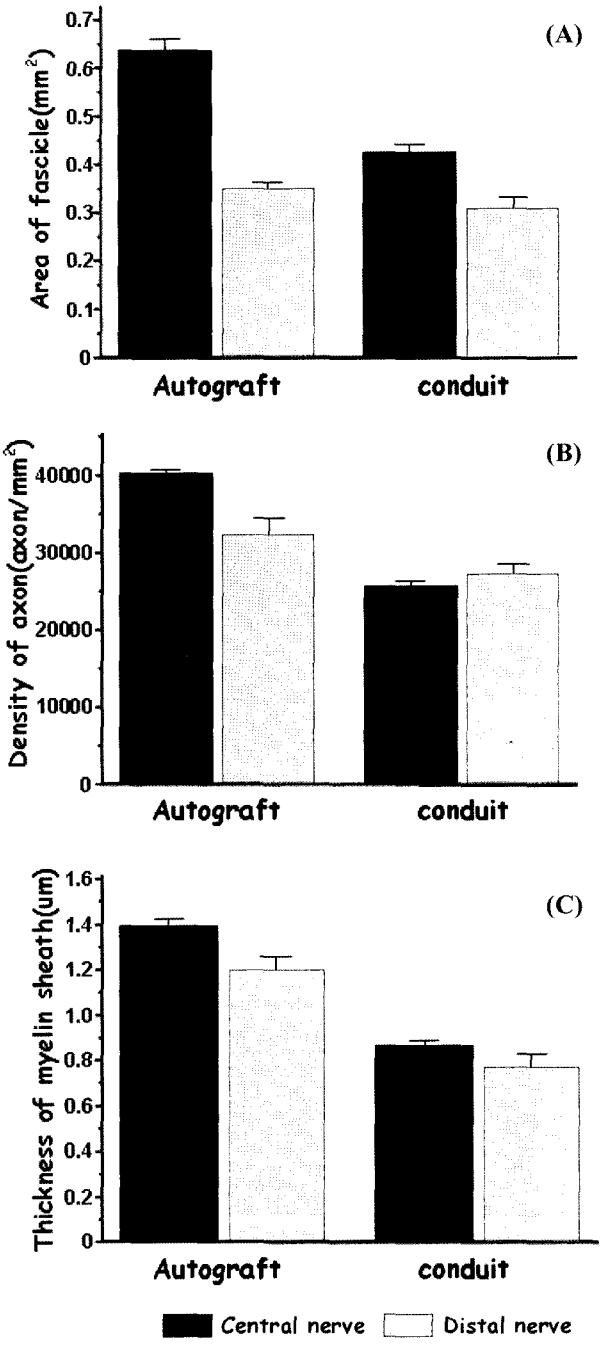

Figure 9. (A) Area of fascicle, (B) Density of axon, and (C) Thikness of myelin sheath in regenerated sciatic nerve.

was increased in the distal section, and under the consideration the thickness of myelin sheath, these results show that lots of thin and long regenerated nerve fiber was concentrated in distal. Comparing experimental groups to control group, in midpoint section large difference exits but such differences diminish in distal section, so these results express the application of nerve conduit for the repair of long damaged nerve. From the histological view, we can conclude that damaged nerve can be recovered through nerve conduit from proximal to distal.

Conclusions

In this study, sodium alginate and type I collagen (all natural polymers) as base materials were used for the manufacturing nerve conduit through previously mentioned. By animal test using rat, damaged nerve tissue regeneration was evaluated and we could get some results of the nerve regeneration which was convinced with obvious observation of reconnected nerve between distal and proximal site in the exposure after 2 months later of surgery and bioabsorption of polymeric conduit materials was also observed after 2 months of surgery. By the histological evaluation, myelinated nerve fibers were observed in distal and proximal sections.

Although the area of fasciculus, density of axon and thickness of myelin sheath of harvest nerve were not sufficient to those of control (autograft), axon regeneration induced the recovery of nerve connectivity.

Nerve damage gives to human a fatal lesion and the therapeutic methods for nerve disease and damage was not established except autotransplantation till now. To overcome such problems, nerve regeneration through nerve conduit shows good latent possibilities. In near future, combining such nerve conduit with nerve growth factors which activate the proliferation of nerve cell will offer very effective therapeutic tool for the various inveterate nerve disease.

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