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Original Article

Facial nerve regeneration with bioabsorbable collagen conduits filled with collagen filaments: An experimental study

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A R T I C L E   I N F O

A B S T R A C T

Introduction: A bioabsorbable collagen conduit (Renerve™) filled with collagen filaments is currently approved as an artificial nerve conduit in Japan and is mainly used for connecting and repairing peripheral nerves after traumatic nerve injury. However, there are few reports on its applications for reconstructing and repairing the facial nerve. The present study evaluated the efficacy of the conduit on promoting nerve regeneration in a murine model with a nerve defect at the buccal branch of the facial nerve.
Methods: Under inhalational anesthesia and microscopic guidance, the buccal branch of the left facial nerve in an 8-week-old Lewis rat was exposed, and a 7 mm gap was created in the nerve. The gap was then connected with either the nerve conduits (NC group) or an autologous nerve graft (the autograft group). At 13 weeks after the procedure, we compared the histological and physiological regenerations in the both groups.
Results: We found compound muscle action potential amplitude is significantly larger in the autograft group (2.8 ± 1.4 mV) than in NC group (1.3 ± 0.5 mV) ($p < 0.05$). The number of myelinated fibers of the autograft group was higher (3634 ± 1645) than that of NC group (1112 ± 490) ($p < 0.01$). The fiber diameter of the autograft group (4.8 ± 1.9 μm) was larger than that of NC group (3.8 ± 1.4 μm) ($p < 0.05$). The myelin thickness of the autograft group was thicker than that of NC group (0.6 ± 0.3 μm vs. 0.4 ± 0.1 μm) ($p < 0.05$). G-ratio of the autograft group (0.74 ± 0.19) was lower than that of NC group (0.79 ± 0.10) ($p < 0.05$).
Conclusion: This study demonstrated the efficacy of collagen nerve conduit for facial nerve reconstruction following nerve injury. However, the effectiveness of the conduit on the promotion of nerve regeneration was inferior to that of the autograft.

1. Introduction

Facial nerve palsy can be divided into facial paralysis and paresis [1]. Facial paralysis appears after (1) the surgical resection of malignant head and neck tumors such as parotid gland malignancy and (2) facial trauma, and the paralysis also gives a facial asymmetry, which is due to the paralysis of facial mimetic muscles, and hypersalivation during food intake, which significantly reduce the patients' social activities and quality of life (QOL) [2]. In facial paresis, Waller degeneration appears in the axons distal to the site of injury, followed by remyelination along the Schwann tube starting from the proximal side, which is expected to lead to nerve regeneration [3]. The treatment of facial paralysis, however, requires various surgical reconstructive techniques such as nerve reconstruction with nerve grafts and dynamic reconstruction by free tissue transfer [4]. Although autologous nerve grafting procedure, where sensory nerves such as the sural nerve and the greater auricular nerve are harvested and transplanted into the defect, has been used as a nerve reconstruction technique [5], the procedure also gives unavoidable and significant complications

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such as sensory disturbance and scar formation caused by the nerve harvesting [5,6]. For solving these problems, Lundborg et al. report an "artificial nerve grafting" technique connecting two separated nerves in a silicone artificial conduit [7]. Currently, for replacing non-bioabsorbable materials such as silicone, artificial nerve conduits made of absorbable materials are used in clinical practice, and their efficacy is reported extensively, mainly in the treatment of sensory neuropathies such as traumatic digital nerve defects [8].

As absorbable materials for the outer-layer of artificial nerve conduit, polyvinylidene difluoride (PVDF), polyglactin mesh, Gore-Tex, arterial cuffs, mesothelial tubes, collagen, various synthetic polyesters such as polylactic acid (PLA), polyglycolic acid (PGA), and *poly*-L-lactic acid (PLLA) are used [3]. As scaffolding materials, proteins, collagen, gelatin, fibronectin, keratin, and silk fibroin are also used [9]. Among these materials, collagen is known to be one of the suitable bioabsorbable materials for nerve conduits in terms of biocompatibility. In clinical practice, collagen nerve conduits are mainly used for treating sensory nerve injuries and reported to provide good outcomes as long as the length of the defect less than does 30 mm [10].

To date, no clinical or basic research investigate the efficacy of collagen artificial nerve conduit for treating the facial nerve injuries. In this study, the efficacy of collagen nerve conduit on the regeneration of the facial nerve was investigated by implanting the conduit into an experimental defect in the buccal branch of the facial nerve in rats, observing the facial nerve regeneration histologically, and comparing the physiological characteristic functions of regenerated nerves in the conduit and those of transplanted autograft nerves.

2. Materials and methods

All animal care and handling procedures were performed in accordance with the Principles of Laboratory Animal Care of Tokyo Women's Medical University Animal Experimentation Committee. Eight-week-old male syngeneic Lewis rats (body weight: 200–250 g) (n = 20) were purchased from Charles River Laboratories (Yokohama, Japan).

2.1. Nerve conduits

Bioabsorbable collagen conduits filled with collagen filaments (Renerve®) (Nipro, Osaka, Japan) used in this study are commercially available and approved as a medical device for clinical practice by Japanese Government since 2016. The tube-wall of the conduit was made by wrapping collagen fibers around a metal mandrel, which was removed before the next process. Enzymatically solubilized collagen (a mixture of collagen type I and III) was dissolved in water to prepare an aqueous solution, which was extruded into a coagulating liquid for making collagen filaments for the lumen of the conduit, resulting in collagen fibers having a longitudinal alignment. Prepared conduits having an inner diameter of 1 mm and a length of 10 mm were frozen and then lyophilized, resulting in dry conduits containing 10v/v% collagen filaments (Fig. 1A and B). Due to the longitudinally aligned collagen fibers in the lumen, the strength and flexibility of the prepared conduits are known to be superior to those of conventional artificial conduits. The conduits were sterilized with gamma-ray irradiation (Fig. 1C and D). The conduits passed several safety tests including genotoxicity, carcinogenicity, reproductive toxicity tests (ISO10993-3), in vitro tests for cytotoxicity (ISO10993- 5), effect after implantation test (ISO10993-6), test for irritation and skin sensitization (ISO10993-10), and systemic toxicity.

2.2. Surgical procedure and experimental design

Rats (n = 20) were anesthetized with 4% isoflurane through a nasal mask attached to a Univentor 400 Anesthesia Unit (Narcobit-E) (Natsume Seisakusho, Tokyo). A preauricular incision with a marginal mandibular extension was made on the left side of the face for exposing the buccal and marginal mandibular branches of the facial nerve and parotid gland (Fig. 2A and B). The marginal
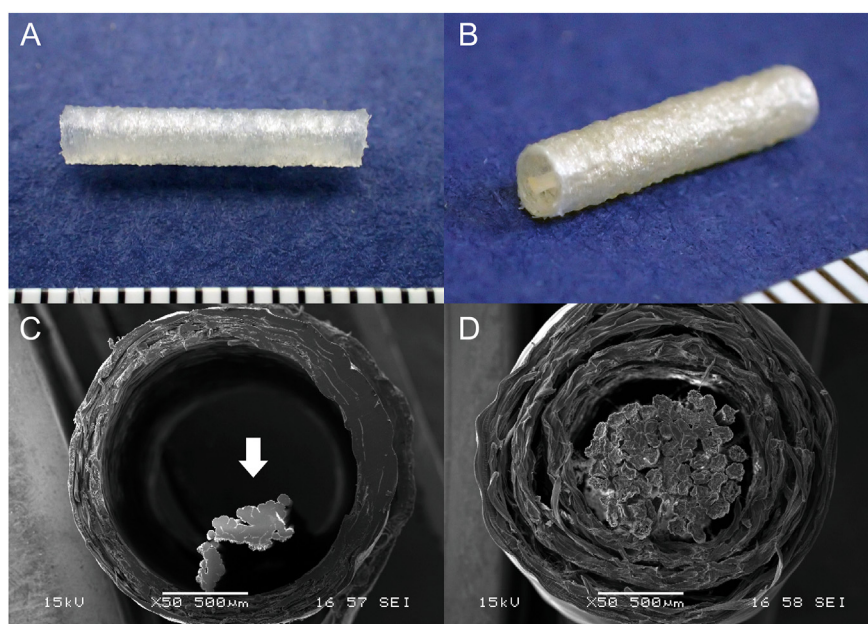

Fig. 1. A collagen artificial nerve conduit used in this study. Photograph A shows the overall view of a collagen artificial nerve conduit filled with collagen filaments. The zebra patter in the bottom of photograph shows the part of scale, and the distance between tick marks indicates 1 mm. Photograph B shows the upper-left view of the conduit. Electron microphotograph C and D show the cross-sectional view of the conduit before and after collagen filaments were filled in the lumen, respectively. In photograph C, the white arrow indicates dry collagen filaments, which swell after being immersed in physiological saline. In photograph D, the outer cylinder also swells, and the lumen is filled with the swollen filaments. Scale bars in photograph C and D represent 500 μm.
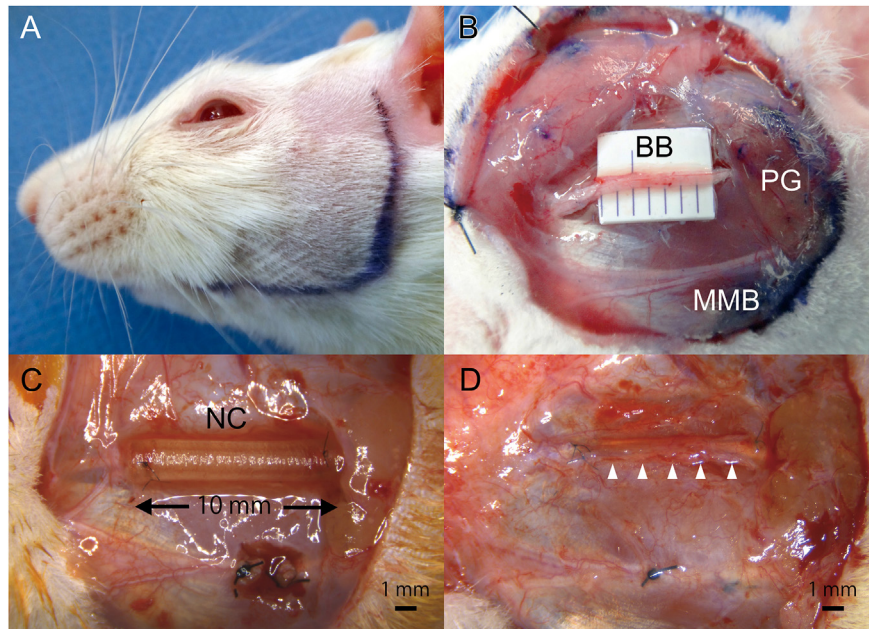
Fig. 2. Creating procedure of facial nerve defect in a rat and the implanting procedure of an artificial nerve conduit (A) An anterior auricular incision was made to expose the buccal branch of the facial nerve and the parotid gland (B) The photograph shows the expanded view of surgical site. BB indicates the buccal branch of facial nerve; MMB, the marginal mandibular branch of the facial nerve; PG, the parotid gland (C) A 10-mm artificial nerve conduit (NC) was implanted into a 7-mm defect of the buccal branch and fixed with a two-point mattress suture (D) At 13 weeks after implantation, the regenerated nerve was exposed, and the middle portion of the regenerated nerve marked with the white arrow-heads was excised. The zebra patterns in the bottoms of photograph C and D show the parts of scales, and the distance between tick markers indicate 1 mm.

mandibular branch was transected with microsurgical scissors (MB-51-10) (Natsume Seisakusho) and ligated with 7–0 nylon sutures (Ethilon®, Ethicon) (Johnson & Johnson, New Brunswick, NJ, USA). A 7-mm defect was made in the buccal branch because of rat face's physical limitation. The excised portion of the left facial nerve was then re-sutured after the inversion of distal and proximal portions as an autologous graft (n = 10) (the autograft group) with epineural sutures with 9–0 nylon sutures (Ethilon). A collagen nerve conduit having a length of 10 mm and an inner diameter of 1 mm was flushed with physiological saline for 10 min before grafting. The nerve defects were individually connected by the nerve conduits (NC group, n = 10), which were secured in place by the two-point mattress suture method reported previously (Fig. 2C). A 1-mm nerve stump was then inserted into the conduit by pulling both sutures. Surgery was performed with a microscope (M60) (Leica Microsystems, Wetzlar, Germany). At 13 weeks after implantation, the conduit was carefully excised with the nerve stump on the graft bed (Fig. 2D). A 13-week period yielded sufficient neural regeneration, allowing the physiological functions of regenerated nerve in the conduit to be evaluated by compound muscle action potential (CMAP) measurement.

2.3. CMAP recordings of the vibrissal muscles

At 13 weeks after surgery, CMAP was recorded for assessing the function of regenerated nerves. Rats were anesthetized with intraperitoneal urethane at a dose of 1.2 g/kg and immobilized with a stereotaxic apparatus. Depth of anesthesia was confirmed by observing the disappearance of eyelid reflex and the absence of whisker movement. Stainless-steel microelectrodes with an impedance of 9–12 MΩ at 1 kHz (UESMGCSELNNM-type) (FHC, Bowdoin, ME) were inserted into the vibrissal muscles of rows C and D for recording CMAP by stimulating the regenerated buccal branch of the facial nerve or autologous nerve graft. Standard electrodes (TN204-089B) (Unique Medical, Tokyo) were placed caudally on the skull. After the regenerated buccal branch was

exposed, a tandem hook-shaped stimulation electrode (IMC-220224) (InterMedical, Nagoya) was attached to the regenerated nerves in both groups. The nerves were stimulated with supra-maximal stimulation pulses (~2 mA, 100-μs monopolar pulses) at a frequency of 0.2 Hz through an isolator (SS-202J) (Nihon Kohden, Tokyo). Recorded signals were processed with a multichannel amplifier (MEG-6100) (Nihon Kohden) at 15 to 10,000 Hz and then digitized at 40 kHz with PowerLab 4/30 and LabChart 7 systems (ADInstruments, Dunedin, New Zealand). An upward trace indicated a negative deflection (depolarization), and 10 consecutive traces were averaged. CMAP amplitude was measured as the difference between maximum and baseline voltages (Fig. 3A and B). Duration was calculated as the time between the two points where the baseline was crossed by the rising and declining CMAP curves (Fig. 3A and B). Latency was estimated as the time between the stimulus artifact and the point where the baseline was crossed by the rising CMAP curve (Fig. 3A and B).

2.4. Histological analysis of myelinated fibers

At 13 weeks after transplantation, the center of the regenerated buccal branch of the rat facial nerve was resected and stained with toluidine blue for counting the number of myelinated fibers. Actually, the specimens were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L cacodylate buffer at pH 7.4 at 4 °C overnight, then washed 3 times with 0.1 mol/L cacodylate buffer for 30 min, and postfixed with 2% osmium tetroxide in 0.1 mol/L cacodylate buffer for 3 h at 4 °C. Graded ethanol solutions (50, 70, 90, and 100%) were used for dehydration with the following schedule: 50% and 70% for 30 min individually at 4 °C, 90% for 30 min at room temperature, and 4 repetitions of 100% for 30 min at room temperature. Dehydration was then continued overnight with 100% ethanol at room temperature. Infiltration of resin into the specimens was performed by washing in propylene oxide (PO) 2 times for 30 min and then incubating in a 70:30-mixture of PO and epoxy resin (Quetol-812) (Nisshin EM, Tokyo) for 1 h. A sample
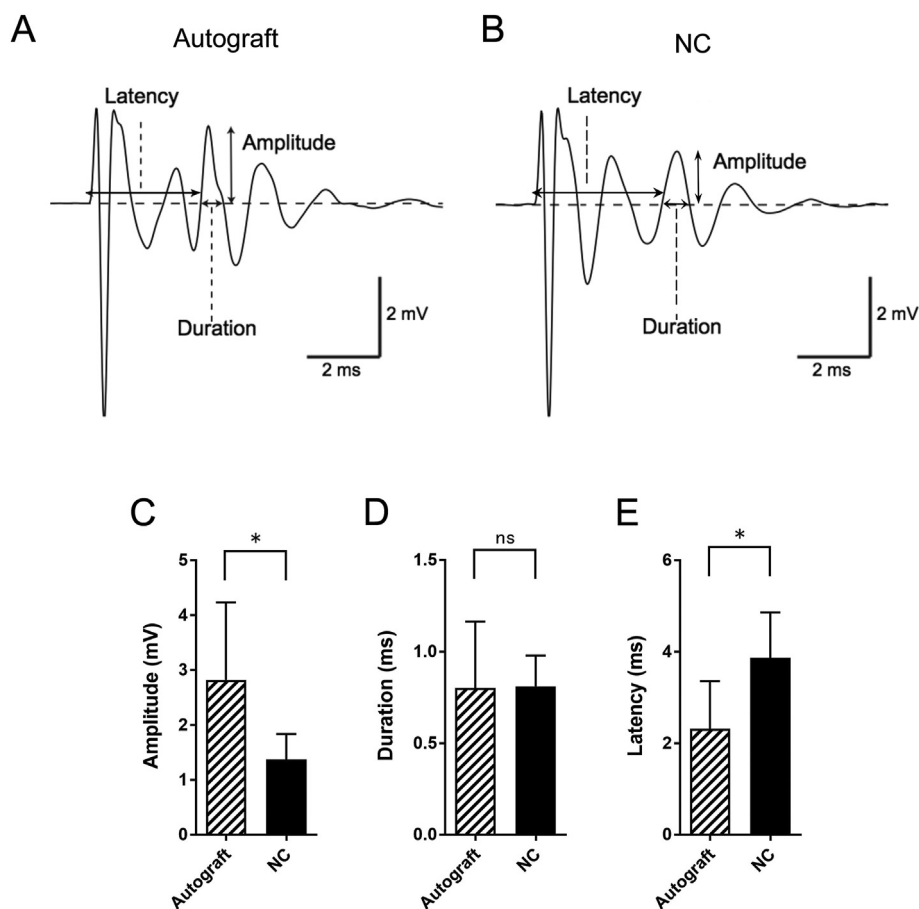
Fig. 3. Compound muscle action potential (CMAP) analysis. The left (A) and right (B) graphs show the waveforms of CMAP recorded from the whisker pads of rats in the autograft and collagen nerve conduit (NC) groups after maximal stimulation, respectively (C) Amplitude was higher in the autograft group than in NC group (D) No significantly different in duration was found between the autograft and NC groups (E) Latency was longer in NC than in the autograft groups. Vertical lines above the columns represent standard deviations (SDs). One asterisk (*) indicates that the probability is less than 0.05 (p < 0.05). The abbreviation ns indicates non-significance.

tube containing the specimen was left without closure for allowing PO to volatilize overnight, resulting in the concentration of the resin. Specimens were transferred into a fresh 100% resin solution, which was allowed to polymerize for 48 h at 60 °C. Ultra-thin 1.5-µm sections were prepared from the polymerized resin with a glass knife and an ultramicrotome (Ultracut UCT) (Leica, Vienna, Austria) and stained with 0.5% toluidine blue.

2.5. Transmission electron microscopy of the regenerated nerves

For observing the morphology of regenerated nerve axons and myelin, three specimens were randomly collected from the autograft and NC groups individually and cut into 70-nm sections, which were mounted on grids (EM fine-grid F-200) (Nisshin EM). The sections were then stained with 2% uranyl acetate and lead staining solution (Sigma–Aldrich, St. Louis, MO) and observed with a transmission electron microscope (TEM) (JEM1200EX) (JEOL, Tokyo) with an accelerating voltage of 80 kV. Fiber diameters, axon diameters, and the myelin thicknesses of axons randomly selected from five TEM images at a magnification of 3550 were measured using Photoshop CC 2018 software (Adobe Systems, San Jose, CA). All numerical values of individual groups collected by two-stage sampling described above were used for comparison analysis.

2.6. Statistical analysis

The number of myelinated fibers, fiber diameter, axon diameter, myelin thickness, g-ratio (axon diameter/fiber diameter), CMAP amplitude, duration, and latency in both groups were analyzed by unpaired Student's t-test in GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA). Numerical data are expressed as mean ± SD. A probability less than 0.05 (p < 0.05) was considered statistically significant.

2.7. Data availability

The authors declare that data supporting the findings in this study are available within the paper.

3. Results

CMAP amplitude was found to be significantly larger in the autograft group (2.8 ± 1.4 mV) than in NC group (1.3 ± 0.5 mV) (p < 0.05) (Fig. 3C). No significant difference was observed in duration between the autograft (0.8 ± 0.4 ms) and NC (0.8 ± 0.2 ms) groups (Fig. 3D). Latency was found to be significantly shorter in the
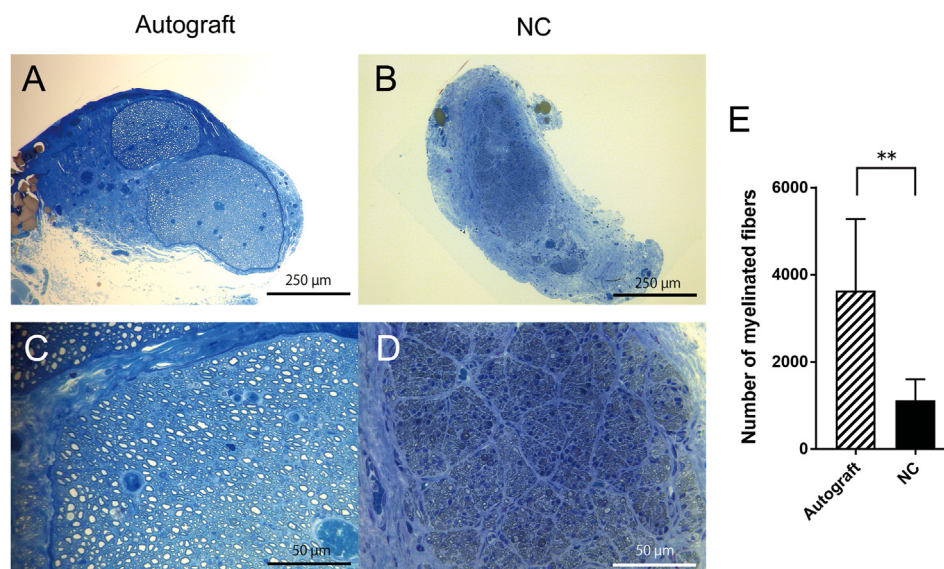
Fig. 4. Optical microphotographs of the cross-sections of the middle portions of the regenerated nerves at 13 weeks after implantation. Photographs in the left (A) and right (B) of upper row show the cross-sections of regenerated nerves of the autograft and nerve conduit (NC) groups at a magnification of 100, respectively. Photographs in the left (C) and right (D) of lower row show those of the autograft and NC groups at a magnification of 400, respectively. Scale bars in the upper and lower rows are 250 μm and 50 μm, respectively. Column graph E shows that the number of myelinated fibers the autograft group was significantly higher than that of nerve conduit (NC) group. Two asterisks (**) indicate that the probability is less than 0.01 (p < 0.01).

autograft group (2.3 ± 1.0 ms) than in NC group (3.9 ± 1.0 ms) (p < 0.05) (Fig. 3E).

Toluidine blue stained specimens showed that regenerated nerve fibers in the autograft group were thicker than those in NC group (Fig. 4A–D). The number of myelinated fibers of the autograft group was larger (3634 ± 1645) than that of NC group (1112 ± 490) (Fig. 4E).

Electron microphotographs (Fig. 5A and B) showed that the fiber diameter of the autograft group (4.8 ± 1.9 μm) was larger than that of NC group (3.8 ± 1.4 μm) (Fig. 5C). The axon diameter of the autograft group was thicker than that of NC group (3.6 ± 1.5 μm vs. 3.0 ± 1.2 μm) (Fig. 5D). The myelin thickness of the autograft group was thicker than that of NC group (0.6 ± 0.3 μm vs. 0.4 ± 0.1 μm) (Fig. 5E). G-ratio of the autograft group (0.74 ± 0.19) was lower than that of NC group (0.79 ± 0.10) (Fig. 5F).

4. Discussion

Similar to autografts, the nerve conduit is known to be effective for repairing the sensory nerve defects of which length is less than 30 mm [10]. In an animal facial-nerve defect model in this study, the motor-nerve regeneration ability of collagen conduit was also confirmed.

Several factors are known to affect disadvantageously the regeneration of facial nerves: facial nerve regeneration requires the regeneration of acetylcholine receptors at the nerve endings; the facial mimetic muscles have fewer muscle bodies but are more prone to atrophy; and the motor nerves have a lower regeneration ability than the sensory nerves [11]. However, in this study, the facial nerve regeneration was achieved with the artificial nerve conduit because of the positive effect of collagen in the lumen of conduit. Collagen is composed of a family of 28 proteins with a triple helical structure and the most abundant protein in mammals [12] making up approximately 25% of the total protein content. Type I collagen, which is most abundantly found in the human body, is used as a biomaterial for artificial organs and skin [13] and considered a suitable material for making a nerve conduit due to its low immunogenicity, excellent biocompatibility, and cytocompatibility [14]. Okamoto et al. implant collagen artificial nerve conduit

into an experimental 30-mm sciatic nerve defect in dogs, evaluate the morphological and physiological characteristics of the regenerated nerve, and find that the regenerated myelinated nerve fibers show a bimodal distribution comparable to that of the normal nerve fibers [15]. This indicates that (1) the regenerated nerves recover their functional communication with the target organs and (2) the artificial collagen conduit is an effective scaffold for nerve regeneration [16]. Waitayawinyu et al. implant two different artificial nerve conduits made of type I collagen and polyglycolic acid (PGA) into an experimental 10-mm sciatic nerve defect in rats and find that the collagen conduit is significantly superior to PGA conduit [17]. They speculate that the superiority of collagen conduits is originated from the structure of collagen as an extracellular matrix and its biological properties as a cellular scaffold promoting cell proliferation.

In this study, the efficacy of the collagen nerve conduits was found to be inferior to that of the autologous nerve grafts, and as a possible reason, the implanted collagen nerve conduits might be degraded and absorbed before the conduit expressed its nerve-regeneration promoting ability. Another artificial nerve conduit (Nerbridge®) currently used in Japan is found to be degraded and absorbed for several months in vivo, because the artificial conduits are made of PGA. Since the conduit used in this study was made of collagen, the degradation and absorption rates are higher than those of PGA conduits [15]. Collagen is degraded by proteases, gradually digested, and absorbed by the tissues surrounding the conduit, and the rate of absorption varies by the implantation site and temperature. In this study, the conduit was implanted under the facial skin where a high blood flow accelerates the tissue absorption of collagen of the conduit. The necessity for the immobilization of nerve regeneration site after artificial conduit implantation is proposed by Lohmeyer et al., suggesting that a 2-week-immobilization is essential [18]. In this study, however, the body movements of rats caused by chewing and swallowing food immediately after implantation were unable to give a stable environment for nerve regeneration, probably resulting in the suppressed neuroregenerative effect of collagen conduits. Therefore, in humans, resting for a specific period of time after surgery could be effective for enhancing the nerve regeneration in the implanted
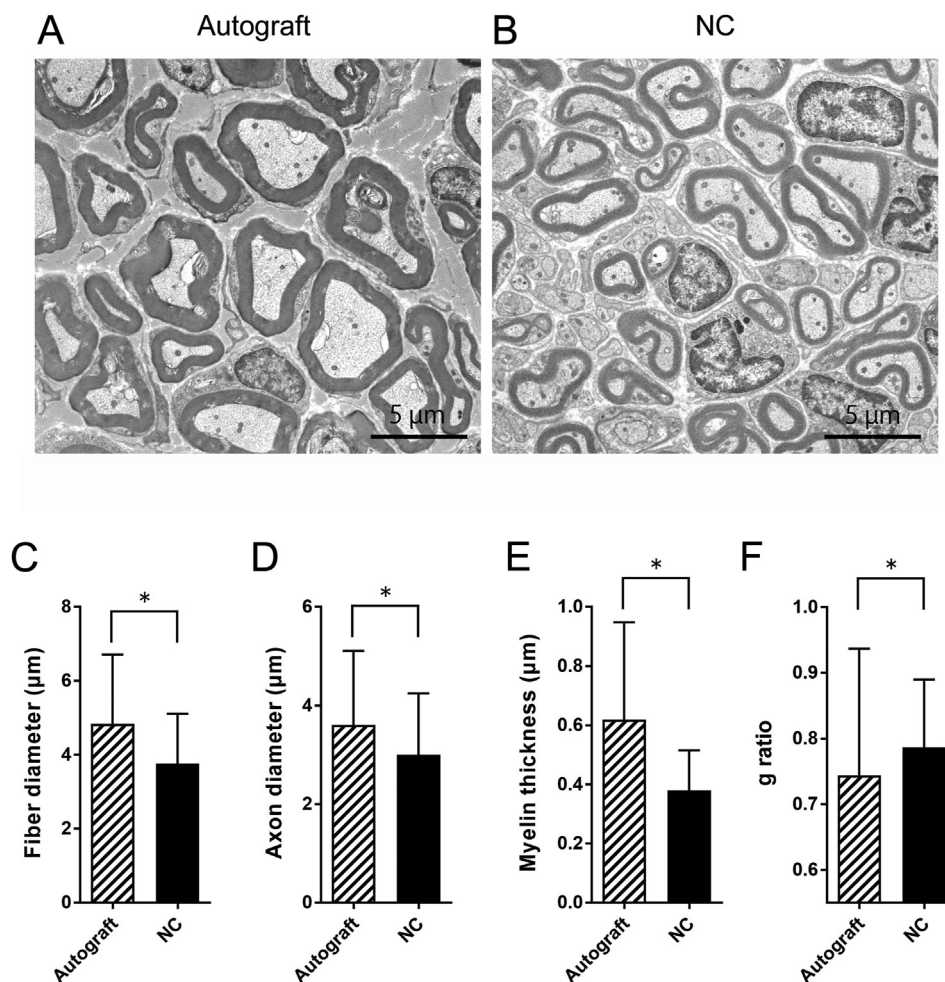
Fig. 5. Electron microphotographs of the cross-sections of the middle portions of the regenerated nerves at 13 weeks after implantation. Left (A) and right (B) electron microphotographs show the cross-sections of regenerated nerves in the autograft and nerve conduit (NC) groups, respectively. Scale bars in both left and right photographs are 5 μm at a magnification of 3555. Column graph C, D, E, and F show the fiber diameter, axon diameter, myelin thickness, and g-ratio (axon diameter/fiber diameter) of the regenerated nerves in the autograft and NC groups, respectively. Vertical lines above the columns represent standard deviations (SDs). One asterisk (*) indicates that the probability is less than 0.05 (p < 0.05).

collagen conduits. In addition, the amount of collagen filled in the lumen of the nerve conduit used in this study could be insufficient to induce Schwann cell proliferation.

Autologous nerves are an ideal nerve regeneration-inducing material for motor nerve reinnervation, because they have a strong connective tissue covered with the basement membrane, which makes the autologous nerve extremely strong, and they contain many Schwann cells enhancing nerve regeneration. Therefore, for finding ideal materials for artificial nerve conduits, specific cells as replacements for Schwann cells and growth factors are studied [19]. Various studies report that the injections of stem cells and growth factors into the lumen of artificial nerve conduits improve their regeneration-inducing abilities [8,20,21]. Maki et al. create an artificial nerve conduit of which lumen is filled with a cocktail of cytokines secreted by skeletal muscle-derived stem cells, implant the conduit into a sciatic nerve defect in mice, and find an enhanced nerve regeneration [22]. In the authors' previous studies, for improving the neuroregenerative ability of the conduits, hybrid-type artificial nerve conduits of which lumen is filled with adipose-derived stem cells, stromal vascular fraction, and/or dedifferentiated fat cells are prepared, transplanted into a defect of the buccal branch of the facial nerve in rats, and observed to enhance the nerve regeneration [23,24]. Although the highly

neuroregenerative ability of conduits containing adipose-derived stem cells is attractive for treating the nerve defects, the preparation process of the stem-cell conduits is quite complex and requires long time and high production cost, which could reduce a willingness to apply the conduits to clinical practice. On the other hand, collagen, which is one of the main materials for making the myelin sheath, plays an important role in the process of nerve regeneration [25], and collagen conduits filled with collagen filaments are found to give a suitable environment for nerve regeneration [10], and the production cost of the collagen conduits is expected to be reasonable as medical devices. Therefore, the collagen conduit used in this study is expected to be a handy medical device for reconstructing pheripheral nerves. Since the neruoregeneration ability of the collagen conduit in this study was inferior to that of the autologous nerve graft, for enhancing the neruoregeneration ability, various low-cost stem cells and growth factors will be investigated for incorporating them in the lumen of the conduits. Although silicone never conduits are reported to be used with showing the efficacy [7], silicone nerve conduits are known to be non-bioabsorbable and require the secondary surgery for removing the implanted silicone conduit in which the facial nerve regenerates. Therefore, no non-bioabsorbable nerve conduits including silicone conduits were thought to be used in this study.

As the limitations of this study, the number of rats might be small, and further studies with a larger sample size would be expected. The length of the defect was 7 mm, which was short for human and large animals. Translational research with large animals such as sheep will be planned before the future clinical applications [26,27].

5. Conclusions

This study demonstrated the efficacy of collagen nerve conduit for facial nerve reconstruction. Despite its lower neuroregenerative ability than the autograft, the collagen conduits were found to have various advantages such as reasonable production cost, no requirement of harvesting site for autografts, and easy-handling.

Declaration of competing interest

None.

Acknowledgments

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