Evaluation of dual release of stromal cell-derived factor-1 and basic fibroblast growth factor with nerve conduit for peripheral nerve regeneration: An experimental study in mice

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Running Title: Dual release of SDF-1 and bFGF for nerve regeneration

Abstract

Background: The development of drug delivery systems has enabled the release of multiple bioactive molecules. The efficacy of nerve conduits coated with dual controlled release of stromal cell-derived factor-1 (SDF-1) and basic fibroblast growth factor (bFGF) for peripheral nerve regeneration was investigated.

Materials and Methods: Sixty-two C57BL6 mice were used for peripheral nerve regeneration with a nerve conduit (inner diameter, 1 mm, and length, 7 mm) and an autograft. The mice were randomized into five groups based on the different repairs of nerve defects. In the group of repair with conduits alone (n=9), a 5-mm sciatic nerve defect was repaired by the nerve conduit. In the group of repair with conduits coated with bFGF (n=10), SDF-1 (n=10) and SDF-1/bFGF (n=10), it was repaired by the nerve conduit with bFGF gelatin, SDF-1 gelatin and SDF-1/bFGF gelatin, respectively. In the group of repair with autografts (n=10), it was repaired by the resected nerve itself. The functional recovery, nerve regeneration, angiogenesis and TGF-β1 gene expression were assessed.

Results: In the conduits coated with SDF-1/bFGF group, the mean sciatic functional index value (-88.68±10.64, P=0.034) and the axon number (218.8±111.1, P=0.049) were significantly higher than the conduit alone group, followed by the autograft group; in addition, numerous CD34-positive cells and micro vessels were observed. TGF-β1 gene expression relative values in the conduits with SDF-1/bFGF group at 3 days (7.99±5.14, P=0.049) significantly increased more than the conduits alone group.

Conclusion: Nerve conduits coated with dual controlled release of SDF-1 and bFGF

promoted peripheral nerve regeneration.

Introduction

Tissue engineering has developed notably in recent years and many researchers have been able to design the natural microenvironment using cells and bioactive substances.[1] In particular, bioactive molecules such as growth factors and cytokines/chemokines have been extensively applied in the multidisciplinary approach. The development of drug delivery system (DDS) has allowed the well-timed release of bioactive molecules, tissue engineering, which regenerates damaged tissue by promoting the activity of endogenous cells without exogenous cell transplantation, has been remarkable. [2-6] We also reported that peripheral nerve regeneration with nerve conduit was accelerated by basic fibroblast growth factor (bFGF) incorporated into gelatin hydrogels that enhanced angiogenesis and Schwann cell proliferation.[7]

   In more recent years, the development of DDS has made it possible to release multiple biological molecules simultaneously. Dual release of bioactive molecules using DDS has been demonstrated toeffectively promote tissue regeneration such as bone formation and wound healing as reported in many studies.[8-18] However, there has been a few reports about peripheral nerve regeneration using nerve conduit with dual controlled release of the bioactive molecules.

   Stromal cell-derived factor-1 (SDF-1), which is a CXC family chemokine that repairs damaged tissue by recruiting stem cells, progenitor cells and immune cells into the injured site, has been widely used in the field of tissue engineering.[6,9,13,19-22] Several researchers have reported that SDF-1 is an important factor in nerve regeneration, in

both central and peripheral nervous systems, and facilitates regeneration by the recruited cells.[23,24]

The purpose of this study is to construct the nerve conduit coated with both bFGF, which has been already used in our previous study, and SDF-1 incorporated into gelatin hydrogels and to investigate the efficacy of the nerve conduit on peripheral nerve regeneration in mouse sciatic nerve defect models.

Materials and Methods

For this sduty, sixty-two male C57BL6 mice (6 weeks old) were operated for functional recovery evaluation, electrophysiological examination, histological evaluation, histomorphometry and gene expression analysis. All experimental protocols and animal maintenance procedures were approved by the Animal Ethics Research Committee of Osaka City University Graduate School of Medicine.

Preparation of nerve conduit

We have previously reported biodegradable peripheral nerve conduits composed of poly L-lactide (PLA) and polycaprolactone (PCL).[7,25-30] Since this nerve conduit comprise an outer layer, which was composed of a PLA multifilament fiber mesh, and an inner layer, which was composed of a 50% PLA and 50% PCL porous sponge with pores of 10 to 50 mm, it can maintain its tubular structure while staying very flexible, additionally supportive cells and growth factors can be added into it. The dimensions used in this study were as follows:; outer diameter: 2mm, inner diameter: 1mm, and length: 7mm to adjust the size and gap of nerves.

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Preparation of gelatin hydrogel microspheres incorporating SDF-1 and bFGF

Biodegradable gelatin hydrogel microspheres were prepared by glutaraldehyde cross-linking of acidic gelatin (isoelectric point=5.0; Nitta Gelatin Inc., Osaka, Japan) as previously reported.[2,6,13] Carrier-free human recombinant SDF-1 alpha (R&D systems Inc., Minneapolis, MN) and bFGF (Kaken Pharmaceutical Co., Tokyo, Japan) were obtained. The solution of gelatin hydrogel incorporating SDF-1 (SDF-1 gelatin; 10μl (500ng) of SDF-1 in phosphate-buffered saline (PBS) was dropped onto 1mg of freeze-dried gelatin and gelatin hydrogel incorporating bFGF (bFGF gelatin; 10μl (10μg) of bFGF in PBS dropped onto 1mg of the freeze-dried gelatin were impregnated at 4 °C overnight to obtain the gelatin hydrogel microspheres incorporating SDF-1 and bFGF (SDF-1/bFGF gelatin; SDF-1 gelatin and bFGF gelatin were mixed directly before injecting them into the nerve conduit) (Fig. 1). Evaluation of SDF-1 and bFGF release from gelatin hydrogels in vivo has been previously described using the ^{125}I-labeled SDF-1 and bFGF.[2,6,13] The release of SDF-1 and bFGF was consistently maintained until a few weeks.

Surgical Procedures and animal grouping

Male C57BL6 mice (6 weeks old; Japan SLC, Hamamatsu, Japan) were anesthetized by subcutaneous injection of ketamine (50 mg/ml) and 2% xylazine into the dorsal back. A left sciatic nerve of the mouse was carefully exposed and complete 5-mm defects were made. The mice were randomly assigned to one of the following five groups: (1) nerve conduits alone without bFGF and SDF-1 gelatin (C group, n=9), (2) nerve conduits with

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bFGF gelatin (F group, n=10), (3) nerve conduits with SDF-1 gelatin (S group, n=10), (4) nerve conduits with SDF-1/bFGF gelatin (S/F group, n=10), (5) autogenous nerve grafts (A group, n=10). One mouse in the nerve conduits alone group died before the evaluation at 12 weeks post-surgery. In the nerve conduits alone group, nerve conduits with bFGF gelatin group, nerve conduits with SDF-1 gelatin group, and nerve conduits with SDF-1/bFGF gelatin group, both proximal and distal ends of the nerve were pulled into the lumen of the nerve conduit at 1mm length, and sutured to it with 9-0 nylon sutures under a microscope (Fig. 2). In the autogenous nerve grafts group, the defect was reconstructed by inserting the resected nerve itself between the two nerve stumps. The nerve was turned 180 degrees and sutured with 9-0 nylon sutures under a microscope.

Evaluation of functional recovery

The mice in each group were evaluated at 12 weeks after surgery using a sciatic functional index (SFI).[31] Pawprints were recorded three times by painting the hind paws of each mouse with India ink and having them walk unassisted on a white paper along a 6×80 cm corridor; we then selected the clearest pawprint from each of the three walks and calculated the average of measurements (Fig. 3). The measurements of toe spread and print length were made on the experimental side; experimental toe spread (ETS), experimental print length (EPL) and the contralateral control side; normal toe spread (NTS), normal print length (NPL). SFI was calculated from the values of ETS, NTS, EPL and NPL as follows: SFI=118.9×((ETS-NTS)/NTS)-51.2×((EPL-NPL)/NPL)-7.5. The SFI value which is close to 0 indicates normal nerve function.

Electrophysiological examination

To evaluate the electrophysiological function of the sciatic nerve, the nerve was exposed, and a bipolar stimulator was placed proximal to the reconstructed area (supramaximal electrical pulses; duration 0.1 msec; frequency 1 Hz; square wave) (VikingQuest; Natus Neurology, Middleton, WI). The amplitude of the compound muscle action potential (CMAP) of the gastrocnemius muscle was recorded by inserting the recording electrode into the central portion of the muscle (Fig. 4).

Histological evaluation and histomorphometry

The nerve conduit and grafted nerves were harvested at 12 weeks post-surgery, and immersed in 4% paraformaldehyde overnight at 4 °C. The specimens were embedded in paraffin and five-micrometer-thick central transverse sections were examined by immunohistochemistry. To evaluate the regenerating axons at the central transverse section, the neurofilament antibody-positive axons (anti-neurofilament protein antibodies (1:100; DAKO, Glostrup, Denmark)) were analyzed using computer-assisted imaging. An image of the transverse section, in which the greatest number of regenerating axons was found, was photographed at a magnification of ×400 with an Olympus DP70 camera (Olympus, Tokyo, Japan). The number of the neurofilament antibody-positive axons were counted automatically using ImageJ software, wherein the axons were stained clearly (National Institutes of Health, http:// imagej.nih.gov/ij/). Also, to examine angiogenesis, the specimen of the central transverse section in the nerve conduits with bFGF gelatin group and nerve conduits with SDF-1/bFGF gelatin

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group were immunohistochemically stained using anti-CD34 antibody (1:200; eBioscience, San Diego, CA).

Evaluation of expression of anti-inflammatory cytokine genes

To evaluate inflammatory responses during peripheral nerve regeneration, gene expression levels of IL-10 and TGF-β1 in the nerve conduits were evaluated. 12 male C57BL6 mice (6 weeks old; Japan SLC, Hamamatsu, Japan) were randomly assigned to nerve conduits alone group (n=6) and nerve conduits with SDF-1/bFGF gelatin group (n=6). The nerve conduits were taken out at 3 days (n=3 in both groups) and 7 days (n=3 in both groups) post-surgery, immersed in RNA-storing reagent (RNAlater solution; Ambion, Austin, TX) and stored at -20 °C. The total RNA from the tissues including the nerve conduit was isolated using QIAzol Lysis Reagent and RNeasy Plus Universal Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturers' protocol. After the concentration and purity were measured, the cDNA was synthesized using a synthesis kit (iScript, Bio-Rad, Hercules, CA). Then, quantitative real-time PCR kits (PowerUp SYBR Green Master Mix, Thermo Fisher Scientific) were used to determine RNA levels in the nerve conduits. The reaction mix contains 10μL of PowerUp SYBR Green Master Mix with 0.8μL of forward and reverse primers, 8.4μL of diluted reverse transcription products and nuclease-free water. All reactions were performed in triplicate on a 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA) with the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. HPRT-1 was used as an endogenous control after preparation of four other prospective endogenous genes in the tissue from the

nerve conduits (HPRT-1, data not shown). The threshold cycle (Ct) value is defined as the cycle number at which the fluorescence exceeds the given threshold, and comparative Ct was used for evaluation of gene expression. The expression of anti-inflammatory genes (IL-10, TGF-β1) of the nerve conduits with SDF-1/bFGF was normalized to that of the nerve conduit alone which was assigned a value of 1.[13,32] The primers are listed in Table1.

Statistical Analysis

All data are expressed as the mean ± standard deviation (SD). The Mann-Whitney U-test with Bonferroni correction was used as a post hoc test after statistically significant differences were detected with the Kruskal-Wallis H-test, using Excel for statistical analysis (ystat 2013; Igakutosho, Tokyo, Japan). All differences were considered significant at $p < 0.05$.

Results

Functional recovery and electrophysiological examination

The mean values of the SFI in the autogenous nerve grafts group (-81.81±6.48) were significantly higher than the nerve conduits alone (-116.09±11.08, P=0.017), nerve conduits with bFGF gelatin (-111.41±7.45, P=0.012), and nerve conduits with SDF-1 gelatin (-99.91±7.68, P=0.024) groups at 12 weeks post-surgery (Fig. 5(A), Table 2). The mean values of the SFI in the nerve conduits with SDF-1/bFGF gelatin group (-88.68±10.64) were the second highest after the autogenous nerve grafts group and tended to be higher than the nerve conduits with bFGF gelatin and nerve conduits with

SDF-1 gelatin groups. The amplitude of the CMAP of the gastrocnemius muscles in the autogenous nerve grafts group (31.57±8.08) was the highest among all 5 groups; the nerve conduits alone (6.28±3.66, P=0.017), the nerve conduits with bFGF gelatin (7.60±2.95, P=0.012), the nerve conduits with SDF-1 gelatin (13.58±4.10, P=0.024) and the nerve conduits with SDF-1/bFGF gelatin groups (7.84±4.09, P=0.014) (Fig. 5(B), Table 2).

Histological evaluation of peripheral nerve regeneration and angiogenesis

Neurofilament protein-positive axons in each group, are shown in Figure 6. The axon number (562.5±241.2) in the autogenous nerve grafts group were the highest among all 5 groups; the nerve conduits alone (59.6±42.4, P=0.030), the nerve conduits with bFGF gelatin (94.6±51.7, P=0.023), the nerve conduits with SDF-1 gelatin (52.8±19.2, P=0.023) and the nerve conduits with SDF-1/bFGF gelatin groups (218.8±111.1, P=0.13) (Fig. 6(F,G) , Table 2). The axon areas in the autogenous nerve grafts group (435.19±267.91) were also highest among all 5 groups; the nerve conduits alone (37.25±33.81, P=0.023), the nerve conduits with bFGF gelatin (50.54±42.26, P=0.023), the nerve conduits with SDF-1 gelatin (21.47±13.16, P=0.023) and the nerve conduits with SDF-1/bFGF gelatin groups (101.50±53.27, P=0.034). The number and the areas of the axons in the nerve conduits with SDF-1/bFGF gelatin group were the second highest followed by the autogenous nerve grafts group, and higher than the nerve conduits alone, nerve conduits with bFGF gelatin and nerve conduits with SDF-1 gelatin groups (p < 0.05; Fig. 6(F,G), Table 2). The axons in the nerve conduits alone groups were sparse, whereas they were packed closely together forming funicles like the

autogenous nerve grafts group and exhibited preservation of their morphological

properties in the nerve conduits with SDF-1/bFGF gelatin groups (Fig. 6(A,B,E-G)).

The CD34-positive micro vessels and cells in the nerve conduits with bFGF gelatin

group were present in some places but were present all over the place in the nerve

conduits with SDF-1/bFGF gelatin group (Fig. 7(A,B)).

Cytokine gene expression in the nerve conduit

With regard to inflammation responses during peripheral nerve regeneration, TGF-β1

gene expression relative values in the nerve conduits with SDF-1/bFGF gelatin group at

3 days post-surgery (7.99±5.14, P=0.049) significantly increased more than the nerve

conduits alone group (Fig. 8(A)), but those at 7 days post-surgery (0.27±0.15, P=0.27)

were decreased similar to the nerve conduits alone group (Fig. 8(B)). In the nerve

conduits with SDF-1/bFGF gelatin group, IL-10 gene expression relative values at 3

days (0.16±0.10, P=0.16) and 7 days (0.20±0.10, P=0.049) post-surgery were not

significantly increased than the nerve conduits alone group. (Fig. 8(A,B)).

Discussion

In this study, it was shown that the nerve conduit with dual controlled release of SDF-1

and bFGF accelerated the peripheral nerve regeneration and functional recovery through

promoting CD34-positive recruited cells and angiogenesis. TGF-β1 gene expression

levels in the nerve conduit with SDF-1/bFGF gelatins were elevated in the early phase

of peripheral nerve regeneration.

   In neural tissue engineering, various exogenous cell transplantations such as cultured

Schwann cells, bone marrow mesenchymal stem cells (BMSCs) and adipose-derived

stem cells (ADSCs) have been applied to accelerate peripheral nerve regeneration.[33-37]

Specifically, we have also reported that the same nerve conduit as used in the present

study coated with induced pluripotent stem cell (iPSc)-derived neurospheres accelerated

peripheral nerve regeneration and functional recovery in mice.[7,26-28,30] Recently, tissue

engineering using the drug delivery system (DDS), which promotes the activity of

endogenous cells without exogenous celltransplantation, has developed and many

researchers have reported the usefulness of this system for tissue regenerations.[2-6] In

particular, since the development of drug delivery technology has allowed not only

single release of bioactive molecules but dual release, the dual release of drugs using

DDS has been increasingly remarkable and its robust effects have been   demonstrated

in many studies.[8-18] In bone formation, the efficacy of dual release such as bone

morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF),

BMP-2 and bFGF, BMP-2 and SDF-1, and BMP-7 and SDF-1 have been demonstrated

in previous studies.[8-12] Other studies have concluded that the controlled combination

release of the following bioactive molecules, bFGF, hepatocyte growth factor (HGF),

keratinocyte growth factor(KGF), SDF-1, and sphingosine-1 phosphate agonist

(SEW2871) have been effective for wound healing.[13,16,17] In the peripheral nerve

regeneration, the combination release of nerve growth factor (NGF) and glial

cell-derived neurotrophic factor (GDNF), NGF and VEGF, and NGF and bFGF have

also been reported in previous studies.[14,15,18] Recently, several types of nerve conduits

have been clinically used for peripheral nerve regeneration; however, there is no nerve

conduit aimed for the release of these bioactive molecules. Furthermore, to date, little

was known regarding the use of nerve conduits with the dual controlled release of these

bioactive molecules for peripheral nerve regeneration even in animal studies.

Stromal cell-derived factor-1 (SDF-1), a bioactive molecule designated as CXCL12,

promotes the recruitment of circulating immune cells, stem cells and progenitor cells

including MSCs, hematopoietic stem cells (HSCs) and endothelial progenitor cells

(EPCs) to enhance tissue regeneration through G-protein-coupled receptor

CXCR4.[6,19,20,22,38-40] SDF-1 is also known to contribute to cell-based

vascularization.[6,20] In the present study, an increase of regenerative axons

accompanyied with more micro vessels and CD34-positive cells were found at the

center of nerve conduits in the nerve conduits with SDF-1/bFGF gelatin group

compared to the nerve conduits with bFGF gelatin group. These results suggest that

SDF-1 might promote recruitment of CD34-positive cells, such as MSCs, endothelial

cells, progenitors for myeloid, lymphoid and hematopoietic lineages, leading to axonal

growth. These results also support the previous study described by Sheu ML et al. that

application of SDF-1 increased the migration of the CD34-positive cells and the effects

were consistent with improvement of rat sciatic nerve regeneration.[23]

The present results concerning cytokine gene expression showed that levels of

TGF-β1 gene expression at 3 days post- surgery were up-regulated, but those of IL-10

were not up-regulated, although anti-inflammatory monocytes generally produce

IL-10.[32] Actually, some researchers advocated that the effect on angiogenesis of SDF-1

associated with anti-inflammatory monocytes such as M2 macrophages.[41,42] Krieger JR

et al.[22] also reported the localized SDF-1, which promotes selective recruitment of monocytes along peri-implant arterioles, accelerated growth and maturation of micro vessel networks in skin injury animal models. On the other hand, Kim YH et al.[13] suggested that SDF-1 alone did not have the enhancing effect of macrophage migration, whereas the migration of MSCs was promoted by SDF-1, and increasing amount of IL-10 produced would be due to the cross-talk between MSCs and macrophages. Similarly, in the present study, the acceleration of axonal regeneration and angiogenesis promoted by SDF-1 was probably associated with the recruitment of stem and/or progenitor cells including MSCs, rather than of anti-inflammatory monocytes like M2 macrophages. Furthermore, the levels of TGF-β1 gene expression were elevated only at 3 days post- surgery, but not at 7 days. These results indicated that SDF-1 promotes the recruitment of CD34-positive cells in the early phase of peripheral nerve regeneration.

bFGF generally promotes proliferation of fibroblasts and developing neural cells and also stimulates angiogenesis.[4] Moreover, bFGF contributes to the proliferation of undifferentiated Schwann cells and nerve regeneration at the early phase of peripheral nerve injury.[43,44] Successful peripheral nerve regeneration with nerve conduit depends on the formation of a new extracellular matrix scaffold, over which blood vessels, fibroblasts, recruitment of stem and/or progenitor cells, and Schwann cell migration.[45,46] In the present study, it is assumed that the combinatorial effects of SDF-1 and bFGF further promoted nerve regeneration. There are several limitations in this study. A 5-mm sciatic nerve defect model in mice was investigated at a single time

point: 12 weeks post-operation. A literature review of artificial nerve conduits revealed that an average 5 mm (range 4 -6 mm) gap was used as the sciatic nerve regeneration model in mice.[47] Since the nerve conduit (1 mm inner diameter) was suitable for the mouse sciatic nerve diameter (approximately 0.3-0.5 mm) as with our previous studies using mouse sciatic nerve gaps, the 5 mm sciatic nerve gap was used in the present study in accordance with the literature and the size of conduit according to our previous studies.[7,28,30,47] Although regeneration would have likely intensified if the evaluation was conducted for a longer period, we believe that the evaluation of nerve regeneration for 12 weeks postoperatively is adequate because nerve regeneration using the nerve conduit at 12 weeks was maintained for as long as 48 weeks after implantation in a previous study.[28] Therefore, in the present study, experiments were performed according to previous reports.[28]

Our present results showed that the nerve conduit with DDS did not match the performance of autogenous nerve grafts. However, the key point to emphasize in the present study was that the addition of multiple bioactive molecules such as SDF-1 and bFGF to the nerve conduits designed for controlled drug release system is beneficial for the acceleration of nerve regeneration. As Fukuda T et al. reported that the bFGF slow-release DDS combined to a nerve conduit improved the nerve regeneration, the slow-release DDS of multiple growth factors with nerve conduits would also attract more attention in the future.[48] The disadvantage of the current nerve conduit is, however, still its poorer nerve regeneration ability than that of the autograft. To overcome poor nerve regeneration with nerve conduits, we first applied the addition of

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dual release of bioactive molecules to the nerve conduit and showed acceleration of

axonal growth. These processes used in the present study are novel. Moreover, we used

bFGF and SDF-1 as bioactive molecules that were commercially available. We believe

that the release of multiple suitable factors for axonal growth through DDS dramatically

enhances the nerve regeneration by the additive or synergistic effects. Finally, the

combination of exogenous cell transplantation and the multiple release of bioactive

factors through DDS would greatly increase the success of tissue engineering.

Conclusion

Combined release of SDF-1 and bFGF promoted peripheral nerve regeneration. Nerve

conduits coated with dual controlled release of effective bioactive molecules present an

attractive strategy for inducing nerve regeneration.

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Figure Legends

Fig. 1.

The biodegradable nerve conduit coated with SDF-1 and bFGF incorporated into gelatin hydrogels. **(A)** The gelatin hydrogels were injected into the nerve conduit. **(B)** Cross section of the nerve conduit coated with gelatin hydrogels.

Fig. 2.

Intraoperative gross findings in the experimental model. Reconstructed sciatic nerve gap with the nerve conduit in the Nerve conduits alone, Nerve conduits with bFGF gelatin, Nerve conduits with SDF-1 gelatin, and Nerve conduits with SDF-1/bFGF gelatin groups. Both the proximal and distal ends of the nerve were pulled into the nerve conduit at a length of 1 mm and the nerves were sutured on the lumen wall.

Fig. 3.

Walking track analysis. Typical walking tracks were obtained from each mouse.

Fig. 4.

Electrophysiological evaluation. The amplitude of the compound muscle action potential derived from the gastrocnemius muscles was recorded by stimulating the sciatic nerve on proximal to the reconstructed area.

Fig. 5.

Recovery of function and electrophysiological results 12 weeks postoperatively. **(A)** Functional recovery was assessed by SFI 12 weeks postoperatively **(B)** Amplitude of compound motor action potential of gastrocnemius muscle 12 weeks postoperatively. *$p < 0.05$. Two-tailed Mann–Whitney U-test with Bonferroni correction. A group: Autogenous nerve grafts group, C group: Nerve conduits alone group, F group: Nerve conduits with bFGF gelatin group, S group: Nerve conduits with SDF-1 gelatin group, S/F group: Nerve conduits with SDF-1/bFGF gelatin group.

Fig. 6.

Representative histological sections of axons immunohistochemically stained with anti-neurofilament antibody. **(A)** Autogenous nerve grafts group (A group): numerous regenerated axons were observed. **(B)** Nerve conduits alone group (C group): regenerated axons were sparse. **(C)** Nerve conduits with bFGF gelatin group (F group): regenerated axons were locally packed together. **(D)** Nerve conduits with SDF-1 gelatin group (S group): axonal regeneration was induced, but the axons were not closely packed together. **(E)** Nerve conduits with SDF-1/bFGF gelatin group (S/F group): axonal regeneration was abundantly induced and the axons were packed together to form the nerve bundle. Scale bar indicates 20 μm.

(F) Axon numbers 12 weeks postoperatively **(G)** Axon areas 12 weeks postoperatively. *$p < 0.05$. Two-tailed Mann–Whitney U-test with Bonferroni correction.

Fig. 7.

Representative histological sections of vessels and cells immunohistochemically stained with anti-CD34 antibody 12 weeks postoperatively. **(A)** Nerve conduits with bFGF gelatin group (F group): vessels and cells stained with anti-CD34 antibody were observed but not numerous. **(B)** Nerve conduits with SDF-1/bFGF gelatin group (S/F group): numerous vessels and cells stained with anti-CD34 antibody were observed. Scale bar indicates 20 μm.

Fig. 8.

The gene expression levels of IL-10 and TGF-β1. (A) 3 days postoperatively. (B) 7 days postoperatively. $*p < 0.05$. One-tailed Mann–Whitney U-test. C group: Nerve conduits alone group, S/F group: Nerve conduits with SDF-1/bFGF gelatin group.

Tables

Table 1. Primers used for Real-Time PCR Analysis

Table 2. Analysis of SFI, compound motor action potential amplitude of gastrocnemius muscle, axon numbers, and axon area

Figure 1.

Figure 2.

Figure 3.

NPL

NTS

EPL

ETS

Walking direction

Figure 4.

Figure 5.

A

B
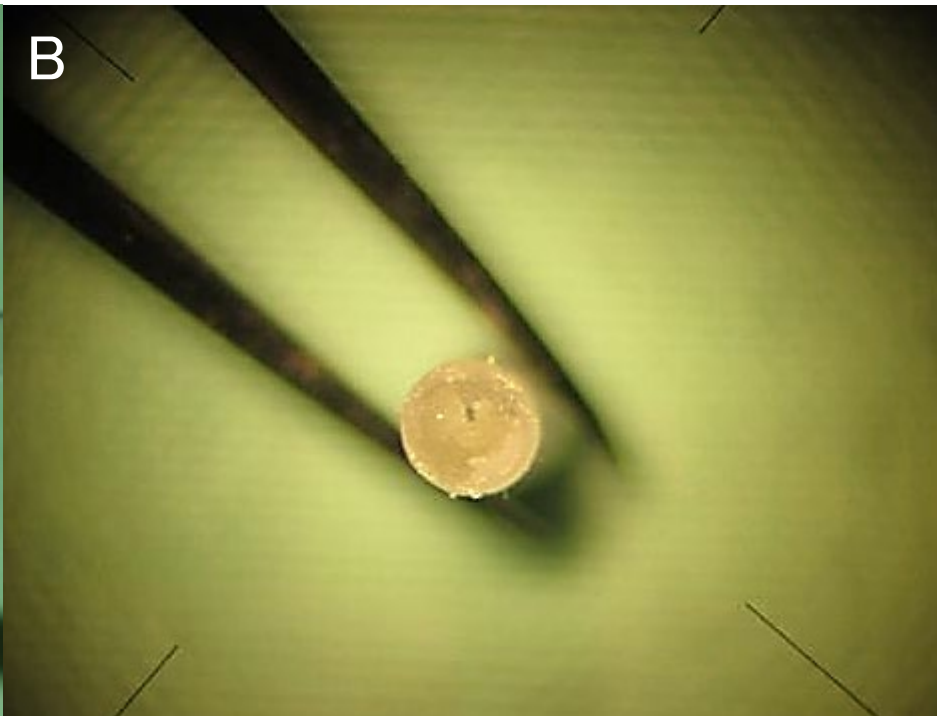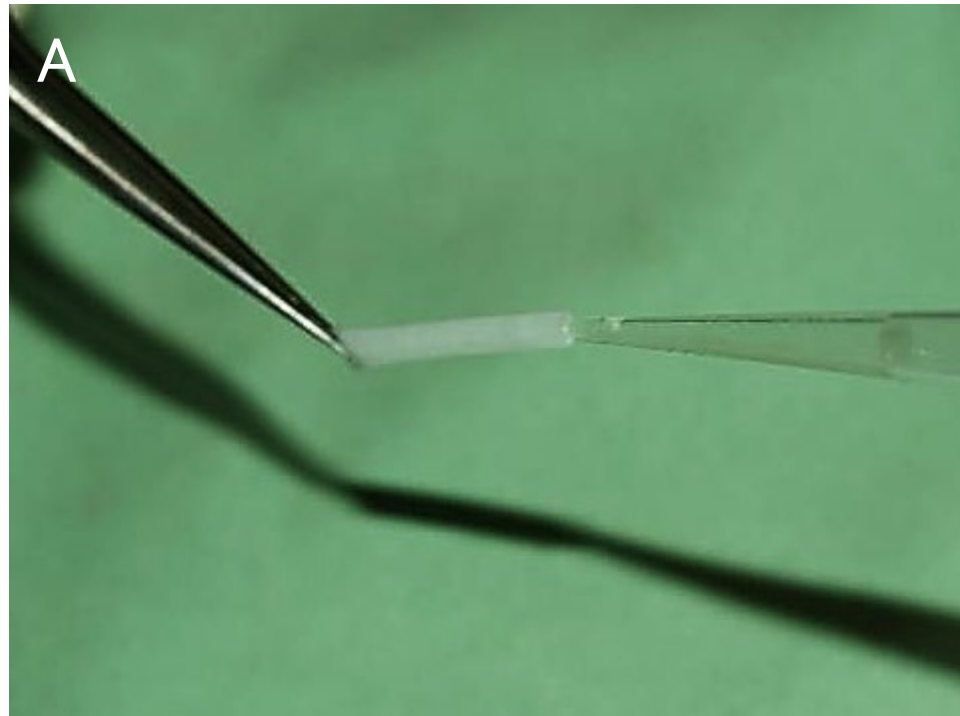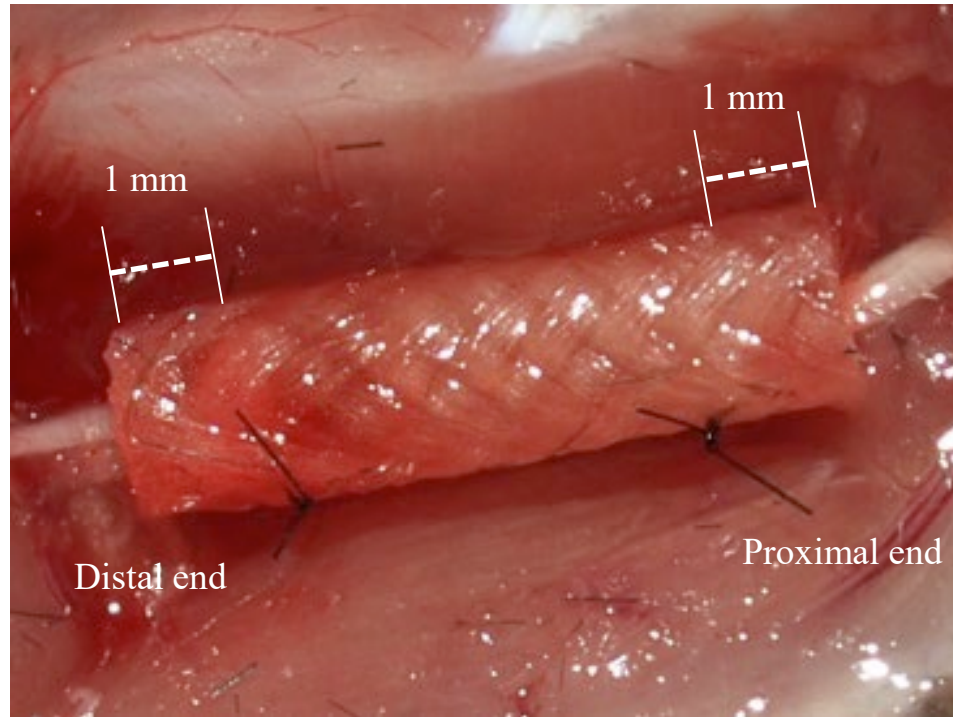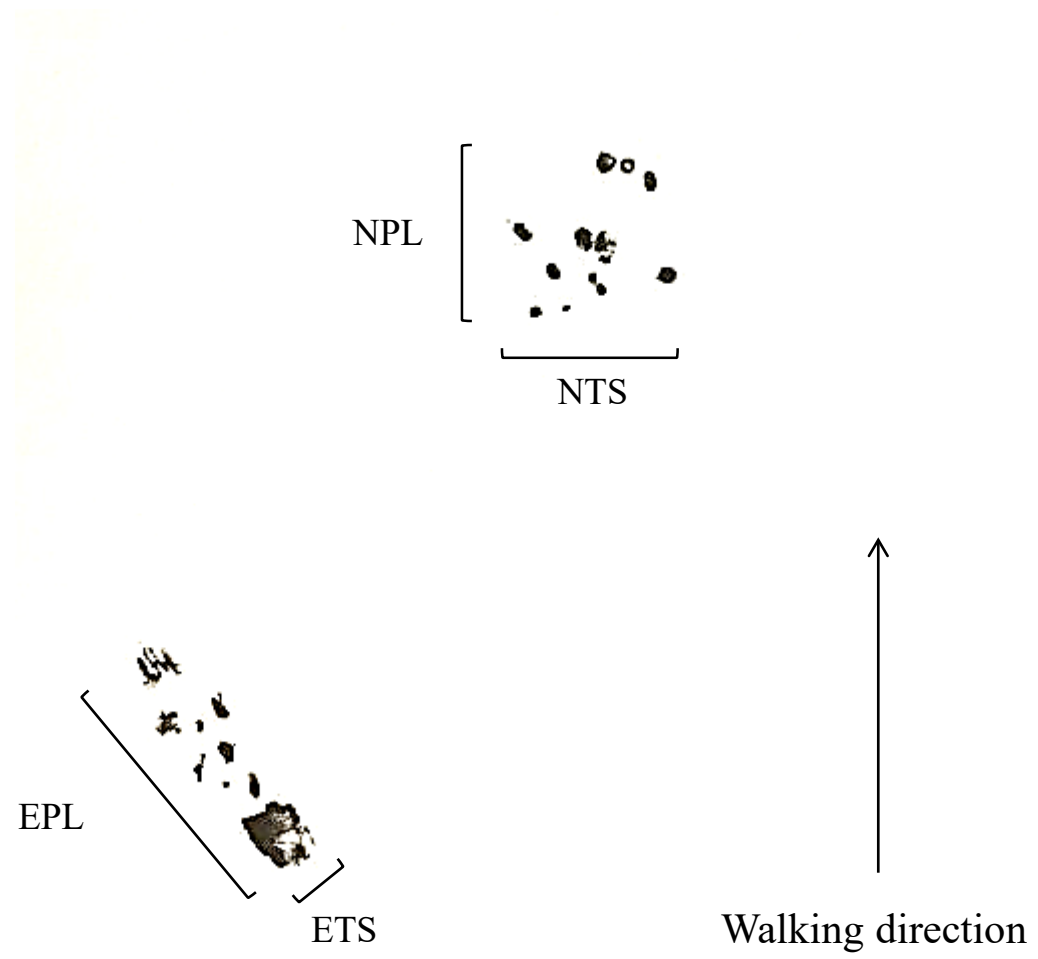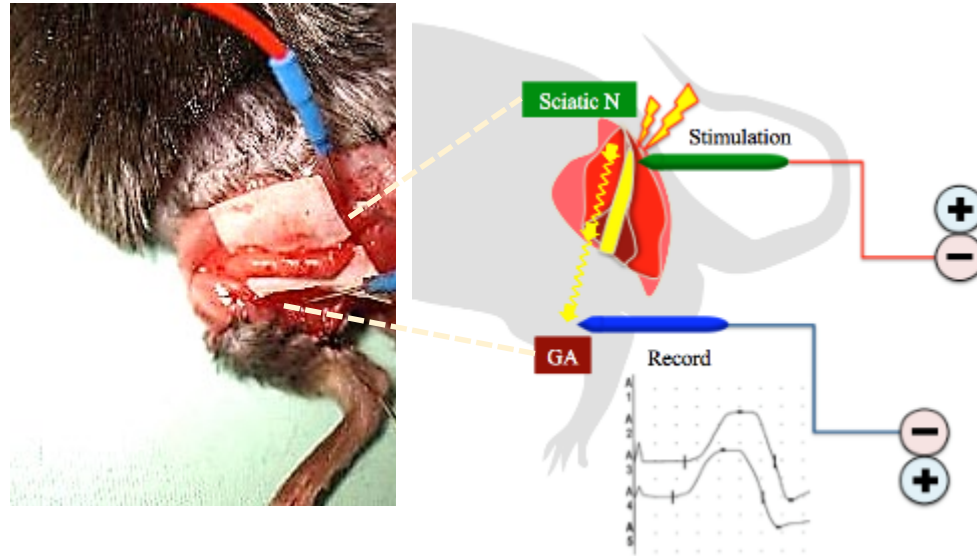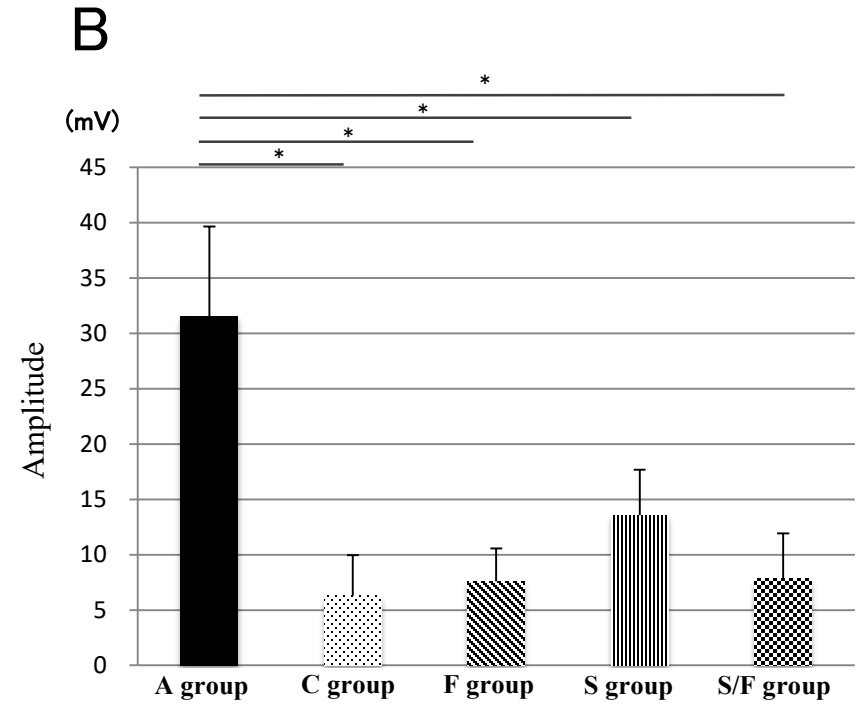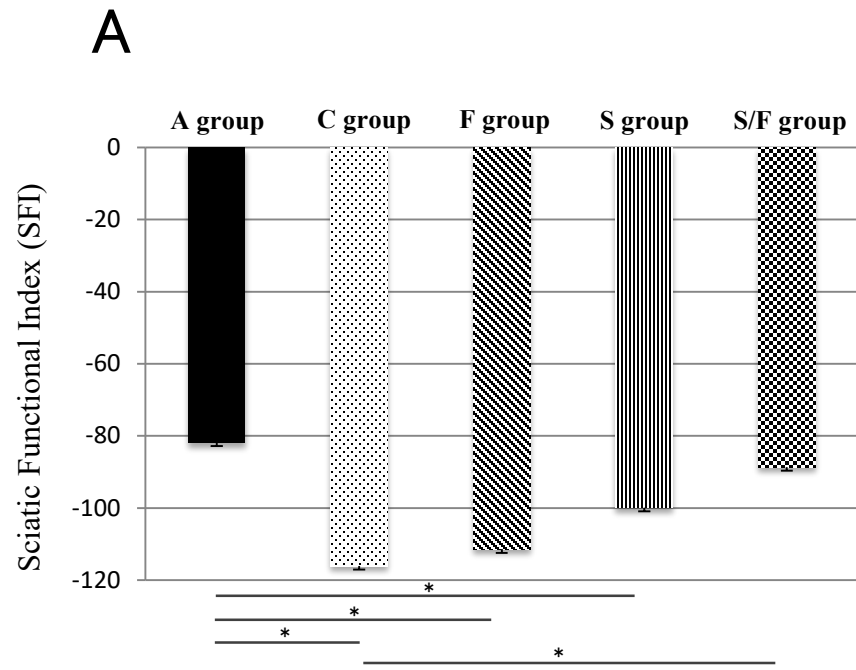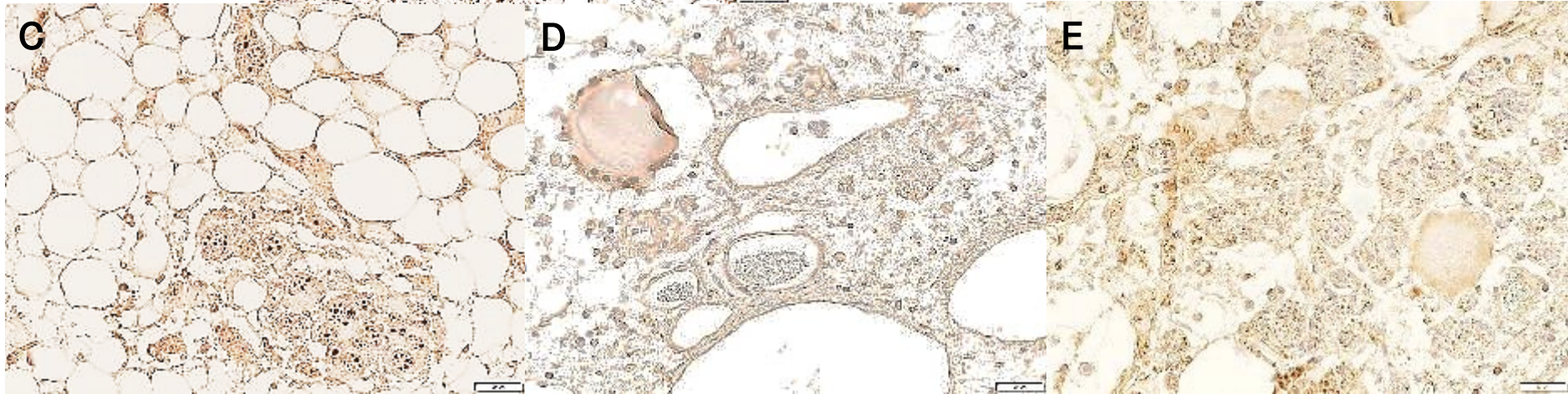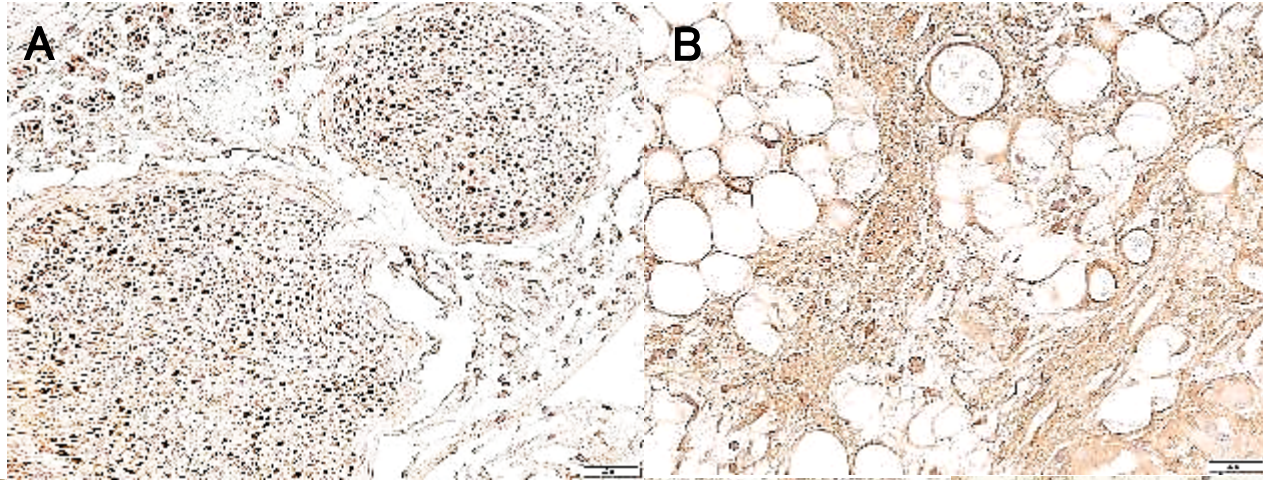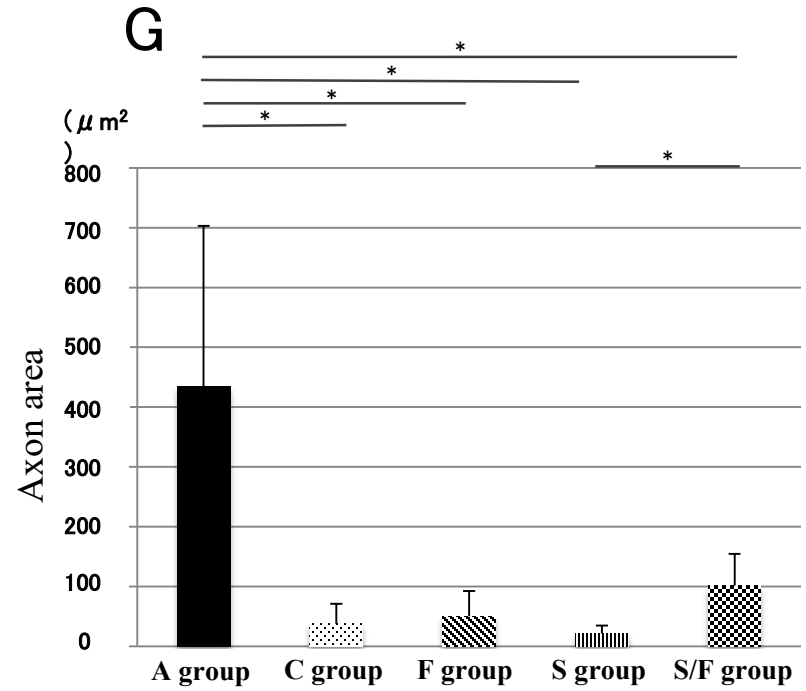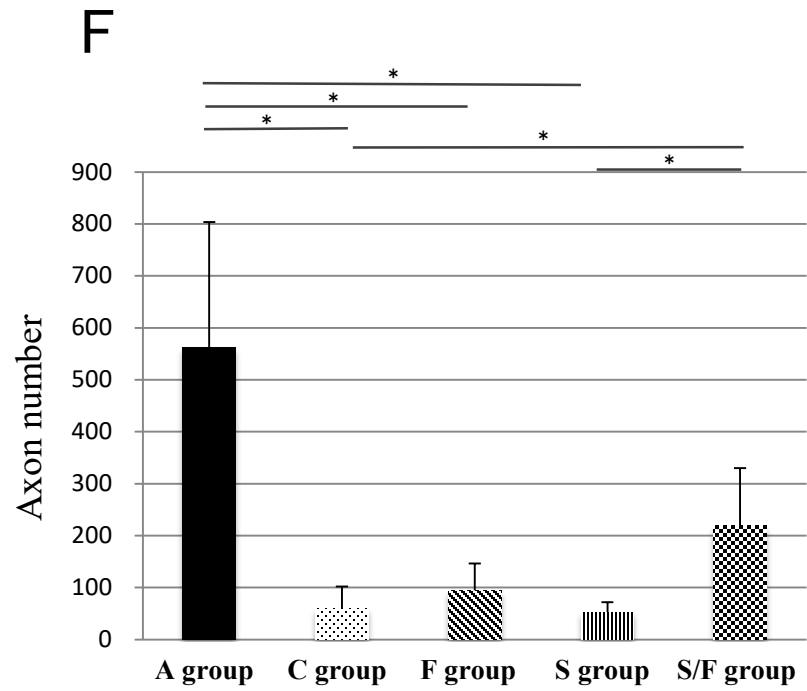
Figure 6.

Figure 6.

Figure 7.
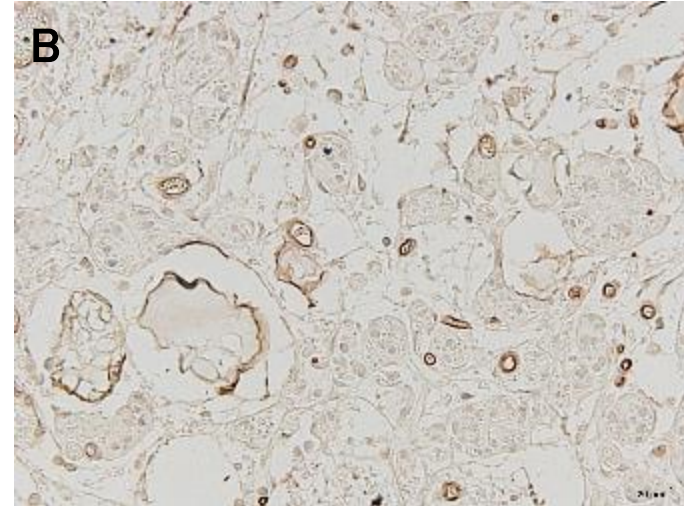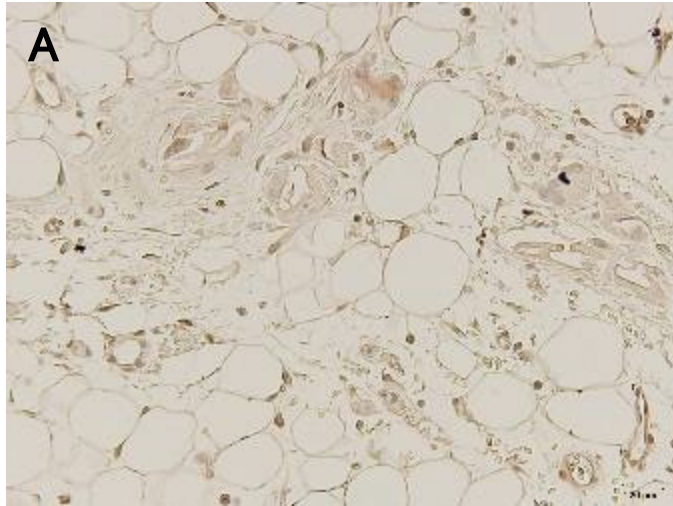
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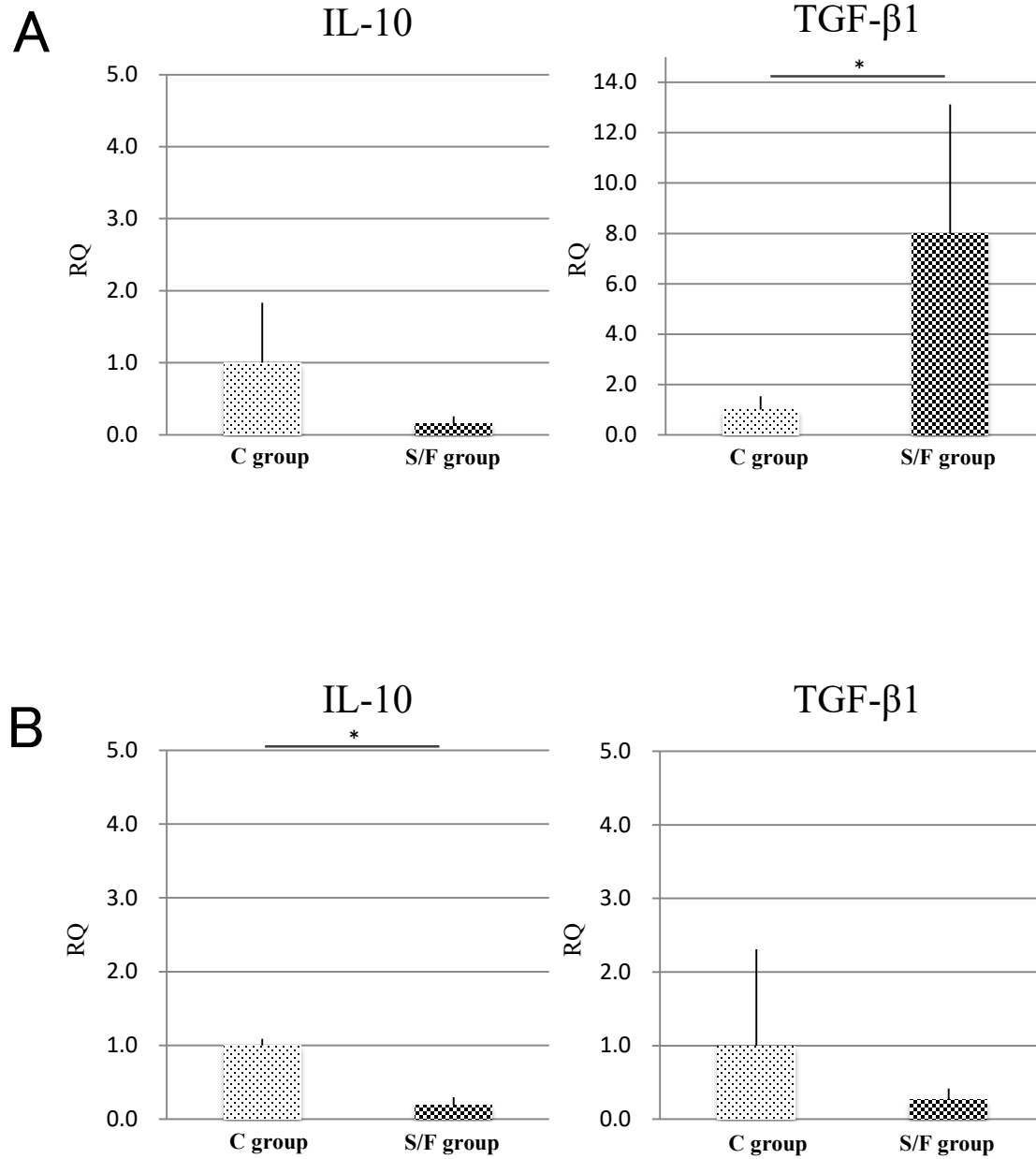
Table 1.

mRNA	Forward	Reverse
IL-10	CTTTAAGGGTTACTTGGGTTG	AAATGCTCCTTGATTTCTGG
TGF-β1	GGATACCAACTATTGCTTCAG	TGTCCAGGCTCCAAATATAG
HPRT-1	AGGGATTTGAATCACGTTTG	TTTACTGGCAACATCAACAG

Table 2.

Group	SFI	Amplitude (mV)	Axon number	Axon area (μm^2)
A group	-81.81±6.48	31.57±8.08	562.5±241.2	435.19±267.91
C group	-116.09±11.08	6.28±3.66	59.6±42.4	37.25±33.81
F group	-111.41±7.45	7.60±2.95	94.6±51.7	50.54±42.26
S group	-99.91±7.68	13.58±4.10	52.8±19.2	21.47±13.16
S/F group	-88.68±10.64	7.84±4.09	218.8±111.1	101.50±53.27