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EXPERIMENTAL

A Nanofiber Sheet Incorporating Vitamin B12 Promotes Nerve Regeneration in a Rat Neurorrhaphy Model

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Background: Outcomes of peripheral nerve repair after injury are often suboptimal. Therefore, developing biological approaches to augment nerve regeneration is important. In this in vivo study, we tested the hypothesis that augmentation with an electrospun nanofiber sheet incorporating methylcobalamin (MeCbl) would be effective for regeneration after peripheral nerve transection and repair.

Methods: Rats were divided into 3 groups that either underwent sciatic nerve repair with or without the MeCbl sheet, or a sham operation. At 4 and/or 8 weeks after the operation, sensory and motor functional recovery, along with histological findings, were compared among the groups using the toe-spreading test, mechanical and thermal algesimetry tests, tibialis anterior muscle weight measurements, electrophysiological analyses, which included nerve conduction velocity (NCV), compound muscle action potential (CMAP), and terminal latency (TL), and histological analyses involving the myelinated axon ratio, axon diameter, and total axon number.

Results: Compared with the repair group without the MeCbl sheet, the repair group with the MeCbl sheet showed significant recovery in terms of tibialis anterior muscle weight, NCV and CMAP, and also tended to improve in the toe-spreading test, mechanical and thermal algesimetry tests, and TL. Histological analyses also demonstrated that the myelinated axon ratios and axon diameters were significantly higher. Among these findings, the repair group with the MeCbl sheet demonstrated the same recovery in NCV as the sham group.

Conclusion: This study demonstrated that electrospun nanofiber MeCbl sheets promoted nerve regeneration and functional recovery, indicating that this treatment strategy may be viable for human peripheral nerve injuries. *(Plast Reconstr Surg Glob Open 2019;7:e2538; doi: 10.1097/GOX.0000000000002538; Published online 12 December 2019.)*

INTRODUCTION

Peripheral nerve injuries are common, affecting up to 2.8% of trauma patients and resulting in uncertainty while waiting for an often unpredictable and marginal level of recovery.[1–3] Despite significant neurobiological research and numerous microsurgical advances in terms of the management of these injuries, direct nerve repair with epineural microsutures represents the current gold standard for severe neurotmesis injuries that occur when the nerve is transected with a sharp object or when a small gap between nerve edges exists.[4] However, this methodology often has suboptimal outcomes due to the difficulties involved in correctly aligning and approximating the transected nerve segments to allow for the reinnervation of target organs and the achievement of functional recovery.[5] Therefore, there has been growing interest in alternative biological approaches to augment nerve regeneration after injury and improve functional outcomes, in addition to microsurgical techniques to enhance the repair of peripheral nerves.

We have studied the influence of methylcobalamin (MeCbl) on peripheral nerve regeneration by elucidating the underlying molecular mechanism. MeCbl, an analog of vitamin B12, promotes nerve regeneration and is

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effective for neuronal cell survival; however, a high concentration of MeCbl is required to maximize its effectiveness.[6–9] Therefore, we developed a novel electrospun nanofiber sheet incorporating MeCbl to locally deliver a high-concentrated compound to the peripheral nerve injury site, and showed its effectiveness both in vitro and in vivo in the axonal outgrowth of neurons and the differentiation of Schwann cells.[10] In this study, the local administration of MeCbl promoted nerve regeneration and functional recovery in a rat sciatic nerve crush injury model, assuming the case of nerve injury was in continuity such as entrapment neuropathy.[10]

In the present study, we hypothesized that the electrospun nanofiber sheet incorporating MeCbl may also be effective for nerve regeneration after peripheral nerve transection and repair. To test this hypothesis, we aimed to investigate the impact of the MeCbl sheet on nerve regeneration and functional recovery using a quantitative measure in a rat sciatic nerve transection model. To this end, we measured regeneration after sheet-augmented repair at 8 weeks, and predicted that this method would differ from conventional methods in terms of the resulting functional recovery. In addition, we evaluated whether the local microenvironment would support axonal regeneration, including a myelinated axon population.

MATERIALS AND METHODS

Animals

Wistar rats (6-week-old males; 180–220 g) were used for this in vivo experiment. Animals were housed under a 12/12h light/dark cycle (lights on, 08:00–20:00 h). All animals had free access to food (MF, Oriental Yeast, Osaka, Japan) and tap water. All experimental procedures involving animals were conducted in accordance with the guidelines of the Animal Care Committee of our institution, and this study was approved by our institutional review board (registration number, 29-006-000). In addition, maximum effort was employed to minimize the number of animals used and to limit any suffering.

Surgical Procedure

Each rat was deeply anesthetized by subcutaneous injection of a mixture of 2 mg/kg midazolam, 2.5 mg/kg butorphanol, and 0.15 mg/kg medetomidine. The left sciatic nerve was exposed from the sciatic notch to its bifurcation into the tibial and peroneal nerves, and sharply transected with microscissors. Thereafter, the sciatic nerve was repaired by end to end epineural microsutures with 10-0 nylon. Finally, the wounds were closed in layers and the animals were allowed to recover for 8 weeks. All surgeries were performed by the same surgeon. Twenty-five rats were divided into 3 groups: (1) microsurgical repair group (n = 10, *Repair*), where the sciatic nerve was transected and repaired; (2) MeCbl sheet placement group (n = 10, *Repair + Sheet*), where the transected and repaired nerve was wrapped by a 10 × 10 mm of nanofiber sheet containing 3% MeCbl; and (3) sham operation group (n = 5, *Sham*), where the sciatic nerve was only exposed. The MeCbl sheet was fabricated by the electrospinning method, as previously described.[10] Biodegradable nanofibers were produced through electrospinning poly (ε-caprolactone) and MeCbl with a voltage of 20 kV (Nanon-01A, MECC Co., Ltd., Fukuoka, Japan). Figure 1 shows the experimental pictures and illustrations.

Functional Analysis

Mechanical and thermal algesimetry tests were performed to evaluate sensory function at 4 and 8 weeks after the operation.[11,12] Paw withdrawal thresholds were measured to assess mechanical sensitivity with calibrated von Frey filaments (TouchTest, North Coast Medical Inc, Gilroy, Calif.). Rats were placed on elevated metal mesh and then a series of von Frey filaments were gently applied to the plantar surface of the hindpaw. The nociceptive threshold was expressed as the force at which the rat withdrew the paw in response to the stimulus. Thermal algesimetry tests were performed using a hot plate (Ugo Basile, Varese, Italy) at a temperature of 52.5°C. Rats were placed in a plastic box with an elevated glass floor. The beam of a lamp was focused on the paw plantar surface, until the rat withdrew the paw, and sensors in the device shut off the lamp. The threshold was defined as the latency time until withdrawal.[12] Mechanical and thermal thresholds in the sciatic territory of the ipsilateral hindpaw typically display higher values in rats with injured nerves, while they return to basal levels when sensory function recovery occurs.[11]

To assess the motor functional recovery of the injured limb, the toe-spreading test was performed at 4 and 8 weeks. By lifting their tails, the rats were induced to spread their toes by briefly lifting the hindlimbs off the bench, and the appearance of a toe-spreading reflex was graded (2, normal spreading; 1, intermediate spreading; 0, no spreading). Toe-spreading requires the innervation of the extensor digitorum and the intrinsic muscles of the foot, including the interossei. Accordingly, following sciatic nerve lesion, a simple and meaningful measure of muscle reinnervation is the return of the toe-spreading reflex. As such, the foot cannot be dorsiflexed at the ankle and the toes remain close together until the reinnervation of the intrinsic muscles of the foot is restored.[13,14]

Tibialis Anterior Muscle Weight Measurement

The wet weight of the tibialis anterior muscle from the experimental side was measured to determine a gross estimation of muscle reinnervation. The weight of the muscles distal to the injured and regenerated nerve was considered proportional to the degree of innervation, given that the denervated muscles suffer progressive atrophy, and this was proposed as a parameter for functional recovery.[15]

Electrophysiological Analysis

Electrophysiological analysis was performed 8 weeks after the operation.[8] Rats were deeply anesthetized as described above, and the sciatic nerve and the tibialis anterior muscle were exposed. To record the compound

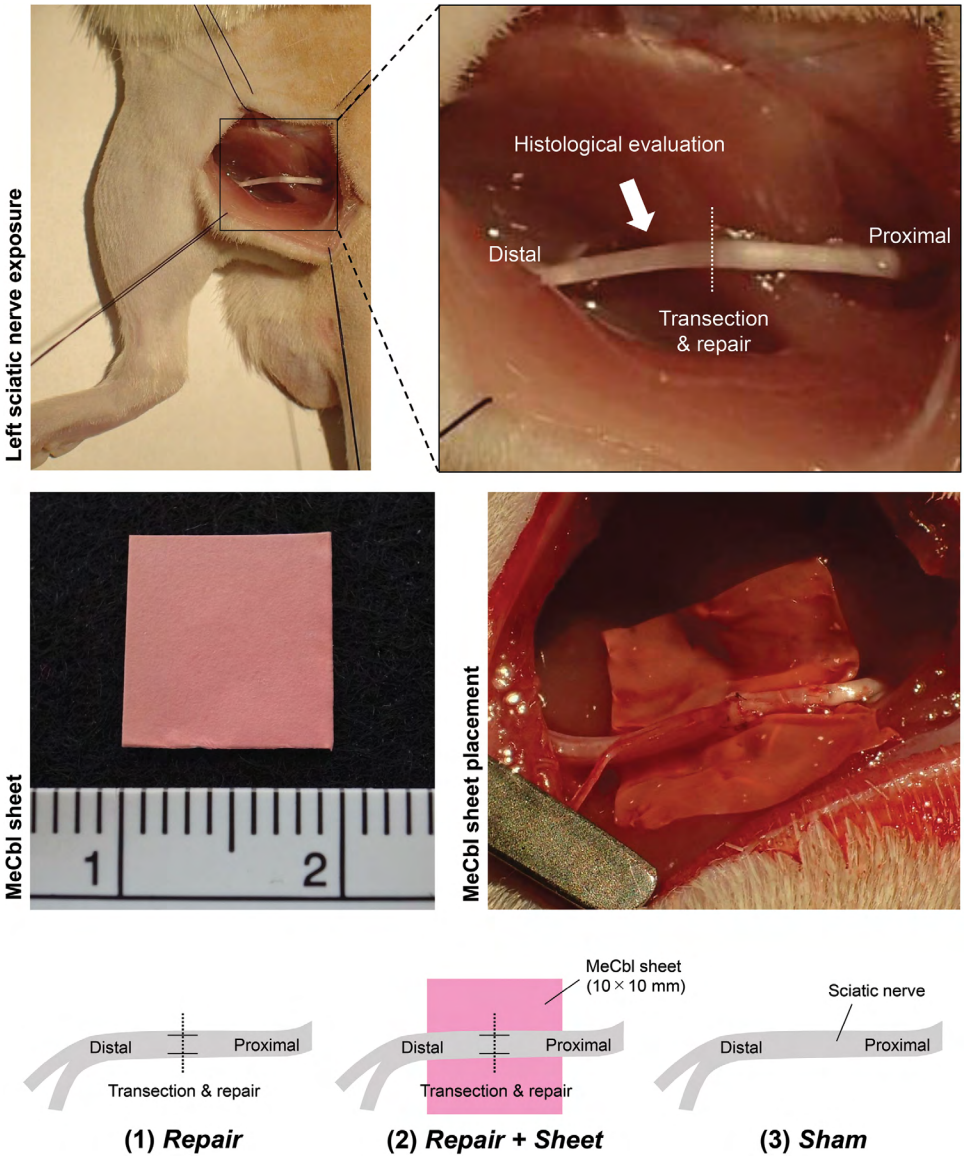

Fig. 1. Experimental design. The left sciatic nerve is exposed and freed from surrounding tissues (*above-left*). The nerve is transected, and then repaired by end to end epineural microsutures with 10-0 nylon (*above-right*). Gross view of the electrospun nanofiber sheet incorporating MeCbl sheet (*middle-left*), and an intraoperative photo (*middle-right*) are shown. The sheet is placed around the sciatic nerve without anchoring to the surrounding tissue after microsurgical repair. Rats are divided into 3 groups (*below*): (1) microsurgical repair group (*Repair*, n = 10), where the sciatic nerve was transected and repaired; (2) MeCbl sheet placement group (*Repair + Sheet*, n = 10), where transected and repaired nerve was wrapped around by 10 × 10 mm of nanofiber sheet containing MeCbl; and (3) sham operation group (*Sham*, n = 5), where sciatic nerves were only exposed.

muscle action potentials (CMAP) and the terminal latency (TL), a recording electrode was placed in the tibialis anterior muscle and bipolar stimulating electrodes were noninvasively placed on the proximal side 5 mm from the sutured site. Nerve conduction velocity (NCV) was calculated using 2 different points across the sutured site, and was stimulated on the distal side 5 mm from the sutured site. The NCV, CMAP, and TL were detected and measured using the PowerLab device and associated software (AD Instruments, Bella Vista, NSW, Australia).

Histological Analysis

Rats were sacrificed at the appropriate endpoints (after completion of all operative procedures), and the sciatic nerve containing the sutured site was harvested at a length of 10 mm. Specimens were fixed with 4% paraformaldehyde for 5 days, and then stored in 20% sucrose in 0.01 M PBS for 24 h. Subsequently, specimens were placed and frozen in appropriate frozen section compound (Leica Biosystems), sectioned axially at 2 mm-distal from the sutured site, and mounted on glass slides. Thin

sections were permeabilized with 100% methanol for 30 min, blocked with PBS, 0.2% TritonX, and 5% bovine serum albumin for 30 min, and incubated overnight with primary antibodies against MBP (1:1,000; Calbiochem NE1018) and NF200 (1:1,000; Sigma–Aldrich N4142) at 4°C inside a wet chamber. The fluorescence-conjugated secondary antibodies used were an Alexa Fluor 488 goat anti-rabbit IgG antibody (1:1,000; Life technologies) and an Alexa Fluor 568 goat anti-mouse IgG antibody (1:1,000; Life technologies), which were allowed to react for 1 h.

Stained sections were viewed and photographed at ×444 magnification on a KEYENCE BZ-X700 microscope (Keyence Corporation). Images were captured using a KEYENCE BZ-X700 BZ-X Analyzer. The number of total axons, myelinated axons, and axon diameters were measured, along with the calculation of the axon diameter frequency. The myelinated axon ratio was then calculated as the number of both MBP- and NF200- positive axons (myelinated axons) to the number of NF-200 positive axons (total axons).

Statistical Analysis

All statistical analyses were performed using IBM SPSS, Version 23.0 (IBM Corp., Armonk, N.Y.), with the significance level set at $P < 0.05$. The normality of all the variables in any analysis was tested by the Shapiro-Wilk parametric test.

To detect differences among the *Repair*, *Repair + Sheet*, and *Sham* groups, data were analyzed using a single 1-way analysis of variance and unpaired *t*-tests (if variables were normally distributed) or Kruskal-Wallis test and the Mann-Whitney *U* test (if variables were not normally distributed), with Bonferroni adjustments for multiple comparisons.

RESULTS

Functional Recovery

Bar charts for the sensory and motor functional analyses are shown in Figures 2 and 3. Sensory function was measured by both mechanical and thermal algesimetry tests, and their thresholds of all 3 groups were recorded at 4 and 8 weeks, postoperatively. For the *Repair* group, the mechanical threshold values were 96.5 ± 43.2 and 73.2 ± 30.9 g; for the *Repair + Sheet* group, they were 43.1 ± 32.2 and 53.2 ± 14.3 g; and for the *Sham* group, these values were 53.2 ± 15.2 and 46.4 ± 18.6 g, respectively. There was a significant difference detected between the *Repair* and *Repair + Sheet* groups at 4 weeks after the operation ($P = 0.042$) (Fig. 2A). With regard to the thermal thresholds recorded at 4 and 8 weeks postoperation, the values were 13.2 ± 2.5 and 13.1 ± 1.4 seconds for the *Repair* group; they were 11.4 ± 2.2 and 11.8 ± 0.8 seconds for the *Repair + Sheet* group; and they were 12.0 ± 0.6 and 12.3 ± 0.3 seconds for the *Sham* group, respectively. A significant difference between the *Repair* and *Sham* groups was detected at 4 weeks after the operation ($P = 0.042$) (Fig. 2B).

Motor recovery after sciatic nerve injury was evaluated using the toe-spreading test. At 4 and 8 weeks, scores for the *Repair + Sheet* group (0.80 ± 0.79 and 1.00 ± 0.47) tended to be higher than those in the *Repair* group (0.10 ± 0.32; $P = 0.060$ and 0.50 ± 0.71; $P = 0.168$), although these differences did not reach those observed in the *Sham* group (2.00 ± 0.00 and 2.00 ± 0.00) (Fig. 3).

Tibialis Anterior Muscle Weight Recovery

The muscle weight in the *Repair + Sheet* group (672.0 ± 113.3 g) was significantly higher than that in the *Repair* group (455.4 ± 59.5 g; $P < 0.001$), although they did not reach that in the *Sham* group (1,350.1 ± 141.1 g) (Fig. 4).

Electrophysiological Recovery

Data collected for the electrophysiological analyses are shown in Figure 5. The NCV of the *Repair + Sheet* group (63.5 ± 22.4 m/s) was significantly faster than that of the *Repair* group (25.7 ± 9.8 m/s; $P < 0.001$), and was of the same magnitude as that of the *Sham* group (63.2 ± 8.7 m/s) (Fig. 5A). Values of CMAP in the *Repair + Sheet* group (9.25 ± 3.86 mV; $P = 0.048$) and the *Sham* group (23.48 ± 10.16 mV; $P = 0.042$) were significantly higher than that of the *Repair* group (5.05 ± 3.14 mV) (Fig. 5B). TL in the *Repair* group (2.86 ± 0.79 ms) tended to be retarded compared with that in the *Repair + Sheet* group (2.60 ± 0.24 ms), although in the *Sham* group (2.08 ± 0.30 ms) it was the shortest (Fig. 5C).

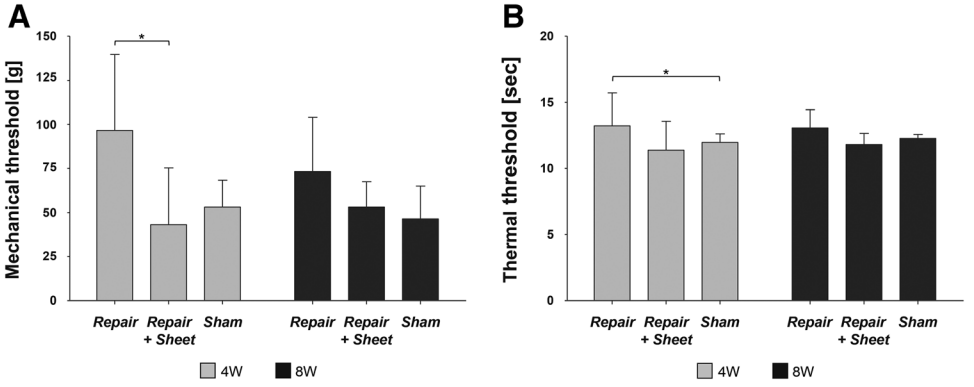

Fig. 2. Sensory functional analyses by mechanical (A) and thermal (B) algesimetry tests. * indicates statistically significant *P* value with Bonferroni adjustment: $P < 0.05$.

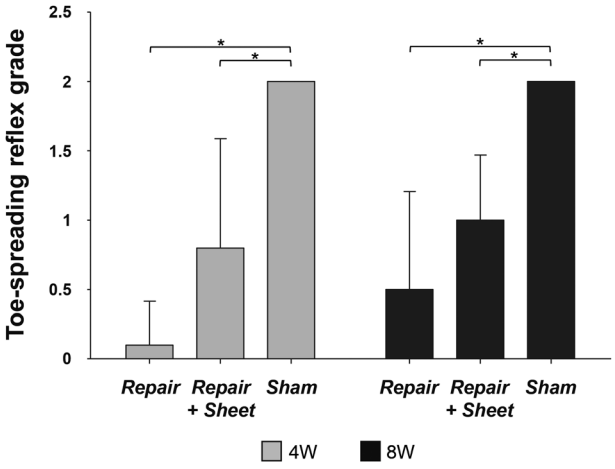

Fig. 3. Motor functional analysis by toe-spreading test. * indicates statistically significant *P* value with Bonferroni adjustment: $P < 0.05$.

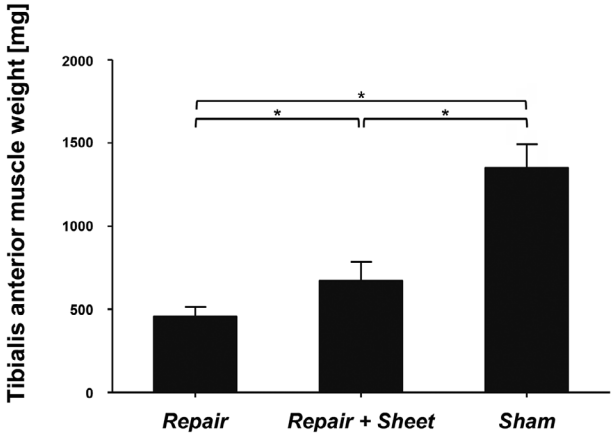

Fig. 4. Tibialis anterior muscle weight measurement. * indicates statistically significant *P* value with Bonferroni adjustment: $P < 0.05$.

Histological Recovery

Results obtained from histological analyses are shown in Figure 6. The ratios of myelinated axons and axon diameter in the *Repair + Sheet* group (79.2 ± 3.0 % and 5.16 ± 0.23 μm) were significantly higher than those calculated in the *Repair* group (60.9 ± 3.7 %; $P < 0.001$ and 4.05 ± 0.38 μm; $P < 0.001$), although they did not reach those observed in the *Sham* group (92.3 ± 0.8 % and 6.30 ± 0.34 μm) (Fig. 6, *second row*). The population of larger regenerated axons was higher in the *Repair + Sheet* group compared with the *Repair* group (Fig. 6, *below-right*), although we did not detect any statistical difference in the total number of axons among the 3 groups, with the *Repair* group at 21,027 ± 5,947 axons/mm^2, the *Repair + Sheet* group at 20,973 ± 4,086 axons/mm^2, and the *Sham* group at 16,007 ± 1,836 axons/mm^2 (Fig. 6, *below-left*).

DISCUSSION

In this study, we investigated the impact of an electrospun nanofiber sheet incorporating MeCbl on nerve regeneration and functional recovery using quantitative measures in a rat sciatic nerve transection model. Our results demonstrated that augmentation with the MeCbl sheet at peripheral nerve injury sites enhanced functional recovery, and this was further supported by sensory and motor functional tests, along with electrophysiological findings (Figs. 2–5). Histological analyses also revealed that locally administrated MeCbl significantly promoted regeneration distal to the injury site (Fig. 6).

Challenges in nerve regeneration, particularly in transected nerve repair, arise due to the disruption of axonal continuity and the resulting inhibition of axonal regeneration to the distal nerve and end-organ targets. However, in vitro and in vivo models using MeCbl offer opportunities to examine the underlying enhancement of nerve regeneration. Previous in vitro studies have shown that MeCbl promotes neurite outgrowth in neurons,[8] differentiation and myelination in Schwann cells,[16] and proliferation and migration in myoblasts.[17] Furthermore, the systemic administration of MeCbl with an osmotic pump has been shown to accelerate functional, electrophysiological, and histological recoveries in a rat sciatic nerve transection model,[8] and remyelination in a focal demyelination rat model.[16] Suzuki et al[10] fabricated electrospun nanofiber sheets incorporating MeCbl to deliver enough MeCbl locally to the peripheral nerve injury site, and showed effectiveness in promoting nerve regeneration in a rat sciatic nerve crush injury model, without affecting plasma concentrations of MeCbl. In this article, they also demonstrated a nanofiber sheet not incorporating MeCbl did not affect the function and the morphology of peripheral nervous system.[10] In addition, nanofiber sheets themselves have been shown to enhance axonal regeneration at local sites by modulating inflammatory responses and reducing scarring/fibrosis.[18,19] However, to our knowledge, no

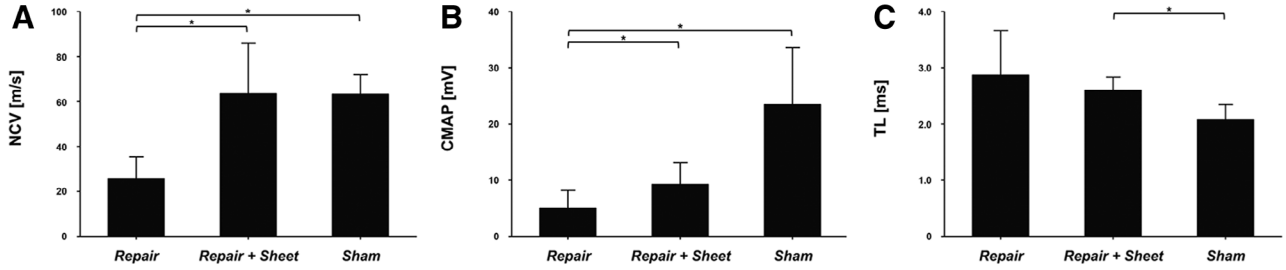

Fig. 5. Electrophysiological analyses of NCV (A), CMAP (B), and TL (C). * indicates statistically significant *P* value with Bonferroni adjustment: $P < 0.05$.

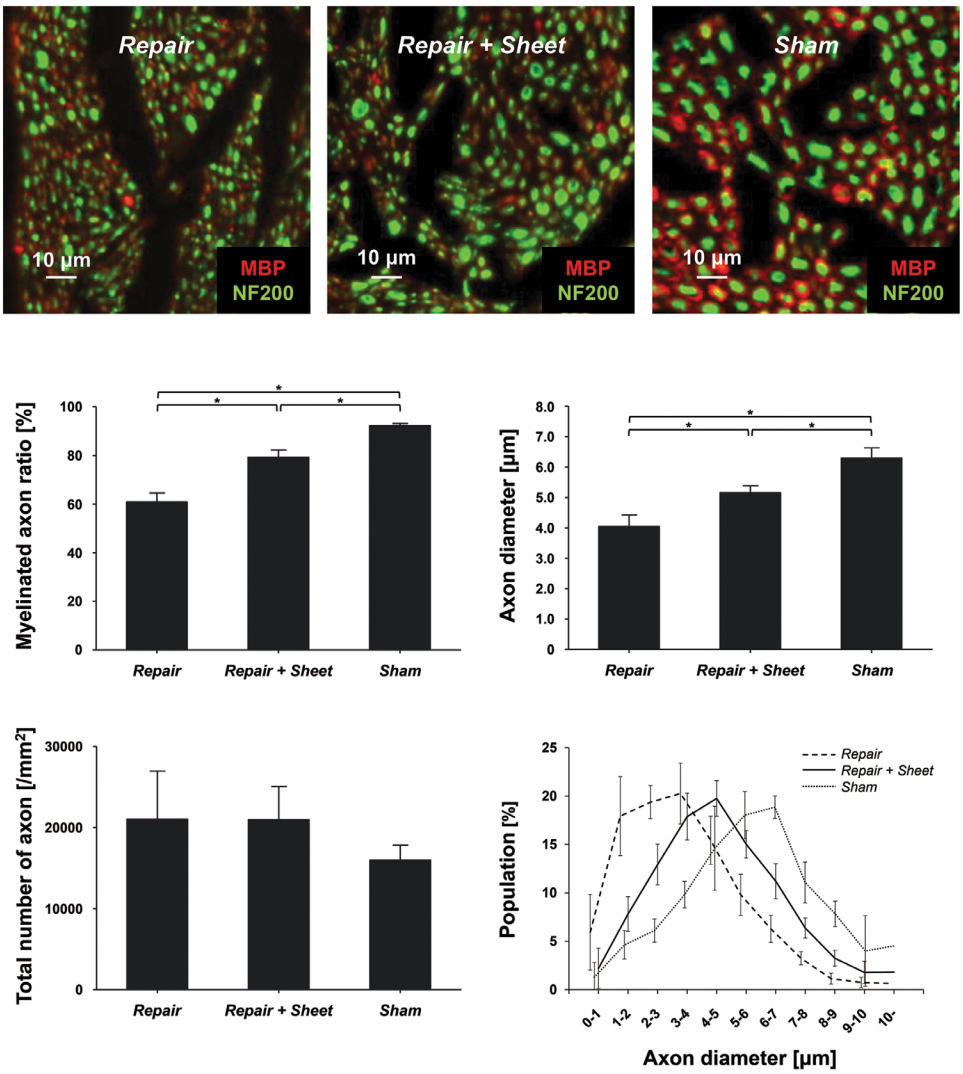

Fig. 6. Histological analyses of sciatic nerve distal to the repair site. Fluorescent microscopic images show cross-sectional slices of sciatic nerves labeled for MBP (red) and NF200 (green) in 3 groups (high-magnification, × 444; scale bar = 10 μm) (above). Quantitative measurements are shown in the graphs below: myelinated axon ratio (middle-left); axon diameter (middle-right); total axon number (below-left); and population of axons with regard to the diameter (below-right). * indicates statistically significant *P* value with Bonferroni adjustment: $P < 0.05$.

attempt has been made to investigate the efficacy of local administration of MeCbl using the drug releasing material on transected nerve repair, which is considered a more severe condition compared with nerve crush injury. Therefore, this report adds important information to the previous studies.

Our findings revealed functional and electrophysiological recoveries in the *Repair + Sheet* group (Figs. 2–5). Histologically, this group also displayed increases in the ratio of myelinated axon, the diameter of new axons, and populations of regenerated axons with a large diameter (Fig. 6). These results supported our study hypothesis that local administration of MeCbl through electrospun sheets would contribute to axonal maturation during peripheral nerve regeneration, resulting in improved neurological recovery.

The value of NCV, which is a local indicator of nerve regeneration across injury sites, in the *Repair + Sheet* group recovered to the same degree as that in the *Sham* group (Fig. 5A). This likely is attributed to the progression of nerve regeneration at the repair site, presumably due to the recovery of myelinated axons, which may explain the trend we observed on sensory functional tests where measurement values recovered to the same degree as the *Sham* group. On the other hand, insufficient recovery of CMAP, which is determined by the number of muscle fibers that are innervated and is regarded as a total indicator from the nerve injury site to the target organ (Fig. 5B), suggested that the neuromuscular recovery we observed was still premature in several weeks after the injury. This supports the findings of the toe-spreading test and tibialis anterior muscle weights that the *Repair + Sheet* group recovered

more than the *Repair* group, but not as much as the *Sham* group (Figs. 3 and 4). Overall, this kind of sensory dominant recovery would be compatible with the widely known belief that sensibility return occurs before motor function recovery during the early phase of regeneration,[20,21] and the MeCbl sheet was considered to promote this phase.

The current study had several potential limitations. First, measurements were performed at 4- and 8-week time points, which represent the early phase for peripheral nerve regeneration. However, even in this premature phase, we found meaningful differences between the *Repair + Sheet* and the *Repair* groups. Second, using a stronger negative control such as allograft model could be of more useful. However, using MeCbl sheet and comparing this to a simple repair, we expected only small changes, making a statistically significant findings harder to detect compared with challenging models. If a statistical difference would be found in recovery this would be more meaningful for the study hypothesis. Other limitations include the lack of a negative control group with a nanofiber sheet not incorporating MeCbl in this nerve neurorrhaphy model. These limitations notwithstanding, all outcome measurements support and enhance the findings of previous experimental studies. Hence, we believe that MeCbl sheets may be effective for nerve regeneration in nerve transection and repair cases.

CONCLUSION

The current study revealed the impact of electrospun nanofiber sheets incorporating MeCbl on nerve regeneration and functional recovery. We found that these sheets were effective for nerve regeneration in peripheral nerves injuries by delivering MeCbl locally around the injured site. Therefore, the present findings add a potential treatment option for human peripheral nerve injuries.

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