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Title: Regenerative rehabilitation: Exploring the synergistic effects of rehabilitation and implantation of bio-functional scaffold in enhancing nerve regeneration

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Abstract

Clinically, rehabilitation is the one of the most common treatment option for traumatic injuries. Despite that, recovery remains suboptimal and recent breakthroughs in regenerative approaches may potentially improve clinical outcomes. To date, there have been numerous studies on the utilization of either rehabilitative or regenerative strategies for traumatic injury treatment. However, studies that document the combined effects of rehabilitation with regenerative tissue engineering options remain scarce. Here, in the context of traumatic nerve injury treatment, we use a rat spinal cord injury (SCI) model as a proof of concept to evaluate the synergistic effects of regenerative tissue engineering and rehabilitation. Specifically, we implanted a pro-regenerative hybrid fiber-hydrogel scaffold and subjected SCI rats to intensive rehabilitation. Of note, the rehabilitation session was augmented by a novel customized training device that imparts normal hindlimb gait movements to rats. Morphologically, more regenerated axons were observed when rats received rehabilitation (~ 2.5 times and ~ 2 times enhancement after 4 and 12 weeks of recovery respectively, $p < 0.05$). Besides that, we also observed a higher percentage of anti-inflammatory cells ($36.1 \pm 12.9\%$ in rehab rats vs. $3.31 \pm 1.48\%$ in non-rehab rats, $p < 0.05$) and perineuronal net formation in rehab rats at Week 4. Physically, rehab animals were also able to exert higher ankle flexion force (~ 0.779 N vs. ~ 0.495 N at Week 4 and ~ 1.36 N vs. ~ 0.647 N at Week 12 for rehab vs. non-rehab rats, $p < 0.001$) and performed better than non-rehab rats in the open field test. Taken together, we conclude that coupling rehabilitation with regenerative scaffold implantation strategies can further promote functional recovery after traumatic nerve injuries.

Keywords: regeneration; rehabilitation; traumatic injury; electrospinning; biomaterials

Introduction

Rehabilitation is currently the clinical gold standard for improving the functions of patients following traumatic injuries^{1,2} as it is by far the most effective treatment for preventing further deterioration of tissue structures and functions. While this approach is widely adopted and allows patients to regain function over time, recovery for those severely injured remain suboptimal. Besides improving the rehabilitative regime, it is recently recognized that tissue regeneration, coupled with rehabilitation, would be the path to achieve better recovery.³

Termed 'regenerative rehabilitation', this paradigm involves the synergistic integration of principles and approaches from both the regenerative medicine and rehabilitation fields in order to maximize the recovery of the patients in form and function.³ Significant advances in regenerative medicine might be able to improve survival from catastrophic injuries, but often fail to fully restore function.⁴⁻⁷ Similarly, treatment with rehabilitation alone is insufficient.^{8,9} Accordingly, this implies that in many trauma cases, neither regenerative medicine nor rehabilitation alone can result in complete recovery, especially for patients who sustained more complex traumatic injuries.³

In the context of traumatic nerve injuries, currently available clinical treatments mainly rely on preserving and preventing further decline of the injured nerve.¹⁰ Fortunately, tissue engineering approaches for attaining neural regeneration have shown promising outcomes in the last few decades amidst the immense technical hurdles. One example is the groundbreaking work by Lu *et al.* where they demonstrated the efficacy of using neural stem cells embedded in fibrin matrices containing growth factor cocktails for promoting spinal cord

repair.¹¹ Besides that, Li *et al.* also had considerable success with neural regeneration by implanting Neurotrophin-3 (NT-3) loaded chitosan scaffolds in fully transected rats¹² and in hemi-transected monkeys.¹³

However, enhanced morphological regeneration does not equate to enhanced functional recovery. This was evident in the study by Lu *et al.* where even though axonal growth was extremely robust, the animals did not show weight bearing capabilities.¹¹ Moreover, while these studies were able to elegantly improve spinal cord regeneration, none evaluated rehabilitation as a treatment option for promoting motor function recovery. Conversely, works that conducted rehabilitation on spinally injured animals¹⁴⁻¹⁶ did not explore scaffolds for tissue regeneration purposes. Since spinal cord injury (SCI) is a complex phenomenon, combinatorial therapies that include both regeneration and rehabilitation are most likely required. As such, this work explored this strategy by using a rat SCI model as a proof of concept. Specifically, the effects of rehabilitation were evaluated on spinally injured rats with an implanted pro-regenerative hybrid scaffold.

Here, an established NT-3 loaded fiber-hydrogel scaffold¹⁷⁻²⁰ was implanted into T9-T10 fully transected (2 mm gap) spinal cords of Sprague-Dawley rats. Thereafter, the effects of body weight-supported treadmill training (BWSTT) on nerve regeneration was explored. We custom-designed and built a rehabilitative apparatus for intensive gait rehabilitation. This construct was armed with a pair of hindlimb manipulation device which could be clamped onto the rats' ankle to actuate pre-programmed normal rat hindlimb gait during training.²¹ We show that administration of NT-3, a reputed neurotrophin for enhancing neuronal survival,²² coupled with rehabilitation for spinally injured rats promoted axonal ingrowth into

scaffolds, enhanced perineuronal nets formation within the thoracic region and improved plantarflexion in the rat hindlimbs. Our results demonstrate the potential of combining neural rehabilitation with regenerative medicine to provide a unique solution to improve tissue regrowth following traumatic nerve injuries.

Methods

Materials

Rat tail collagen type 1 was purchased from Corning, USA. Neurotrophin-3 (NT-3) was procured from PeproTech, USA. Heparin and bovine serum albumin (BSA) were obtained from Sigma, USA. Chicken anti-NF200 (822601) was procured from Biolegend, USA. Rat anti-MBP (MCA409S) and mouse anti-CD68 (ED1, MCA341GA) was purchased from Biorad, USA. Rabbit anti-CD206 (ab64693) was obtained from Abcam, UK. Mouse anti-iNOS/NOS type II (iNOS, 610329) was purchased from BD Biosciences, USA. Fluorescein-conjugated *Wisteria floribunda agglutinin* (WFA, FL1351) was bought from Vector Laboratories, USA. Goat anti-chicken Alexa Fluor 555, goat anti-rat Alexa Fluor 488, goat anti-mouse Alexa Fluor 555, goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 633 and 4',6-diamidino-2-phenylindole (DAPI) were acquired from Life Technologies, USA.

NT-3 loaded PCLEEP scaffold fabrication

The scaffold fabrication schematic and its characterizations were shown in our previous works.¹⁸⁻²⁰ Briefly, poly (ϵ -caprolactone-co-ethyl ethylene phosphate)(PCLEEP, M_w = 59,102 g/mol), a synthetic copolymer consisting of ϵ -caprolactone and ethyl ethylene phosphate,²³ was electrospun using an air gap electrospinning technique to obtain PCLEEP fibers. These fibers were then rolled into a bundle and placed within a mold. Thereafter, 200

µl of rat tail collagen type 1 was prepared at a concentration of 3 mg/ml following manufacturer's protocol. Following that, 1 µl of NT-3 from a stock concentration of 2 µg/µl (2 µg per scaffold) constituted in 0.1% BSA and heparin (400 µg/µl) (BSA : heparin v/v ratio = 1:1) was mixed with the collagen before adding into the mold (4 mm in diameter). After gelation, the scaffolds were frozen at - 20 °C for 3 h before being lyophilized overnight. Finally, the scaffolds were cut into 2 mm long pieces and sterilized under UV light for 45 min before *in vivo* implantation.

Animals

A total of 26 adult female Sprague Dawley rats (7-9 weeks, 180-230 g)(n = 4 each for rehab and non-rehab group at Week 4 and n = 9 each for rehab and non-rehab group at Week 12) were obtained from In Vivos Pte Ltd (Singapore). All animals procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanyang Technological University and approved by the Animal Ethics Committee, Institutional Animal Care and Use Committee (IACUC, Protocol number: A0309). Animals were housed under temperature controlled conditions, with a normal 12:12 h light/dark cycle with ad libitum access to water and food.

Surgical procedures for severe spinal cord injury

Buprenorphine (0.05 mg/kg) was first administered to each animal subcutaneously 30 min before surgery. They were then placed in a chamber consisting 4-5% mixture of isoflurane in air and injected with Ketamine (75 mg/kg) and Xylazine (10 mg/kg) intraperitoneally. Once the animals were well anaesthetized, the dorsal region of the rats (thoracic) were shaved and cleaned with 70% ethanol and betadine. A midline incision was made at the disinfected area

and the overlying muscles were bluntly dissected to expose the vertebrae. A laminectomy procedure was further performed to reveal the underlying spinal cord. Following that, the dura was sliced open and a 2 mm full transection was made on the spinal cord (T9-T10). The scaffold (2 mm in length) was then implanted into the lesion site, with its fibers aligned with the direction of growth of axons in the spinal cord, once the bleeding had subsided. After the implantation, the muscles were sutured using Prolene 4/0 single stitches and the skin was closed using wound clips. Post-operative treatments and care included addition of Meloxicam (5 mg/ml) into the drinking water for a duration of ten days post-surgery and expression of bladder twice a day until recovery.

Animal rehabilitation

One week before surgery, tested animals were acclimatized, once every day, following a progressive duration (5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 30 min), to run on a motor driven treadmill belt at a speed of 12 cm/s (speed was determined based on the endurance of the rats). Body weight-supported (65% of the rat's weight) treadmill rehabilitation training for the rats was conducted on a custom-made rehabilitation machine²¹ (Supplemental Figure 1) starting one week after surgery to allow for sufficient recovery. Rehab rats adopted a quadrupedal position while training and were imparted with a pre-programmed normal hindlimb gait by the manipulation devices, which, to the best of our knowledge, have not been implemented in other neural rehabilitation studies. Training was conducted on every Monday, Wednesday and Friday for 30 min at a treadmill belt speed of 3 cm/s.²¹

Immunohistochemical analysis

At designated time points post-implantation (Week 4 and 12), animals were euthanized in CO₂ before being transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA). The implanted scaffolds and some of the surrounding spinal cord tissues were then harvested and further transferred into 4% PFA for at least 3 h, followed by 15% sucrose solution for 24 h and finally 30% sucrose solution. Thereafter, tissues were embedded in Tissue-Tek® O.C.T. Compound and cut into 20 µm thick horizontal sections using a cryostat (Leica CM1950). Immunofluorescent staining was performed to evaluate the effects of the treatment on various cell types of interest. Sections were first permeabilized with 0.3% Triton X-100, followed by blocking with 10% goat serum for 1 h and incubating with primary antibodies overnight at 4 °C. Primary antibodies used for recognizing neurofilaments and myelin basic protein (MBP) included chicken anti-neurofilament 200 (NF200, 1:1000 dilution) and rat anti-MBP (1:200 dilution) respectively. After primary antibody incubation, the samples were washed thrice with PBS before secondary antibody incubation for 1 h at room temperature using goat anti-chicken Alexa Fluor 555 (1:700 dilution) and goat anti-rat Alexa Fluor 488 (1:700 dilution) respectively.

For macrophage staining, a sequential staining protocol was adopted. Specifically, sections were first post-fixed briefly for 10 min with 4% PFA, washed with 1 x PBS thrice and placed in 50 ml of 1 x citrate buffer (pH 6) in the oven at 85 °C for 45 min for antigen retrieval. Thereafter, the samples were then removed from the oven and left to cool at room temperature for 30 min before blocking with 10% goat serum for 1 h. After blocking, mouse anti-ED1 (1:50 dilution) and rabbit anti-CD206 (1: 200 dilution) were added to the sections and incubated at 37 °C for 30 min. Following that, sections were washed thrice with 1 x PBS before secondary antibody incubation for 1 h at 37°C using goat anti-mouse Alexa Fluor 555

(1:700 dilution) and goat anti-rabbit Alexa Fluor 488 (1:700 dilution). After the first round of primary and secondary antibody incubation, samples were washed thrice with 1 x PBS and further incubated with mouse anti-iNOS (1:100 dilution) at 37 °C for 30 min. Samples were then subsequently washed thrice with 1 x PBS and incubated with goat anti-mouse Alexa Fluor 633 (1:500 dilution) for 1 h at 37 °C.

For perineuronal nets (PNNs) staining, sections were first blocked with 10% goat serum for 1 h, stained with *Wisteria floribunda agglutinin* (WFA, 1:1000 dilution) for 4 h, washed thrice with 1 x PBS and incubated with mouse anti-NeuN (1:500) at 4 °C overnight. Thereafter samples were washed thrice with 1 x PBS before incubating with goat anti-mouse Alexa Fluor 555 (1: 700) for 1 h at room temperature. Nuclei were labelled with DAPI (1:1000 dilution) for all immunohistochemically stained samples during secondary antibody incubation and all slides were coverslipped with Fluoromount-G.

The area percentage of NF200 staining within the scaffold was quantified with ImageJ (NIH, USA) using fluorescently-labelled stitched collage consisting of several 10x magnified images (Leica DMI8). A total of three sections per animal were used for the quantification. The area percent of NF200 staining was calculated using the following equation:

$$\text{Area percent of NF200 in scaffold (\%)} = \frac{\text{Area occupied by NF200}^+ \text{pixels}}{\text{Area of scaffold}} \times 100\%$$

To quantify myelination index within the scaffold, 40x magnified confocal z-stack images (Zeiss LSM800) were analyzed using ImageJ (NIH, USA) and a customized colocalization plugin. For each sample section, five images were taken at different regions of

the scaffold (top left, top right, middle, bottom left and bottom right). A total of three sections were quantified per animal and the myelination index was defined by the following equation:

$$\text{Myelination Index (\%)} = \frac{\text{Number of colocalized NF200 and MBP pixels}}{\text{Number of NF200 pixels}} \times 100\%$$

Macrophages were manually quantified using 40x magnified images obtained from the confocal microscope (Zeiss LSM800). One image was acquired at each pre-defined region per section. In total, three sections were quantified per animal. The pre-defined regions were namely, the rostral implant interface (Interface (Rostral)), the rostral side within the scaffold (In Scaffold (Near Rostral)), the middle of the scaffold (In Scaffold (Middle)), the caudal side within the scaffold (In Scaffold (Caudal)) and the caudal implant interface (Interface (Caudal)). The density of ED1⁺ cells was calculated using the following equation:

$$\text{Density of ED1}^+ \text{ cells (cells/mm}^2\text{)} = \frac{\text{Number of DAPI}^+ \text{ED1}^+}{\text{Area of ROI (mm}^2\text{)}}$$

The percentage of ED1⁺ cells was calculated using the following equation:

$$\text{Percent of ED1}^+ \text{ cells (\%)} = \frac{\text{Number of DAPI}^+ \text{ED1}^+ \text{ cells}}{\text{Number of DAPI}^+ \text{ cells}} \times 100\%$$

The percentage of ED1⁺iNOS⁺ or ED1⁺CD206⁺ cells was calculated using the following equation:

$$\begin{aligned} &\text{Percentage of ED1}^+ \text{iNOS}^+ \text{ or ED1}^+ \text{CD206}^+ \text{ cells (\%)} \\ &= \frac{\text{Number of DAPI}^+ \text{ED1}^+ \text{iNOS}^+ \text{ or ED1}^+ \text{CD206}^+ \text{ cells}}{\text{Number of DAPI}^+ \text{ED1}^+ \text{ cells}} \times 100\% \end{aligned}$$

In the spinal cord, perineuronal nets (PNNs) were identified as a halo surrounding NeuN⁺ cells.²⁴ Quantification was conducted using 20x magnified images (Leica DMI8) acquired at increasing distances (1 mm, 2 mm and 3 mm) from the scaffold interface rostrally

and caudally. In total, three sections were quantified per animal. The percent of NeuN⁺ cells with PNN was calculated with the following equation:

$$\text{Percent of NeuN}^+ \text{ cells with PNN (\%)} = \frac{\text{Number of NeuN}^+ \text{ cells encircled by WFA}}{\text{Number of NeuN}^+ \text{ cells}} \times 100\%$$

Behaviour test

The hindlimb recovery of animals was evaluated through a weekly open field test using the Basso, Beattie, Bresnahan (BBB) scoring system.²⁵ Animals were placed within a circular enclosure (diameter of 1.5 m) and allowed to move around freely. Each rat was filmed for a duration of 3 min and their scores were assessed by two independent observers blinded to group identity.

The plantarflexion strength of animals was measured using a custom-made device that imparts dorsiflexion on the rats' hindlimb. The amount of force exerted by the rats when they resist through plantarflexion was detected through a force-sensing resistor placed underneath the hindlimb. In order to conduct the test, rats were first secured onto the harness with one of their hindlimbs strapped to a base plate, which was then further slotted in place above a force sensor. At the default starting position, the base plate is completely parallel to the ground (0°). Once the motor is activated, the base plate will be pivoted to a 45° angle in 2 s (inclination phase). This position is then maintained for 10 s (steady phase) before pivoting back to the normal default position in 2 s (declination phase). Measurements of the force (N) as a function of time throughout the entire cycle were obtained for both hindlimbs (three measurements per limb) of the rehab and non-rehab rats at Week 6 and Week 12. These values were also converted to a frequency plot using Gaussian distribution for statistical analysis.

Statistical analysis

Data was first tested for normality and homogeneity of variances using Shapiro-Wilk and Levene's test respectively. Those with homogenous variances were analysed using one-way ANOVA followed by Tukey's post-hoc test. For non-parametric data, Kruskal-Wallis test followed by Mann-Whitney post-hoc test were used instead. All error bars represent standard deviation.

Results

Rats that underwent rehabilitation had better axonal infiltration into site of injury

We observed that at Week 4, animals that received rehabilitation had better axonal ingrowth into the injury site as shown by the increased amount of NF200 expression within the scaffolds (Figure 1, $2.00 \pm 0.79\%$ for rehab rats vs. $0.79 \pm 0.14\%$ for non-rehab rats). By Week 12, this difference became more significant (Figure 1, $4.05 \pm 0.65\%$ for rehab rats vs. $2.07 \pm 0.45\%$ for no rehab rats, $p < 0.05$). However, quantification of the myelin index yielded no prominent difference between rehab and non-rehab animals at both time points (Supplemental Figure 2A and 2B). Additionally, from Figure 1A, it was observed that the PCLEEP fibers (average diameter = $1.25 \pm 0.13 \mu\text{m}$),²⁰ based on polarized images, were not degraded at Week 12 and importantly, rehabilitation did not appear to affect their degradation rates.

Figure 1. Rats that underwent rehabilitation had better axonal infiltration into scaffold. (A) Representative images of spinal cord sections stained with NF200 and images of scaffolds taken under polarized light to identify aligned PCLEEP fibers. **(B)** Quantification of the area percent of NF200 staining in scaffold. ANOVA, **: p-value < 0.01; *: p-value < 0.05. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats at Week 4; n = 7 and 9 for rehab and non-rehab rats respectively at Week 12.

Density and percentage of pan-macrophages within the injury site were unaffected by rehabilitation

Macrophages are instrumental in reflecting the immunological state within the injured region. As such, we wanted to know if rehabilitation will bring about any changes in their relative abundance within and outside the scaffold. Through ED1 staining (a pan-macrophage

marker in rats), we observed that there was no significant difference in the density of ED1⁺ cells within the scaffold and at both the rostral and caudal implant interfaces at Week 4 between rehab and non-rehab rats (Figure 2A and 2B). This trend was consistent as well when we looked at the percentage of cells that were ED1⁺ (Figure 2A and 2C). Although there was no notable disparity in the amount of macrophages between rehab and non-rehab rats in each region quantified, there were, however, significant differences between the regions analyzed. Specifically, higher percentage of cells were ED1⁺ at the centre of the scaffold as compared to the caudal implant interface for both rehab ($23.1 \pm 4.72\%$ at middle of scaffold vs. $12.9 \pm 2.87\%$ at caudal interface, $p < 0.05$) and non-rehab animals ($26.6 \pm 7.04\%$ at middle of scaffold vs. $15.3 \pm 3.82\%$ at caudal interface, $p < 0.05$) (Figure 2A and 2C).

Figure 2. Density and percentage of pan-macrophages within the injury site were unaffected by rehabilitation. (A) Representative images of ED1⁺ cells around and within the injury site at

Week 4. Quantification of the **(B)** density and **(C)** percentage of ED1⁺ cells. ANOVA, **: p-value < 0.01; *: p-value < 0.05; N.S. = Not Significant. All data are shown as mean ± SD; n = 4 for both rehab and non-rehab rats.

Injury region in rehab rats had relatively lower percentage of ED1⁺iNOS⁺ cells and higher percentage of ED1⁺CD206⁺ cells.

Next, we wanted to know if rehabilitation would lead to the skewing of macrophage phenotype (pro-inflammatory (M1-like) and anti-inflammatory (M2-like)) within the scaffold as well as the rostral and caudal regions of the host-implant interface. We observed that, in all the regions surveyed around the injury, there was a higher percentage of ED1⁺iNOS⁺ (M1-like) cells in non-rehab animals (Figure 3A and 3B). When comparing M2-like cells, there appeared to be a significantly larger percentage of ED1⁺CD206⁺ cells in the rostral and caudal implant interface of rehab rats as compared non-rehab rats ($p < 0.05$)(Figure 3A and 3C). Moreover, the percentage of ED1⁺CD206⁺ cells at the caudal implant interface were also significantly higher in rehab rats ($36.1 \pm 12.9\%$) than non-rehab rats ($3.31 \pm 1.48\%$)($p < 0.05$)(Figure 3A and 3C).

Figure 3. Injury region in rehab rats had relatively low and high percentage of ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells respectively. (A) Representative images of ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells around and within the injury site at Week 4. White and green arrows denote ED1⁺iNOS⁺ and ED1⁺CD206⁺ cells respectively. Graph depicting the percentage of **(B)** ED1⁺iNOS⁺ cells and **(C)** ED1⁺CD206⁺ cells. ANOVA, *: p-value < 0.05. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats.

Scaffold implantation did not affect rehabilitation-induced perineuronal nets formation

We next proceeded to stain for plasticity-associated perineuronal nets (PNNs) using WFA, a broad PNN marker,²⁶ with the hypothesis that its expression would be increased due to exercise training-induced regulation within the spinal cord,²⁴ regardless of the presence of the scaffold. As expected, we observed a clear trend where the percentage of NeuN⁺ cells with PNN increased as the distance from the injury site increased rostrally and caudally. This

was especially significant at the caudal end of rehab animals comparing distances at 3 mm ($15.4 \pm 3.41\%$) vs. 1 mm ($6.93 \pm 3.94\%$) ($p < 0.05$) (Figure 4A and 4B). Additionally, there was a trend where animals that underwent rehabilitation had a larger percentage of NeuN⁺ cells that were surrounded by PNNs at all distances away from the injured area rostrally and caudally as compared to non-rehab rats.

Figure 4. Perineuronal nets formation were enhanced rostrally and caudally away from the injured region in rehab rats at Week 4. **(A)** Representative images comparing percent of NeuN⁺ cells surrounded with a perineuronal net (PNN) at 1mm, 2mm and 3mm away from the injured area rostrally and caudally in both rehab and non-rehab rats. White arrows denote

the characteristic halo encircling NeuN⁺ cells when stained with *Wisteria Floribunda* agglutinin (WFA). **(B)** Graph depicting the percentage of NeuN⁺ cells enclosed by a PNN. Kruskal-Wallis, *: p-value < 0.05. All data are shown as mean $\pm$ SD; n = 4 for both rehab and non-rehab rats.

Rats that underwent rehabilitation had better motor recovery than rats that did not undergo rehabilitation

The custom-made device that exerts dorsiflexion on the rats' hindlimbs and subsequently measure the force of plantarflexion is shown in Supplemental Figure 3. At Week 6, we observed that rehab rats (0.779 ± 0.113 N) had significantly stronger plantarflexion strength as compared to non-rehab animals (0.495 ± 0.0673 N, $p < 0.001$) (Figure 5A). In addition, rehab rats were more able to maintain this plantarflexion over the duration of the steady phase, as seen by the various higher value blue shades (rehab animals) of lines depicted as compared to the red shades (non-rehab animals) of lines, where each line corresponded to the measurement of an animal (Figure 5A). This trend remained consistent at Week 12 (1.36 ± 0.133 N by rehab rats vs. 0.647 ± 0.111 N by non-rehab rats, $p < 0.001$) (Figure 5B). Comparing the compiled plantarflexion test results for both rehab and non-rehab rats at Week 6 and 12, we observed that rats which underwent rehabilitation had improved hindlimb strength as compared to rats that did not undergo rehabilitation ($p < 0.001$ for plantar flexion) (Figure 5C). Moreover, in just 6 weeks, rehab rats were already able to outperform non-rehab rats from Week 12. Although the BBB results validated that rehab animals performed better than non-rehab animals at Week 12, this was not reflected at Week 6 (Figure 5D).

Figure 5. Rats that underwent rehabilitation had better motor recovery than rats that did not undergo rehabilitation at Week 6 and Week 12. Graphs showing the profile of vertical force exerted with respect to time during steady phase (2-12s) at (A) Week 6 and (B) Week 12. Blue and red shaded measurements belong to rehab and non-rehab rats respectively. Each line represents the measurements from an animal. (C) Comparison of the force exerted in the vertical direction between rehab and non-rehab rats during steady phase (2-12s) at Week 6 and Week 12. (D) Comparison of the BBB locomotor score between rehab and non-rehab rats over a period of 12 weeks. Kruskal-Wallis test, **: p-value < 0.01; *:p-value < 0.05 between rehab and non-rehab rats. All data are shown as mean $\pm$ SD; n = 6 for both groups at Week 6 and n = 3 for both groups at Week 12.

Discussion

Currently, rehabilitation is the most widely utilized treatment option for patients with traumatic injuries. However, this strategy only serves to prevent the decline of tissue integrity

and function and has limited success in more severe cases, which reflects the reality that rehabilitation alone cannot completely restore functions. On the other hand, regenerative medicine may offer new options, but on its own, results are not optimal as well. In response to these gaps, it has recently been proposed that regenerative rehabilitation, the marriage of regenerative medicine and rehabilitative efforts for restoring tissue form and function respectively, is the potential way to go for an effective and all-rounded clinical treatment.³

This practice is particularly applicable in the context of traumatic nerve injury as similar trends were observed in this field. For example, while sizeable morphological improvements were achieved through cellular^{11,27} or biomaterial scaffolding^{12,13} methods for regenerative purposes, locomotor functions remained disappointing.^{11,12} Importantly, even though there have been many studies exploring regenerative medicine^{11-13,27} and neural rehabilitation¹⁴⁻¹⁶ for treating traumatic nerve injuries, not many have applied both of these principles concurrently in their work.

Therefore in this study, we opted for the combinatorial approach of neuroregeneration and rehabilitation, capitalizing on the advancement of biomaterial scaffolds and body weight-supported treadmill training (BWSTT) concept, to promote nerve regeneration and functional recovery using SCI model as a proof of concept. Additionally, to further improve the rehabilitative process, our customized treadmill also contained a pair of pre-programmed manipulators for actuating the injured rat's hindlimb according to the normal gait.

Experimentally, animal models for traumatic SCI, such as contusion and incomplete laceration, are most relevant in clinical settings as injuries in humans are typically caused by physical impact.²⁸ Although rare, patients with a completely lacerated spinal cord can survive but their chances of regaining motor function below the injured region is extremely slim.²⁸ Correspondingly, in order to demonstrate the efficacy of our treatment strategy, we opted for the most stringent full spinal cord transection model. Animals were first spinally transected at the T9-T10 region with a 2 mm gap before implantation with a NT-3 loaded hybrid fiber-hydrogel scaffold between the rostral and caudal stumps to facilitate axonal growth across the injury. This platform was established in our previous study¹⁷⁻²⁰ and was also demonstrated to be effective for sustained drug and gene delivery after SCI.^{17,19} Consistent with these studies, we also observed neurofilament signals penetrating into the interior of the scaffold. Surprisingly, we also noticed that in one of the rehab rats, a bundle of neurofilaments were able to traverse across the entire length of the scaffold at week 12 (Figure 1A), suggesting rehabilitation-induced regeneration. Although crossing of neurofilaments across the scaffold in full spinally transected animals have been reported,¹² due to the lack of similarity in the experimental methodology and limited literature, it is not known exactly how rehabilitation might have led to such robust axonal regeneration. Additionally, while enhanced axonal regeneration was seen in rehab animals, we did not observe significant differences in myelinated axon formation between the two groups within the scaffold (Supplementary Figure 2). Taken together, future studies could explore how rehabilitation might induce axonal regeneration, where the axonal projections originate and also if the inclusion of pro-myelinogenic factors, such as miR-219/miR-338,¹⁷ could further enhance functional recovery.

The CNS is an immune privileged region²⁹ and as a result, it is ill-equipped to resolve the protracted immune responses mounted following traumatic SCI.³⁰ While part of the reason for these prolonged reactions can be attributed to the continual recruitment and activation of leukocytes and glia, the persistence (dwell time) of various leukocyte subpopulations, such as macrophages/microglia, around the lesion site (up to months) are responsible as well.³⁰ This slow rate of clearance was also observed in our results where ED1⁺ macrophages were still present within and outside the scaffold 4 weeks (chronic time point³¹) following SCI. Additionally, regardless of rehabilitation, the density and percentage of macrophages present remained unaffected. On the other hand, rehabilitation might have affected macrophages functionally, which was evident from the reduction of pro-inflammatory ED1⁺iNOS⁺ cells within and outside the scaffold, and the increment of anti-inflammatory ED1⁺CD206⁺ cells outside the scaffold. As anti-inflammatory macrophages have been shown to improve the morphological aspects of the injured spinal cord (reduce axonal dieback, reduce demyelination, promote tissue repair),³² such skewing elicited through rehabilitation might be related to the enhanced axonal ingrowth into the scaffold in rehab animals.

Perineuronal nets (PNNs) are structures formed by the extracellular matrix (ECM) which enwrap the soma and proximal dendrites of neurons³³ and is highly associative with neuronal plasticity, a key mechanism for functional recovery following SCI. Within the spinal cord, the expression of PNNs increases with exercise in both injured¹⁶ and uninjured rats,²⁴ albeit in the absence of scaffolds. As such, we proceeded to evaluate if rehabilitation-induced modulation of PNNs will be dysregulated in this model when an implanted pro-regenerative scaffold is present. Not surprisingly, our results are in agreement with the literature²⁴ where

rehab animals had a higher percentage of neurons with PNNs as compared to non-rehab animals in the thoracic spinal region since the effects induced by exercise training are far-reaching and could even evoke beneficial responses in the brain.^{34,35}

Although rhythmic hindlimb activity induced by ascending sensory inputs can be achieved even when the adult spinal cord is isolated from the brain, it is generally unable to provide weight bearing functions,³⁶ except in cats that have undergone intensive training.^{37,38} This meant that the BBB test, which utilizes a non-linear, ordinal grading scale, might not be fully compatible for certain injury models,^{39,40} such as an extremely severe SCI (e.g. 2 mm complete transection injury gap) in animals. Specifically, the inability of the animals to bear their own weight would have limited their attainable score to a maximum of 7, and consequently resulted in reduced resolution for accessing recovery.³⁹ Therefore, to further augment the functional assessment, we developed a customized device for quantifying the animals' plantarflexion strength based on the resistive force exerted during forced dorsiflexion of the hindlimb. Similar method of measurements have also been obtained from humans clinically^{41,42} and more recently, animals,⁴³⁻⁴⁵ for studying maximum muscle contractility through electrical stimulation. Although we do not incorporate electrical stimulation, the range of rat plantarflexor muscles' force values obtained in our study (~ 0.5 N to ~ 1.4 N) were consistent with those animal studies,^{44,45} verifying that these measurements could be an indicator of hindlimb recovery.

Conclusion

In conclusion, we demonstrated that there are beneficial effects by combining regenerative medicine and rehabilitation. The implantation of a pro-regenerative NT-3 loaded

scaffold and intensive gait-controlled exercise training could enhance tissue regeneration (axonal growth), PNNs formation and functional recovery in spinally transected rats. Furthermore, we found that the immunological state within the scaffold and implant interface at chronic time points are drastically different and that rehabilitation might play a role in modulating the presence of anti-inflammatory macrophages for better recovery. Taken together, this study provides a proof of concept of the benefits of regenerative rehabilitation.

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Contributions

S. Y. Chew generated the hypothesis. J. Lin, D. Anopas, U. Milbreta, J. S. Chin, P. H. Lin and N. Zhang performed experiments. J. Lin wrote and edited the manuscript. W. Wu, S. K. Wee, A. Tow, W. T. Ang and S. Y. Chew edited and proofread the manuscript. S. K. Wee, A. Tow, W. T. Ang and S. Y. Chew guided the project. S. Y. Chew provided the funding.

Competing interests

The authors declare that they have no competing interests.

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