

Doxorubicin-Immersed Skeletal Muscle Grafts Promote Peripheral Nerve Regeneration Across a 10-mm Defect in the Rat Sciatic Nerve

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Abstract

Background The treatment of peripheral nerve defects requires bridging materials. Skeletal muscle grafts have been studied as an alternative to nerve autografts because they contain longitudinally aligned basal laminar tubes that are similar to axons. Several pretreatment methods for muscle grafts have promoted axonal regeneration. Here, a new method of doxorubicin pretreatment was used, and the efficacy of the pretreated muscle graft was evaluated in a rat model of a sciatic nerve defect.

Methods A rat model of a 10-mm sciatic nerve defect was analyzed in three settings: muscle grafts with and without doxorubicin pretreatment (M-graft-w-Dox and M-graft-w/o-Dox groups, respectively) and a nerve autograft group (N-graft) ($n = 6/\text{group}$). The M-graft-w-Dox group was immersed in a doxorubicin solution for 10 minutes and rinsed with saline. Analyses of target muscle atrophy, electrophysiology, and histology were performed 8 weeks after grafting.

Results Electrophysiological parameters and target muscle atrophy were significantly superior in the M-graft-w-Dox group compared with the M-graft-w/o-Dox group. Histological assessment revealed the presence of a significantly greater number of regenerated axons in the M-graft-w-Dox group versus the M-graft-w/o-Dox group, while there were no significant differences between the M-graft-w-Dox and N-graft groups. The diameter of myelinated axons of the regenerated nerve in the M-graft-w-Dox group was significantly larger than that in the M-graft-w/o-Dox group, while it was not significantly different compared with the N-graft group.

Conclusion Pretreatment of muscle grafts with doxorubicin promoted significant peripheral nerve regeneration. This method may represent a new option for the treatment of peripheral nerve defects.

Keywords

- peripheral nerve
- doxorubicin
- pretreatment
- muscle
- graft
- nerve regeneration

Peripheral nerve injury can lead to lifelong issues or loss of the function that is mediated by the injured nerve.¹ A peripheral nerve that is lacerated or completely severed requires acute surgical repair.² Direct tensionless end-to-end repair, if possible, achieves the most predictable outcome. However, often the

nerve ends cannot be approximated without tension. Thus, bridging the defect with a nerve autograft is a method that is widely used for nerve reconstruction.^{3,4} Sensory nerves are donor candidates and the sural nerve was used most commonly in previous studies.⁵ In such cases, donor-site morbidity,

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including sensory loss, painful neuroma at the proximal nerve end, complaints of irritating hypoesthesia, and unpleasant sensations at the corresponding nerve area, occurs often.⁵ To overcome these issues, artificial materials, other autologous tissues, and various alternative methods have been studied as substitutes for autologous nerve grafts (N-grafts) in the treatment of nerve defects.^{3,5-9}

Autologous muscle tissue is a candidate bridging graft for nerve defects because the topological structure of muscle fibers includes basal lamina tubes, which are considered to provide a suitable scaffold for nerve regeneration.¹⁰⁻¹⁶ Moreover, the abundance of muscle tissue in the body resolves donor-site problems. Several clinical studies reported a good sensory recovery using this method, whereas motor recovery remained poor.^{4,17-19} Various pretreatment methods have been developed because removing or emptying the myoplasm that fill the internal space of the basal lamina tube in as early a phase as possible was essential before axonal elongation.^{14,16} Freezing, anesthetic infiltration, microwaving, heating, and alcohol treatments were previously reported methods to make an “empty tube” from muscle tissue.^{10,13,14,20,21} The rationale behind these pretreatments is to degenerate the muscle fibers and accelerate macrophages to phagocyte the myoplasm inside the basal lamina tube.^{14,16} Even with these pretreatments, however, muscle grafts do not yield satisfying outcomes in clinical applications, and the number of studies using muscle grafts for nerve defects has decreased in the past decade.

Doxorubicin is a major anticancer drug for breast cancer, sarcomas, childhood solid tumors, and leukemia.²² Doxorubicin also causes the cardiac and skeletal muscle tissues to undergo degenerative change by inducing muscular cell apoptosis, however.²³⁻²⁵ The mechanism underlying this toxicity is the interaction between doxorubicin and iron, which generates reactive oxygen species that target topoisomerase 2 and impair mitochondria.²⁶ In the field of nerve regeneration using muscle grafts, pretreatments have been performed to promote degenerative change to the muscle tissue. In this context, we arrived at the idea that muscle grafts can be degenerated by doxorubicin toxicity.

In the current study, we hypothesized that the immersion of muscle grafts in a doxorubicin solution promotes and advances macrophage migration in an early phase of neural regeneration through degenerative changes to the muscle tissue caused by doxorubicin pretreatment. Furthermore, these advantages in an early phase enhance axonal regeneration and result in improved motor nerve functional recovery. Thus, the efficacy of doxorubicin-immersed muscle grafts was assessed using a 10-mm defect of the sciatic nerve in rats, which cannot be bridged naturally.²⁷

Methods

Animals and Experimental Plan

F344 rats (age, 9–10 weeks; weight, 200–250 g; CLEA, Tokyo, Japan) were used in this study. Each rat was housed in a separate cage and given food and water without restriction. The surgical procedure was performed after acclimation to that environment.

In this study, first, assessments of the grafts immediately after pretreatment were performed, which included hematoxylin and eosin staining as well as length and weight measurement. Next, we assessed the pretreated graft 1 week after transplantation with TUNEL (TdT-mediated dUTP Nick End Labeling) staining and immunofluorescent studies. The aim of TUNEL staining was to confirm the degenerative change induced by doxorubicin pretreatment, and the aim of immunofluorescent studies was to assess whether doxorubicin pretreatment enhanced macrophage migration in an early phase of axonal regeneration. Finally, to assess the efficacy of the pretreatment of muscle grafts with doxorubicin, three types of graft were compared: muscle grafts with and without doxorubicin pretreatment (M-graft-w-Dox and M-graft-w/o-Dox, respectively) and an autologous N-graft. This study was planned to collect data from six rats in each group. Eight weeks after transplantation, electrophysiological, histological, and morphometric analyses were performed, and target muscle atrophy was assessed.

Peripheral Nerve Defect Model, Graft Harvesting, and Doxorubicin Pretreatment

General anesthesia was performed via intraperitoneal injection of 40 mg/kg of pentobarbital sodium (Kyoritsuseiyaku, Tokyo, Japan) after inhalation of isoflurane (Mairanseiyaku, Osaka, Japan), with rats in the prone position. A straight skin incision was made from the right hip joint to the popliteal portion. Muscle fibers were harvested from the biceps femoris, splitting along with the direction of the muscle fibers into a long narrow shape. A muscle segment of ~15 mm was harvested and trimmed down to 10 mm after the pretreatment. After exposing the right sciatic nerve, the middle part of the nerve segment was removed to generate a nerve defect, and a 10-mm graft was transplanted between the nerve stumps using 9-0 nylon sutures. For nerve autografting, a 10-mm nerve segment was turned in the opposite direction and sutured (reversed autograft). After each procedure, intraperitoneal fentanyl (0.02 mg/kg; Janssen Japan, Tokyo, Japan) was administered for postoperative analgesia, and rats were returned to their cages and allowed to walk and feed freely. All animal protocols were performed in accordance with the Guidelines of the Animal Experimentation Committee, Kyoto University.

Doxorubicin pretreatment was performed as follows: the harvested muscle tissue was immersed in a doxorubicin solution of 100 μ mol/L (Doxorubicin, Nippon Kayaku, Tokyo, Japan) for 10 minutes, followed by rinsing with a saline solution for 10 minutes. In the M-graft-w/o-Dox group, the rinsing procedure alone was performed before transplantation.

Hematoxylin and Eosin Staining

To assess the structural change by pretreatment, hematoxylin and eosin staining was performed. The samples were fixed immediately after pretreatment with 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin blocks. Semithin sections (5 μ m) of these samples were obtained and assessed by light microscopy (BZ-X700; KEYENCE, Osaka, Japan), after being stained with hematoxylin and eosin solution.

Graft Measurement (Length and Weight) and Histological Analysis of Doxorubicin-Immersed Muscle Grafts

In the M-graft-w/o-Dox and M-graft-w-Dox groups, graft length and weight were measured before and after pretreatment with the drug. Graft length was measured to assess graft shrinkage after pretreatment. This measurement was performed via photography and length was calculated with 0.1 mm precision using the Image J software (National Institutes of Health, Bethesda, MD). Graft weight was measured using a digital weighing scale with 0.001 mg precision.

TUNEL Staining and Immunofluorescence Studies for the Assessment of Macrophage Migration in an Early Phase of Axonal Regeneration

Six rats in each group were used to assess whether doxorubicin pretreatment induced degenerative change to muscular cells and promoted macrophage accumulation in an early phase of axonal regeneration. Using a 10-mm nerve defect model, each rat was transplanted a M-graft-w-Dox ($n = 3$) or M-graft-w/o-Dox ($n = 3$) pretreatment. One week after surgery, grafts were removed and segments were fixed in 4% paraformaldehyde overnight. After cryoprotection in 20% sucrose for 48 hours at 4°C, 14- μ m-thick transverse frozen sections were obtained by cryosection cutting.

TUNEL staining was performed using a kit (In situ Apoptosis Detection Kit, TAKARA Bio, Shiga, Japan). Briefly, frozen sections were washed by phosphate-buffered saline (PBS) and permeabilization buffer was added. The sections were treated with terminal deoxynucleotidyl transferase enzyme and labeling buffer, and then slides were viewed using a confocal microscope (BZ-X700; KEYENCE). Negative controls were treated solely by labeling buffer (data not shown).

For the immunofluorescence studies, after the antigen retrieval procedure with proteinase K (PK; Sigma-Aldrich) at room temperature (RT) for 15 minutes, blocking and permeabilizing were performed for 1 hour at RT with donkey serum (Sigma-Aldrich) and Triton X-100 (Sigma-Aldrich), followed by incubation with primary antibodies at 4°C for 24 hours: CD68 (mouse immunoglobulin G [IgG], 1:50) and laminin (rabbit IgG, 1:50) (Bio-Rad). After washing with PBS, slides were incubated with secondary antibodies at RT for 1 hour (donkey antimouse IgG [H + L], CFTM543 antibody, Sigma-Aldrich; donkey antirabbit IgG [H + L], CFTM488 antibody, Sigma-Aldrich). Mounted with bicarbonate-buffered glycerol (pH 8.6) and cover slips, slides were viewed using a confocal microscope (BZ-X700; KEYENCE). The negative controls included the omission of primary or secondary antibodies on parallel sections (data not shown). The number of CD68-positive cells was counted from five random high-power fields per section.

Electrophysiological Assessments

Electrophysiological studies were performed 8 weeks after surgery under general anesthesia, as described previously, with the rats in the prone position. The sciatic nerve was stimulated at the point of the distal pisiforms (S1) and the popliteal fossa (S2) using an electromyogram device (Neuro-

pack S1 MEB-9404, Nihon Kohden, Tokyo, Japan). To assess the compound muscle action potentials (CMAPs) of the pedal adductor muscles, which are the most distal muscles innervated by the sciatic nerve, two needle electrodes were inserted into that muscle. The CMAPs of the contralateral, healthy side were evaluated before the right side. The stimulation was performed 20 times (1 Hz) at the minimum voltage at which the maximum amplitude was observed. The amplitude of the CMAPs was calculated from the wave of the S1 (peak to peak), and the motor nerve conduction velocity (MNCV) was calculated from the time difference between the distal latencies of the S1 and S2 stimulations and the distance between the two stimulation points. The bilateral sciatic nerves were assessed and the amplitudes of the CMAPs and MNCV were expressed as a percentage of the values corresponding to the uninjured side.

Assessment of Target Muscle Atrophy

The bilateral tibialis anterior muscles were harvested, including the attachment of the tendinous part, and the wet muscle was weighed on a digital scale with 0.001 g precision. The results were expressed as a percentage of the value from the uninjured side.

Histological and Morphological Analyses of the Regenerated Axons

After exposure of the right sciatic nerve, nerve segments with a thickness of 1 mm were obtained at the middle portion of the regenerated nerve and at 5 mm from the distal nerve stump. Specimens were fixed in 1.44% paraformaldehyde and 1% glutaraldehyde, postfixed in 1% osmic acid, and embedded in epoxy resin. Transverse semithin sections collected at the midportion of the graft were stained with a 0.5% (w/v) toluidine blue solution and assessed using a light microscope (ECLIPSE 80i, Nikon, Tokyo, Japan). Ultrathin sections of the same tissue were stained with uranyl acetate and lead citrate and were examined using a transmission electron microscope (TEM; Model H-7000; Hitachi High-Technologies, Tokyo, Japan). Assessments consisted of counting the total number of myelinated axons and measuring the shortest axon diameter and the shortest diameter of myelinated axons using the ImageJ software for morphometric analysis.²⁸ The total number of myelinated axons was calculated as follows: the whole neural area (a) was measured on an image at a magnification of $40\times$. Randomly, six or seven fields were selected for analysis in each specimen to represent more than 20% of the total area. For each field, the number of myelinated axons was counted, and the measurement of neural areas was performed at a magnification of $400\times$. The number of myelinated axons in (b), and the neural areas (c) of the six or seven fields were summed. The total number of myelinated axons in each field was estimated as $b \times a/c$.

Supplemental Study to Evaluate an Alternative Doxorubicin Pretreatment Concentration

A supplemental study was performed to evaluate the efficacy of pretreatment with a higher concentration of doxorubicin (2,000 μ mol/L) using the rat model described previously. Six

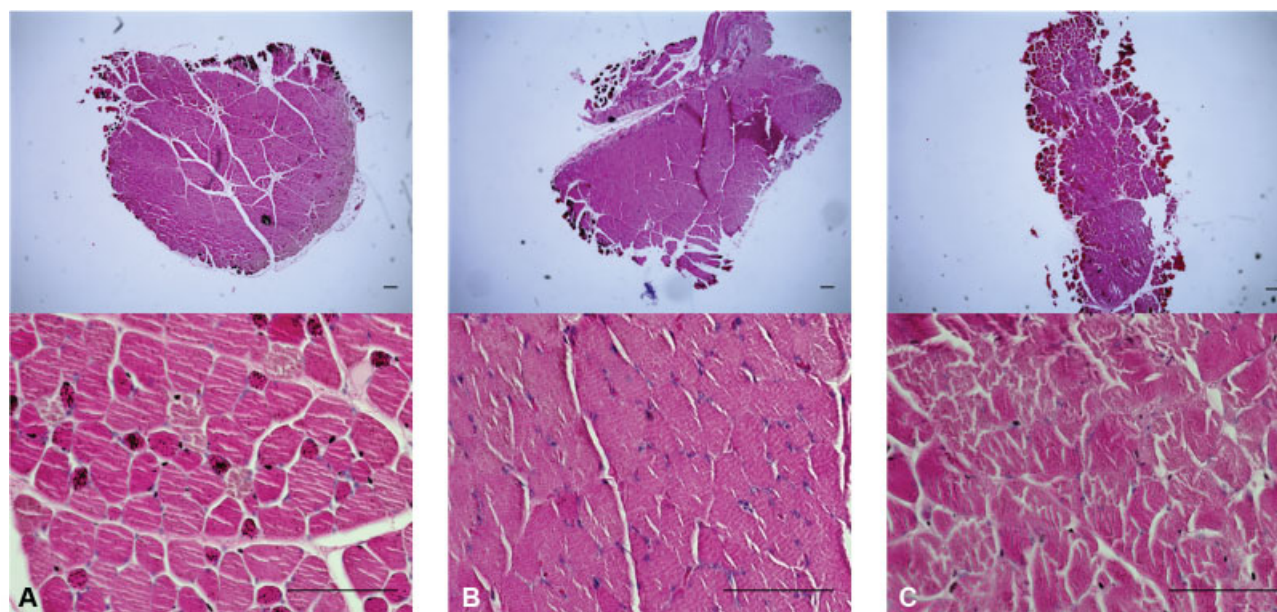


Fig. 1 Assessments of the grafts just after pretreatment stained with hematoxylin and eosin. (A) The borders between muscle fibers were clear (M-graft-w/o-Dox). (B) However, in M-graft-w-Dox group, the spaces were less clear or each fiber was swelled, indicating that muscle tissue was damaged by doxorubicin pretreatment. (C) Moreover, in 2,000 µmol/L group, the borders were diminished (scale bar, 100 µm). M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin.

additional rats were added as the 2,000 µmol/L group and the electrophysiological and histological studies and target muscle atrophy assessments were performed in a manner that was similar to that described for the main study.

Statistical Analysis

Data were expressed as means $\pm$ standard deviation. The two-group analysis was performed by nonpaired *t*-test, whereas the multigroup analysis including three groups was performed via one-way analysis of variance followed by Tukey's honest significant difference test (JMP 8; SAS Institute, Cary, NC). Significance was set at $p < 0.05$.

Results

Hematoxylin and Eosin Staining

In the sections of the M-graft-w/o-Dox group, muscle fibers were regularly aligned and fibers were evenly distributed with space between each other; the borders between fibers were clear (**Fig. 1A**). In the M-graft-w-Dox group, however, muscle fibers were densely aligned or each fiber was swelled and the spaces between fibers were less clear than those in the M-graft-w/o-Dox group, indicating that muscle tissue was damaged by doxorubicin pretreatment. The borders

between fibers were also unclear (**Fig. 1B**). Moreover, in the section from the 2,000 µmol/L group, the basal lamina tube of the muscle fibers was severely damaged and the borders between fibers were diminished (**Fig. 1C**).

Graft Measurement (Length and Weight)

The mean graft length was 1.52 ± 0.16 mm (before) and 1.44 ± 0.14 mm (after) in the M-graft-w/o-Dox group, and 1.52 ± 0.12 mm (before) and 1.42 ± 0.15 mm (after) in the M-graft-w-Dox group. No significant differences were observed in inter- and intragroup analyses. The mean graft weight was 0.092 ± 0.023 mg (before) and 0.101 ± 0.027 mg (after) in the M-graft-w/o-Dox group, and 0.091 ± 0.098 mg (before) and 0.098 ± 0.039 mg (after) in the M-graft-w-Dox group. Significant differences were observed in inter- and intragroup analyses ($p < 0.01$, **Table 1**).

TUNEL Staining and Immunofluorescence Studies for the Assessment of Macrophage Migration in an Early Phase of Axonal Regeneration

TUNEL-positive cells were detected in both the M-graft-w/o-Dox group (**Fig. 2A**) and the M-graft-w-Dox group (**Fig. 2B**). These cells were more obvious in the M-graft-w-Dox group, however. These results indicate that muscular cell apoptosis was induced by doxorubicin pretreatment.

Table 1 Graft measurement (length and weight) before and after doxorubicin pretreatment

	Graft length (mm)		Graft weight (mg)	
	Before pretreatment	After pretreatment	Before pretreatment	After Pretreatment
M-graft-w/o-Dox	1.52 ± 0.16	1.44 ± 0.14	0.091 ± 0.023	0.101 ± 0.027
M-graft-w-Dox	1.52 ± 0.12	1.42 ± 0.15	0.091 ± 0.037	0.098 ± 0.039

Abbreviations: M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin.

Note: Graft shrinkage was not obvious after pretreatment and graft weight was slightly increased.

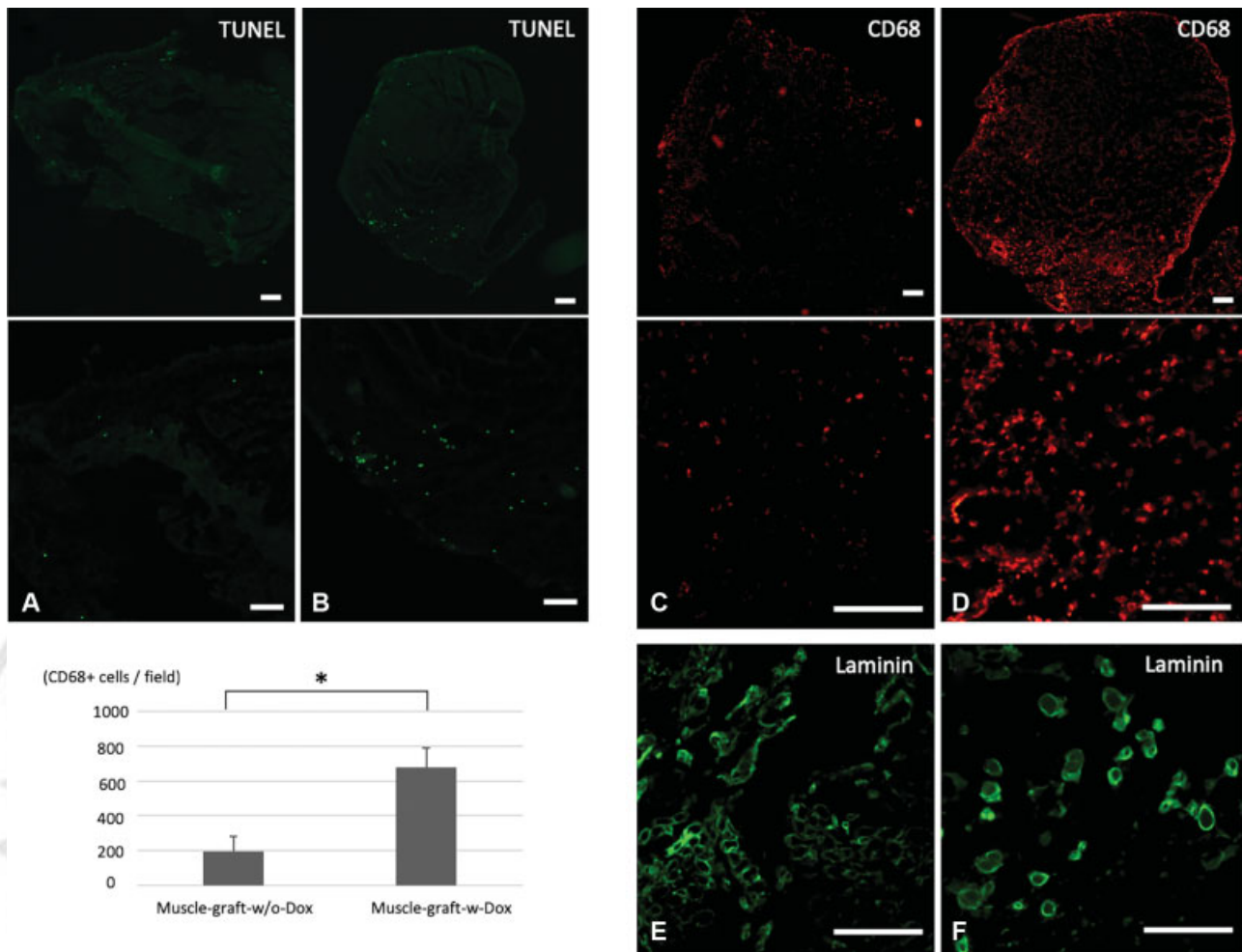


Fig. 2 TUNEL staining and immunofluorescence studies for the assessment of macrophage migration in an early phase of axonal regeneration. TUNEL-positive cells were shown in both groups ([A] M-graft-w/o-Dox group and [B] M-graft-w-Dox group) (scar bar, 100 μ m). However, these cells were more obvious in the M-graft-w-Dox group. Expression of CD68-positive cells and laminin were expressed in both groups ([C, E] M-graft-w/o-Dox group and [D, F] M-graft-w-Dox group) (scar bar, 100 μ m). The left panel showed the number of CD68-positive cells, which was significantly higher in M-graft-w-Dox group ($p < 0.05$). M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin.

In the immunofluorescence studies, CD68-positive cells were expressed in both groups in an early phase of axonal regeneration. The number of CD68-positive cells was 195 ± 83 cells/field (M-graft-w/o-Dox, ▶Fig. 2C) and 676 ± 115 cells/field (M-graft-w-Dox, ▶Fig. 2D), however, and significant differences were seen between the groups ($p < 0.05$) (▶Fig. 2, left panel). These results suggest that macrophage migration was enhanced by doxorubicin pretreatment. Laminin was also expressed in both groups, suggesting that the extracellular matrix was preserved even after doxorubicin pretreatment (▶Fig. 2E, F).

Macroscopic Examination of the Grafts

Macroscopic examination performed at 8 weeks after grafting showed the continuity of the nerve defect in all cases in the M-graft-w-Dox and N-graft groups. ▶Fig. 3 shows representative cases from the M-graft-w/o-Dox (▶Fig. 3A), M-graft-w-Dox (▶Fig. 3B), and N-graft groups (▶Fig. 3C). It is of note that no obvious changes surrounding the graft, such as adhesiveness, were observed in the doxorubicin-treated group.

Electrophysiological Assessment

The CMAPs of the pedal adductor muscles were detected in all animals in the M-graft-w-Dox and N-graft groups, whereas no valid waveform was obtained from any of the six animals in the M-graft-w/o-Dox group. The mean percentage of the amplitude of the CMAPs from the injured side compared with the uninjured side of the M-graft-w-Dox group ($4.61 \pm 3.08\%$) was significantly larger than that from the M-graft-w/o-Dox group (0%) ($p < 0.05$) and significantly smaller than that from the N-graft group ($10.04 \pm 3.59\%$) ($p < 0.05$). A significant difference was also observed between the M-graft-w/o-Dox and N-graft groups ($p < 0.05$). The mean percentage of the nerve conduction velocity compared with the uninjured side in the N-graft group was $49.8 \pm 14.4\%$, whereas it was $39.4 \pm 19.8\%$ in the M-graft-w-Dox group, which was not significantly different ($p = 0.284$) (▶Fig. 4).

Assessment of the Target Muscle Atrophy

The percentage of target muscle atrophy in the grafted side compared with the uninjured side was $35.7 \pm 6.0\%$ (M-graft-w/o-Dox), $52.2 \pm 5.7\%$ (M-graft-w-Dox), and $54.5 \pm 4.5\%$ (N-

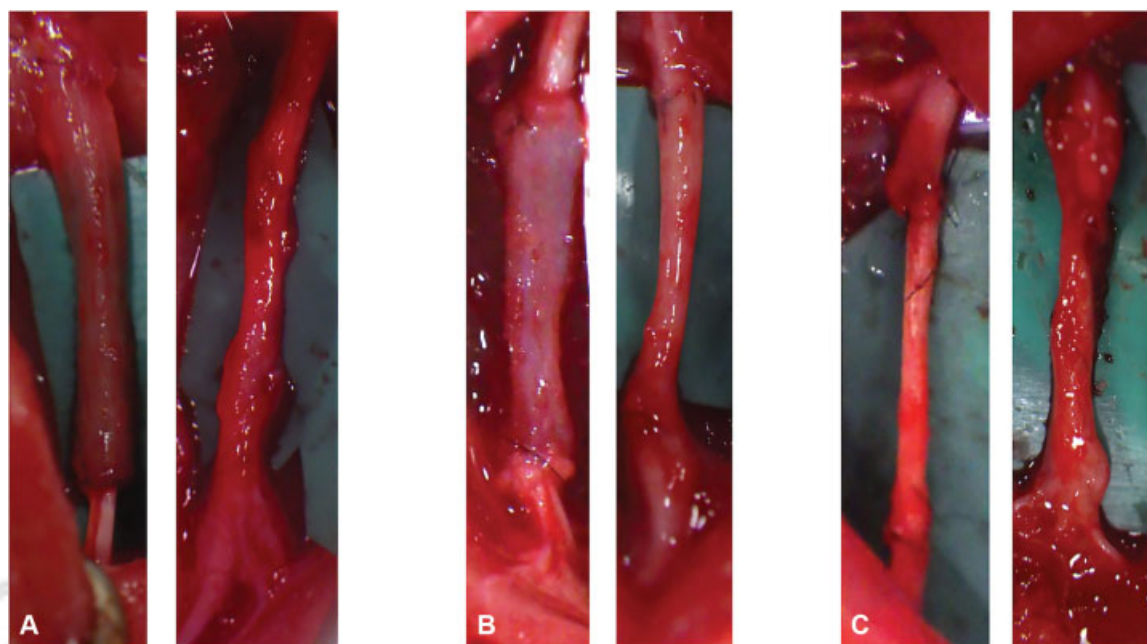


Fig. 3 Macroscopic findings of muscle grafts with/without doxorubicin pretreatment and nerve autografts. Grafts just after (left) and 8 weeks after (right) transplantation. (A) M-graft-w/o-Dox group, (B) M-graft-w-Dox group, and (C) N-graft group. M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

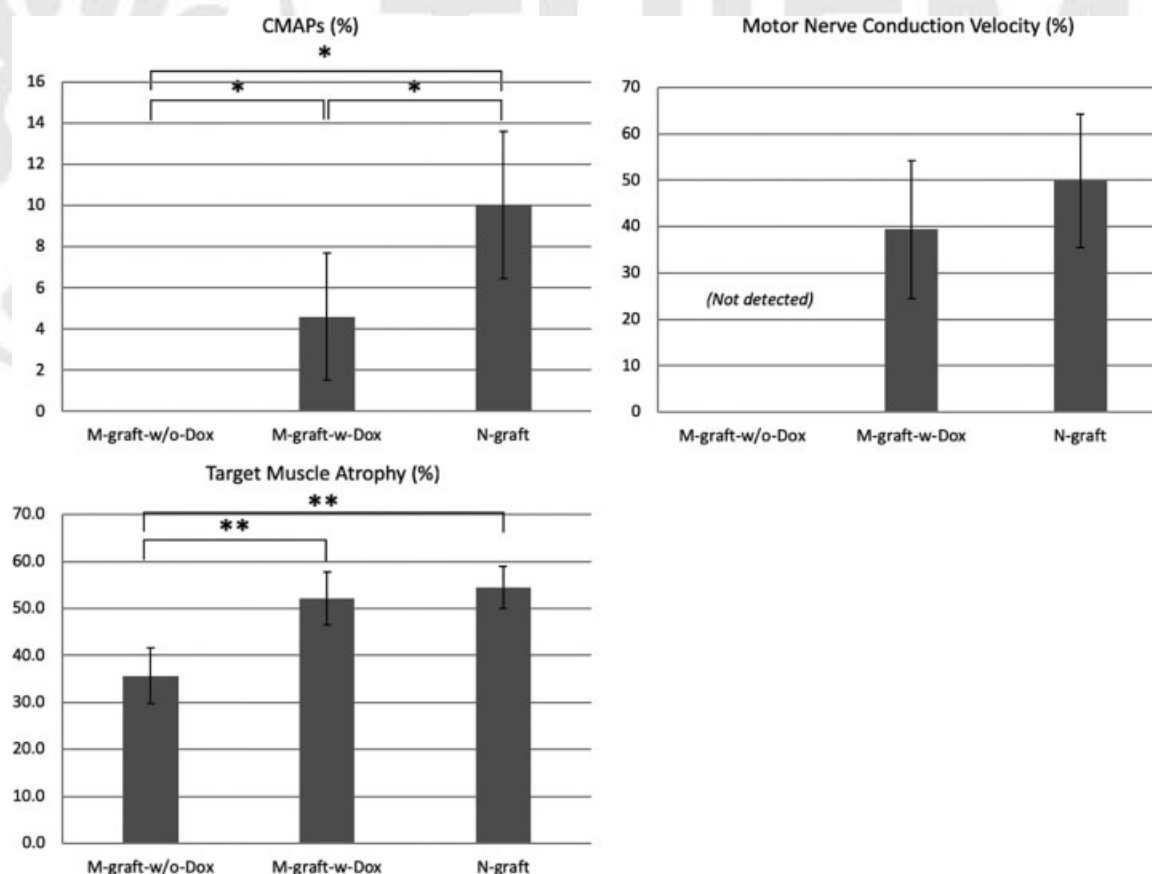


Fig. 4 Electrophysiology and target muscle atrophy (percentage of the uninjured side). No valid waveform was obtained from any of the animals in the M-graft-w/o-Dox group. The CMAPs in the N-graft group were significantly larger than those in the M-graft-w-Dox group ($p < 0.05$) (top-left panel). There were no significant differences in motor nerve conduction velocity between the M-graft-w-Dox and N-graft groups (top right). The wet muscle weight percentage (bottom) in the M-graft-w-Dox and N-graft groups was significantly greater than that in the M-graft-w/o-Dox group ($*p < 0.001$), whereas, no significant difference was observed between the M-graft-w-Dox and N-graft groups. CMAPs, compound muscle action potentials; M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

graft). Target muscle atrophy was significantly greater in the M-graft-w-Dox and N-graft groups compared with the M-graft-w/o-Dox group ($p < 0.001$ and $p < 0.001$, respectively), whereas no significant difference was observed between the M-graft-w-Dox and N-graft groups ($p = 0.778$) (►Fig. 4).

Histological and Morphological Analyses of the Regenerated Axons

The mean number of myelinated axons from the middle portion of the regenerated nerve was $1,763 \pm 1,216$ in the M-graft-w/o-Dox group, $7,942 \pm 1,796$ in the M-graft-w-Dox group, and $10,344 \pm 1,662$ in the N-graft group. The M-graft-w-Dox and N-graft groups had a significantly larger number of myelinated axons than did the M-graft-w/o-Dox group ($p < 0.0001$ and $p < 0.0001$, respectively), whereas no significant difference was observed between the M-graft-w-Dox and N-graft groups ($p = 0.0907$) (►Fig. 5). In the 5-mm distal portion from the distal nerve stump, the number of myelinated axons was $812 \pm 1,197$ in the M-graft-w/o-Dox group, $6,147 \pm 1,736$ in the M-graft-w-Dox group, and $6,859 \pm 1,109$ in the N-graft group. The M-graft-w-Dox and N-graft groups had a significantly larger number of myelinated axons than did the M-graft-w/o-Dox group ($p < 0.0001$ and $p < 0.001$, respectively), whereas no significant difference was observed between the M-graft-w-Dox and N-graft groups ($p = 0.362$) (►Fig. 5).

The histological analysis of the M-graft-w/o-Dox group revealed that the structure inside the graft was loose and that the regenerated axon units were separated by a large amount of connective tissue (►Fig. 6A). In the M-graft-w-Dox group, many regenerated axons were observed; they were organized into many units, which were clustered close together (►Fig. 6B). In the N-graft group, a considerable number of regenerated axons were densely organized (►Fig. 6C). The morphological analysis performed using TEM revealed the presence of myelinated axons in each group (►Fig. 6D–F). The histological analysis of the region 5 mm distal from the

distal nerve stumps showed the presence of regenerated axons in each group. However, regenerated axons were more densely observed in the N-graft and M-graft-w-Dox groups compared with the M-graft-w/o-Dox group (►Fig. 7A–C). TEM revealed the presence of regenerated axons with myelin sheaths at the 5 mm distal from the distal nerve stumps (►Fig. 7D–F).

The results of the morphometric analysis of the regenerated axons in the middle portion of the graft were as follows: the mean myelinated axon diameter was $2.56 \pm 0.23 \mu\text{m}$ in the M-graft-w/o-Dox group, $3.27 \pm 0.15 \mu\text{m}$ in the M-graft-w-Dox group, and $3.73 \pm 0.24 \mu\text{m}$ in the N-graft group. The diameter of the myelinated axons in the M-graft-w-Dox and N-graft groups was significantly larger than that of the myelinated axons in the M-graft-w/o-Dox group ($p < 0.05$ and $p < 0.01$, respectively), whereas no significant difference was observed between the M-graft-w-Dox and N-graft groups ($p = 0.147$) (►Fig. 8). The mean axon diameter was $1.76 \pm 0.24 \mu\text{m}$ in the M-graft-w/o-Dox group and $2.27 \pm 0.14 \mu\text{m}$ in the M-graft-w-Dox group. The mean axon diameter in the N-graft group ($2.42 \pm 0.22 \mu\text{m}$) was significantly larger than that in the M-graft-w/o-Dox group ($p < 0.05$). There was no significant difference in the mean axon diameter between the M-graft-w-Dox and N-graft groups ($p = 0.110$) (►Fig. 8).

Evaluation of Pretreatment with a Higher Concentration of Doxorubicin—Supplemental Study

A macroscopic observation performed at 8 weeks after transplantation showed the continuity of the right sciatic nerve (►Fig. 9A). However, histological analysis of the graft showed a complete absence of axonal regeneration, and muscle tissue was replaced by fibrotic tissue in all cases (►Fig. 9B). In the electrophysiological study, no valid waveforms were observed in any of the cases examined. The mean percentage of target muscle atrophy was $31.3 \pm 6.2\%$, which was significantly smaller than that of the M-graft-w-Dox group ($p < 0.05$) (►Fig. 9C).

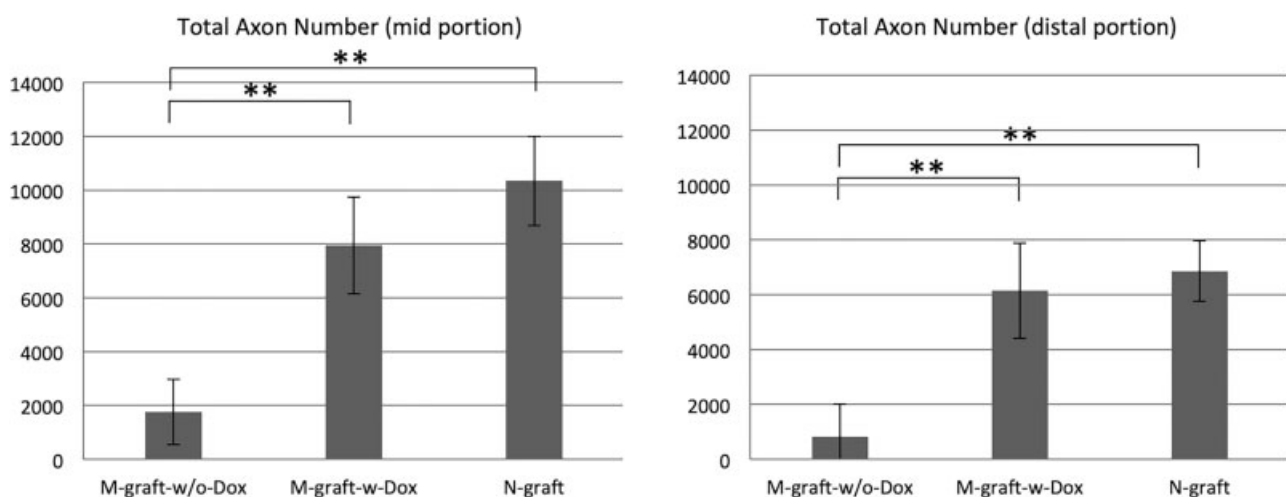


Fig. 5 Histological and morphological analyses. Total number of axons at the midportion of the graft (left) and the 5-mm distal portion from the distal nerve stump (right). In both portions, the M-graft-w-Dox and N-graft groups had a significantly higher number of axons than the M-graft-w/o-Dox group ($p < 0.0001$), while no significant difference was observed between the M-graft-w-Dox and N-graft groups. M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

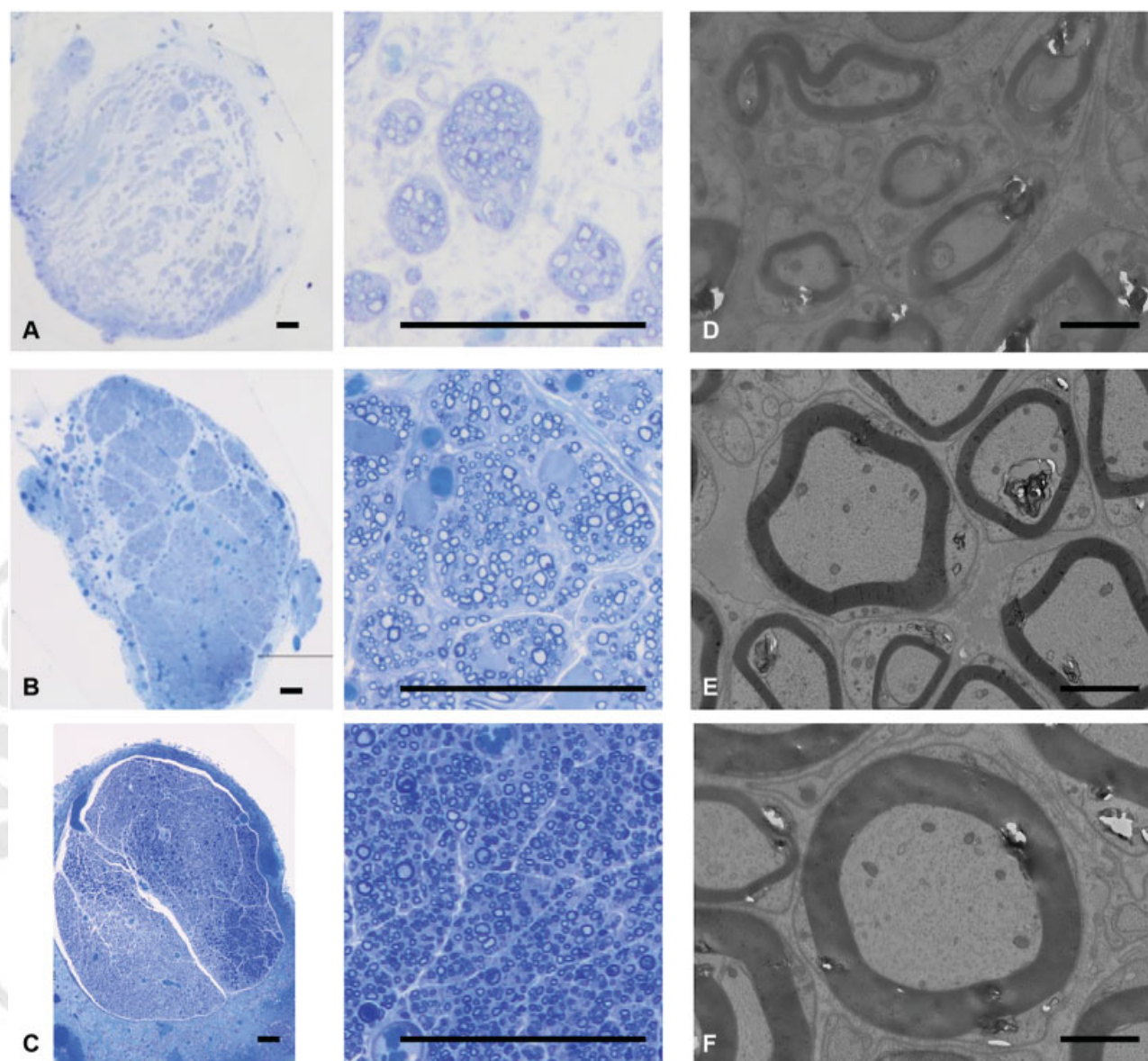


Fig. 6 Histology of the muscle graft sections (8 weeks after transplantation). (A) In the M-graft-w/o-Dox group, regenerated axons were sparsely observed compared with (B) the M-graft-w-Dox group. (C) In the N-graft group, abundant regenerated axons were prominent in the entire field (A–C: toluidine blue staining; scale bar, 100 μ m). Transmission electron microscopy showed regenerated axons with myelin sheaths in the (D) M-graft-w/o-Dox, (E) M-graft-w-Dox, and (F) N-graft groups (E–F: transmission electron microscopy; scale bar, 2 μ m). M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

Discussion

Skeletal muscle tissue has been used as a bridging material for the treatment of nerve defects because muscle fibers provide a basal lamina scaffold, which is similar to the cross-sectional structure of a nerve. Moreover, the abundance of this tissue, the absence of secondary functional loss, the safety of the immunological compatibility, and the low cost of the procedure represent additional advantages. Previous clinical studies that used skeletal muscle grafts for nerve defects have reported good outcomes; however, improvements in this area are warranted.²⁹ To improve the clinical outcome of muscle grafts, various types of degenerative pretreatment have been suggested, such as heating or the freeze-and-thaw technique. These procedures are aimed at disrupting the cytoplasm of muscle cells while

retaining the coaxially aligned basal lamina structural tube.³⁰ However, these pretreatments may have failed to retain the tubular structure of the muscle because graft shrinking just after pretreatment was observed.¹² Another pretreatment method consisted the use of a detergent procedure, which was reported to have excellent outcomes even in a 2-cm defect.^{10,14} However, this procedure is performed over 4 days, which is an obstacle to its clinical application because of the risk of contamination. Moreover, the toxicity of the detergent was not determined.

Here, we report good outcomes for the use of a M-graft-w-Dox pretreatment in a model of peripheral nerve defect regarding electrophysiology, target muscle atrophy, and the number of regenerative axons. Immediately after pretreatment with doxorubicin, muscle graft shrinkage was not observed

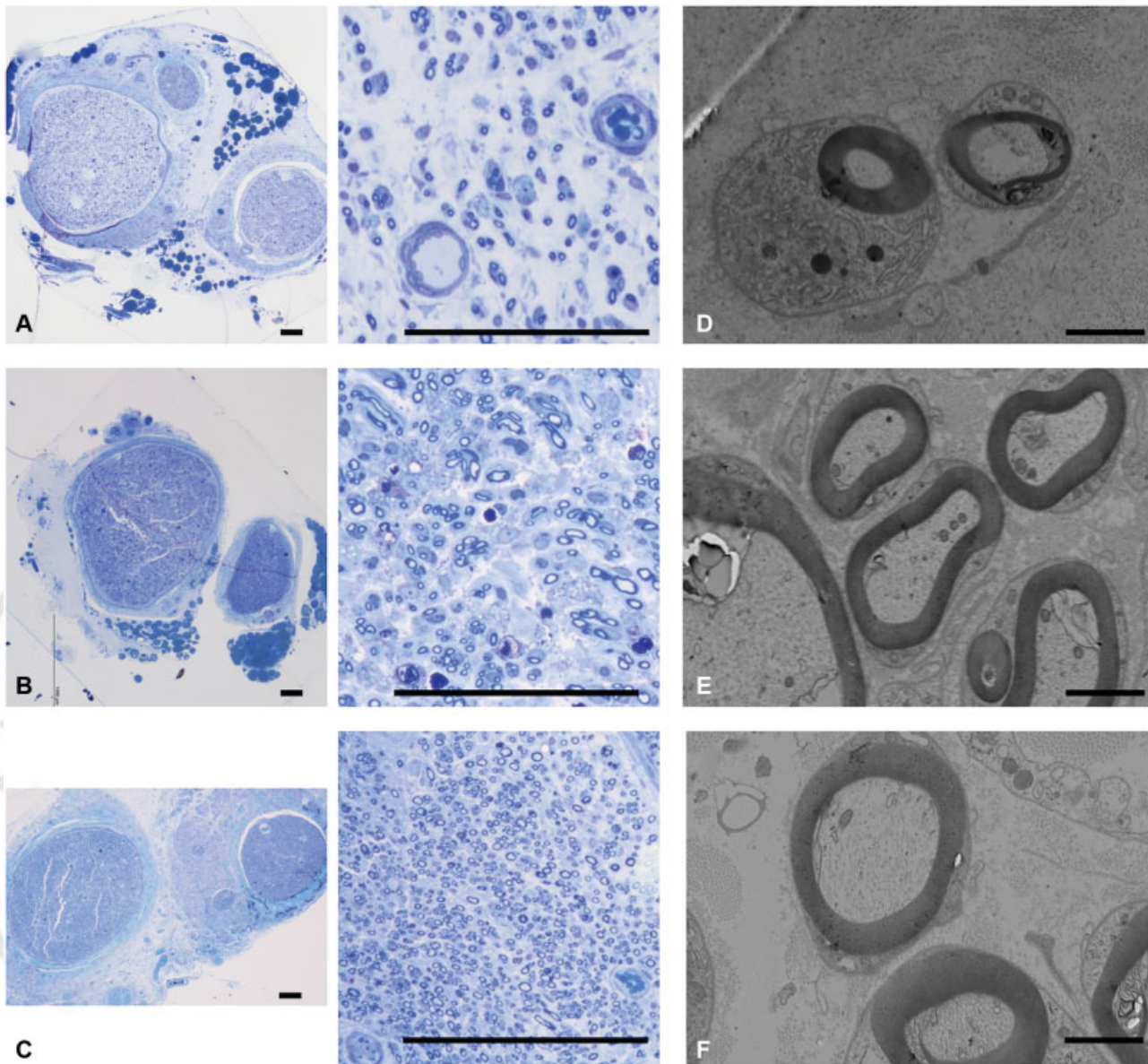


Fig. 7 Histology of the sciatic nerve at the 5-mm distal portion from the distal nerve stumps (8 weeks after transplantation). (A, D) M-graft-w/o-Dox group, (B, E) M-graft-w-Dox group, and (C, F) N-graft group (A–C: toluidine blue staining; scale bar, 100 μ m; E–F: transmission electron microscopy; scale bar, 2 μ m). M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

compared with saline pretreatments, suggesting that no massive structural change occurred. The results of hematoxylin and eosin staining also showed structural change in muscle tissue that was pretreated with doxorubicin. Moreover, we confirmed that there were more apoptotic cells in the M-graft-w-Dox group with TUNEL assay. These results indicate that degenerative change was induced by doxorubicin pretreatment. Furthermore, macrophage migration was enhanced by doxorubicin pretreatment. Recruited macrophages were considered to phagocytose the myoplasm, which was degenerated by doxorubicin pretreatment to make an “empty tube” in an early phase of nerve regeneration, which resulted in better recovery of muscle functions such as CMAPs or muscle weight. In addition, macrophages are also known to aid axonal regeneration by secreting tissue-repairing factors or neurotrophic factors

as well as by promoting Schwann cell infiltration.^{31–33} Additionally, macrophages expressed the specific receptor to bind the apoptotic cells to phagocytose them, and induced anti-inflammatory responses.^{34,35} Further studies are needed to assess the relationships among doxorubicin, macrophages, and peripheral nerve regeneration.

In vitro experiments using cardiomyocytes were reported previously. In those studies, doxorubicin was added to the medium to evaluate its adverse effects on myocytes.²⁶ Thus, soaking a muscle graft in doxorubicin would result in adverse effects. As the efficiency of doxorubicin delivery to the cells in the muscle graft is considered to be lower than that in the in vitro experiments, the concentration of doxorubicin in this study was set to 100 μ mol/L, which is $\sim$ 100 times greater than that applied in the cell culture experiments.²⁶ In previous

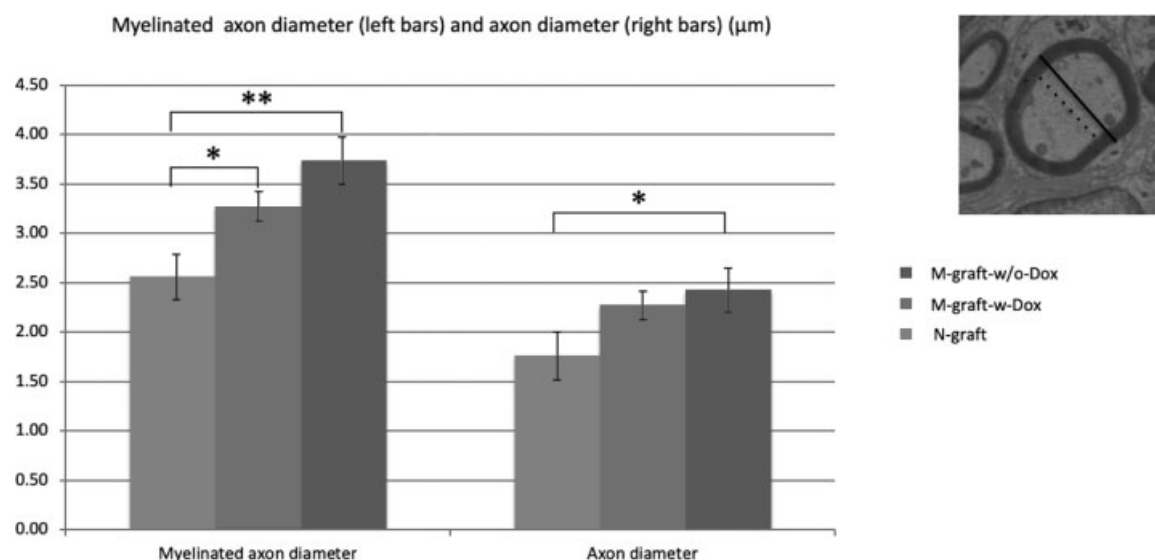


Fig. 8 Morphometric analysis of the regenerated axons. Myelinated axon diameter (left bars) and axon diameter (right bars) of the regenerated axons at the midportion of the graft. The solid and dotted lines indicate the myelinated axon diameter and axon diameter, respectively. The myelinated axon diameter of the M-graft-w-Dox and N-graft groups was significantly larger than that of the M-graft-w/o-Dox group. The axon diameter of the N-graft group was significantly larger than that of the M-graft-w/o-Dox group ($p < 0.05$, $^*p < 0.01$). M-graft-w-Dox, muscle grafts with doxorubicin; M-graft-w/o-Dox, muscle grafts without doxorubicin; N-graft, nerve graft.

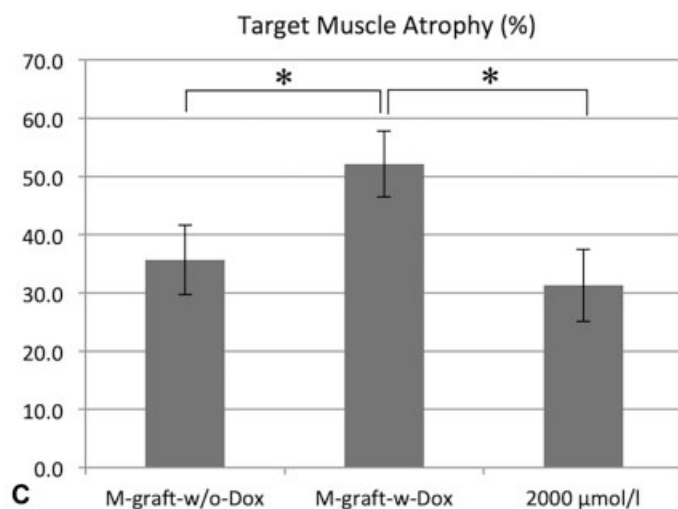
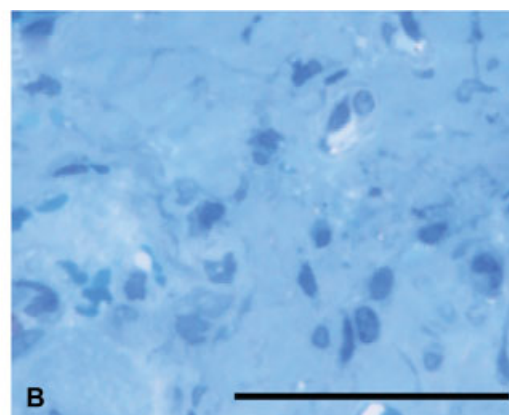
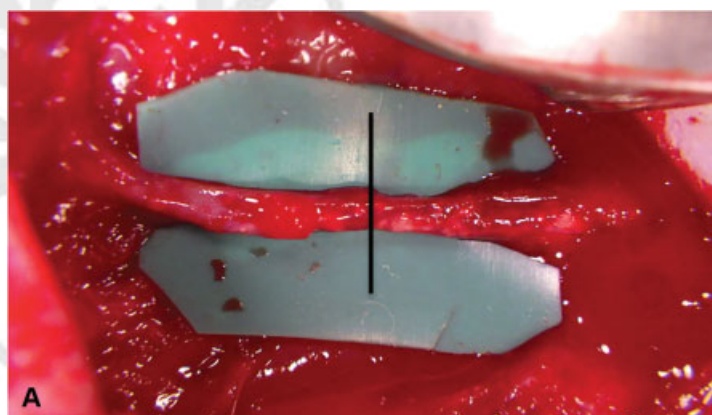


Fig. 9 Results of the pretreatment with 2,000 $\mu\text{mol/L}$ of doxorubicin. (A) A macroscopic observation performed at 8 weeks after transplantation showed the continuity of the right sciatic nerve. (B) However, a histological analysis of the graft showed a complete absence of axonal regeneration and muscle tissue was replaced by fibrotic tissue (scale bar 50 μm). (C) The mean percentage of the target muscle atrophy was $22.7 \pm 5.7\%$, which was significantly smaller than that of the M-graft-w-Dox group ($p < 0.05$).

studies, a relationship between the duration of doxorubicin treatment and mitochondrial injury or cell apoptosis was reported.^{36–38} In the case of pretreatment of M-graft-w/o-Dox, both the concentration of the drug and the duration of the exposure to its effects are considered to be important. The current doxorubicin concentration of 100 $\mu\text{mol/L}$ with a 10-minute exposure provided a favorable outcome. On the contrary, the combination of a higher concentration (2,000 $\mu\text{mol/L}$ of doxorubicin) with a 10-minute exposure resulted in a poorer result. This result indicated that doxorubicin pretreatment at 100 $\mu\text{mol/L}$ enhanced axonal regeneration via the suitable degeneration of the muscle tissue, which was not observed in the pretreatment with 2,000 $\mu\text{mol/L}$ doxorubicin. However, another optimal combination of concentration and exposure time might exist.

This study had several limitations. First, in our experiments, a 10-minute rinse with saline was applied after the doxorubicin pretreatment. The safety of this type of graft is an important issue, and adverse effects on the body should be assessed. However, doxorubicin is a drug that is used clinically to treat cancer; thus, its safety to the human body has been established. Moreover, according to the Food and Drug Administration drug information, a systemic adverse effect was observed at a total cumulative dose of 300 mg/m^2 . Considering this dosage, the residual quantity of this drug within the graft seemed to be too low to induce systemic adverse effects.

Second, comparing the results of N-graft and M-graft-w-Dox, our results showed significant difference only in the CMAPs, and not in muscle atrophy or axon counts. This discrepancy can be explained as follows: First, muscle atrophy was assessed by the tibialis anterior muscle, which was innervated more proximally than the pedal adductor muscle that CMAPs were measured. Our results revealed that assessments with proximally innervated muscle showed similar values, so assessments with the distal one were appropriate to compare these groups. Second, our results did not show a significant difference in axon counts despite the results of CMAPs. The underlying reason for this was the difference in the number of regenerated motor units. We speculate that the regenerated axons in N-graft reinnervated more muscle fibers than those in M-graft-w-Dox. Moreover, the influence of axonal sprouting must be considered, as it may result in an overestimation of the number of regenerating axons. The counting of motor neurons by the application of dyes to the regenerated site is a method to overcome these problems.

Finally, the study included a small number of subjects. A significant difference between the M-graft-w/o-Dox and M-graft-w-Dox groups was detected in many assessments. However, a significant difference between the M-graft-w-Dox and N-graft groups was found only in the electrophysiological study. A study with a larger number of samples may reveal the true differences or similarities between these groups.

Conclusion

In conclusion, a good efficacy of the doxorubicin-immersed muscle graft was shown in terms of the number of accumulated macrophages, regenerated axons and their morphology,

electrophysiological evaluation, and target muscle atrophy. Although autologous nerve grafting is a gold-standard method for the treatment of nerve defects, our results provide a new possibility of using muscle grafting as an alternative method to nerve grafting.

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None.

Conflict of Interest

None declared.

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