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Evaluation of the effectiveness of biodegradable electrospun caprolactone and poly(lactic acid-ε-caprolactone) nerve conduits for peripheral nerve regenerations in a rat sciatic nerve defect model

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Background/aim: The aim of this study was to compare electrospun caprolactone (EC) and poly(lactic acid-ε-caprolactone) (PLCL) nerve conduits with nerve graft in a rat sciatic nerve defect model.

Materials and methods: A total of 32 male Wistar albino rats were divided into 4 groups, with 8 rats in each group. A nerve defect of 1 cm was constructed in the left sciatic nerve of the rats. These defects were left denuded in the sham group, and reconstructed with nerve grafts, PLCL, and EC nerve conduits in the other groups. After 3 months, nerve regenerations were evaluated macroscopically, microscopically, and electrophysiologically. The numbers of myelinated axons in the cross-sections of the nerves were compared between the groups.

Results: Macroscopically, all nerve coaptations were intact and biodegradation was detected in nerve conduits. Electromyographic assessment and count of myelinated axons in the cross-sections of the nerves displayed the best regeneration in the nerve graft group ($P < 0.001$) and similar results were obtained in the PLCL and EC nerve conduit groups ($P = 0.79$). Light and electron microscopy studies demonstrated nerve regeneration in both nerve conduit groups.

Conclusion: EC nerve conduits and PLCL nerve conduits yielded similar results and may be alternatives to nerve grafts as they biodegrade.

Key words: Nerve regeneration, nerve graft, electrospun caprolactone nerve conduits, poly(DL-lactide-ε-caprolactone) nerve conduits

1. Introduction

Primary repair without stretching is the gold standard in peripheral nervous system injuries. Nerve autografting is the most common surgical procedure to repair nerve defects when primary repair is not possible. However, comorbidity in the donor area and the usually limited size of available tissue limit its usage. For this reason, nerve conduits could be used to bridge the gap when an injured nerve could not be primarily repaired in peripheral nerve system injuries (1).

Nerve conduits can be obtained from autogenous and nonautogenous materials. Limited success is reported in the literature with autogenous materials such as vein, artery, muscle fiber, tendon, and fascia, except for the autogenous nerve. Nonabsorbable synthetic materials such as silicone, Gore-Tex, polytetrafluoroethylene (ePTFE), or conduits composed of nylon tubes provide good lumen support, but long term results are poorly related to foreign body reaction against these materials. Lately, the usage of nerve conduits produced from biodegradable materials has become widespread because they degrade following reconstruction. Various natural purified extracellular matrix (ECM) components such as collagen and fibronectin; synthetic polymers such as polyglycolic acid (PGA), poly(ε-caprolactone) (PCL), and poly(DL-lactide-ε caprolactone) (PLCL); and chitosan,

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which is a polysaccharide obtained from N-deacetylation of chitin, are used to produce commercial conduits for use in clinical settings (1–15).

The electrospinning process is a method of polymeric filament production by using electrostatic forces. The electrospinning process enables the production of large amounts of nanofibers from all polymers in a simple, quick, and continuous way. The surface to volume ratio of caprolactone fibers produced by the electrospinning method is 1000 times greater than standard caprolactone fibers, which enables softer consistency, increased controlled porosity, high permeability, and early resorption (16–18).

In the present study, we compared electrospun caprolactone (EC) nerve conduits with PLCL nerve conduits and autogenous nerve grafts in a rat sciatic nerve defect model. To the best of our knowledge, there are no previous reports in the literature describing the production and usage of EC as nerve conduits and the comparison of EC nerve conduits with PLCL nerve conduits and nerve grafts.

2. Materials and methods

All the procedures adopted in the present study were approved by the Institutional Animal Care and Use Committee and conformed to the Helsinki Declaration. A total of 32 male Wistar albino rats weighing 200–280 g were used in the study.

2.1. Membrane preparation

The details of polymer synthesis and the preparation and properties of the electrospun caprolactone membranes were similar to those described by Bölgen et al. (16,17). In short, to produce an electrospun caprolactone sheath, a mixture of polycaprolactone, chloroform, and dimethylformamide is exposed to jet current, and electrospun caprolactone membrane films form in a lamellar shape as the solvent evaporates during the jet current (16–18) (Figure 1a).

2.2. Electrospun caprolactone conduit preparation

The conduits were prepared from electrospun caprolactone sheets. Unidirectional fiber oriented sheets (8 × 14 mm) were rolled around a 16-cannula to form a 14-mm-long tube with an internal diameter of 1.6 mm (16 G Slicone Branul, Abbott, Ireland). The rolled sheets were attached longitudinally with cyanoacrylate glue (Histoacry, Braun, Aesculap, Germany) (19–21) (Figure 1b).

2.3. Surgical procedure

All animals underwent surgery on 2 consecutive days. They received anesthesia with 50 mg/kg intraperitoneal ketamine (Ketalar 2%, Pfizer, İstanbul, Turkey) and 10 mg/kg xylazine (Rompun, Bayer, İstanbul, Turkey) injections. On the second day of surgeries, only intraperitoneal ketamine (50 mg/kg) was applied to prevent a disturbance in the electromyographic measurements. The right limbs of the rats were trimmed and cleaned with iodine solution (Batikon, Adeka, Samsun, Turkey). Under an operating microscope (Carl Zeiss, f170 OPMI Pico, Aalen, Germany) the right sciatic nerve was exposed via a gluteal muscle splitting incision. All of the surgical procedures were performed by the same surgeon (MD) using a sterile microsurgical technique with 10× magnification. The left sciatic nerves were left untouched. The nerve defect was produced by cutting out a 10-mm segment of the sciatic nerve from 0.5 cm distal to the sciatic notch (Figure 2a).

Four groups with 8 rats in each group were created. Group 1 was the sham group; a 1-cm sciatic nerve segment was resected without any repair. Group 2 was the nerve graft group; a 1-cm sciatic nerve segment was resected, reversed, and resutured in the gap with 8/0 nylon sutures (Ethicon, Johnson and Johnson Intl., Diegem, Belgium) (Figure 2b). In Group 3 the defect was repaired with a PLCL nerve conduit (Nerolac Polyganics, Groningen, the Netherlands). The gap was bridged using a 14-mm

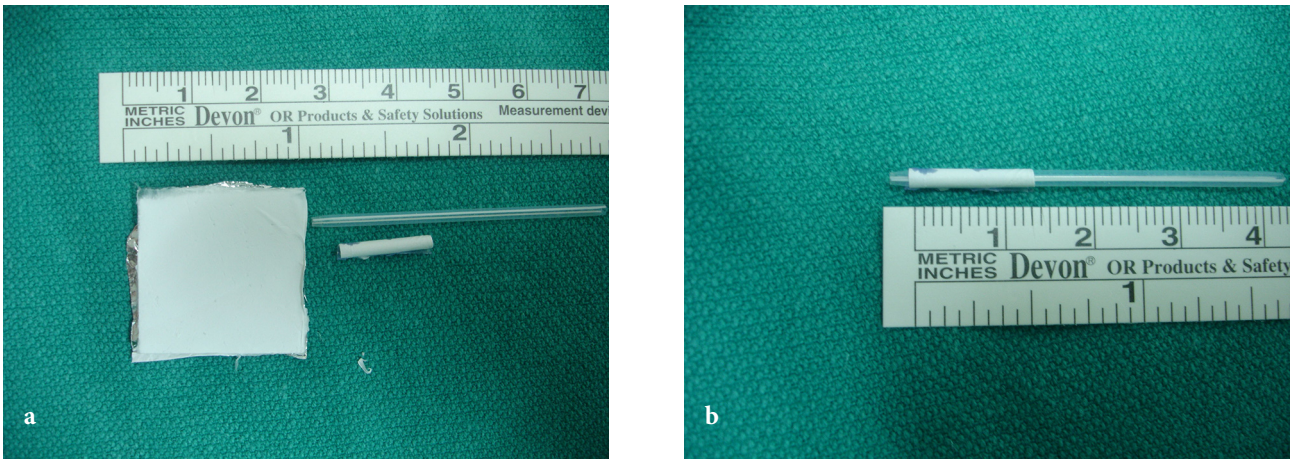

Figure 1. Electrospun caprolactone membranes (a) and conduit preparation (b).

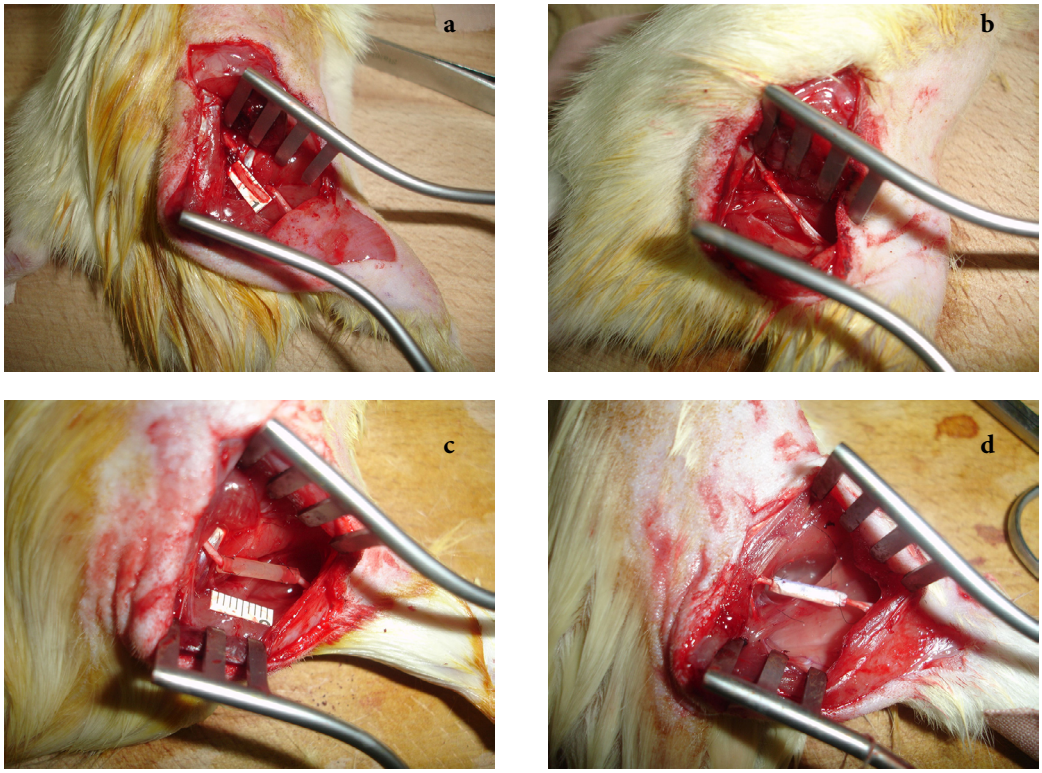

Figure 2. a) Creation of sciatic nerve defect; b) repair with autogenous nerve graft; c) repair with PLCL nerve conduit; d) repair with EC nerve conduit.

PLCL conduit, entubulating 2 mm of the nerve stump at each end. Four 8/0 nylon sutures (Ethicon, Johnson and Johnson Intl.) were used to anchor the conduit to the epineurium at each end (Figure 2c). Group 4 was the EC nerve conduit group; the defect was repaired with an EC nerve conduit. The gap was bridged using a 14-mm EC conduit, entubulating 2 mm of the nerve stump at each end. Four 8/0 nylon sutures (Ethicon, Johnson and Johnson Intl.) were used to anchor the conduit to the epineurium at each end (Figure 2d).

The lumens of the conduits were empty. In all groups, muscle and skin incisions were closed with 5/0 polyglactin and 3/0 polypropylene sutures, respectively.

2.4. Assessment of regeneration

All rats were evaluated for nerve regeneration at postoperative week 12 with macroscopic, microscopic, and electromyographic methods.

2.4.1. Macroscopic assessment

Atrophy of leg flexor muscles and dominance of dorsiflexion in all rats were evaluated by comparing them with their counterparts in the contralateral legs prior to the application of anesthesia.

Moreover, the presence of wound infections and/or pressure ulcers was evaluated for each rat. The right sciatic nerve was reexposed and the conduit was carefully dissected from surrounding tissues, observing its macroscopic appearance, adhesion of the sciatic nerve trace, and nerve conduit condition.

2.4.2. Electromyographic assessment

Electromyography was performed on all animals at postoperative week 12 before their sacrifice. The sciatic nerve was exposed at the sciatic notch proximal to the regenerated nerve, under anesthesia. A conventional electromyography device (Medelec Synergy, Surrey, UK) was used for electromyographic measurements and records. A recording needle electrode was placed in the gastrocnemius soleus muscle group at 1 cm distal to the tibial tubercle. An electrode with a hook shaped tip was used for nerve stimulation by elevating the nerve up to 2–3 mm. The 25–35 mA current given proximal to the coaptation line was compared with the evoked compound muscle action potential (CMAP) and the area under the CMAP curve was analyzed with statistical methods.

2.4.3. Microscopic assessment

After completion of the electromyographic assessment, 0.5-cm samples were excised from the middle to the distal part of the neural conduits and nerve grafts. Nerve structure, nerve regeneration, myelin structure, arrangement of axons, and nerve guide channels were assessed with a light microscope. Sections were stained with hematoxylin–

eosin and Kluver–Barrera stains. The Kluver–Barrera method was used for staining of myelin and nerve cells. With this method myelin and phospholipids were stained in the spectrum of blue to green and cell nuclei were stained purple. Additionally, the samples were coated with 100 Å thickness and evaluated by a SEM ASID-10 scanning electron microscope with an acceleration voltage of 80 kV and photographs were recorded.

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). CMAP and the areas under the CMAP curves were compared between groups with Kruskal–Wallis tests and binary comparisons were made by the Conover test. Myelinated fiber counts were compared between groups with Kruskal–Wallis tests and binary comparisons were made by Mann–Whitney U tests with Bonferroni corrections. $P < 0.05$ was considered significant.

3. Results

All the animals survived surgery.

3.1. Macroscopic assessment

The healing of the surgical wounds was good and no clinical signs of infection or pressure wounds were found in the operated legs. Atrophy of leg flexor muscles, dominance of dorsiflexion, and adhesions in the sciatic nerve trace were evaluated by comparing them with those in the contralateral legs for all rats prior to the application of anesthesia.

Flexor muscle atrophy and dominance of dorsiflexion was high in Groups 3 and 4, but minimal in Group 2. The atrophy was highest in the sham group and the rats in this group could not use their legs.

The right sciatic nerve was reexposed and the conduit was carefully dissected from surrounding tissues, observing its macroscopic appearance. In Groups 3 and 4, the conduits were not totally degraded and retained their integrity to a large extent. In Group 2, adhesions were minimal, the nerve could be easily dissected from the surrounding tissues, and none of the cases had neuroma in the coaptation lines (Figure 3a). Severe fibrotic reaction and adhesions were observed around the conduits (Figures 3b and 3c). In Group 1, adhesions were minimal and neuromas were formed in the proximal nerve stumps.

3.2. Electromyographic assessment

CMAP values and the areas under the CMAP curves obtained by electromyography were measured for all rats (Table 1). The results were compared between the groups. In the nerve graft group (Group 2), nerve regeneration was better when compared with the conduit groups (Groups

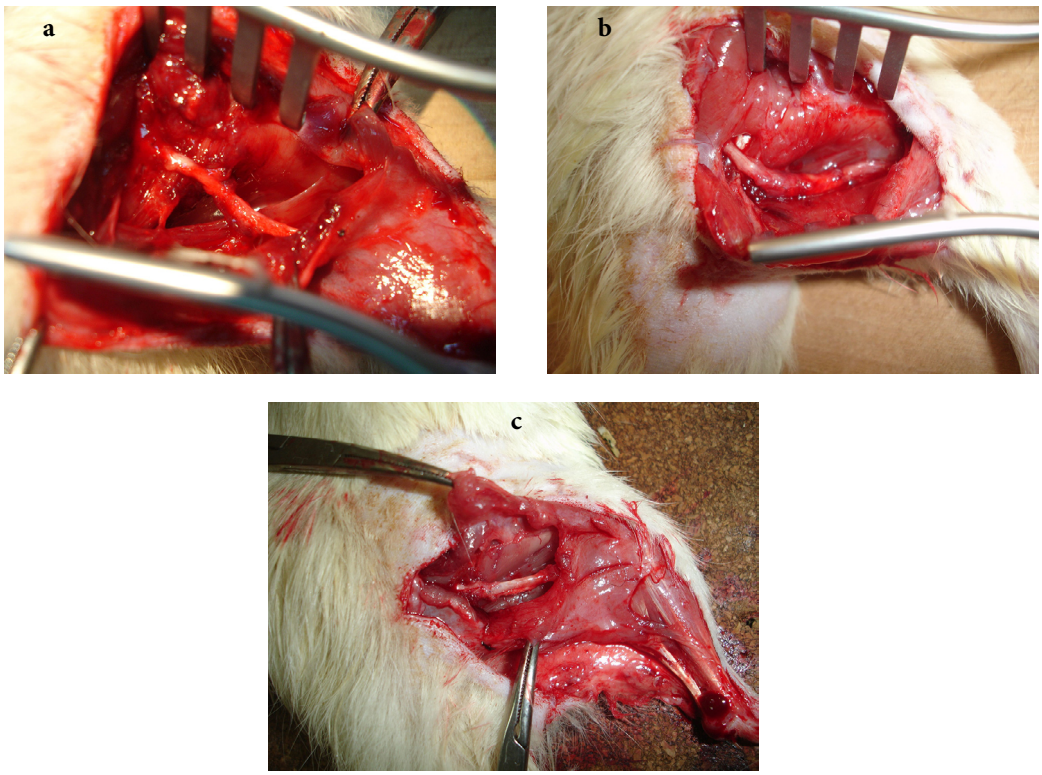

Figure 3. Macroscopic appearances at postoperative week 12: a) nerve graft group; b) PLCL nerve conduit group; c) EC nerve conduit group.

Table 1. Electromyographic assessment.

Rats	CMAP* (ms/mV)					CMAP area (m/mV)				
	Normal	Group 1	Group 2	Group 3	Group 4	Normal	Group 1	Group 2	Group 3	Group 4
1	33.6	0	5	3.1	2.6	51.4	0	11.3	6.7	3.5
2	27.3	0	7.1	2.8	3.9	45	0	15	5.9	7.5
3	35.5	0	9.3	2.6	1.3	53.2	0	15.5	3.2	2.5
4	25	0	9.8	2.5	2.5	39.4	0	15.5	3.4	3.2
5	34	0	8.5	1.5	1.7	49.8	0	13.1	3.6	2.9
6	36.5	0	6.2	1.1	2.8	54.6	0	11.4	2.9	5.7
7	22	0	12.4	2.3	1.1	31.9	0	22	3	2.7
8	32.2	0	8.1	1.8	2.3	48.5	0	12.4	3.1	3.5
Average	30.76	0	8.3	2.21	2.275	46.72	0	14.52	3.97	3.94

*CMAP: compound muscle action potential.

3 and 4) ($P < 0.05$). There were no statistically significant differences between Group 3 and Group 4 ($P < 0.05$).

3.3. Microscopic assessment

No nerve regeneration was observed in Group 1 by light microscopy. Fibroadipose tissue was observed in the sections. In Group 2, there was extensive nerve regeneration inside the perineurium (Figure 4a). Signs of nerve regeneration were observed in addition to extensive foreign body reactions around the PLCL nerve conduit in Group 3 (Figure 4b). There were multinuclear giant cells near the inner surface of nerve conduits and the thickness of conduits changed in different sections. In the EC nerve conduit group (Group 4), the signs of nerve regeneration and foreign body reactions were similar to Group 3, but the conduit did not lose its integrity (Figure 4c). Foreign body reactions, which were absent in Group 2, were intensively observed in Groups 3 and 4.

When the numbers of myelinated axons in the cross-sections of nerves were compared between the groups, there were significant differences between Group 2 and Groups 3 and 4 ($P < 0.001$). There was no significant difference between Group 3 and Group 4 ($P = 0.79$) (Table 2) (Figure 5).

In electron microscopic evaluation, the highest count of myelinated nerves per unit area was in the nerve graft group. Thick myelin structures and axons wrapped by them were observed in this group (Figure 6a). In Groups 3 and 4, the counts of myelinated nerves were low and they had smaller diameters and thinner walls (Figures 6b and 6c).

4. Discussion

The standard technique for bridging a peripheral nerve defect is repairing it with an autologous nerve graft if the nerve stumps can be sutured together without tension. In the reconstruction phase, the best results are expected by repairing with an identical tissue. Alternative graft materials are vein grafts, degradable and nondegradable tubes, vein grafts filled with various materials and tissues, and growth factors. Silicone has been the most widely used material for experimental tubulization and has also been applied clinically. However, due to the lack of degradation of the silicone implants, it has been advocated that the next step in nerve gap repair should be the use of a bioabsorbable synthetic conduit that would elicit most of the inflammatory reactions and would remain in situ long enough to support regeneration. There is still a need for materials that are biodegradable, sterile, and capable of controlled drug release when needed; have nontoxic biodegradation materials; and lead to minimal reactions in order to be used in nerve repair (1,5,22,23).

The first nerve conduits made from biodegradable materials were produced by Mackinnon and Dellon (8) from polylactic and polyglycolic acids, which are poly(α-hydroxyl acids). By copolymerization, the high penetration feature of polycaprolactone unites with the good degradation property of polylactide, forming a commonly used combination in nerve guide channels. PLCL conduits have a widespread use in clinics. Studies reporting favorable effects of this combination on nerve regeneration are present in the literature (11,13,14). Den Dunnen et al. (14) reported better results by PLCL conduits when compared with nerve grafts, but it is widely accepted that nerve graft is the most successful method for bridging nerve defects.

Electrospun nonwoven materials have a small size, high porosity, and high surface area, which enhance their use in a wide variety of biomedical applications, such as scaffolds in tissue engineering (16–18,24–26). Yoshimoto et al. (24) used an electrospinning process to

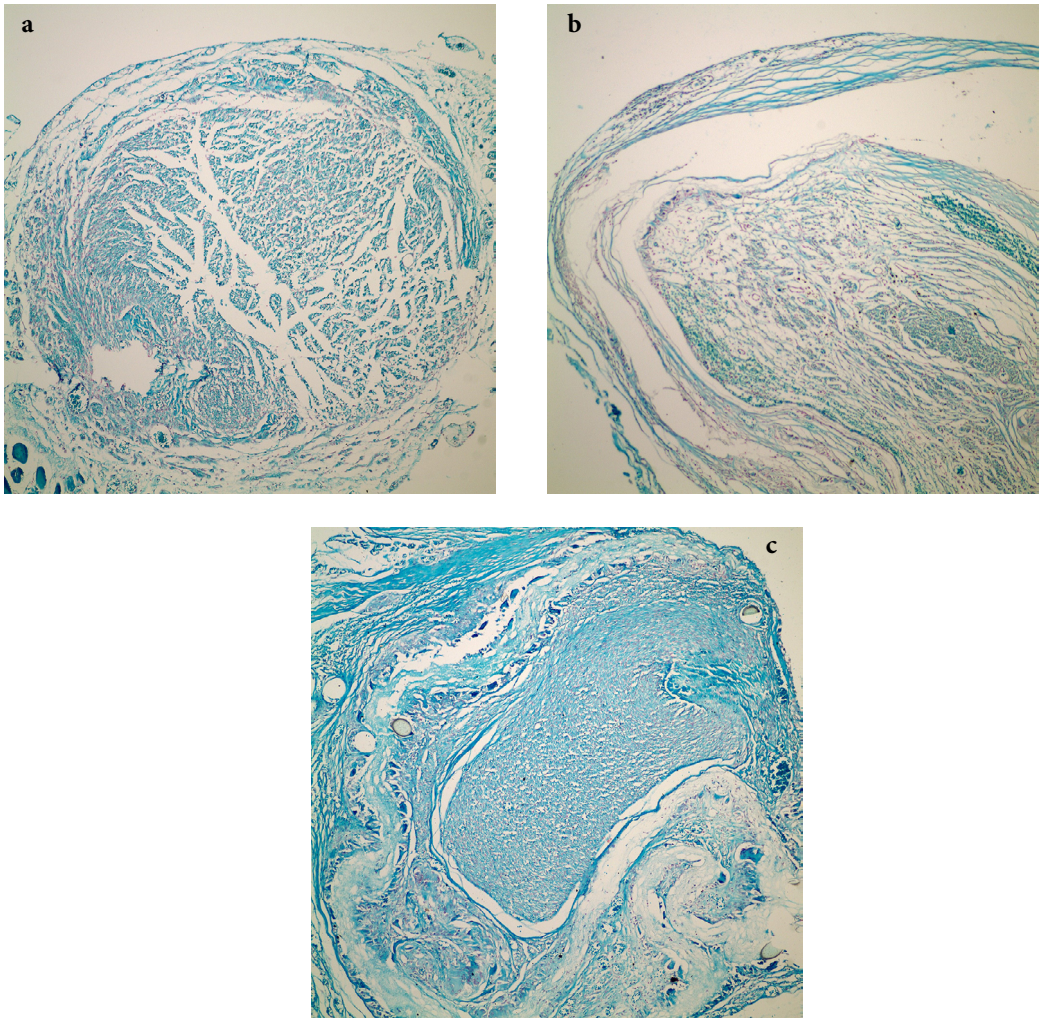

Figure 4. Microscopic photomicrographs of transverse sections through the peripheral nerves of all groups (Kluver–Barrera method, magnification 40×): a) nerve graft group; b) PLCL nerve conduit; c) EC nerve conduit.

Table 2. Myelinated fiber count.

Rats	Group 1	Group 2	Group 3	Group 4
1	0	5854	5113	4455
2	0	7821	3645	6369
3	0	9281	3303	1815
4	0	8745	3215	3514
5	0	8473	2254	2254
6	0	6536	1612	3991
7	0	10,284	3109	1748
8	0	7819	2435	2945
Average	0	8101 (8147*)	3085 (3162*)	3386 (3229*)

*Median count.

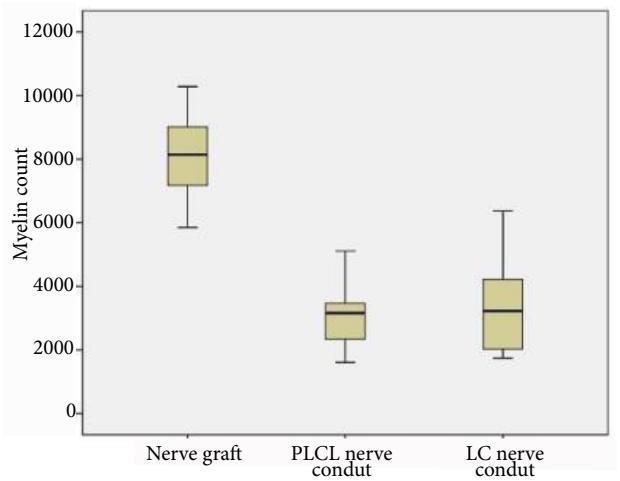

Figure 5. Myelinated fiber counts.

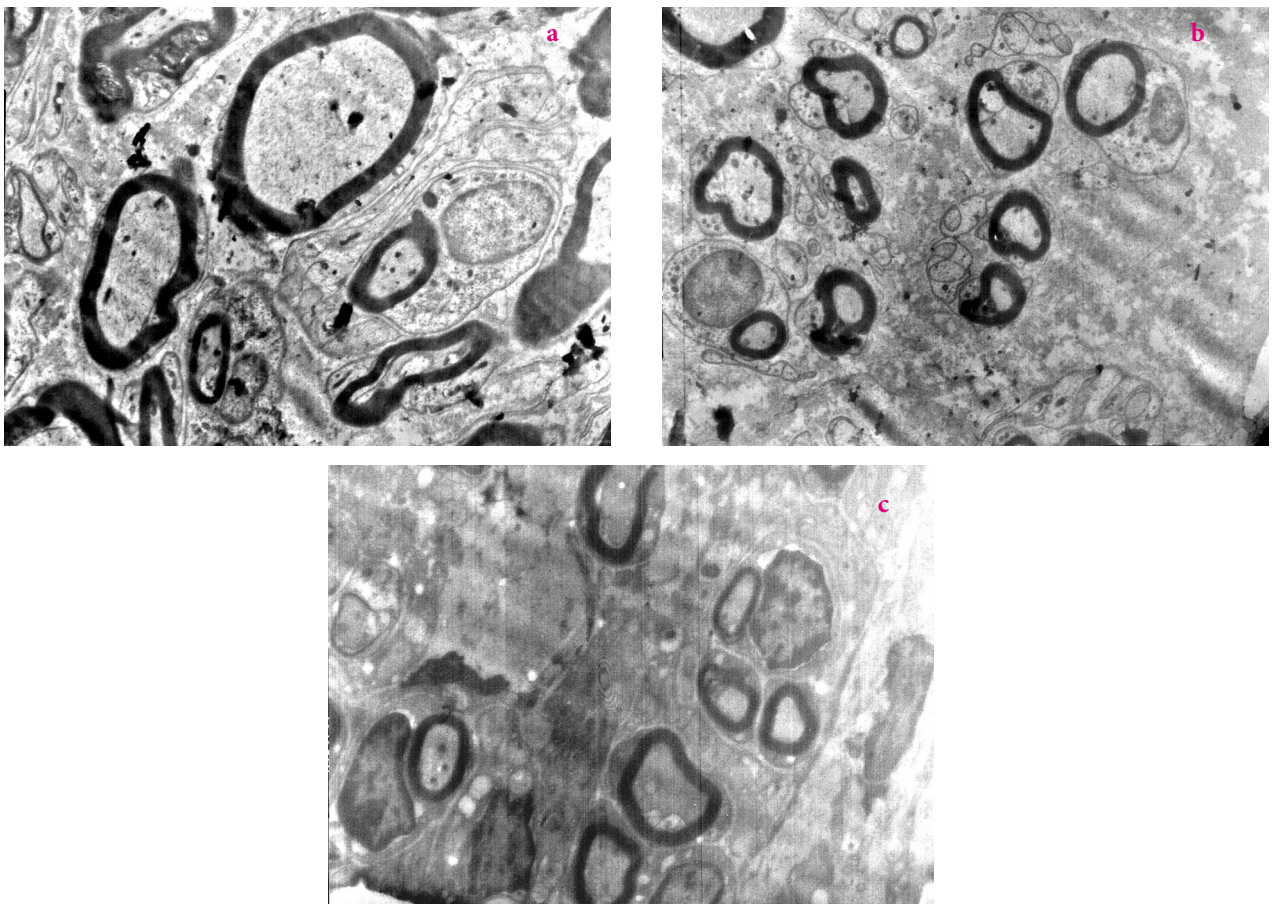

Figure 6. Electron microscopic appearance (SEM ASID-10 scanning electron microscope, magnification 5000×): a) nerve graft group; b) PLCL nerve conduit; c) EC nerve conduit.

prepare PCL based microporous nonwoven scaffolds and cultured mesenchymal stem cells derived from the bone marrow of neonatal rats on these electrospun scaffolds in bioreactors; they suggested that these scaffolds may be potential candidates for bone tissue engineering. Kim et al. (25) described the use of electrospun caprolactone in

tissue engineering for scaffolds and characterized their morphology, crystallinity, and mechanical properties with different techniques. Shao et al. (26) suggested that the PCL nanofibrous structure may be a suitable candidate scaffold for cartilage tissue engineering.

Bölgen et al. (16) explained in detail the properties of poly(ε-caprolactone) nanofibers prepared by an electrospinning process under different conditions and their in vitro and in vivo degradations in their study. They found that the fiber diameter significantly influenced both in vitro degradation (performed in ringer solution) and in vivo biodegradation (conducted in rats), and the in vivo degradation was found to be faster than the in vitro one. As electrospun membranes were more hydrophobic than PCL solvent-casted ones, their degradation was much slower. Bölgen et al. (17) prepared nonwoven materials from poly(ε-caprolactone) PCL and their antibiotic containing forms by electrospinning, so as to prevent postsurgery induced abdominal adhesions in rats. The antibiotic (metronidazole) embedded membranes significantly eliminated postsurgery abdominal adhesions and improved healing. Intense porosity is favorable for controlled drug elimination. This property permits installing growth factors and neurotrophins into nerve conduits.

The favorable effect of permeable tubes may be related to the metabolic exchange across the tube wall, the diffusion of growth promoting factors generated in the external environment into the guide lumen, and the retention of trophic factors secreted by the nerve stumps. Hence, tube wall pores and their stability over time seem to be important factors determining the flow of different constituents that may promote or inhibit regeneration (1,27,28). We thought that electrospun caprolactone conduits would affect nerve regeneration positively because the small and controlled pore structure related to their nanofiber constitution permitted more diffusion and was suitable for cell penetration from outside. Their soft consistency facilitated bringing them into the tube shape and we had no problems in the suturing process.

The PLCL conduits used in our study had to be softened by keeping them in hot water before the surgery. The stiffness of these conduits made the suturing process difficult by microsurgical methods with 8/0 sutures. Needle deformation led to suture loss and increased the cost. Another important disadvantage was the cooling and stiffening of the conduits during surgery. In EC conduits there was no need for warming in hot water, and we had no problems in the suturing process due to their high porosity and soft consistency (11,13,14).

Wetting and preserving tube formation and lumen aperture inside the tissue are important properties of conduits. Wall thickness is another important factor: if the conduit wall is too thick, it would degrade slowly, thus lengthening the time of possible foreign body reactions; and if the wall is too thin, it would degrade too quickly, resulting in the loss of supportive structures. Usually, a conduit with a 1.5-mm internal diameter and a 0.3-mm-thick wall gives optimal results in peripheral nerve regeneration (1,8–14). In the EC conduits, the tube formation was preserved intraoperatively and in the histologic sections 3 months after the operation it was seen that nerve fibers passed through it. There were no problems with tube formation because of the rigid consistency of PLCL conduits, whose transparent structure allowed the appearance of nerve stumps inside the lumen. The absence of lumen collapse was an important advantage of the bioartificial nerve grafts when compared with vein grafts. Moreover, it was thought that its nanofiber structure would enable resorption in the early postoperative period. We observed intense fibrosis secondary to foreign body reaction in the EC and PLCL nerve conduits at postoperative week 12. We also observed that both conduits had preserved their integrity and had different areas of resorption in histologic sections in the microscopic evaluation. The giant cells observed in the histologic sections indicated the continuation of degradation and there was no difference in foreign body reactions between the two nerve conduit groups. There was no foreign body reaction in the nerve graft group, and the graft preserved its integrity, including the perineurium. We wanted to research the innovations that nanofiber structures bring to nerve conduit production, usage, and functional results. In our study, we found similar effects of both PCLC and EC conduits on nerve regeneration and there were no significant differences between them.

In our study, atrophy in the leg flexor muscles and dominance of dorsiflexion were more prevalent in the nerve conduit groups when compared with the nerve graft group. The results of EMG measurements and myelinated nerve fiber counts were correlated with the macroscopic findings. The best results of Cmap and Cmap area measurements and myelinated nerve fiber counts were obtained in the nerve graft group, which is in accordance with the literature. There were no significant differences between PCLC and EC nerve conduits, but both nerve conduit groups had lower nerve regeneration when compared with the nerve graft group. To the best of our knowledge, there are no previous reports in the literature describing the production and usage of EC as nerve conduits.

The most important properties of an ideal nerve guide channel include not only biodegradation or high permeability, but also the controlled release of growth factors and neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3-4-6, ciliary neurotrophic factor, leukemia inhibitory

factor, glial cell derived neurotrophic factor, insulin-like growth factor 1, and platelet-derived growth factor (PDGF)). There are studies in the literature reporting a positive impact of incorporating support cells and lining of the interior part of nerve guide channels with Schwann cells on nerve regeneration. In this way Schwann cells may provide support and have a positive impact on nerve regeneration. Moreover, electrical activity and nerve substratum oriented inside the intraluminal channels have been shown to have favorable effects in nerve regeneration (1,28–43).

The increased understanding of the underlying mechanisms of peripheral nerve regeneration will allow the development of better nerve conduits with integrated growth factor delivery systems and/or cellular components. The potential to release growth or trophic factors, and to improve the outgrowth of axons after nerve injury, is an area in which considerable development will be expected. The combination of two or more growth factors or neurotrophins will likely exert synergistic effects and the combination of Schwann cells with growth factors may further improve nerve regeneration. Despite multiple studies on the utilization of growth-promoting factors inside the lumens of nerve conduits, they have not been introduced in clinical practice yet.

In conclusion, although EC conduits had advantages such as controlled porosity, pliability, rapid degradation, and compatibility with surgery, we observed no significant differences in nerve regeneration when compared with PCLC conduits. However, the EC material, which is composed of nanosized particles that provide appropriate pore width for controlled drug release and are convenient for culturing, makes EC conduits suitable for further studies.

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