

Protective effect of biodegradable nerve conduit against peripheral nerve adhesion after neurolysis

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OBJECTIVE Peripheral nerve adhesion caused by extraneural and intraneural scar formation after neurolysis leads to nerve dysfunction. The authors previously developed a novel very flexible biodegradable nerve conduit composed of poly(L-lactide) and poly(ϵ -caprolactone) for use in peripheral nerve regeneration. In the present study, they investigated the effect of protective nerve wrapping on preventing adhesion in a rat sciatic nerve adhesion model.

METHODS Rat sciatic nerves were randomly assigned to one of the following four groups: a no-adhesion group, which involved neurolysis alone without an adhesion procedure; an adhesion group, in which the adhesion procedure was performed after neurolysis, but no treatment was subsequently administered; a nerve wrap group, in which the adhesion procedure was performed after neurolysis and protective nerve wrapping was then performed with the nerve conduit; and a hyaluronic acid (HA) group, in which the adhesion procedure was performed after neurolysis and nerve wrapping was then performed with a 1% sodium HA viscous solution. Six weeks postoperatively, the authors evaluated the extent of scar formation using adhesion scores and biomechanical and histological examinations and assessed nerve function with electrophysiological examination and gastrocnemius muscle weight measurement.

RESULTS In the adhesion group, prominent scar tissue surrounded the nerve and strongly adhered to the nerve biomechanically and histologically. The motor nerve conduction velocity and gastrocnemius muscle weight were the lowest in this group. Conversely, the adhesion scores were significantly lower, motor nerve conduction velocity was significantly higher, and gastrocnemius muscle weight was significantly higher in the nerve wrap group than in the adhesion group. Additionally, the biomechanical breaking strength was significantly lower in the nerve wrap group than in the adhesion group and HA group. The morphological properties of axons in the nerve wrap group were preserved. Intraneural macrophage invasion, as assessed by the number of CD68- and CCR7-positive cells, was less severe in the nerve wrap group than in the adhesion group.

CONCLUSIONS The nerve conduit prevented post-neurolysis peripheral nerves from developing adhesion and allowed them to maintain their nerve function because it effectively blocked scarring and prevented adhesion-related damage in the peripheral nerves.

<https://thejns.org/doi/abs/10.3171/2017.4.JNS162522>

KEY WORDS nerve conduit; peripheral nerve; adhesion; scar; nerve wrapping; rat

NEUROLYSIS of peripheral nerves is an effective and frequently performed surgical procedure for entrapment neuropathy. However, postoperative perineural scarring and adhesion often cause secondary neuropathy.^{44,52} Once peripheral nerves are injured by trauma

or neurolysis, scar tissue with infiltration of fibroblasts and myofibroblasts develops and peripheral nerve adhesion is completed. If the peripheral nerves are compressed or tethered by the scar tissue and adhesion, they become dysfunctional.^{1,7,32,35,36} Extraneural scarring also suppresses

ABBREVIATIONS HA = hyaluronic acid; PCA = poly(ϵ -caprolactone); PLA = poly(L-lactide).

SUBMITTED October 10, 2016. **ACCEPTED** April 14, 2017.

INCLUDE WHEN CITING Published online October 20, 2017; DOI: 10.3171/2017.4.JNS162522.

blood flow affecting the peripheral nerves and thickens the epineurium and perineurium, leading to intraneural scarring that further suppresses the blood flow.^{1,7,35,44,65}

For neurolysis-treated peripheral nerves, various procedures that involve wrapping veins or free or pedicled flaps with muscle or adipose tissue have been reported to prevent adhesion.^{12,14,16,34,38,45,47,50,58,62} However, because these methods involve the use of autologous normal tissues, they have major drawbacks, including donor-site morbidity and a high risk of surgical complications. Many nerve protective materials including silicone sheets, collagen tubes, gels, and fluids have been developed in recent years to overcome these drawbacks.^{2,3,6,9,13,15,19–21,23–26,29,31,39–41,45,46,60,64}

We previously developed a novel biodegradable nerve conduit composed of poly(L-lactide) (PLA) and poly(ϵ -caprolactone) (PCL) to treat peripheral nerve injury and confirmed that axonal regeneration was induced within the conduit.^{22,30,40,56,57} This nerve conduit comprises 2 layers: an outer layer composed of PLA multifilament fiber mesh and an inner layer composed of a PLA- and PCL-containing porous sponge with pores of 10 to 50 μ m. It is soft and flexible enough to cover the peripheral nerve with minimal damage, and it is degraded and absorbed very slowly in vivo (> 1 year) (Fig. 1).⁵⁵

We hypothesized that this biodegradable nerve conduit is useful for both nerve regeneration and protective wrapping of the nerve to prevent scar and adhesion formation. In the present study, we used a rat nerve adhesion model to compare protective nerve wrapping with this nerve conduit versus hyaluronic acid (HA), which has been shown to protect nerves from adhesion formation, by examining the efficacy of each materials to prevent nerve adhesion.

Methods

Nerve Conduit

We used the same biodegradable polymer tube for the treatment of peripheral nerve defects as previously reported.^{22,30,55–57} The conduit (outer diameter 3 mm; inner diameter 2 mm; length 15 mm) was sized so that it was 1 to 2 mm larger than the diameter of the rat sciatic nerves, which were less than 1 mm, to ensure that no nerve constriction was induced.^{25,29} The nerve conduit comprises an outer layer composed of a PLA multifilament fiber mesh and an inner layer composed of a 50% PLA and 50% PCL porous sponge with pores of 10 to 50 μ m (Fig. 1). This nerve conduit can maintain its tubular structure while staying very flexible. In particular, the PLA and PCL copolymer sponge of the inner layer has a honeycomb structure that is minimally damaging to the nerve.^{22,30,55–57}

Animal Model

All experimental protocols and animal maintenance procedures were approved by the Animal Ethics Research Committee of Osaka City University Graduate School of Medicine. Lewis rats (body weight approximately 250 g at the time of the operation [SLC, Inc.]) were anesthetized by subcutaneous injection of 1 ml of ketamine (50 mg/ml) and 0.3 ml of 2% xylazine into the dorsal back. The adhesion procedures were performed in accordance with a previously described sciatic nerve adhesion model.^{1,11,21} In

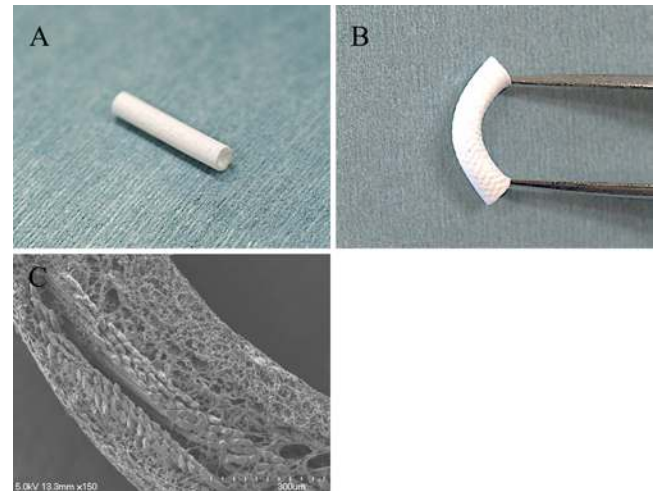


FIG. 1. The biodegradable nerve conduit. **A and B:** Gross appearance of the flexible nerve conduit. **C:** Scanning electron microscope image of a transverse section of the nerve conduit. The outer layer is composed of PLA and 50% PCA. The inner layer is a honeycomb structure containing pores.

short, the left sciatic nerve was carefully exposed and released from the surrounding tissues, including the neural bed, without injury to the nerves or vessels. Scarring and adhesion around the surgically released nerve was produced by repeatedly burning the biceps femoris muscle that composed the neural bed over a 10-mm length using a bipolar coagulator (ConMed Excalibur Plus PC; Aspen Surgical) to stimulate a local fibrotic response around the sciatic nerve.

The nerves were randomly assigned to one of the following four groups: a no-adhesion group ($n = 8$), which involved neurolysis alone without the adhesion procedure; an adhesion group ($n = 12$), which involved performing the adhesion procedure after neurolysis but with no treatment; a nerve wrap group ($n = 12$), which involved performing the adhesion procedure after neurolysis and subsequent wrapping with the nerve conduit; and an HA group ($n = 8$), which involved performing the adhesion procedure after neurolysis and wrapping with 1% sodium HA viscous solution (Artz; Kaken). Approximately 0.5 to 1.0 ml of HA was applied around the surgically released nerve after the adhesion procedure (Fig. 2).^{13,21} Six weeks after each operation, the extent of scar adhesion and the peripheral nerve function were evaluated.

Gross Evaluation: Wound Healing and Adhesion Scores

The quality of wound healing of the skin and fascia was assessed using previously described wound healing scores.⁴¹ The scores ranged from 1 to 3 (1, skin or muscle fascia entirely closed; 2, skin or muscle fascia partially open; and 3, skin or muscle completely open). The quality of nerve adhesion was also assessed using previously described adhesion scores.⁴¹ The scores ranged from 1 to 3 (1, no dissection or mild blunt dissection; 2, some vigorous blunt dissection required; and 3, sharp dissection required during neurolysis to the area of adhesion). Both the wound healing score and adhesion score were calculated in each rat.

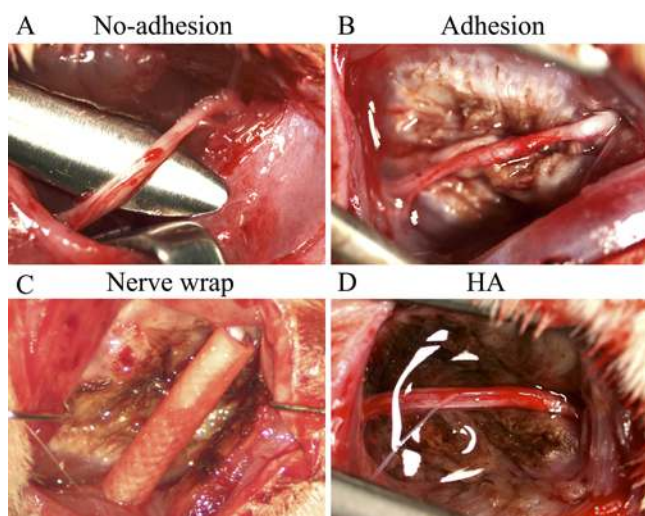


FIG. 2. Intraoperative gross findings in the experimental model. **A:** No-adhesion group, which involved neurolysis alone without the adhesion procedure (burning of the neural bed using a bipolar coagulator). **B:** Adhesion group, which involved performing the adhesion procedure after neurolysis but no subsequent treatment. **C:** Nerve wrap group, which involved performing the adhesion procedure after neurolysis and wrapping with the nerve conduit. **D:** HA group, which involved performing the adhesion procedure after neurolysis and wrapping with a 1% sodium HA viscous solution.

Biomechanical Examination

To evaluate the extent of adhesion, the ultimate breaking strength of the nerve that adhered to the neural bed was assessed, as previously described.³⁹ With the operated limb fixed on the table, the nerve was ligated with 1-0 silk thread 5 mm proximal to the adhesion area and dissected at the proximal end. The proximal stump was then mounted on a digital force gauge (FGP-0.2; Shimpo) and subjected to traction at a rate of 2 cm/min until the nerve was completely detached from the neural bed.

Electrophysiological Examination

To evaluate the electrophysiological function of the adherent nerve, the sciatic nerve was exposed and a bipolar stimulator was placed proximal to the adhesion area (supramaximal electrical pulses; duration 0.1 msec; frequency 1 Hz; square wave) (VikingQuest; Natus Neurology). The compound muscle action potential of the gastrocnemius muscle was recorded by inserting the recording electrode into the central portion of the muscle. A bipolar stimulator was also placed distal to the adhesion area, 15 mm distal to the level of proximal stimulation, to calculate the nerve conduction velocity.

Wet Weight and Histological Evaluation of Gastrocnemius Muscle

To evaluate the functional recovery of the adherent nerve, the gastrocnemius muscles on both the affected and unaffected sides were resected and their wet weight was measured (no-adhesion group, $n = 6$; adhesion group, $n = 8$; nerve wrap group, $n = 8$; and HA group, $n = 6$). The specimens were immersed in 4% paraformaldehyde over-

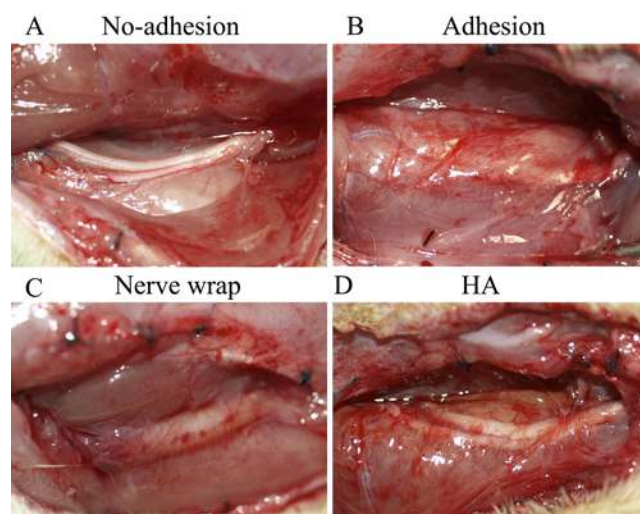


FIG. 3. Gross findings 6 weeks postoperatively. **A:** No-adhesion group: little scar tissue was present around the sciatic nerve. **B:** Adhesion group: a large amount of scar tissue covered the sciatic nerve. **C:** Nerve wrap group: scar tissue was present around the nerve conduit; however, the sciatic nerve was protected from scar tissue by the nerve conduit. **D:** HA group: adherent scar tissue was present between the sciatic nerve itself and the neural bed.

night and embedded in paraffin. Five-micrometer-thick transverse sections at the level of the largest area of the muscle were stained with H & E to evaluate the muscle atrophy.

Histological Evaluation of Nerve Adhesions

The sciatic nerve with its surrounding soft tissue, including the neural bed, was harvested. The specimens were immersed in 4% paraformaldehyde overnight and embedded in paraffin. Five-micrometer-thick central transverse sections were stained with Masson's trichrome to evaluate areas of scarring surrounding the nerve. They were also immunohistochemically stained using anti-neurofilament antibody (1:100, mouse; DAKO) to evaluate the axons. The neurofilament antibody-positive axons were morphometrically analyzed using computer-assisted imaging. An image of the transverse section of each adherent nerve was photographed at a magnification of $\times 400$ with an Olympus DP70 camera, and the number and area of the neurofilament antibody-positive axons were counted automatically using ImageJ software (National Institutes of Health) (each group, $n = 4$). To examine the inflammatory process in the areas of intraneural scar formation, the transverse sections of the nerve adhesions were also immunohistochemically stained using anti-CD68 antibody (1:500, rabbit immunoglobulin G; Abcam) for total activated macrophage markers and anti-CCR7 antibody (1:100, rabbit immunoglobulin G; Abcam) for proinflammatory M1 macrophage markers. The rats' spleen tissue was used as a positive control.^{4,7,18,22,63}

Statistical Analysis

All data are expressed as the mean \pm standard deviation. The Mann-Whitney U-test with Bonferroni correction was used as a post hoc test after statistically signifi-

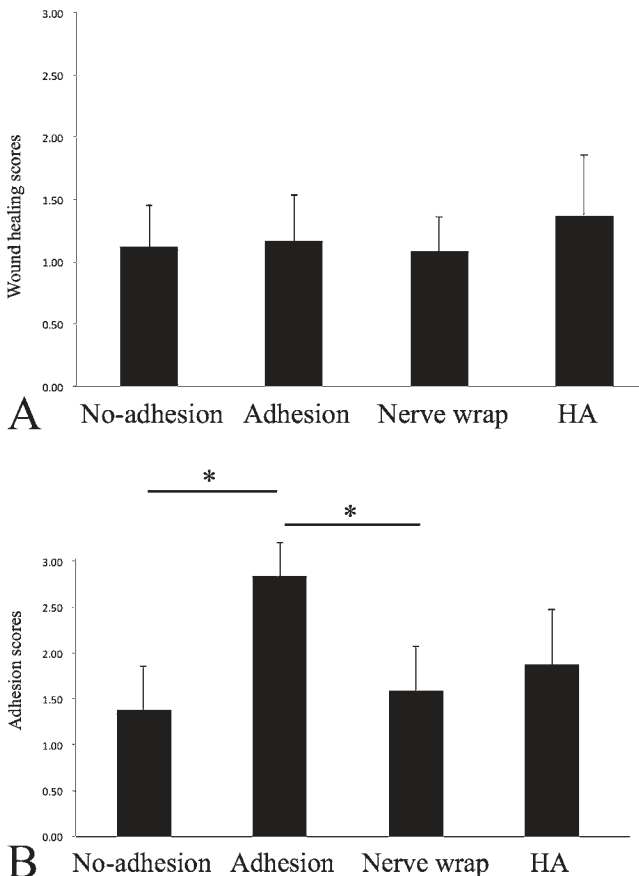


FIG. 4. Wound healing and adhesion scores 6 weeks postoperatively. **A:** There were no significant differences in the wound healing scores among the 4 groups. **B:** The adhesion scores were significantly higher in the adhesion group than in the no-adhesion and nerve wrap groups. * $p < 0.05$.

cant differences were detected with the Kruskal-Wallis H-test, using Excel for statistical analysis. All differences were considered significant at $p < 0.05$.

Results

Gross Evaluation (wound healing and adhesion scores)

The gross appearance of the adherent nerves 6 weeks postoperatively is shown in Fig. 3. There were no significant differences in the wound healing scores among the 4 groups, indicating that both the superficial layer and fascia had healed without damage in all groups (Fig. 4). In contrast, the adhesion scores in the no-adhesion group and nerve wrap group were significantly lower than those in the adhesion group ($p < 0.05$). The adhesion scores in the nerve wrap group were the same as those in the no-adhesion group. Although the adhesion scores in the HA group tended to be lower than those in the adhesion group, no significant differences were found between these 2 groups ($p = 0.41$).

Biomechanical Examination

The ultimate breaking strength of the adherent nerves in the no-adhesion group was the lowest among the 4

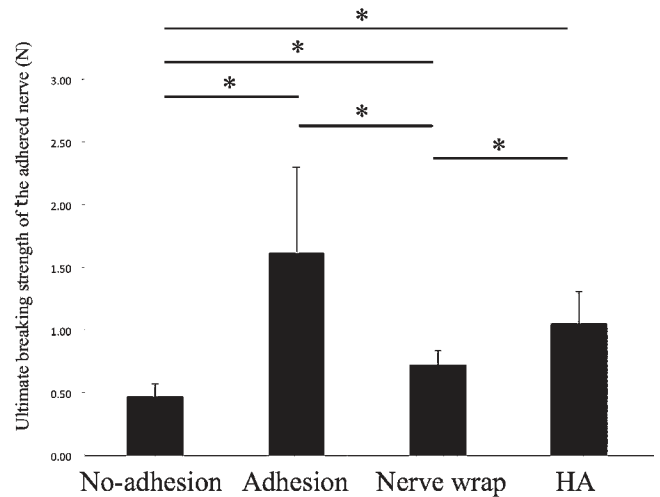


FIG. 5. The ultimate breaking strength of the adherent nerves 6 weeks postoperatively was significantly higher in the order of the adhesion, HA, nerve wrap, and no-adhesion groups. * $p < 0.05$.

groups, with statistical significance (adhesion group, $p = 0.009$; nerve wrap group, $p = 0.015$; and HA group, $p = 0.024$) (Fig. 5). That in the nerve wrap group was the next lowest and was significantly lower than those in the adhesion and HA groups. While the ultimate breaking strength of the adhered nerves in the HA group tended to be lower than that in the adhesion group, no significant difference was seen between these 2 groups ($p = 0.41$).

Electrophysiological Examination

The amplitude of the compound muscle action potential of the gastrocnemius muscles in the no-adhesion group was the highest among all 4 groups, with statistical significance (adhesion group, $p = 0.009$; nerve wrap group, $p = 0.025$; and HA group, $p = 0.024$) (Fig. 6). Although that in the nerve wrap group tended to be higher than that in the adhesion and HA groups, there were no significant differences among them. The motor nerve conduction velocity in the nerve wrap group was significantly higher than that in the adhesion group and the same as that in the no-adhesion group. These results indicate that nerve wrapping with the nerve conduit preserved the nerve function electrophysiologically.

Wet Weight and Histological Evaluation of Gastrocnemius Muscle

Representative images of gross appearance and histological appearance of the gastrocnemius muscle are shown in Fig. 7. The gastrocnemius muscles on the affected side atrophied in the order of the adhesion, HA, nerve wrap, and no-adhesion groups. The wet weights of the gastrocnemius muscles in the nerve wrap group were significantly greater than those in the adhesion group and HA group and the same as those in the no-adhesion group.

Histological Evaluation of Adhered Nerve

Scar tissue stained with Masson's trichrome was found around the nerve, especially between the nerve and neu-

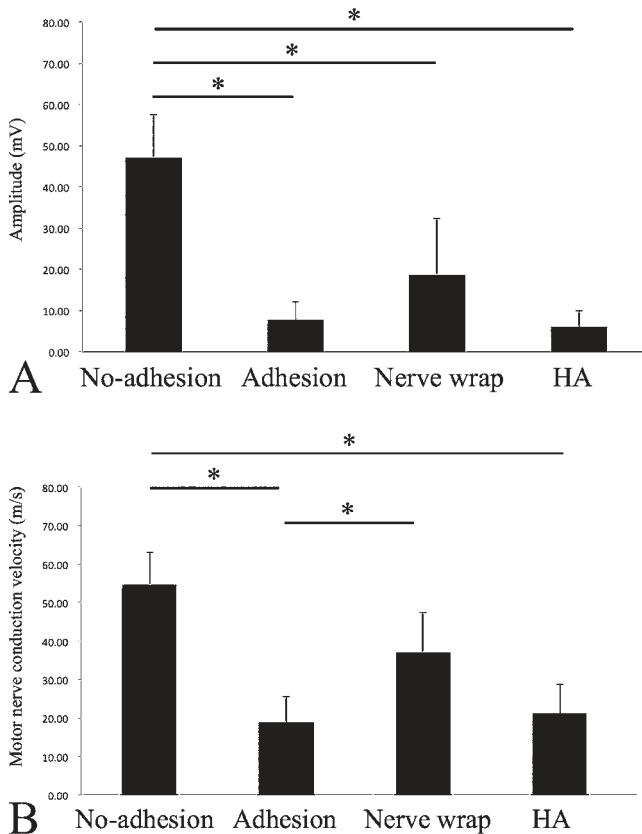


FIG. 6. Electrophysiological results. **A:** Amplitude of compound motor action potential of gastrocnemius muscle 6 weeks postoperatively in the adhesion group was lowest and that in the no-adhesion group was highest among all 4 groups. **B:** Motor nerve conduction velocity 6 weeks postoperatively in the adhesion group was lowest among all 4 groups and that in the nerve wrap group was significantly higher than that in the adhesion group. * $p < 0.05$.

ral bed, in each group (Fig. 8). In the adhesion group, the most notable scar tissue surrounding the nerve had adhered strongly to the nerve. In contrast, a tiny amount of the scar tissue had loosely adhered to the neural bed in the no-adhesion group. In the nerve wrap group, although scar tissue was seen between the nerve conduit and neural bed, little perineural scar formation was present around the nerve because the nerve conduit had blocked it. Fibrous bridging was present between the nerve and the neural bed in the HA group, but adhesion of the nerve was looser than that in the adhesion group.

Axons in adherent nerves, which were positive for neurofilament protein, are shown in Fig. 9. The axons in the adhesion and HA groups were sparse, whereas they were packed extremely closely together and exhibited preservation of their morphological properties in the no-adhesion and nerve wrap groups. Morphometric analysis showed that the number and areas of axons that were positive for anti-neurofilament protein antibodies tended to be greater in the no-adhesion and nerve wrap groups than in the adhesion and HA groups (Fig. 9).

The highest number of CD68-positive activated macrophages was found in the intraneural scar tissue in the adhesion group, and the lowest number was found in the

no-adhesion group. There were fewer CD68-positive activated macrophages in the intraneural scar tissue in the nerve wrap group than in the adhesion and HA groups. The number of CCR7-positive proinflammatory M1 macrophages was higher in the adhesion and HA groups and lower in the no-adhesion and nerve wrap groups. These findings indicate that the nerve conduits protected against macrophage invasion, especially M1 macrophage invasion (Fig. 10).

Discussion

The effect of the nerve conduit on protecting neurolysis-treated nerves from scarring and adhesion was evident in the gross appearance and in the biomechanical, electrophysiological, and histological finding of the adherent nerves in the present rat sciatic nerve adhesion model.

Once inflammation occurs in the soft tissue surrounding peripheral nerves injured by neurolysis, extraneural and intraneural scarring is established during the wound healing process, leading to adhesion of the nerve.^{48,54} Generally, local inflammation causes hyperpermeability of capillary vessels, migration of leukocytes including macrophages, and fibrin deposition. The activated macrophages produce transforming growth factor- β , insulin-like growth factor, macrophage-derived growth factor, and fibroblast growth factor, allowing fibroblasts and vascular endothelial cells to migrate to the site of injury.^{3,28,33,43,49,59,61} Adhesion of the soft tissues occurs secondary to granular and scar formation by fibroblasts and collagen fibers, wound contraction,^{17,37} and the replacement of connective tissues by fibrin.⁴² In this proliferation phase, which lasts for several weeks, it is important for nerves to be guarded from scar tissue invasion to prevent nerve adhesion.

Many methods by which to protect peripheral nerves and prevent scar tissue formation have been devised. In the clinical setting, nerves that have undergone neurolysis may be covered by autologous vein wraps and adipose tissue or muscle flaps; however, these are associated with donor-site morbidity and a risk of surgical complications.^{12,14,16,34,38,45,47,50,58,62} Various nonbiodegradable or biodegradable materials such as silicone sheets, collagen, porcine extracellular matrix, biodegradable glass fiber wrap, PLA film, gel, and fluid have recently been used to cover neurolysis-treated nerves.^{2,3,6,9,13,15,19–21,23–26,29,31,39–41,45,46,60,64} NeuroGen (Integra LifeSciences Corporation), which is a nerve conduit for peripheral nerve injuries, is used as a biodegradable wrapping material for peripheral nerves and effectively induces axonal growth and protects peripheral nerves.^{2,9,31}

The PLA-PCL material used in the present study has also been used for nerve conduits and has suitable flexibility and gentleness for nerves.^{22,30,55–57} As mentioned above, it is important to protect peripheral nerves from scar formation during the initial healing stage, which continues for several weeks after neurolysis. The present study proved that the morphological properties of axons in the nerves wrapped with the nerve conduits were preserved because the PLA-PCL conduit prevented scarring and adhesion of the nerve beyond the initial healing stage without being completely absorbed and while maintaining its flexibility.

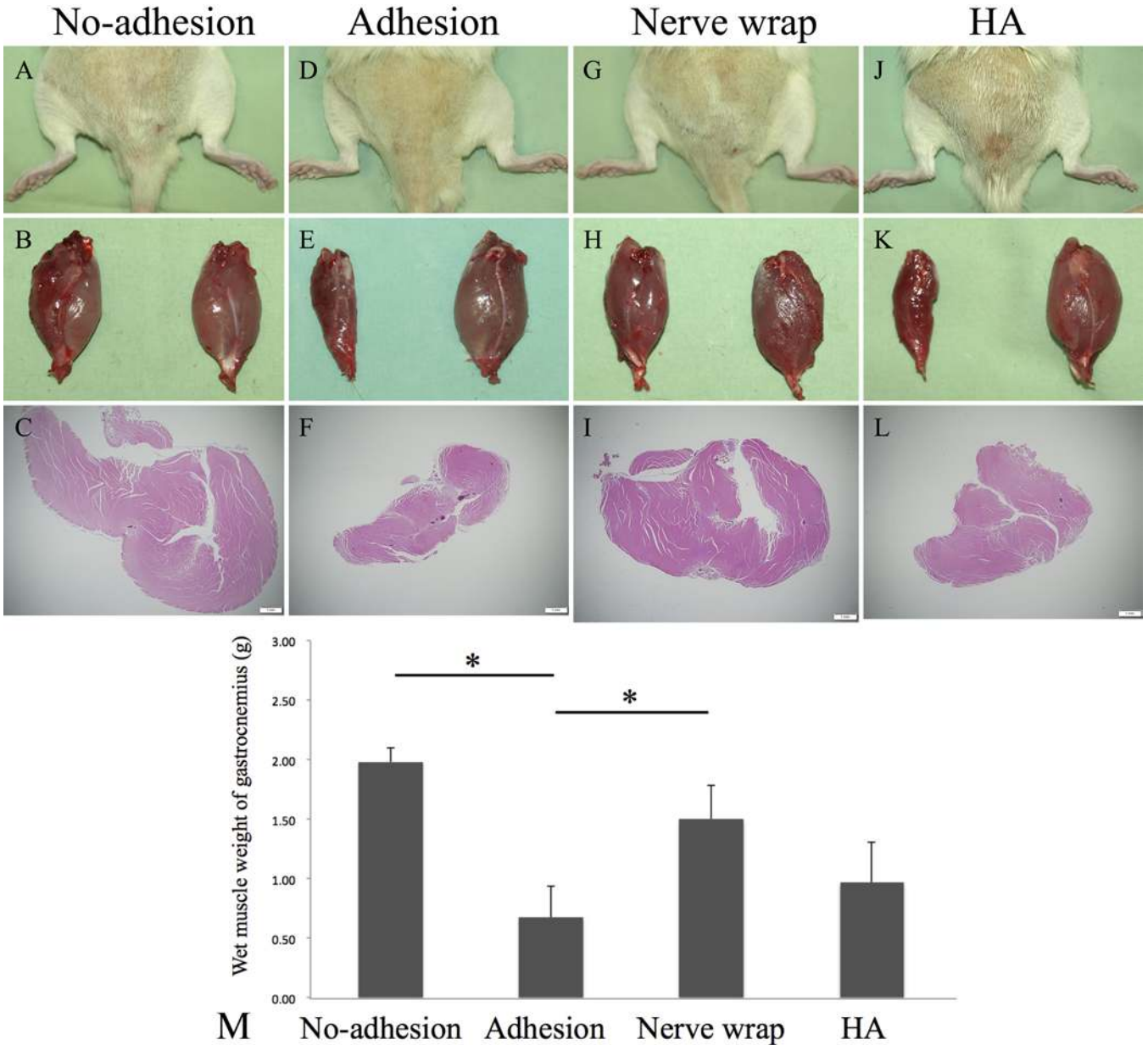


FIG. 7. Representative gross appearance of hind limbs (**A, D, G, and J**) and gastrocnemius muscles (**B, E, H, and K**) and photomicrographs (**C, F, I, and L**) of gastrocnemius muscle stained with H & E (scale bar 1 mm). Wet weight of gastrocnemius muscle (**M**) on the affected side atrophied in the order of the adhesion, hyaluronic acid, nerve wrap, and no-adhesion groups. **p* < 0.05.

While HA was less effective for protection of the nerve from adhesion in the present study, we speculated that it would be absorbed earlier. PLA material for prevention of adhesions has also been recently described.^{15,46,60} PLA material is hydrophobic and less adhesive to biological tissue. We previously reported that PLA material was slowly absorbed for several years without side effects.⁵⁵ We believe that PLA material effectively preserves the peripheral nerves until end of scar formation.

The herein-described PLA-PCL nerve conduit can also be used as a scaffold for supportive cells and growth factors. Some growth factors, such as fibroblast growth factor, which has been shown to be effective in the prevention of scar formation, can be added to the conduit as a drug de-

livery system.^{10,27,51,53,66} Such conduits with growth factors may provide a more effective barrier to adhesion and have greater potential for healing from the damage induced by scarring and adhesion. No reports have yet described these hybrid protective materials that contain added factors for peripheral nerve adhesion.

The mechanism of adhesion-induced peripheral nerve dysfunction remains unclear. Masear and Colgin³⁴ have suggested that mechanical constriction of scar tissue, loss of nerve gliding and traction, and nerve ischemia cause nerve dysfunction. Murakami et al.³⁸ have suggested that establishment of a barrier that protects nerves, inhibition of adhesion, improvement in nerve gliding function, and the presence of several neurotrophic factors that promote

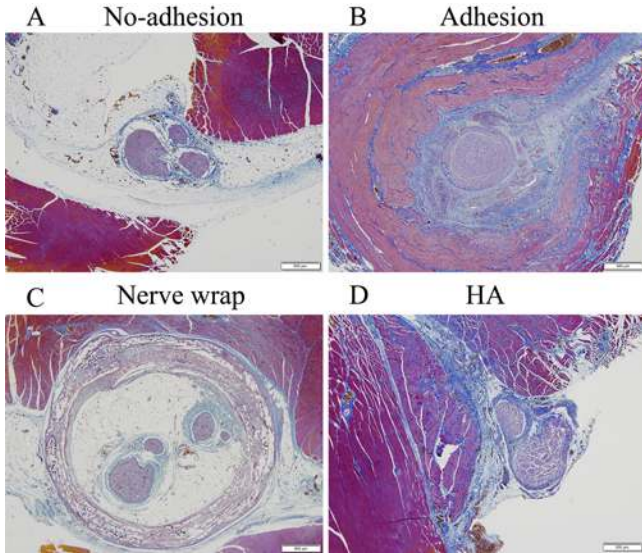


FIG. 8. Representative photomicrographs of the sciatic nerve and neural bed stained with Masson's trichrome 6 weeks postoperatively (scale bar 500 μ m). **A:** Little scar tissue was present between the nerve and neural bed in the no-adhesion group. **B:** Notable scar tissue was surrounding the nerve and strongly adhering to the nerve in the adhesion group. **C:** Perineural scar formation was present in the nerve conduit in the nerve wrap group, although little scar tissue was seen between the conduit and neural bed. **D:** Fibrous bridging scar tissue was found between the nerve and neural bed in the HA group.

axonal growth are important for prevention of adhesion-induced nerve dysfunction. Moreover, in recent years, various studies have evaluated the roles of leukocytes, mast cells, T lymphocytes, and macrophages in scar tissue formation.^{5,8,18,63} In the present study, nerve wrapping with the nerve conduit prevented intraneural macrophage invasion, especially inflammatory M1 macrophage invasion, and scar formation, leading to better nerve function electrophysiologically. We believe that it is important to prevent both extraneural and intraneural scar formation to maintain peripheral nerve function after neurolysis.

The main limitation in the present study is that the only evaluation time point was 6 weeks after the operation. We believe that this time point seems reasonable based on the findings of several well-supported reports. For instance, Crosio et al.¹¹ reported that significant scar tissue-induced adhesion between the nerve and muscles had already formed 3 weeks after the same adhesion procedure as the one performed in the present study. Petersen et al.⁴¹ also evaluated a sciatic nerve adhesion model and reported that a substantial degree of scar formation had been established earlier than 6 weeks postoperatively. Ohsumi et al.³⁹ showed that the blood-nerve barrier had recovered by 6 weeks after neurolysis and provided only a single time point (6 weeks postoperatively) for evaluation of sciatic nerve adhesion. Further studies are needed to evaluate the long-term effects of the nerve conduit because this PLA-PCL material is not only biodegradable but also artificial, and there is a possibility of side effects that interfere with the biological tissues.

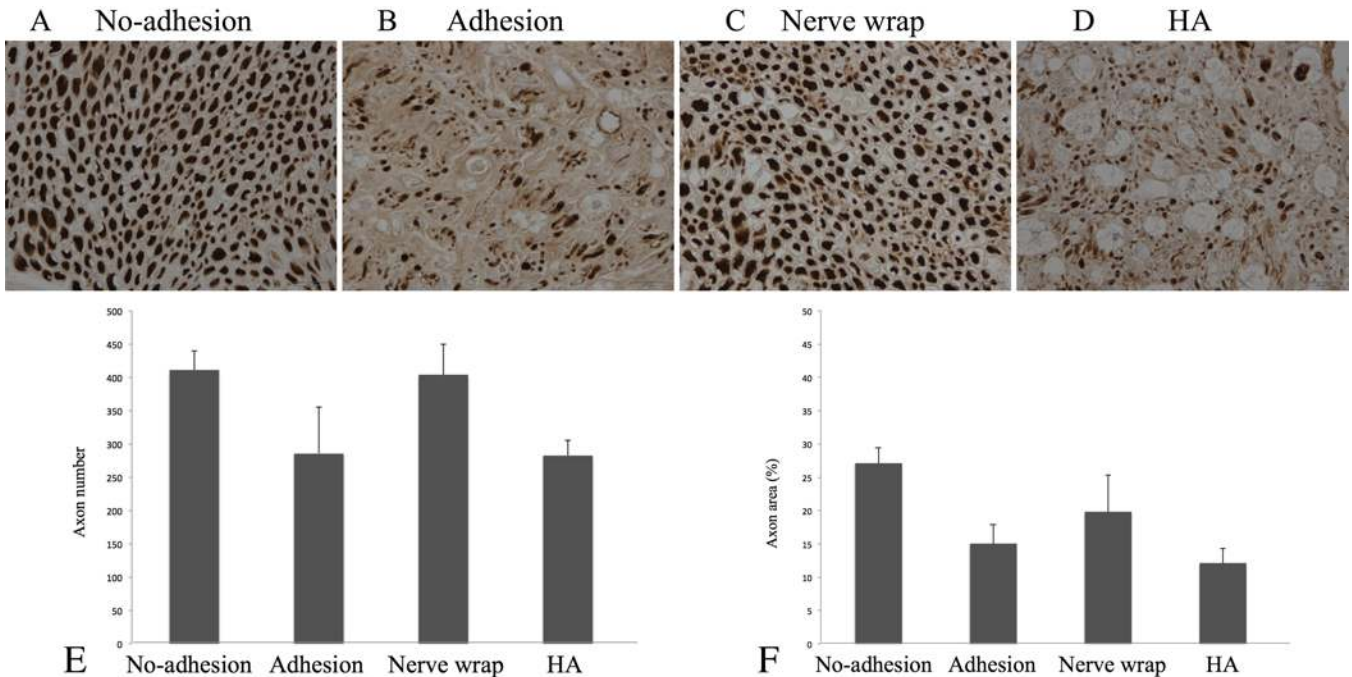


FIG. 9. Representative photomicrographs of axons stained using anti-neurofilament antibody 6 weeks postoperatively (scale bar 20 μ m). **A:** The morphological properties of the axons were preserved in the no-adhesion group. **B:** Numerous but sparse smaller-diameter axons were seen in the adhesion group. **C:** The morphological properties of the axons were relatively well preserved in the nerve wrap group, although the diameter of the axons was smaller than that it was in the no-adhesion group. **D:** Numerous but sparse smaller-diameter axons were seen in the HA group, as in the adhesion group. **E and F:** The axon numbers and areas of neurofilament protein-positive axons were higher in the no-adhesion and nerve wrap groups than in the adhesion and HA groups 6 weeks postoperatively. * $p < 0.05$.

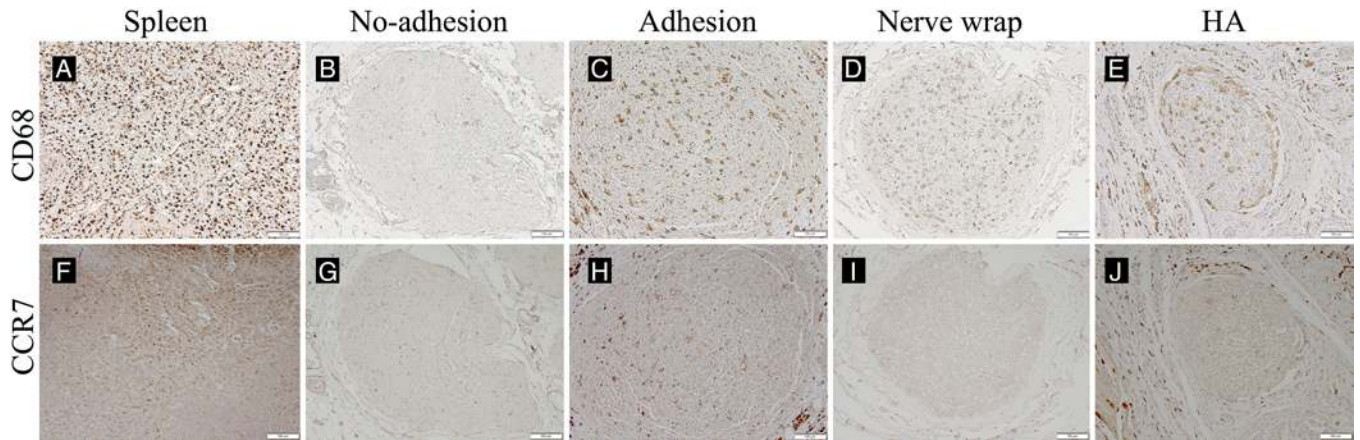


FIG. 10. Representative histological photomicrographs of intraneural scar tissue stained with anti-CD68 antibody and anti-CCR7 antibody 6 weeks postoperatively (scale bar 100 μ m). The highest numbers of CD68-positive activated macrophages and CCR7-positive proinflammatory M1 macrophages were found in the intraneural scar tissue in the adhesion group, whereas the lowest numbers were found in the no-adhesion group. There were fewer CD68-positive activated macrophages and CCR7-positive proinflammatory M1 macrophages in the intraneural scar tissue in the nerve wrap group than there were in the adhesion and HA groups.

Conclusions

This PLA-PCL nerve conduit protected peripheral nerves that underwent neurolysis from the development of adhesion and preserved the nerve function in a rat nerve adhesion model. The nerve conduit effectively blocked scarring and prevented adhesion-related damage in the peripheral nerves.

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Disclosures

The authors report no conflict of interest concerning the materi-

als or methods used in this study or the findings specified in this paper.

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Conception and design: Shintani, Uemura, Takamatsu, Nakamura. Acquisition of data: Shintani, Uemura, Yokoi, Onode. Analysis and interpretation of data: Shintani, Uemura. Drafting the article: Shintani, Uemura. Critically revising the article: Shintani, Uemura. Reviewed submitted version of manuscript: Shintani, Uemura. Approved the final version of the manuscript on behalf of all authors: Shintani. Statistical analysis: Shintani. Administrative/technical/material support: Shintani, Uemura, Yokoi, Onode. Study supervision: Shintani, Uemura, Takamatsu, Okada, Nakamura.

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