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a) Title

A basic fibroblast growth factor (bFGF) slow-release system combined to a biodegradable nerve conduit improves endothelial cell and Schwann cell proliferation: A preliminary study in a rat model

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f) Running title

Biodegradable nerve conduit using bFGF slow-release system

Conflict of interest

None

Abstract

Background: A basic fibroblast growth factor (bFGF) slow-release system was combined to a biodegradable nerve conduit with the hypothesis this slow-release system would increase the capacity to promote nerve vascularization and Schwann cell proliferation in a rat model. **Materials and Methods**: Slow-release of bFGF was determined using ELISA. A total of 60 rats were used to create a 10 mm gap in the sciatic nerve. A polyglycolic acid-based nerve conduit was used to bridge the gap, either without or with a bFGF slow-release incorporated around the conduit (n=30 in each group). At 2 (n=6), 4 (n=6), 8 (n=6), and 20 (n=12) weeks after surgery, samples were resected and subjected to histological, immunohistochemical, and transmission electron microscopic evaluation for nerve regeneration. **Results**: Continuous release of bFGF was found during the observation period of 2 weeks. After in vivo implantation of the nerve conduit, greater endothelial cell migration and vascularization resulted at 2 weeks (Proximal: 20.0±2.0 vs.12.7±2.1, p=0.012, Middle: 17.3±3.5 vs.8.7±3.2, p=0.034). Schwann cells showed a trend toward greater proliferation and axonal growth had significant elongation (4.9±1.1 mm vs. 2.8±1.5 mm, p=0.049) at 4 weeks after implantation. The number of myelinated nerve fibers, indicating nerve maturation, were increased 20 weeks after implantation (Proximal: 83.3±7.5 vs.53.3±5.5, p=0.065, Distal: 71.0±12.5 vs.44.0±11.1, p=0.0491). **Conclusion**: These findings suggest that the bFGF slow-release system improves nerve vascularization and Schwann cell proliferation through the biodegradable nerve conduit.

Keywords

Biodegradable nerve conduit, bFGF, slow-release system, nerve regeneration

Introduction

In the treatment of peripheral neurological deficits, autologous nerve grafting is the first choice. The sural nerve, antebrachial cutaneous nerve, terminal branch of posterior interosseous nerve, and other choices are commonly used, however, loss of function and potential morbidity from creation of these donor sites are undesirable effects of their use. In recent years, synthetic nerve conduits and allogeneic nerve grafting materials have been introduced into clinical practice as alternatives to autologous nerve grafting.[1-3)]

The first attempt at nerve conduit applied for nerve regeneration used silicone tubes.[4)] Subsequent studies explored biodegradable materials with open internal structure as the conduit. More recently, filling the conduit with a regeneration-conducive material such as collagen demonstrated improved nerve regeneration, however, the outcome remained inferior to autologous nerve grafting and allogeneic nerve transplantation.[5-7)]

An adequate blood supply to the nerve is important to promote nerve regeneration.[8)] Basic fibroblast growth factor (bFGF) is a cytokine that accelerates angiogenesis.[9)] If an angiogenic stimulus can be induced at the nerve transection sites which are replaced with the biodegradable nerve conduit by bFGF, then nerve regeneration is speculated to accelerate. However, bFGF has a short half-life ($T_{1/2}$ = 1.5 minutes) and is dispersed within the body quickly. To compensate for this shortcoming, Tabata and colleagues developed a bFGF slow-release system using gelatin microspheres as a carrier to facilitate continuous bFGF administration at the site of application.[10)]

In this study, we combined bFGF slow-release system using gelatin to a biodegradable nerve conduit with the hypothesis that the bFGF slow-release system would promote vascularization, endothelial cell and Schwann cell proliferation, and the subsequent axonal elongation through the nerve conduit in a rat model.

Materials and Methods

All the animal experiments were conducted following the regulations of the Animal Experiment Committee of Kindai University. Fourteen-week-old SD rats (mean body weight 280~324 g Harlan Sprague Dawley, Indianapolis, IN, USA) were used as experimental animals (n = 60). Housing was maintained in a clean rack with uniform temperature (22°C) and humidity (50%) and 12-hour light-dark cycle. Radiation-sterilized (3 mG) solid food and water was provided ad libitum.

1) Creation of biodegradable nerve conduit with bFGF slow-release system

A nerve conduit made of biodegradable material (Nerbridge®, 1.5 mm in diameter, 12 mm in length, Toyobo Co., Ltd., Osaka) was used. Onto this biodegradable nerve conduit, the bFGF-impregnated gelatin microspheres were applied uniformly using a micro-pipette. The bFGF slow-release system using gelatin microspheres (60 μg bFGF/60 μL PBS mixed with 6 mg gelatin) was prepared according to the method of Tabata and colleagues.[10] The bFGF-containing gelatin microsphere-applied biodegradable nerve conduit was immersed in PBS (pH 7.4, 37°C, 10 mL), and 100 μL of supernatant was collected at 1 hour and on 2, 4, 6, 8, 10, 12 and 14 days after immersion. The amount of bFGF contained in the supernatant was determined by ELISA analysis for bFGF (RayBio Human bFGF ELISA Kit).

2) Scanning electron microscopy of the biodegradable nerve conduit surface

Observation of the surface of biodegradable nerve conduit after preparation of the bFGF slow-release system (as above) was conducted using scanning electron microscopy. The samples were dried and gold-coated with evaluation using scanning electron microscope (S-900, Hitachi, Ltd., Japan).[11]

3) Establishment of nerve defect model and experimental design

After general anesthesia with urethane and α-chloralose, a 10-mm gap was created by sharp transection of the sciatic nerve on one leg per rat. The ends of the nerve were drawn into the open ends of the 12 mm long biodegradable nerve conduit by 1 mm on each side, using a horizontal mattress stitch of 10-0 nylon suture to secure each end in place. Two study groups were used, one with the bFGF slow-release system (nerve conduit+60 μg-bFGF, n=30) and a control group (nerve conduit only, n=30). In the nerve conduit+60 μg-bFGF group, the bFGF-containing gelatin microspheres were applied after implanting the conduit in place (n=30), whereas the control group had no further treatment after conduit implantation (n=30). Harvest of the implanted nerve conduit in each group was done 2 (n=6), 4 (n=6), 8 (n=6) and 20 (n=12) weeks after the surgery (**Figure 1**).

4) Evaluation of the nerve growth rate using immunohistological method

After harvest, the tissues collected at 2, 4, 8, 20 weeks (n=6 at each point in each group) after implantation were immersion-fixed in 10% neutral buffered formalin solution for 3 days. Paraffin blocks were prepared and 5-μm sections were made in the longitudinal direction with a microtome (LEICA SM2000R). Anti-CD31 antibody (anti-CD31 Rabbit polyclonal

antibodies: Abcam, ab28364) was used for staining vascular endothelial cells, anti-S100 antibody (anti-S100 Rabbit polyclonal antibodies: Dako, Z0311) was used for staining Schwann cells, and anti-NF antibody (anti-neurofilament 68 kDa-Mouse monoclonal antibodies: Dako, M0762) was used for staining axons. In the staining of vascular endothelial cells using CD31 antibody, the number of blood vessels in 3 regions, the proximal region (peripheral side 2.5 mm from the stump of proximal side), middle region and distal region (proximal side 2.5 mm from the stump of distal side) of the biodegradable nerve conduit was counted. Three visual fields of in each group were photographed, and the mean value per unit area (50 μm × 30 μm , 1,500 μm^2) was calculated for number of blood vessels including capillaries. Image J software (National Institutes of Health, MD, USA) was used for analysis.

The migration distance of Schwann cells (S100 antibody) from the proximal and distal sides of the biodegradable nerve conduit was determined. The distance of axonal elongation (anti-NF antibody) from the proximal nerve stump to the inside of the biodegradable nerve conduit was determined. The distances of Schwann cell migration and axonal elongation were determined by Image J software.

5) Evaluation of nerve maturity using transmission electron microscopy (TEM)

To study nerve maturity, TEM images of myelinated nerve fibers for number and the cross-sectional area were evaluated at 20 weeks (n=6 in each group) after implantation. Contralateral native sciatic nerves (n=6 in each group) were transected for comparison with the other two groups. Biodegradable nerve conduits and native sciatic nerves were immersed and fixed in 4% glutaraldehyde for 3 days. The tissues were sectioned (1.5-μm thick) transversely with an ultra-microtome (LEICA EM UC7) and then post-fixed with 4% osmium. The slices were dehydrated by alcohol series, stained with uranium and embedded in epon. They were sliced to ultrathin sections of 700 A in thickness, stained with uranyl and lead acetate and observed using a transmission electron microscope (H-7700, Hitachi, Ltd., Japan). The sites observed were the proximal, middle and distal regions of biodegradable nerve conduit and were observed at the maximum scale possible to identify axons, 50 μm × 40 μm (= 2,000 μm^2). Three (3) visual fields near the center of each cross-section in each group were photographed, and the mean value per unit area of 2,000 μm^2 was calculated for number and the cross-sectional area of myelinated nerve fibers. Image J software was used for analysis.

6) Statistical analysis

All experimental results were indicated as mean ± standard error (SE). Statistical analyses

used Student *t*-test for comparison between two groups and by one-way analysis of variance (one-way ANOVA) and Holm post-test for comparison among 3 or more groups (different levels of the nerve). Data analysis was conducted using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), and $P<0.05$ was used as the criterion for significance.

Results

1) bFGF amount in slow-release system and localization of gelatin microspheres in biodegradable nerve conduit

The surface of the nerve conduit showed that the gelatin microspheres were localized along the outer surface of biodegradable nerve conduit, with some of the microspheres leaching into the adjacent surrounding tissues (**Figure 2A, B**). The amount of bFGF released from the prepared conduits was determined by the ELISA method. The release rate was constant, with 64.9% of the total bFGF released over 14 days. Moreover, accumulation of bFGF was maintained at about 5 μg/day (**Figure 2C**).

2) Findings of gross examination after implantation of the conduit

The control conduits maintained a clear profile within the tissue bed, with little evidence of integration with the surrounding tissue. The wall of the conduits also became thinner by 20 weeks after implantation. In contrast, the conduits in the nerve conduit+60 μg-bFGF group became covered by and subsequently buried within the surrounding tissues, with clear integration and thickening (**Figure 3**).

3) Evaluation of the nerve growth rate using immunohistochemical method

The time course of the development of blood vessels in three regions (proximal, middle and distal regions) of the biodegradable nerve conduit was examined (**Figures 4, 5 and 6**). Blood vessel significantly increased by 2 weeks in the proximal (20.0 ± 2.0 vs. 12.7 ± 2.1, $p=0.012$) and middle (17.3 ± 3.5 vs. 8.7 ± 3.2, $p=0.034$) regions of the nerve conduit+60 μg-bFGF group, compared with controls (**Figure 4 and 6**). By 4 weeks, the control group had achieved similar numbers as the nerve conduit+60 μg-bFGF group in the proximal and middle portions (**Figure 5 and 6**), but the distal region showed significantly increased blood vessels in the nerve conduit+60 μg-bFGF group (27.3 ± 2.1 vs. 10.7 ± 1.5, $p=0.003$). At 20 weeks, the blood vessel channels had decreased in the proximal and middle regions of both groups. In contrast, for the distal region, the control group had significantly increased blood vessel numbers (32.0 ± 2.0 vs. 15.7 ± 2.1, $p=0.001$), whereas these numbers had decreased in the nerve conduit+60

μg-bFGF group (**Figure 6**). These results support trends for both more rapid development of blood vessel and subsequent maturation of these channels (seen as a pruning of channels within the nerve space).

Migration of Schwann cells from both ends of proximal and distal regions of biodegradable nerve conduit was observed in both groups, showing a progressive pattern from 2 (22.0±3.6 vs. 13.5±0.9, p=0.029) to 4 weeks and with complete extension by 8 weeks. The Schwann cell migration-tip had apparently extended farther in the nerve conduit+60 μg-bFGF group at 2 weeks (**Figures 7 and 8**).

Axonal elongation from the proximal region of biodegradable nerve conduit was observed in both groups, with axons extending to the distal region by 20 weeks. This axonal elongation appeared to be more advanced at the earlier time points (4.9±1.1 mm vs. 2.8±1.5, p=0.049 at weeks) in the nerve conduit+60 μg-bFGF group (**Figures 9 and 10**).

The ratio of average axonal elongation distance between the nerve conduit+60 μg-bFGF and control group was summarized in **Table 1**. When the bFGF slow-release system was combined to a biodegradable nerve conduit, the migration distance of vascular endothelial cells and the number of blood vessels are promoted in the early phase (2-4 weeks) of nerve regeneration process, and the subsequent distances of Schwann cells were extended. Finally, axon elongated 1.7 times at 4 weeks after implantation (**Table 1**), and the nerve regeneration was clearly improved.

4) Nerve maturity at 20 weeks assessed by TEM

The number of myelinated nerve fibers significantly increased in the proximal (83.3±7.5 vs. 55.3±5.5, p=0.0065) and distal (71.0±12.5 vs. 44.0±11.1, p=0.0491) regions in the nerve conduit+60 μg-bFGF group (**Figures 11 and 12**). The myelinated nerve fiber area in the nerve conduit+60 μg-bFGF group was comparable with that of the native sciatic nerve at proximal and middle regions, and it was significantly higher at the distal region (243.3±29.4 vs. 128.1±7.1, p=0.0259) (**Figure 13**). Our results demonstrated that the number and area of myelinated nerve fibers were increased, indicating nerve maturation by bFGF slow-release system.

Discussion

Biodegradable nerve conduits present an attractive alternative to autologous nerve grafting. These conduits use a hollow tube structure of various composition such as polyglycolic acid (PGA), poly lactide-caprolactone (P(LA-CL), or collagen of bovine or porcine origin, to allow

nerve regeneration through the channel. Though in use, variable outcomes have been reported.[3, 12-16] The nerve conduit tested in the present experiment is of the biodegradable material obtained by forming PGA fiber to a luminal structure of which the thickness of luminal wall was adjusted to about 500 μm. Different from the biodegradable nerve conduit having conventional hollow structure, the inner lumen is filled with type I and III collagen sponge, and the outer surface is covered with collagen of the same composition. It is reported that the collagen sponge in the inner lumen becomes the scaffold in migration and growth of various cells contributing to nerve regeneration and the nerve regeneration is promoted.[17-19)]

As concerns regeneration of peripheral nerve, the 3-stage regeneration mechanism shown below has been reported; firstly, the damaged nerve becomes hypoxic, and macrophages migrate and secrete vascular endothelial growth factor A (VEGF-A). Secondly, the vascular endothelial cells migrate in the nerve tissues, and pillared vessels are formed in the direction of nerve tract. Finally, Schwann cells migrate along the formed vessels, and axons regrow.[20)] In the process of nerve regeneration, therefore, the angiogenesis prior to nerve regeneration is considered very important. Though 10 mm is considered as the critical deficit of nerve regeneration, it was possible to regenerate the nerve in 25-mm rat sciatic nerve deficit when the blood vessel was induced into the inner lumen of nerve conduit.[21)] From the histological evaluation, muscle weight and electrophysiological study, nerve regeneration was more accelerated by placing the blood vessel close to the lateral surface of nerve conduit if the low-molecular-weight molecules such as albumin are able to penetrate through the nerve conduit wall.[19)] In this research, the bFGF slow-release system was combined for the purpose of creating more vascular networks around the biodegradable nerve conduit of which the permeability of albumin molecule through the luminal wall has already been confirmed.[17)]

bFGF has the important functions by acting on the vascular endothelial cells and vascular smooth muscle cells, proliferating them, adjusting the expression of both VEGF and HGF factors to promote angiogenesis. Moreover, it was reported that the effect is higher than VEGF and platelet-derived growth factor (PDGF).[9), 22)] When bFGF is administered alone, the biological activity is very short as 90 seconds of blood half-life, however, control release of bFGF can be obtained if bFGF is combined with gelatin microspheres. In this bFGF slow-release system, bFGF and acidic gelatin microspheres adsorb and immobilize electrostatically by interaction. According to the degradation rate of gelatin, bFGF is released slowly and the pharmacological activity of bFGF can be maintained.[10)]

This bFGF slow-release system has already been applied clinically for the surgical treatment of finger-tip amputation. Marked therapeutic effects have been reported when the optimal bFGF concentration of 2~10 μg/cm^2/day in tissues was maintained.[23] For the bFGF slow-release system using gelatin microspheres, it was reported that the strongest angiogenesis was observed *in vivo* when the initial concentration of bFGF was set at 1 mg/mL.[24] In this study, the bFGF slow-release system with the initial concentration of 1 mg/mL (actual dosage of bFGF: 60μg, gelatin: 6 mg) was applied outside the biodegradable nerve conduit. Based on a result of examination using the ELISA, it was confirmed that the bFGF concentration was kept at about 5 μg/day during the observation period for 2 weeks. The in vivo study demonstrated that the migration distance for both vascular endothelial cells and Schwann cells, and the elongation distance of axons was greater in the nerve conduit+60 μg-bFGF group in the early phase of nerve regeneration (within 4 weeks after implantation). To our knowledge, this is the first report which has shown evidence that the vascular network formed in the surrounding environment of a biodegradable nerve conduit by bFGF slow-release system has a strong influence on both angiogenesis and the subsequent nerve regeneration in a rat model. Though the axonal elongation in rats is faster (approximately 3-4 mm/day) while it is 2-4 fold slower in humans[25], a bFGF slow-release system offers a promising technique to enhance nerve regeneration with biodegradable nerve conduits in the clinical setting.

Currently, there is a growing interest in the clinical potential of mesenchymal stem cells derived from bone marrow[26]、adipose tissue[27),28)]、and menstrual blood[29]. Among these, bone marrow stem cells are reported to enhance neovascularization by producing angiopoietin-1 and 2[30]. After being implanted into nerve conduits, bone marrow stem cells have been reported to show their remarkable proliferative capacity by producing neurotrophic factors and by differentiating into Schwann cell-like cells[31),32)]. The utilization of stem cells into biodegradable nerve conduits with FGF slow-release system is therefore an important step toward new methods to enhance peripheral nerve repair.

It is clinically accepted that a biodegradable nerve conduit is indicated for a nerve gap length being less than 2 cm, while the majority of peripheral nerve gap length in human measures around 3-5 cm [33]. In order to accelerate axonal regrowth and later myelination of a biodegradable nerve conduit, approach using stem cells and bFGF slow-release system is considered one of the promising methods.

The limitation of this study is the lack of a positive control such as a group with the nerve autograft and functional assessment such as electrophysiological evaluation. In the field of nerve regeneration, axon, action potential, and the gait needs to be recovered to almost the same level as those of autologous nerve transplantation when the biodegradable nerve conduit is used.[34-36] Though the sufficient number and area of myelinated nerve fibers were observed by the introduction of bFGF slow-release system, the evaluation on motor function is not still clear. The future study on motor function is scheduled.

Conclusion

These findings suggest that the bFGF slow-release system acts on both endothelial cells and Schwann cells to improve axonal elongation through a biodegradable nerve conduit.

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Figures and tables

Figure 1: Experimental protocol

Figure 2: Scanning electron microscopic findings

Panel A showed biodegradable nerve conduit (control), while panel B showed biodegradable nerve conduit with the bFGF slow-release system (experimental group). Panel C showed slowly released bFGF level (bFGF level per day) determined by the ELISA method.

Figure 3: Gross examination after implantation of the conduit

To the sciatic nerve gap, the biodegradable nerve conduit with the bFGF slow-release system (experimental group) and the control biodegradable nerve conduit were implanted. Gross examination was conducted over time. P: Proximal region, D: Distal region.

Figure 4: Histological evaluation of blood vessels using immunohistochemistry

The time course of the development of blood vessels in three regions (proximal, middle and distal regions) of the nerve conduit+60 μg-bFGF group was examined.

Figure 5: Histological evaluation of blood vessels using immunohistochemistry

The time course of the development of blood vessels in three regions (proximal, middle and distal regions) of the nerve conduit group was examined.

Figure 6: Quantitative evaluation of angiogenesis

The number of blood vessels indicated by CD31-staining per 1,500 μm^2 in the biodegradable nerve conduit was counted and compared between the nerve conduit+60 μg-bFGF group and the nerve conduit group.

Figure 7: Immunohistochemical evaluation of Schwann cells

Migration of Schwann cells from both ends of proximal (left) and distal region (right) was evaluated by S100-staining.

Figure 8: Quantitative evaluation of Schwann cells in the biodegradable nerve conduit

Figure 9: Immunohistochemical evaluation of axon

Axonal elongation from the proximal region was evaluated by NF-staining.

Figure 10: Quantitative evaluation of axons in the biodegradable nerve conduit

Figure 11: Nerve maturity at 20 weeks assessed by transmission electron microscope

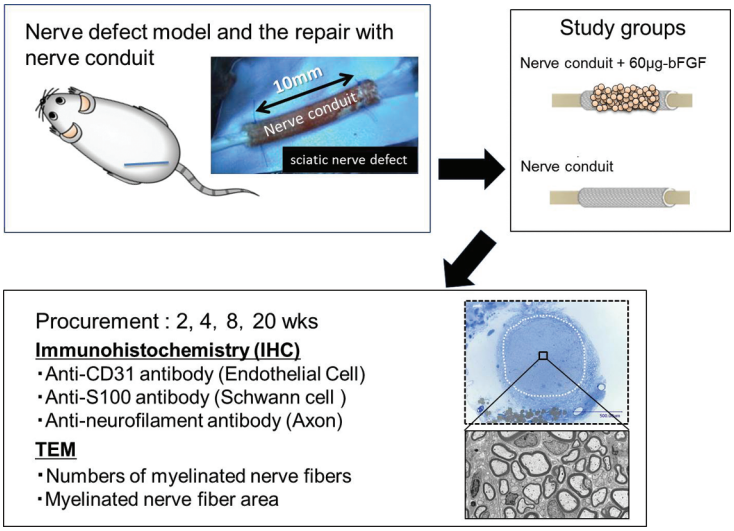
Figure 12: Quantitative evaluation of myelinated nerve fiber numbers 20 weeks after implantation

Total number of myelinated nerve fiber in 2,000 μm^2 in the proximal region (left), middle region (center), and distal region (right) was quantitatively evaluated.

Figure 13: Quantitative evaluation of the myelinated nerve fiber area 20 weeks after implantation

Total area of myelinated nerve fiber in 2,000 μm^2 in the proximal region (left), middle region (center), and distal region (right) was quantitatively evaluated.

Table 1: Axonal elongation distance (mm)



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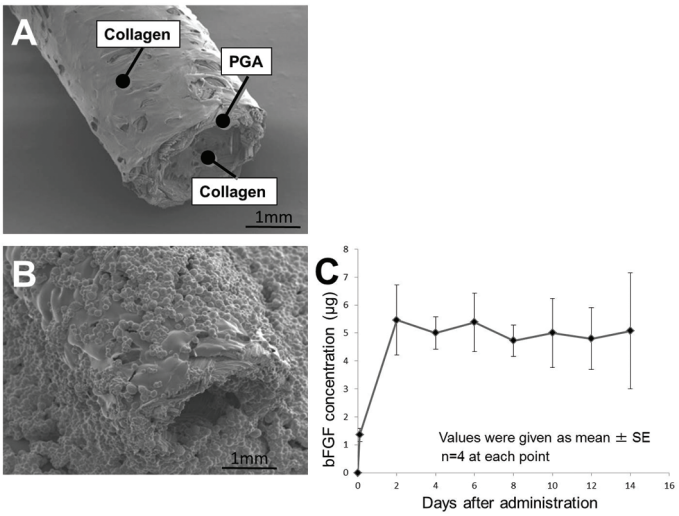

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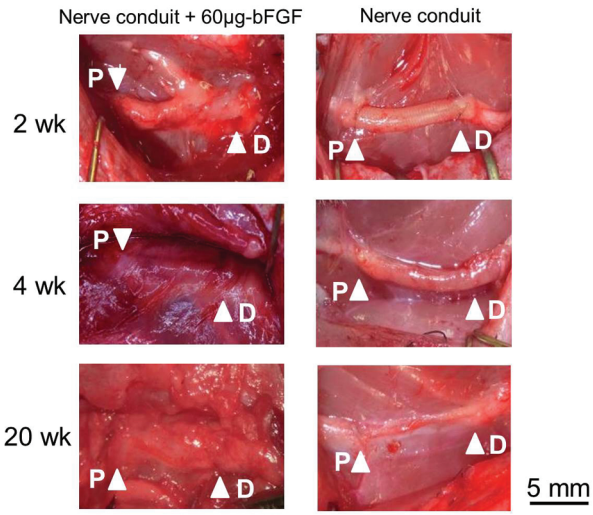

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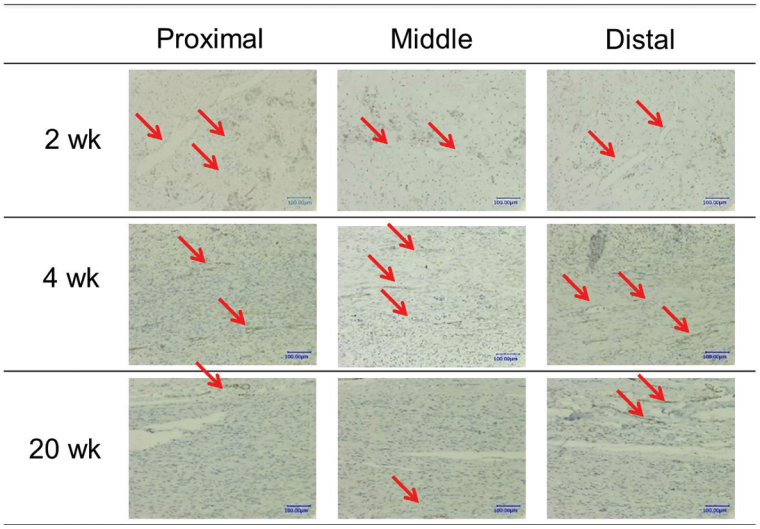

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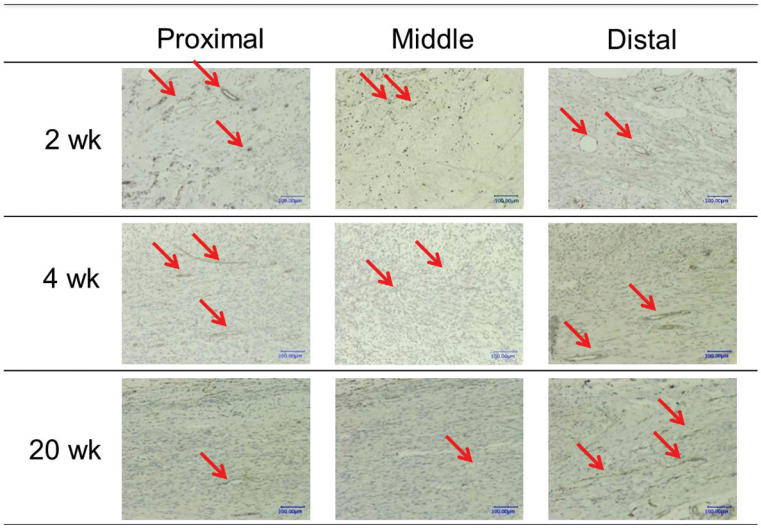

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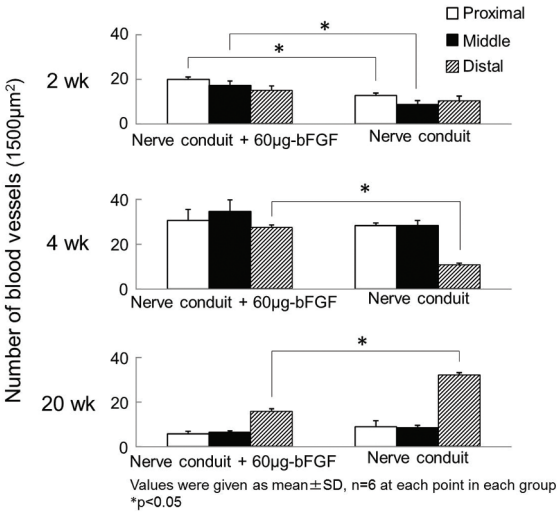

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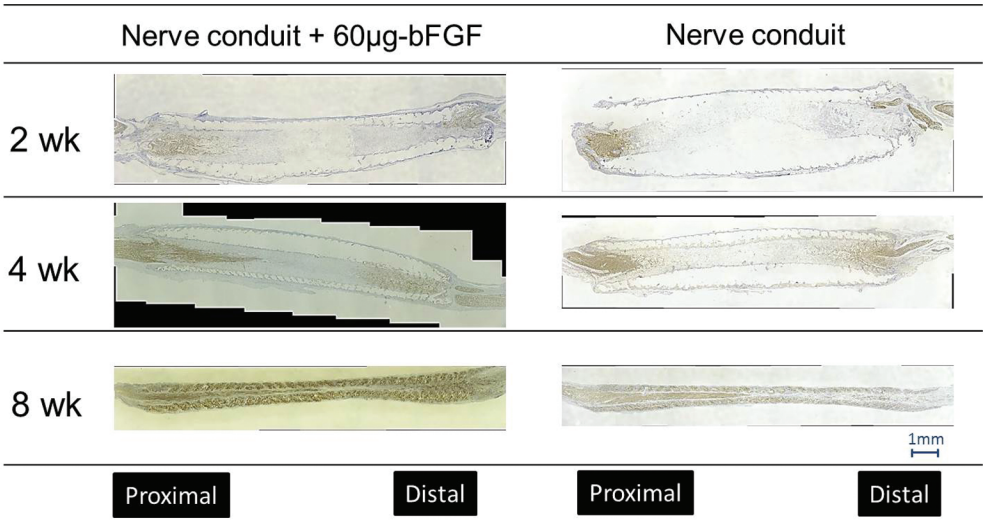

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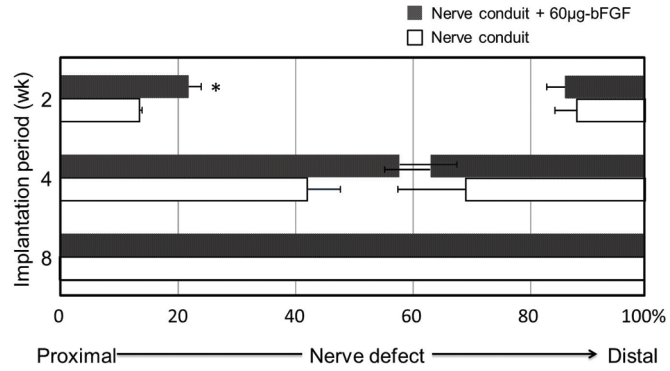

Values were given as mean±SD, n=6 at each point in each group
*p<0.05

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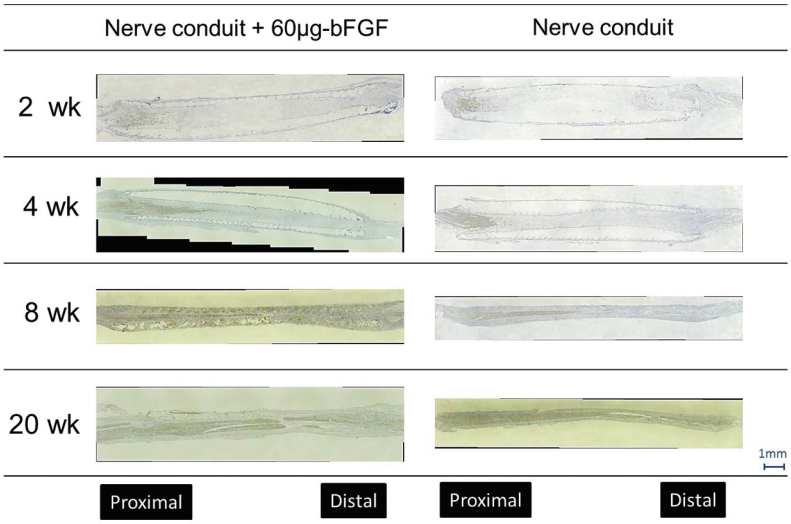

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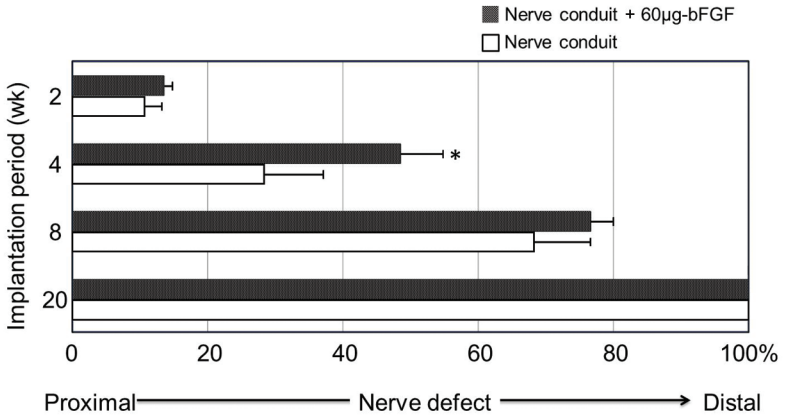

Values were given as mean±SD, n=6 at each point in each group
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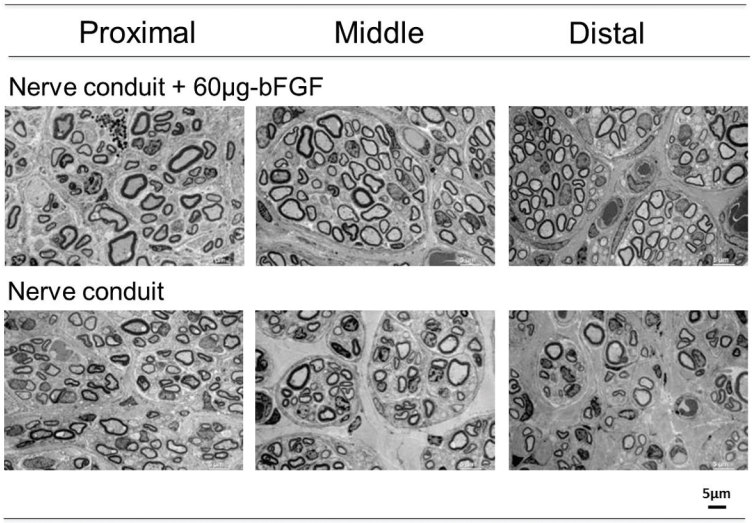

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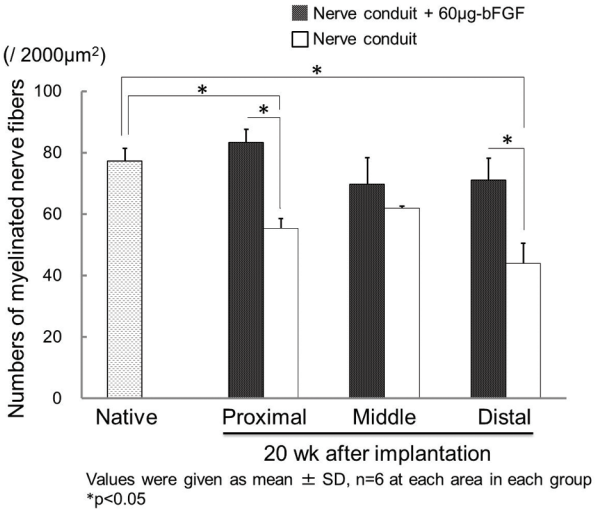

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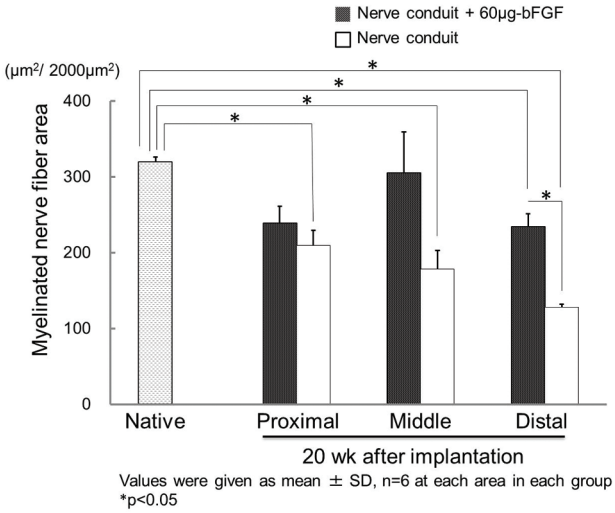

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