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Regenerative Potential of Platelet-Rich Fibrin Releasate Combined with Adipose Tissue–Derived Stem Cells in a Rat Sciatic Nerve Injury Model

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Abstract

Sciatic nerve injuries, not uncommon in trauma with a limited degree of functional recovery, are considered a persistent clinical, social, and economic problem worldwide. Accumulating evidence suggests that stem cells can promote the tissue regeneration through various mechanisms. The aim of the present study was to investigate the role of adipose tissue–derived stem cells (ADSCs) and combine with platelet-rich fibrin releasate (PRFr) in the regeneration of sciatic nerve injury in rats. Twenty-four Sprague-Dawley rats were randomly assigned to four groups, a blade was used to transect the left hindlimb sciatic nerve, and silicon tubes containing one of the following (by injection) were used to bridge the nerve proximal and distal ends (10-mm gap): group 1: untreated controls; group 2: PRFr alone; group 3: ADSCs alone; group 4: PRFr + ADSCs-treated. Walking function was assessed in horizontal rung ladder apparatus to compare the demands of the tasks and test sensitivity at 1-mo interval for a total of 3 mo. The gross inspection and histological examination was performed at 3 mo post transplantation. Overall, PRFr + ADSCs-treated performed better compared with PRFr or ADSCs injections alone. Significant group differences of neurological function were observed in ladder rung walking tests in all treated groups compared to that of untreated controls ($P < 0.05$). This injection approach may provide a successfully employed technique to target sciatic nerve defects in vivo, and the combined strategy of ADSCs with PRFr appears to have a superior effect on nerve repair.

Keywords

adipose tissue–derived stem cells, platelet-rich fibrin releasate, sciatic nerve injury, transplantation

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Introduction

The sciatic nerve is the largest and thickest nerve of the human body. It is the principal continuation of all the roots of the sacral plexus, originating in the lower back and traveling posteriorly through the lower limb as far down as the heel of the foot. This nerve controls several muscles in the lower legs and supplies sensation to the skin of the foot and the majority of the lower leg[1]. A sciatic nerve injury can occur due to trauma to the nerve, which is frequently caused by motor vehicle accidents, falls, stretching exercises, hip or thigh contusions, hip surgery, and/or an intramuscular injection[2]. In some cases, nerve injuries requiring surgical repair may lead to permanent impairment of motor and sensory functions[3]. The surgical approaches rely on methods such as surgery and conduit connection[4], including end-to-end anastomosis, end-to-side anastomosis, allogeneic nerve transplantation[5], autologous nerve transplantation[6], and nerve lengthening[7]. Nowadays autologous nerve transplantation is the gold standard for the treatment of peripheral nerve defects[6]. However, it has some limitations regarding the secondary surgery to obtain the donor nerve: limited donor nerve sources, donor-site infections, and/or painful neuroma formation[8]. The limited sources of donor nerve will provide long segments for grafting without significant sensory losses[5].

Novel therapies resulting from tissue engineering technology and/or regenerative medicine may offer new hope for patients with injuries, end-stage organ failure, or other clinical issues[9]. The main strategies include providing regeneration support and guidance for the damaged neurons by neural scaffolds, inhibiting the formation of glial scars, antagonizing myelin inhibitory signals, and combining cells, drugs, and other substrates[10]. Recently, animal-based studies have shown positive outcomes that the transplantation of Schwann cells (SCs) in combination with nerve scaffolds promotes the repair of injured peripheral nerves[6]. Autologous SC transplantation also offers therapeutic potential and has been employed in human clinical cases[11].

To date, mesenchymal stem cells (MSCs) hold a great promise for cell therapy[12,13]. Bone marrow–derived mesenchymal stem cells (BMSCs) have been identified as an alternative for many therapies for cell or tissue damage, including that of the nervous system, with numerous ongoing clinical trials[14]. Adipose tissue–derived stem cells (ADSCs) are another type of pluripotent adult stem cells that can differentiate into new fat tissue, bone, cartilage, nerve, muscle, and endothelial cells, and they have phenotypic and gene expression profiles similar to those of BMSCs and can be collected from subcutaneous fat tissue using conventional liposuction. Moreover, they can easily be obtained with minimal invasion and are readily available, as the density of MSCs is much higher in adipose tissues than in the bone marrow. ADSCs were used in peripheral nerve grafts showing better results than nerve grafts without the ADSCs[15], and are able to be applied in fibrin glue to stimulate peripheral nerve regeneration[16].

Among natural biomaterials, platelet-rich fibrin (PRF) has recently aroused considerable interest as a biophysical/biochemical milieu that delivers growth factors (GFs), cytokines, and stem-like cells for immunomodulation and wound-healing purposes[17]. The curative effect of PRF is mainly due to the high variety of platelet-derived protein molecules, which include high presence of signaling, membrane proteins, protein processing, cytoskeleton regulatory proteins, cytokines, and other bioactive peptides that initiate and regulate the wound-healing signaling cascade[18]. PRF released autologous GFs that powerfully regulate the cell biological response. Their exogenous addition can further potentiate undifferentiated stem cells to proliferate and differentiate and have been used for tissue regeneration purposes[19]. A recent study has shown that the use of ADSCs in conjunction with PRF extract could enhance tissue regeneration[20]. Our earlier work tested the addition of PRF releasate (PRFr) to ADSCs implantation for osteochondral defect repair, which resulted in positively influencing cartilage repair in terms of the improvement of macroscopic and histological grading scores[21].

The current study hypothesizes that PRFr may serve as a mixture of GFs, act as a unique source/carrier of stem cells, and improve injury repair. This exploratory study investigates the regenerative potential of ADSCs implantation with PRFr through the injection in a rat sciatic nerve transection model with a 10-mm gap, which was repaired with a silicone tube.

Materials and Methods

A total of 24 Sprague-Dawley (SD) rats (BioLASCO Co., Ltd., Yilan, Taiwan), female at 8 to 10 wk of age (200 to 250 g), were used in this study and a 6-mo-old female White New Zealand rabbit (2.5 to 3 kg) underwent blood sampling for the preparation of PRFr. The protocols and surgical procedures of the present study were reviewed and approved by the Institutional Animal Care and Use Committees of National Taiwan University (NTU-104-EL-43). Animals were cared for according to established institutional guidelines and all efforts were made to minimize suffering.

Preparation of PRFr

Blood samples were collected from the experimental rabbit under general anesthesia by injection of a combination of Zoletil 50 (12.5 mg/kg, Virbac Laboratories, Carros, France) and xylazine (5 mg/kg, Lloyd Inc., IA, USA). After adequate skin preparation and sterilization, 6 ml of venous blood from the rabbit's external jugular vein was drawn without anticoagulant and immediately centrifuged in the tubes (#367988, Vacutainer® SST™, BD Biosciences, Franklin Lakes, NJ, USA) using a bench-top centrifuge at 1,000 × *g* for 10 min at room temperature. The resultant jelly-like PRF was present in the middle of the tube, between the top layer of clear yellow serum layer and the bottom layer of red blood cells

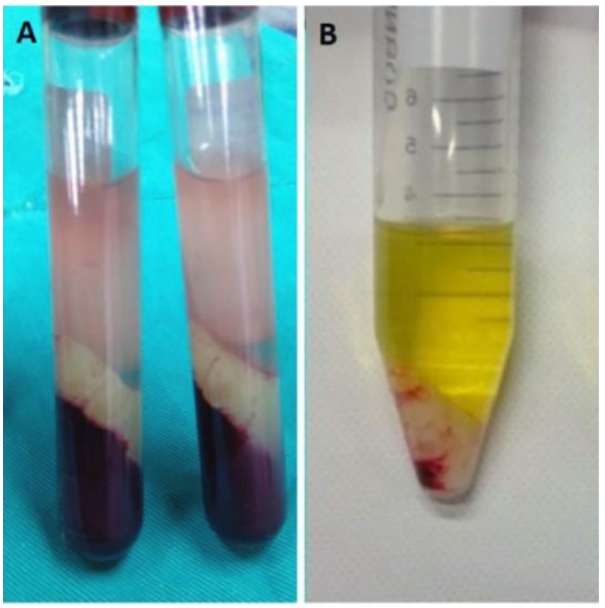

Figure 1. Preparation of PRFr. (A) An initial centrifugation to separate red blood cells and the fibrin clot (PRF) was harvested. (B) The PRF was transferred into sterile centrifuge tube and stood for at least 5 h, the supernatant PRFr was obtained by a second centrifugation to concentrate platelets, which are suspended in the smallest final plasma volume.
PRFr: platelet-rich fibrin releasate

(Fig. 1A). Using forceps, the PRF clots were transferred into a sterile 15-ml centrifuge tube, and stood for at least 5 h. Following centrifugation, the supernatant PRFr (Fig. 1B) was collected in sterile vials and stored at −20°C until use. All preparation and delivery steps were carried out under standard disinfection procedures.

Preparation of ADSCs

Adipose tissues were obtained from inguinal fat pads of 10-wk-old SD rats. The fat was washed with phosphate buffered saline (PBS) to remove blood cells and was cut into fine pieces. The extracellular matrix was digested by a 0.2% collagenase type I solution (C-0130, Sigma-Aldrich, St Louis, MO, USA) gently shaken for 30 min at 37°C to separate the stromal cell fraction from adipocytes. The digestion product was filtered using a 100-μm nylon mesh and centrifuged at 1,000 × *g* for 10 min. The pellet was washed with PBS and erythrocytes were lysed. The cells were collected and cultured in Alpha modified Eagle's minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Santa Clara, CA, USA) and 1% antibiotic (penicillin-streptomycin-amphotericin B, Biological Industries, Cromwell, CT, USA) in an incubator set at 37°C containing 5% CO_2 with 100% humidity. After 3 d, the medium and all floating cells were removed and fresh medium was added to the remaining adherent cells, which were considered as the ADSCs. The medium was replaced every 2 d until the cells reached confluence, following which they were subcultured at a ratio of 1:3. ADSCs from the third passage were used in the present study. To identify the characteristics of the cultured cells, fluorescence-activated cell sorting was performed with the FACSAria Fusion Special Order System (Becton Dickinson, San Jose, CA, USA). Briefly, rat ADSCs were stained with the mouse anti-rat CD31 (#550525), CD73 (#551123), and CD90 (#554895) (all from BD Biosciences); rabbit anti-rat CD45 (ab10558, Abcam, Cambridge, MA, USA). Samples containing 1×10^6 cells/100 μl were incubated with the appropriate antibody at 4°C for 60 min. Then, the cells were washed and stained with phycoerythrin (PE)-conjugated goat anti-mouse Ig (#550589, BD) and DyLight 488-conjugated donkey anti-rabbit IgG (#SA5-10038, Thermo Fisher Scientific, Waltham, MA, USA) secondary antibodies at 4°C for 30 min. Finally, the labeled cells were analyzed by BD FACSAria III cytometer and the analysis was performed using BD FACSDiva 6.1 software.

Sciatic Nerve Injury in Animal Models

All of the rats were anesthetized with a single intraperitoneal injection of Zoletil 50 at a dose of 25 mg/kg mixed with xylazine at a dose of 10 mg/kg. Then, in the prone position, the surgical site was disinfected with povidine-iodine and shaved. After that, a 2-cm incision was made on the left side of the limb in the lateral femoral area, and the subcutaneous tissue and fascia layer were cut open to expose the sciatic nerve. Then, the sciatic nerve was transected and the two ends of the nerve were put into the sleeves of a 14-mm silicone tube (inner diameter 2 mm, wall thickness 0.3 mm). The nerve ends extended into the tube approximately 2 mm; the outer membrane of the nerve ends and the wall of the silicone tube were fixed by 6-0 PDS II (Polydioxanone) sutures (Ethicon Inc., Somerville, NJ, USA). Then, treatment was applied and group 1 served as untreated controls; in group 2 and 3, PRFr (0.1 ml) and ADSCs (1×10^6 cells/0.1 ml) were injected into the silicone tube, respectively; and in group 4, 1×10^6 ADSCs suspended in 0.1 ml of PRFr was injected. Finally, the wounds were closed with 2-0 PDS II sutures subcutaneously and 3-0 nylon sutures in a mattress fashion for the skin (Fig. 2). All animals were housed separately. Walking function was assessed in a horizontal rung ladder apparatus walking at 1-mo intervals for a total of 3 mo. The animals were then sacrificed, tissue samples were obtained, and axon regeneration was evaluated regarding histological criteria.

Behavioral Assessment of Functional Recovery

Rats were trained to perform a behavioral task 1 to 3 mo after surgery. Locomotor movements of rats walking across a ladder rung walking test apparatus (Fig. 3) were quantified using the ladder rung walking test[22]. A ladder rung walking

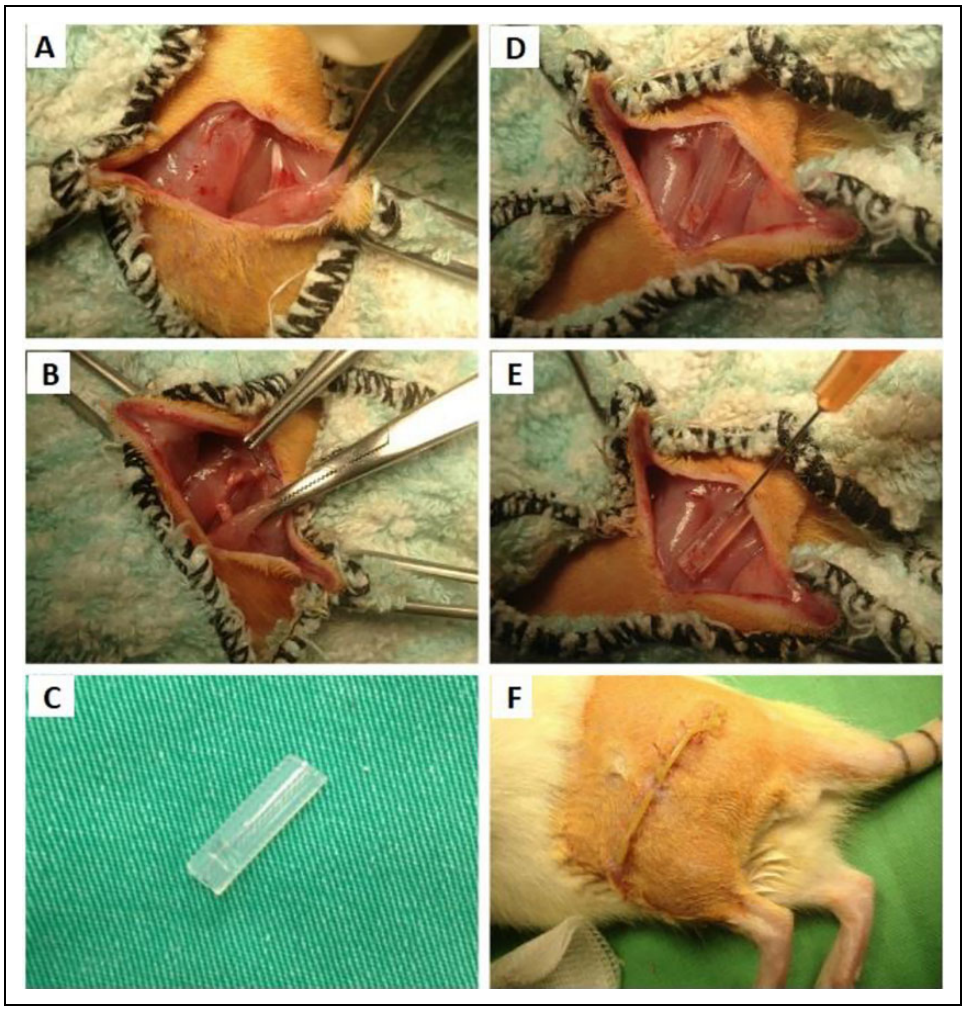

Figure 2. Step-by-step approach to construction of a rat model of sciatic nerve injury. (A) The femoral biceps and gluteus muscles were split to expose the sciatic nerve. (B) A 10-mm nerve segment removed at the midpoint. (C) The silicon tube had a 2-mm inner diameter and were 12 mm in length with a wall thickness of 0.3 mm. (D) The 14-mm silicon tube was placed as an interposition graft with 6-0 PDS II sutures, the nerve ends extended into the tube approximately 2 mm. (E) Subsequently adipose tissue–derived mesenchymal stem cells, alone or in combination with PRFr and PRFr alone, were injected into the silicone tube. (F) The surgical wound was closed in layers, and povidone-iodine gel was spread liberally over the operative site.
PRFr: platelet-rich fibrin releasate.

apparatus consisted of two polymethyl-methacrylate acrylic resin side walls linked by insertion of metal rungs (3 mm diameter, spaced at 1 cm intervals). In so doing, a floor was created that was 1.0 m in length and 20.0 cm in height with variable width that could be adjusted to fit the rat body size. The recorded behavioral data were processed by two experimenters blind to the lesion condition. Videos captured in the ladder rung walking test were observed frame-by-frame at 30 frames/s using Adobe Premiere Pro (Adobe Systems, San Jose, CA, USA) to count the number of hindlimb misplacements, and hindlimb slips and measure the crossing time. A total of three walking tests were required to complete task, and each rat made five runs per test during the entire testing period. Their task performance was then recorded. Finally, the qualitative evaluation of hindlimb placement was performed using a foot fault scoring system as described earlier[22]. Analysis was made by inspection of the video recordings frame-by-frame.

Histology

The sciatic nerves of rats from each group were obtained under anesthesia in an atmosphere saturated with CO_2, and a macroscopic examination of the operated site was performed. The specimens were fixed in neutral-buffered formalin 10% solution and sliced into about 5 μm thicknesses as vertically as possible. All of the slides were stained with hematoxylin and eosin (H&E), and toluidine blue (Sigma-Aldrich) for routine histopathological examinations. Evaluation of axonal degeneration and fibrosis[23] was observed

Figure 3. The skilled ladder rung walking test apparatus in the frontolateral view. Time measurement began after a limb passed the starting line and it ended after all four limbs passed the finish line.

under an optical microscope (CX31RTSF, Olympus Optical Co., Cebu, Philippines) and a digital camera (EOS 500D, Canon Inc., Tokyo, Japan).

Statistical Analysis

Results are presented as means $\pm$ standard derivation. Experimental data were analyzed with a one-way analysis of variance followed by Tukey's post hoc test. A probability (P) value less than 0.05 was considered statistically significant.

Results

The animals survived the entire study period. They all tolerated the surgical procedure planned for each group without complications as well as the follow-up period until the intended date of sacrifice. No wound dehiscence or infection was noticed in any of the rats in any of the groups. All rats showed some weakness in their operated feet, which decreased over time.

Foot Fault Scoring

This study examines the functional recovery after sciatic nerve injury in the rat. The types of foot placement on the rungs were rated on a seven-category scale (Table 1) according to their position and errors that occurred in placement accuracy[22]. (0) Total miss. Zero points are given when a limb completely missed a rung and a fall occurred. (1) Deep slip. One point is given if the limb was initially placed on a rung and then slipped off when weight bearing caused a fall. (2) Slight slip. Two points are scored when a limb was placed on a rung, slipped off when weight bearing, but did not result in a fall. (3) Replacement. Three points are given when the limb was placed on a rung, but before weight bearing was quickly lifted and placed on another rung. (4) Correction. Four points are scored when the limb was aimed at one rung, but then was placed on another rung without touching the first one or recorded if a limb was placed on a rung and was quickly repositioned while on the same rung. (5) Partial placement. Five points are recorded if the heel or toes of the hindlimb were placed on the rung. (6) Correct placement. Six points are assigned when the midportion of the palm of a limb was placed on the rung with full weight support.

Table 1. Rating Scale for Foot Placement in the Ladder Rung Walking Test[22].

Category	Type of foot misplacement	Characteristics
0	Total miss	Deep fall after limb missed the rung
1	Deep slip	Deep fall after limb slipped off the rung
2	Slight slip	Slight fall after limb slipped off the rung
3	Replacement	Limb replaced from one rung to another
4	Correction	Limb aimed for one rung but was placed on another or limb position on same rung was corrected
5	Partial placement	Limb placed on rung with either digits/toes or wrist/heel
6	Correct placement	Midportion of limb placed on rung

Behavior During Walking on the Ladder Rungs

The approach that rats used while walking across the horizontal ladder rung walking test apparatus was noticeably different before injury and after the sciatic nerve damage injury. Before the injury, when the rats were placed at one end of the testing apparatus, they moved to pass the starting line instantly and walked quickly. The step sequence patterns were almost always normal and the foot support was mostly performed diagonally. After the injury, when rats were placed at one end of the ladder apparatus, they stayed just behind the starting line for a while and at times and walked carefully. The step sequence patterns were almost all abnormal. Usually, both forelimbs reached out while both hindlimbs were standing until the forelimbs held onto a rung, and then the hindlimbs moved forward one at a time.

Foot Fault Scores

The results of the foot placement score analysis among individual groups are illustrated in Fig. 4. The scores of five times (runs) were averaged and used for analysis. The average scores for hindlimb placement in controls (presentation order in first, second, and third months) were 2.5/3.1/3.8 points, in PRFr rats 2.9/3.7/4.0 points, ADSCs rats obtained a mean score of 3.0/3.6/4.2 points, and PRFr+ADSCs-treated rats had an average of 2.9/3.7/4.6 points. At the third month, the scores in the PRFr+ADSCs group increased on average by 59% ($P < 0.05$) as compared to the first month, the ADSCs group increased by 40% ($P < 0.05$), and PRFr group increased by 38% ($P < 0.05$). Relative to the PRFr or ADSCs-treated group, functional assessment demonstrated that the PRFr+ADSCs-treated rats had significantly better

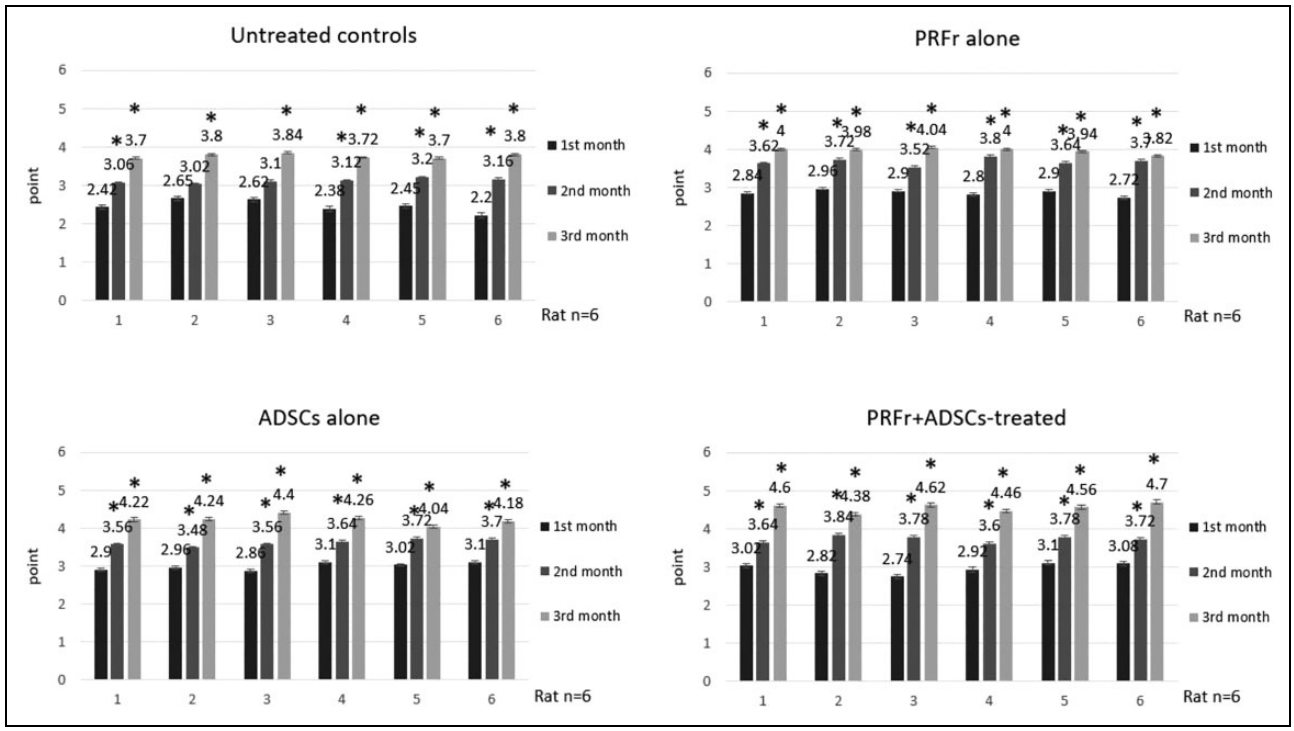

Figure 4. Walking functional evaluation of sciatic nerve injury in rats by ladder rung task at 1-mo interval for a total for 3 mo. Error bars represent mean ± standard deviation for $n = 6$. * Significant differences as compared with the first month (*$P < 0.05$).
ADSCs: adipose tissue–derived mesenchymal stem cells; PRFr: platelet-rich fibrin releasate.

results ($P < 0.05$). Functional recovery was similar in the PRFr-treated and ADSCs-treated group at month 3 postinjury, and there were no significant between-group differences. It was clear that either PRFr+ADSCs-treated rats, or PRFr-/ADSCs-treated alone showed a progressive increase and demonstrated significant recovery outcomes when compared with the control rats.

Gross Appearance of the Repaired Sciatic Nerve

There was no adhesion and inflammation in the site of repair. Trauma was not observed in gross observation. All the sciatic nerves of groups 2, 3, and 4 showed complete healing of the transected site. By contrast, the PRFr and ADSCs groups ($n = 6$ each) had narrower fibers, with mean diameters of 350 and 450 μm, respectively. Nerve fascicles from rats in the PRFr+ADSCs group exhibited large-diameter fibers.

Histological Observations

In the normal rat sciatic nerve cross-sections' centrally placed axons, the surrounding SCs together with the endoneurium were observed. SCs were distinguished by their oval or round nuclei within the endoneurium, as they wrap and compact their membranes around axons to generate the mature myelin sheath. In rats belonging to the untreated group, the axons and myelin sheath presented visible degeneration, the myelin sheath lamellae were separated from each other, and the axons were smaller in some nerve fibers, or found to be completely degenerated in other nerve fibers. We next evaluated the efficacy of ADSCs and/or PRFr-alone therapy in a rat sciatic nerve transection model with a 10-mm gap. Axonal collapse and demyelination were not detected and closely arranged and thinly myelinated regenerating fibers and normal myelinated fibers were evident (Fig. 5). Axon regeneration and myelination in PRFr+ADSCs-treated group was significantly improved compared to untreated control. In addition, the longitudinal sections of the repair site showed neatly arranged nerve fibers running continuously through the anastomotic stoma. In the distal zone, tiny myelinated and nonmyelinated fibers were visible in all treated groups. This result suggested that regenerated axons entered the distal stump and that myelinization gradually occurred. The successfully regenerated nerves had a relatively mature structure, in which a substantial portion of the endoneurial area was occupied by connective tissue with an abundance of myelinated axons. The nerve fiber density was calculated as the number of nerve fibers per square millimeter or another square measure. Macroscopically, the density in untreated nerves was lower compared with regenerated nerves. It can be obviously seen that the PRFr+ADSCs group had a higher fiber density compared with the ADSCs group than in the PRFr group.

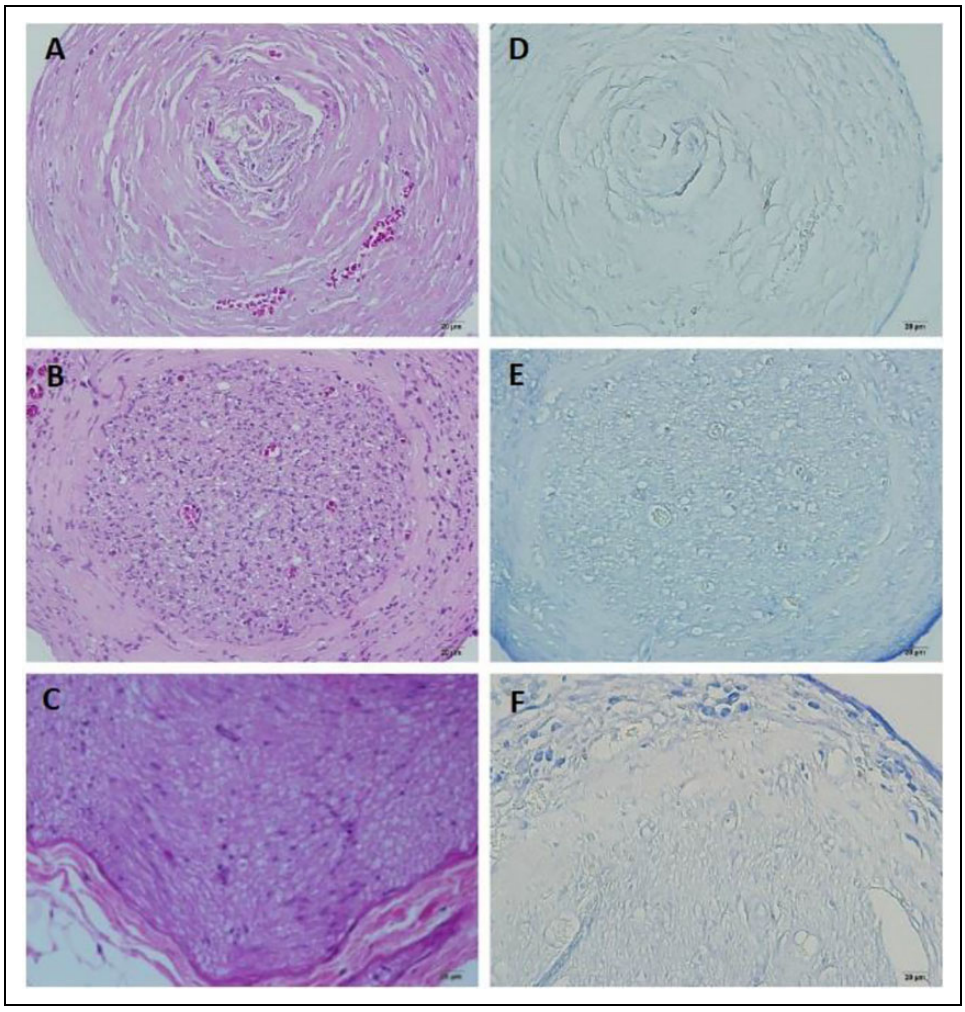

Figure 5. The histological appearances of the regenerate sciatic nerve in representative slides of all experimental groups: (A, D) PRFr group; (B, E) ADSCs group; (C, F) PRFr+ADSCs group, taken 3 mo post transplantation, by means of (A–C) hematoxylin and eosin and (D, E) toluidine blue staining (400×). Bar represents 20 μm.
ADSCs: adipose tissue–derived mesenchymal stem cells; PRFr: platelet-rich fibrin releasate.

Discussion

Nerve regeneration after sciatic nerve injury is a slow process with a limited degree of functional recovery. Accelerating the rate of nerve regeneration and improving the degree of nerve repair is a clinical challenge. In this study, we investigated the potency of MSCs and platelet concentrates as an alternative approach to improving sciatic nerve regeneration in rats. This was achieved by injecting ADSCs and/or PRFr into a silicon chamber that bridged the transection of the nerve. Our data suggested that ADSCs or PRFr alone were able to enter the nerve and migrate mainly retrogradely after transplantation, and had a better result in walking function recovery than that in the untreated control after injury; but when used together, they provided even more effective treatment outcomes.

One of the key issues of successful nerve regeneration after injury is to provide a favorable microenvironment for nerve injury to promote regeneration and functional recovery. The ideal microenvironment of nerve regeneration includes a good blood supply and a chamber that can provide neurotrophic and GF regenerative agents[24]. Findings on the process of peripheral nerve regeneration after severance, as reported by Lundborg[25], confirm that the liquid secreted by the stump contains some growth and neurotrophic factors, which promote axonal and nerve regeneration. Furthermore, in the Lonborg study[26], a silicone tube was used in ulnar nerve repair in 30 patients. In a 5-year follow-up, they observed that the effects of the silicone tube were similar to a nerve graft[27]. The silicone tube regenerative microenvironment provides a reliable tool for the study and application of GFs to promote nerve regeneration.

Various known cytokines and GFs within platelet α-granules such as transforming GF-β1, platelet-derived GF (PDGF), vascular endothelial GF (VEGF), insulin-like GF-1 (IGF-1), basic fibroblastic GF, and epidermal GF (EGF) have been discussed as being crucial for cell proliferation

and differentiation, stimulation of angiogenesis, and scar control in the process of regenerating peripheral nerve structures[28]. The most potent trophic factors secreted by MSCs and used in regenerative therapies are nerve GF (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and IGF-1[29]. Furthermore, MSCs produce angiogenic factors such as VEGF and PDGF[29]. There is convincing evidence that ADSCs release the BDNF and promote axonal regeneration[30]. Fairbairn et al. suggest that transplanted stem cells at the injury site mediate a retrograde neuroprotective effect on adjacent motor and sensory neurons, thereby increasing axon numbers[31]. ADSCs delivered to the injured nerve prevent neuronal loss in the dorsal root ganglia by producing NGF, BDNF, glial-derived neurotrophic factor, and neurotrophin-4[32]. Since cell therapy is thought to activate endogenous SCs, a study reported that ADSCs seeded into a fibrin nerve conduit improve capillary formation in the tube and facilitate nerve regeneration by expressing VEGF-A and angiopoietin-1[33].

In the past several years, studies have already demonstrated positive effects from PRF acting as a pool of GFs, and have shown that concentrations of GFs, protein, and lipids in PRFr are higher than those in supernatant serum[34]. PRFr can be used in a variety of clinical applications, based on the premise that higher GF content should promote better healing. Interestingly, beside the enrichment in platelets and leukocytes, the entrapment of stem-like cells with high regenerative potential within the fibrin network has recently been acknowledged[35], providing an even more solid basis for the use of PRF in regenerative medicine[17]. Literature records concerning the nerve regeneration with stem cell and platelet-rich fibrin therapies are currently limited. Since fibrin can be easily formed under physiological conditions and is characterized by both biocompatibility and cell adhesion, previous studies have reported that transplantation of ADSCs embedded in fibrin glue promotes regeneration through a neuroprotective effect on sensory neurons and stimulation of axon growth[16]. Although stem cell therapy provides a new paradigm in tissue regeneration, they are limitation in clinical application due to the poor survival and differentiation potentials of the transplanted cells[36]. Regarding PRFr, it has significant GF activity so that they can be administered with ADSCs to damaged tissues by either transplanting cells or, as a safer alternative, using the conditioned medium of ADSCs[21].

Numerous tests exist for evaluating nerve regeneration in experimental animal models, and they can be assessed in different ways[37]. We performed transection injuries and measured recovery by ladder rung walking test because it assesses the combination of motor and sensory function[22,38–42] and correlates with the sciatic functional index (SFI)[43]. Though SFI is generally considered to be accurate and reliable in describing sciatic nerve function[43], it has certain disadvantages including frequent footprint artifacts and distortions produced by smearing of the ink when applied to the rats' paws[44]. The footprint key points have to be adequately recognized and analyzed depending on artifacts and operator ability. The precision and thus the discriminative power of the method are limited[45]. Therefore, the parameter was insufficient to describe functional recovery, but it was used to complete the evaluation of functional restoration in association with other kinematical parameters, such as gait-stance duration, ankle angle, and toe-out angle[46].

The rung walking test, also a reliable tool used for sciatic nerve functional assessment, has been shown to be sensitive to chronic movement deficits after adult and neonate lesions to the motor system, including rat models of hypoxia-ischemia[39,47], spinal cord injury[48,49], and Parkinson's disease[50,51]. Moreover, the ladder rung walking test detects changes in fine motor performance induced by physiological variables such as mild stress[42] and even changes in diet[52]. In addition to rat models, the task is also useful for studying skilled walking in mice[39]. Furthermore, there is a lot of evidence that shows even subtle remaining functional motor deficits and compensatory adjustments, so the rung walking test is a reliable tool for assessing functional loss and recovery due to brain or spinal cord injury, and the benefit of treatment approaches[42,48,49].

In the present study, the sciatic nerve regeneration was evaluated according to its functional and histological aspects. The foot fault scores, a reliable and valuable tool for evaluation of post-sciatic-nerve-injury motor function in animal experiments, has been commonly used for recent studies about sciatic nerve functional assessment[38]. Herein, the ADSCs and/or PRFr-treated groups showed better foot fault scores with progressive recovery than those of the untreated group within the 3 mo since injury. Histologically of greater significance than the fiber densities in the proximal regions are the fiber numbers in the distal regions, as these are more suggestive of functional regeneration. Therefore, maintaining a growth-permissive environment in the distal nerve stump following repair is arguably the most important consideration. Due to the limitations of H&E and toluidine blue staining, the authors could not choose a better specimen analysis method due to our experimental conditions. It is certain that more information including numbers and morphological characteristics of the axons might have been obtained if immunochemical evaluation (Caspase-3 or nuclear factor kappa B immunostaining) was used. The better foot fault values in the ADSCs and PRFr-treated groups implied better nerve regeneration, as was consistent with the histological findings. Nonetheless, during walking on the ladder rungs test, we remark that there is a spontaneous walking function recovery in sciatic-denervated rats.

In the literature, it has been suggested that compensatory mechanisms are activated soon after the surgical trauma and they can lead to an adequate recovery in denervated rats due to polyneuronal innervation[53], and the possible activation of other alternative paths to the sciatic nerve[54]. Remarkably, as known in the presence of a spinal cord injury, rats develop alternative locomotor patterns that cannot be discriminated

by the use of qualitative visually based analysis alone[55]. However, it is possible to have good axonal regeneration but poor function[37]. Therefore, morphological evaluation is not thought to be the most reliable way to evaluate the outcome of nerve repair limit because of mismatching, separation, protruding, or kinking between proximal and distal axons. Mechanisms other than simple bridging of axon regeneration across the lesion must be responsible for the improved motor function[56].

The potential limitation of the present study was the variability in the individual healing potential. Moreover, due to this being a pilot and exploratory study, larger subjects and longer periods may provide more definitive and meaningful support for using this therapeutic approach. Despite the limitations of this study, it is significant that the combined strategy of ADSCs with PRFr decreases axonal and myelin damage after sciatic nerve injury and appears to have a positive effect on the recovery of walking function after injury, thus paving the way for nerve damage repair, speeding up the regeneration of nerves and improving recovery quality.

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committees of National Taiwan University (NTU-104-EL-43).

Statement of Animal Rights

All experiments were carried out in accordance with the International Council for Laboratory Animal Science guidelines for the care and use of laboratory animals. All procedures in this study were conducted in accordance with National Taiwan University IACUC-approved protocol (NTU-104-EL-43).

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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