Neural regrowth induced by PLGA nerve conduits and neurotrophin-3 in rats with complete spinal cord transection

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Abstract: Biomaterials and neurotrophic factors represent two promising strategies for spinal cord injury repair. In this study, a combinatorial approach combining the PLGA nerve conduits and the recombinant human neurotrophin-3 (rhNT3) was utilized in a spinal cord injury animal model. After complete transection of the thoracic cord in rats, rhNT3 was administered as a single dose to the host cord caudal to a 2-mm conduit. Axonal regrowth was enhanced, as indicated by immunostaining and neurofilament-positive area measurement. Neural regrowth was further demonstrated via the retrograde tracing across the lesion. The animals implanted with the PLGA scaffold and rhNT3 exhibited significantly improved performance in BBB rating scale and grid walk tests. These observations suggest that PLGA nerve conduits combined with exogenous NT3 may serve as an alternative therapeutic approach for spinal cord injury repair. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 97B: 271–277, 2011.

Key Words: growth factor, PLGA, NT3, spinal cord injury

INTRODUCTION

Spinal cord injury (SCI) results in axonal degeneration and neuronal necrosis. The microenvironment after SCI in adults is much less receptive to regrowth than in neonates,[1] thus hindering the regeneration of damaged nerves. However, accumulating evidence since 1980s has demonstrated that the central nervous system (CNS) in adults can regenerate in a supportive environment following injuries such as SCI.[2] The current research included inhibiting the formation of glial scar or myelin, such as chondroitin sulfate proteoglycans and NOGO[3–7]; supplying growth-promoting cues, such as neurotrophic factors[8]; transplanting cells[9–13]; activating the intrinsic capacity of neuronal regeneration programs.[14] Despite extensive research dedicated to SCI repair, the multifaceted nature of SCI has hampered therapeutic efficacy of current treatment strategies. Consequently, combinatorial measures, including neurotrophic factors and biomaterials,[15] have being investigated in potential protocols for SCI repair.

Neurotrophin family members consist of nerve growth factor, brain derived neurotrophic factor, neurotrophin-3 (NT3), neurotrophin-4/5, and neurotrophin-6. In particular, NT3 plays a crucial role in nerve regeneration in the CNS,[16] especially the corticospinal tracts (CST). CST is one of the most critical motor paths in humans and animals. NT3 enhances both corticospinal neuronal survival and corticospinal fiber sprouting to partially restore the motor and sensory functions.[17–21] In addition, Wallerian degeneration facilitates axonal growth in the presence of NT3, primarily due to the neuroprotective and neuroreparative functions of microglia and macrophages during Wallerian degeneration.[22–25]

Tissue engineering has recently emerged as a promising approach for SCI repair, especially during massive loss of tissue structure. Compared with allografts or autografts, biomaterials of functional scaffolds[26] are readily available and do not require additional operation, thus providing an attractive alternative for SCI repair. In particular, bio-artificial nerve guidance conduits, made of distinct biomaterials, can reconstruct spinal cord tissue architecture and provide guidance for regenerating axons.[27–31]

Poly (lactic-*co*-glycolic acid) (PLGA) is an U.S. Food and Drug Administration approved biomaterial.[32–36] PLGA-based nanoparticles or microsphere,[34–37] nanofibers,[38,39] conduits,[30,40] and membrane[41] have been extensively employed

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for nerve regeneration. Multichannel PLGA nerve conduits with porous architectures were recently manufactured and proved useful for SCI reconstruction.[32] Multichannel nerve conduits are tissue-engineering scaffolds functioning to restrict dispersion of regenerating axons.[42] In multichannel PLGA, the channels are aligned in parallel, and bundles of regular ladder-like inter connecting pores (140 ± 25 μm and 17 ± 5 μm in width) serve to bridge the open channels to achieve more precise orientation. Importantly, the characteristic high porosity and interconnectivity facilitates neurotrophin entry and waste drainage; furthermore, the optimal degradation rate is conducive to nerve regeneration and also prevents cumbersome absorption by surrounding tissues.[32]

In this study, we aimed to evaluate the effect of multichannel PLGA scaffold combined with exogenous NT3 immediately following complete transection of the spinal cord *in vivo*. Motor functional recovery was assessed by the Basso, Beattie, and Bresnahan (BBB) rating scale and grid walk test. Axonal regrowth was examined by nerve fiber immunostaining. Retrograde tracing and electrophysiology of cortical somatosensory evoked potential (CSEP) were also carried out.

MATERIALS AND METHODS

Materials

PLGA nerve conduits were obtained from Sun Yat-Sen University (Guangzhou, China). PLGA with longitudinally parallel-aligned channels and hierarchical pore architecture were manufactured using injection molding, thermally induced phase separation (TIPS) and particulate leaching.[32] Recombinant human NT3 (rhNT3) was prepared in our lab. Briefly, human NT3 gene was cloned from fetal brain cDNA into bacterial expression vector. After transformation of *E.coli* BL21 strain, rhNT3 expression was induced with 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 5 h. The rhNT3 proteins in the inclusion body were renatured at 4°C for three days, then purified with nickel chelate chromatography (Amersham Biosciences). The purity attained was determined above 95% according to the SDS-PAGE result. The biological activity was determined by assaying neurite outgrowth from dorsal root ganglion (DRG) explants, similar to the methods of Ruitenberg et al.[43]

Animal surgery

Fifty female Wistar rats, each weighing 180–220 g, were used in this study. Animal experiments were carried out according to the guidelines approved by Institutional Committee on the Care and Use of Animals in 2006. Animals were anesthetized with intraperitoneal injection of 3.6% chloral hydrate (1 mL/100 g body weight) and randomly divided into five groups. In the first group (PLGA, $n = 12$), rats were subjected to dorsal laminectomy. T8-T10 spinal segments were exposed and completely transected with angled microscissors to form a ~2 mm long cavity. Subsequently, a 2-mm PLGA nerve conduit was implanted into the lesion gap. In the second group (N3, $n = 12$), rhNT3 (200 μg/mL, 15 μL) was administered by a microsyringe into the caudal segment close to the lesion within 20 min. In the third group (PLGA+N3, $n = 12$), animals were implanted with PLGA followed by rhNT3 (200 μg/mL, 15 μLinjection into the adjacent caudal spinal cord. The fourth group consisted of animals that were transected without additional treatment (control, $n = 8$), and animals of the last group were subjected to only laminotomy (sham, $n = 6$). After the operation, surgical sites were sutured and the rats were put back to their cages. The animals received extensive care, including twice daily bladder emptying. Twenty thousand unit penicillin was administered for a week, and food and drinking water were freely accessible.

Neurofilament immunostaining

Sixteen weeks after surgery, all animals were anesthetized and perfused with saline solution (0.9% in sterile water) followed by 4% paraformaldehyde in 0.1*M* phosphate buffered saline (PBS, pH 7.4). The spinal cord encompassing the lesion was carefully dissected and postfixed in the same paraformaldehyde. The spinal cord was then immersed in 20% sucrose buffer for 48 h, and 20-μm frozen sections were obtained and stored at −20°C. Sections were blocked in 10% normal goat serum in PBS at room temperature for 2 h. Samples were then incubated with primary antibodies, including mouse antineurofilament antibody (NF200, monoclonal, 1:200, Millipore) and rabbit anti-glial fibrillary acid protein (anti-GFAP) antibody (polyclonal, 1:200, abcam), overnight at 4°C followed by 1h at 37°C. The sections were incubated with Alexa Flour 594-conjugated anti-mouse IgG (1:100, Invitrogen) and Alexa Flour 488-conjugated anti-rabbit IgG (1:200, Invitrogen) for 1 h at 37°C. Fluorescence was observed under a fluorescence microscope (Leica DM IRE2, Germany); the fluorescence images were digitized and NF200-positive areas were calculated using Image-pro Plus software. The background and nonspecific staining in each digital image was excluded by adjusting intensity threshold. NF200 labeled axons displayed above threshold signals, and the percentage of area labeled with NF200 positive fibers was determined. The investigators were blind to the test.

Hindlimb locomotor function analysis

Functional recovery was evaluated by the BBB rating scale[44,45] and grid walk test[45] for 16 weeks postsurgery. The Basso, Beattie, and Bresnahan (BBB) scale was an open field (overground) locomotor scale to evaluate functional recovery of rats and became a common practical scale. In the BBB rating scale, the movement of the rats' hindlimb was paid great attention. The scale ranged from a score of 0 to 21, in which 0 represented complete paralyses while 21 indicated normal state. This evaluation was performed for 4 min each rat in a blind manner. In the latter experiment, the rats were allowed to climb a 70-cm-high and 45° sloped grids (10 × 10 mm^2 holes). The weight-supported and the plantigrade steps were considered as the right steps. The weight-supported and the plantigrade steps were considered as the right steps. The total number of steps that a rat used to cross the grids and the number of right steps were counted. This experiment was videotaped (Canon S3/S5)

and analyzed off-line in a blind manner in a frame-by-frame way. The percentage of the right steps was calculated by the right number of steps/the total number of steps. The investigators were blind to the tests.

Retrograde tracing

Ten days before being sacrificed, five rats from each of the four groups (control, N3, PLGA, and PLGA+N3) were anesthetized as above. After the transected site was exposed a second time, 0.5 μL retrograde tracer hydroxystilbamidine (4% w/v in saline solution, Molecular Probes) was injected at 5 mm below the injury site within 15 min. After extended retrograde transport of the tracer through CST, animals were sacrificed as described above. Totally, 10 μm sections were used for hydroxystilbamidine labeling. The retrograde tracing of hydroxystilbamidine was examined immediately under a fluorescence microscope (Leica DM IRE2, Germany).

Electrophysiology of CSEP

To investigate whether the sensory signals from below the lesion may be conveyed to the cortex, CSEP was measured immediately before the rats were killed (performed at Sun Yat-Sen University). The sensorimotor cortex (SMC) was exposed with the animal mounted on the stereotaxic apparatus (NARISHIGE, Japan), and electrodes were attached to the exposed sciatic nerves and SMC. Bipolar stimulating electrodes and monopolar recording electrodes were used to induce and record electrical activity respectively. CSEP in response to the Stimulus Isolator (A365, World Prcision Instruments, USA) was recorded by an Isolated Biological Amplifier (ISO-DAM, world precision instruments, USA). Intensity: 5 mV, duration: 0.2 ms.

Statistical analysis

Data were presented as mean values ± standard deviation. The statistical significance of differences between the groups was evaluated by one-way ANOVA with a *post-hoc* test. Results were considered significant at $p < 0.05$.

RESULTS

Implantation of PLGA conduit in combination with exogenous NT3 treatment promoted nerve fiber regrowth

Next, we examined nerve fiber regrowth. As shown in Figure 1(A), the scaffold served as a structural support

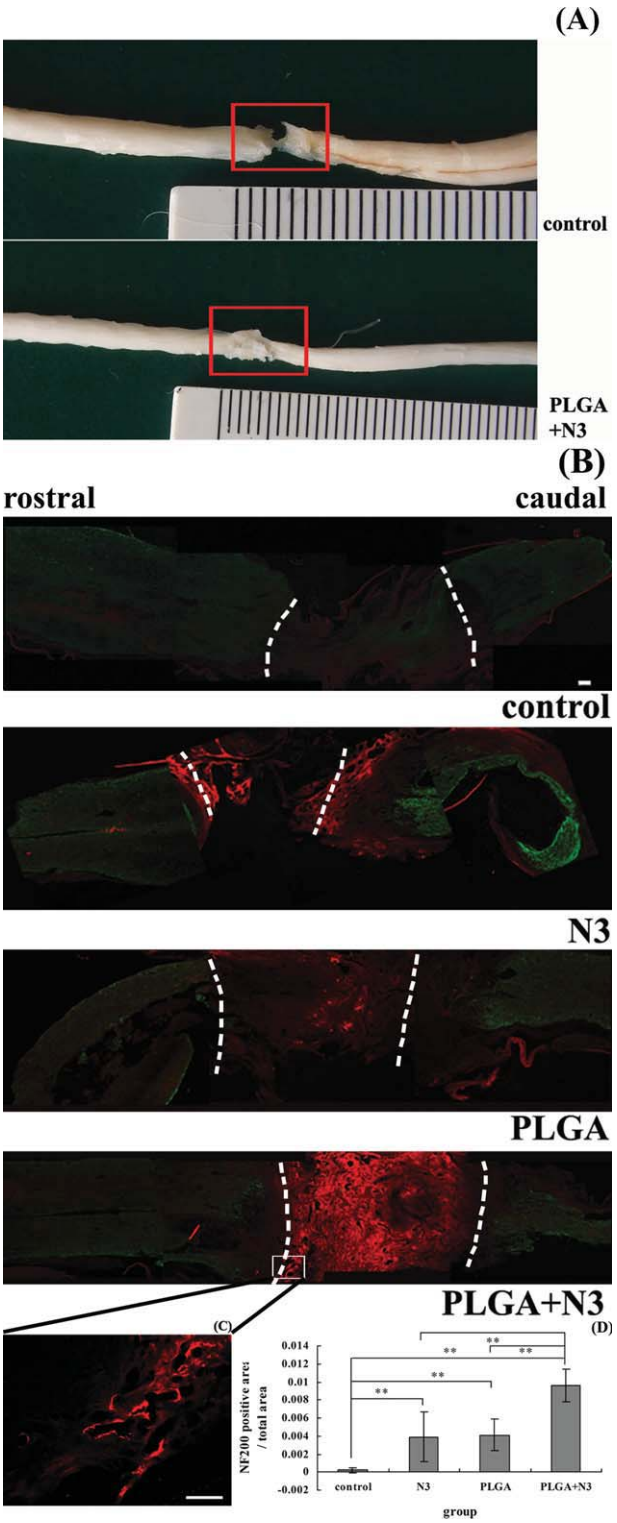

FIGURE 1. (A) The entire spinal cord including the lesion was carefully harvested. The PLGA nerve conduits and NT3 both contributed to tissue structural integrity, whereas a large gap in the lesion site was still present in the control. (B) The longitudinal section of NF200 stained fibers (red) showed robust axonal regrowth in the PLGA+N3 group, with decreasing degrees of regrowth observed in the PLGA, NT3 and control groups. The PLGA scaffold and NT3 supplied more favorable environment to the lesion segment, therefore enhanced more nerve fiber regrowing. While in the rostral or caudal stumps, as the microenvironment was less receptive, more nerve fibers degenerated and reduced greatly. The GFAP-positive cells (green) were found to be trapped at the adjacent spinal cord of the lesion, and indicated slight reactive astrocyte and glial scar formation in the PLGA+N3 group, whereas more GFAP-positive cells invaded into the lesion in the control. The border of the lesion-host tissue is indicated by dash lines. (C) The magnified image of regrowing fibers in the transplant in the PLGA+N3 group. Scale Bars: 50μm in (B), 200μm in (C). (D) The NF200 positive area in the PLGA+N3 group was significantly larger than that of the other groups, with the control being most inferior. There was statistical significance among the four groups ($P = 0.000 < 0.01$). *, $P < 0.05$; **, $P < 0.01$, significance between the groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bridging between the caudal and rostral stumps of the host spinal cord. Neurofilament staining (red) was used to identify regrowing nerve fibers in the lesion site. GFAP-positive cells (green) were found in the rostral and caudal stumps of the host spinal cord [Figure 1(B)]. The NF200 positive fibers revealed that the scaffold and the rhNT3 may greatly promote axonal regrowth [Figure 1(B,C)]. Significantly enhanced axonal growth in the PLGA+N3 group was observed relative to that in the control, PLGA and N3 groups [Figure 1(D)]. These observations indicated that the combination of the PLGA scaffold and the rhNT3 significantly enhanced nerve fiber regrowth.

Implantation of PLGA conduit in combination with exogenous NT3 treatment promoted recovery of hindlimb locomotor function

We first examined the recovery of hindlimb locomotor function in the groups of animals indicated in the Materials and Method. The 21-point system is applied to rats from complete paralysis (0) to normal.[21] Sham-operated animals appeared normal based on the BBB [Figure 2(A), upper panel] and grid walk tests [Figure 2(B)], whereas the rats in the control group showed essentially no functional improvement. The BBB scores in the control group were 0.375 ± 0.518, 0.75 ± 0.886, 0.75 ± 0.886 at the 7th, 12th, 16th week, respectively. In the N3 group, the BBB scores were 0.5 ± 0.756, 0.875 ± 0.835, and 1.25 ± 1.04 at the 7th, 12th, 16th week, respectively. In the PLGA group, the BBB scores were 0.875 ± 0.641, 1.125 ± 0.641, 1.625 ± 0.916 at the 7th, 12th, 16th week, respectively. Animals in the PLGA+N3 group showed no voluntary movement immediately after the surgery, but appeared to gradually recover during the following 16 weeks. The average BBB score for the PLGA+N3 rats group was 2.63 ± 2.45, 2.38 ± 1.69, and 3.50 ± 1.69 at the 7th 12th 16th week, respectively. Significant difference was observed between the PLGA+N3 and the control animals by week 7. Likewise, the BBB score for the PLGA+N3 group was also significantly higher than those of the N3 and PLGA groups [Figure 2(A)]. In the grid walk test, animals were evaluated at 30, 60, and 150 days after the surgery. The performance of the PLGA+N3 animals was significantly superior to that of control animals and the N3 and PLGA animals. The mean percentage in the PLGA+N3 group was 10.50 ± 6.40%, 7.50 ± 7.23%, and 13.50 ± 3.66% at 32, 60, and 150 days after the surgery, respectively [Figure 2(B)]. Taken together, these results indicated that the PLGA+N3-treated animals showed improved performance in hindlimb locomotor function.

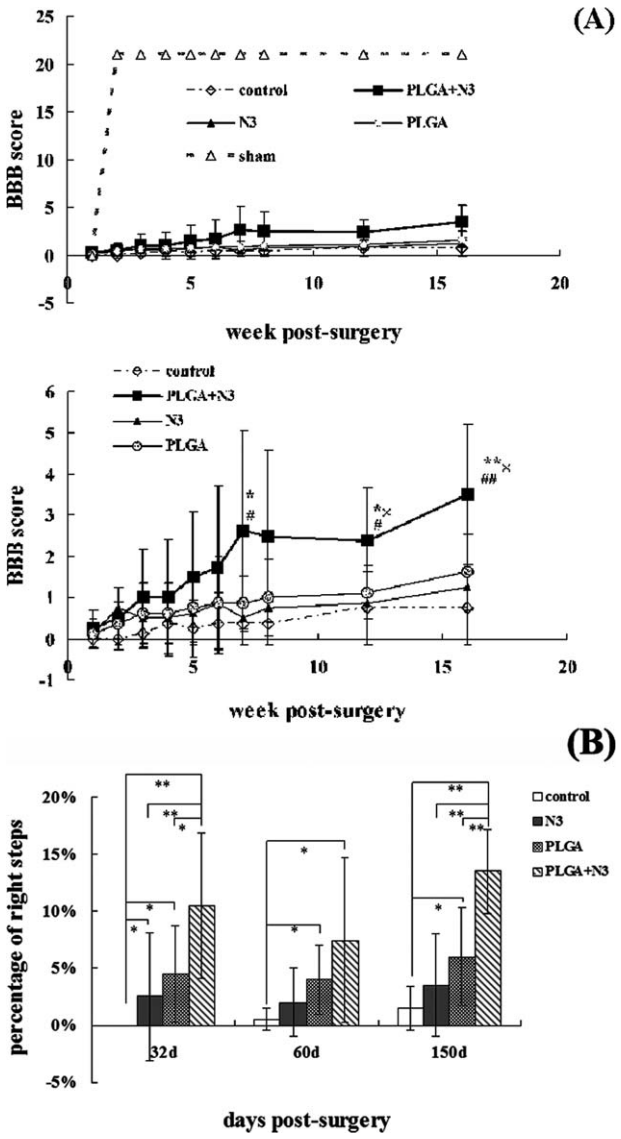

FIGURE 2. Locomotor functional recovery was evaluated by the BBB rating scale and grid walk test. (A) BBB scores at 1, 2, 3, 4, 5, 6, 7, 8, 12, 16 weeks post surgery. The BBB score for the sham group was determined to be 21, much higher than those of the other groups. The value of the sham group and a magnified view of the other values for comparison were given. The statistical significance between the four groups was determined. There was significant difference among the four groups in the 7th, 8th, 12th, 16th week ($p < 0.05$). $^{*}p < 0.05$; $^{**}p < 0.01$; the difference between the control and the PLGA+N3 group. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; the difference between the N3 and PLGA+N3 groups. $^{\times}p < 0.05$, the difference between the PLGA and PLGA+N3 groups (B) Grid walk test results at 32, 60, 150 days post surgery ($p < 0.05$). $^{*}p < 0.05$; $^{**}p < 0.01$; significant difference between the groups.

Retrograde tracing indicated extension of the regrowing nerve fibers beyond the lesion site

Retrograde tracing of hydroxystilbamidine was observed through the lesion in PLGA+N3 group. Hydroxystilbamidine-labeled cells or fibers were markedly reduced in the rostral stumps of the injury site and encompassed a distance of 850 ± 100 μm [Figure 3(A,D)], significantly longer than that of the control, PLGA, N3 groups. Correspondingly, positive hydroxystilbamidine-labeled fibers were seen in the rostral spinal cord [Figure 3(B)], whereas similar positive area extended from the caudal stump through the conduit into the rostral stump was absent in the control, N3 and PLGA groups [Figure 3(A)]. Furthermore, only a few hydroxystilbamidine-labeled cells were found in the brain [Figure 3(C)]. The retrograde observations indicated nerve fibers regrowth across the lesion.

No evoked potential between sciatic nerves and the SMC

CSEP is indicative of sequential activation along the somatosensory pathways when peripheral nerves were electrically stimulated. Our results showed that rats in the sham-operated group exhibited normal response (Figure 4). In contrast, there was no evoked potential recorded in the animals of the control, PLGA, N3, and PLGA+N3 groups (Figure 4).

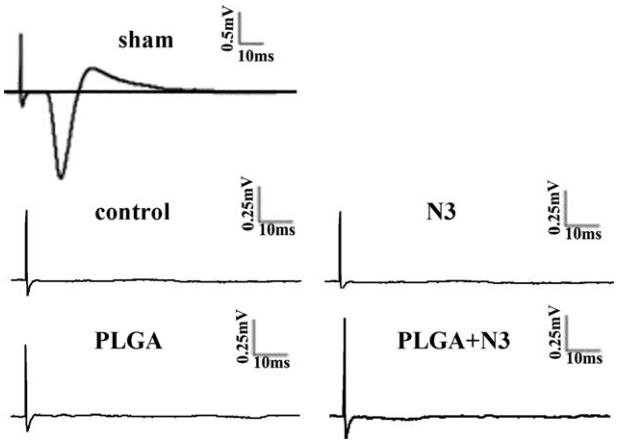

FIGURE 4. CSEP Electrophysiology. The CSEP was evoked in the sham-operated animals, but not in the control, N3, PLGA and PLGA+N3 groups.

DISCUSSION

Synergistic effects of the scaffold and NT3 in axonal regrowth

A single therapeutic approach is generally believed to be insufficient to restore SCI. Growth factors, such as NT3, may stimulate neuronal survival and axonal regeneration. In the rat models of Tuszynski's, the spinal cord tissue of only 1.1 mm ventral to the dorsal cord surface and 1.1 mm to the left of the midline was transected.[19] In our study, however, the T8-T10spinal cord was completely transected. Therefore, not only sensory axons but also motor axons were seriously injured in the animals in this study. Since all the fibers were completely transected, the observed neurofilament positive fibers likely corresponded to axonal regrowth. Axonal

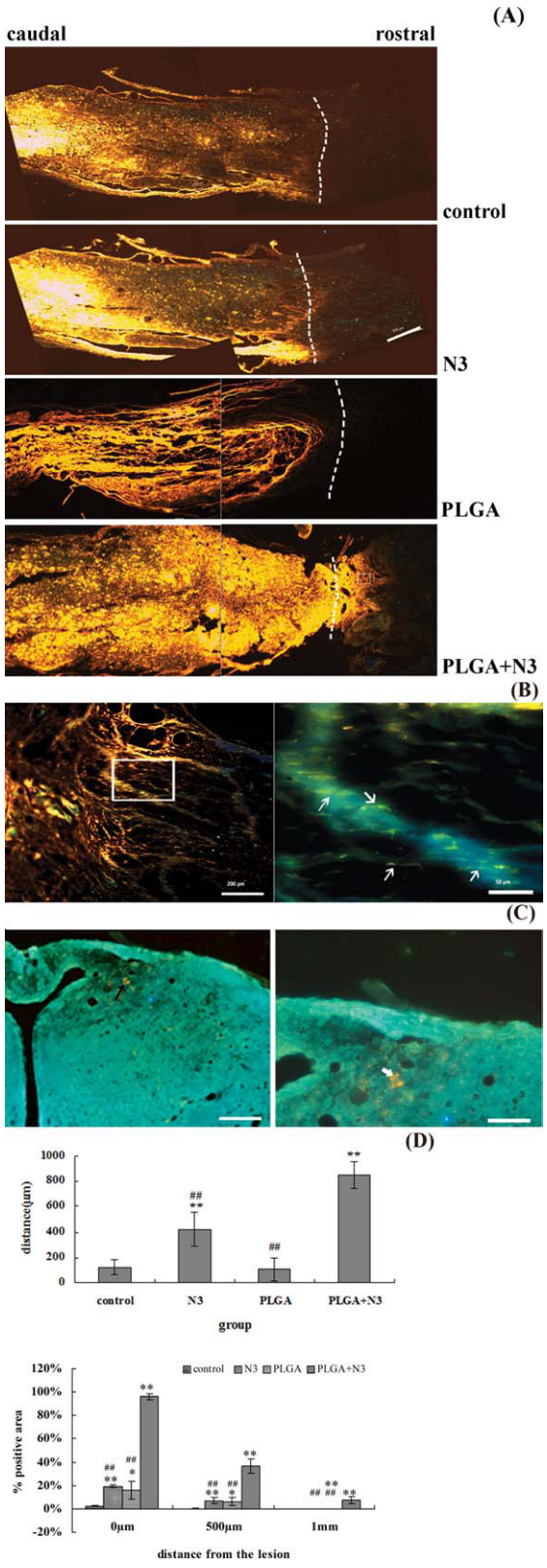

FIGURE 3. (A) There was a great deal of hydroxystilbamidine positive fibers in the caudal to the lesion, while much less in the rostral stump in each group. Retrograde tracing of hydroxystilbamidine showed that it traversed the lesion and extended for a limited distance in the PLGA+N3 group, but not in the other groups. The positive hydroxystilbamidine fibers in the rostral stumps indicated the regenerating ascending fibers. Scale Bar: 100μm. (B) The magnified image showed hydroxystilbamidine-positive fibers in the rostral spinal cord in the PLGA+N3 group. It appeared that the hydroxystilbamidine located along the regenerating nerve fibers in the rostral stump. Scale Bar: 200 μm (left) and 500 μm (right). The arrows indicate hydroxystilbamidine-positive fibers. (C) Only a few and weakly labeled cells were observed in the brain section. Scale Bar: 200 μm (left) and 500 μm (right). The arrows indicate hydroxystilbamidine-positive cells. (D) Statistical analysis of the hydroxystilbamidine-positive area and distance from the lesion in the groups. There was significant difference among the four groups in both hydroxystilbamidine-positive area and distance ($p = 0.000 < 0.01$). $^{*}p < 0.05$; $^{**}p < 0.01$: the difference between the control and the other groups. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$: the difference between the PLGA+N3 and the other groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regrowth has been observed not only in the channels but also in the porous wall.[46] Therefore, the neurofilament-stained axons in the entire transverse section, but not those in individual channels, were quantified, and the results demonstrated an apparently synergistic role of the PLGA nerve conduits and rhNT3.

Previously, Schwann-cell containing multichannel scaffolds had been used to promote axon regeneration.[30] In this study, the enhanced connectivity of the interlumenal surface facilitates inward delivery of rhNT3 and outward diffusion of metabolized substances, thus allowing significant axonal regrowth in the PLGA+N3 group. On the other hand, the adjacent spinal cord displayed a weak response to the injury and subsequently induced slight reactive astrocyte and glial scar formation. In the NT3 alone group, the massive loss of spinal cord tissue may have rendered the animals incapable of robust axonal regrowth. The PLGA alone group may also be deficient in nerve fiber regrowth. While it is difficult to directly compare our study with that by Moore et al.[30] due to different living time of the animals, the scaffold in this study seemed to allow efficient diffusion of growth factors and exhibited synergistic effects in SCI repair. It is possible that the scaffold coupled with exogenous NT3 constituted favorable microenvironment for axonal growth.

Partial hindlimb locomotor functional recovery indicated in the BBB rating scale and the grid walk test

The contribution of the scaffold and NT3 to functional improvement was evaluated by the BBB rating scale and grid walk test. The animals were assigned a BBB score of 3.50 and 13.5% of the right steps in grid walk test. The BBB score showed more dramatic increase over the first seven weeks. Previously, 5–10% of CST was estimated to be required for improved locomotor function after SCI.[47] In this study, however, only about 1% of the total area was NF200 positive in the PLGA+N3 group, the density of nerve fibers relative to the functional native tissue was about 10% (the average percentage of the positive area in the PLGA+N3 and sham groups was 0.96% and 11.67%, respectively); furthermore, axonal regeneration was not expected to take place during this early time window. Since it remained unclear whether the observed functional recovery was caused by regenerating fibers reconnecting to appropriate targets below the lesion,[48] it is possible that plasticity may be crucial for early functional improvement. The regrowing nerve fibers may help establish synapses, eventually leading to functional improvement. Finally, synergistic effects of the PLGA scaffold and growth factors were observed, as either the scaffold or NT3 alone was insufficient in enhancing functional recovery.

Further demonstration of axonal regrowth by retrograde tracing

Growing nerves need to traverse the scaffold and the glial scarring to achieve optimal recovery. Given the well-documented role of NT3 in CST,[16,17,25,49] NT3 was expected to enhance restructuring of ascending and descending axons in this study. In the retrograde tracing, a large number of hydroxystilbamidine-labeled fibers were trapped near the host-transplant border. The retrograde tracing showed extension of a subset of fibers, indicating regrowth of ascending axons. However, little or no retrograde tracing was observed in the brainstem [Figure 3(E)], suggesting that the axonal regeneration of CST was limited.

Limited regeneration as demonstrated with electrophysiology

The result of CSEP demonstrated limited axonal regeneration. It is possible that a greater number of axons were required for the long-distance conduction between the two sites of sciatic nerves and SMC; alternatively, the observed recovery may be partly attributable to the re-established supraspinal input across the transplant[50]

CONCLUSION

In conclusion, our experiments demonstrated beneficial effects of the multichannel and porous PLGA nerve conduits coupled with exogenous rhNT3 in promoting neural regrowth and functional recovery. Future work will aim to optimize the interaction between the scaffold and growth factors while retaining the full activity of the growth factors.

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