No synergistic effect of mesenchymal stem cells and exercise on functional recovery following sciatic nerve transection

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Abstract

The present study examined whether transplantation of mesenchymal stem cells (MSCs) in combination with exercise would have synergistic effects leading to functional recovery that is greater than exercise alone. Sprague-Dawley rats received a sciatic nerve transection and were divided into four groups: denervated (control), denervated + exercise (control+Ex), denervated + MSC transplantation (MSC), and denervated + MSC transplantation + exercise (MSC+Ex). A volume of $1 \times 10^5$ of MSCs was injected into the lesion site in the MSC-treated groups, and culture medium in the control animals. Twelve hours after surgery, a swimming exercise regime was begun: 30 minutes/day for seven days in the MSC+Ex and control+Ex groups. Functional assessments including sciatic function index (SFI), vertical locomotor activity (VA), ankle activity (AA), and electrophysiological studies were performed to monitor the functional recovery. Histological analysis was performed to assess nerve continuity and myelination. No significant differences in SFI, VA, AA and electrophysiological studies were found between the MSC+Ex and control+Ex groups. Also, a morphological study revealed prominent axonal degeneration in the injured nerves of all animals. The results revealed that any synergistic effect of MSC transplantation on functional recovery of swimming exercise-treated transected nerve that may have existed was negligible.

KEY WORDS: exercise, functional recovery, mesenchymal stem cells, sciatic nerve transection, synergistic effects

Introduction

After a peripheral nerve injury, particularly a complete transection, functional recovery in the denervated limb is incomplete or absent (1). Experimental studies in animal models have employed physical exercise training in the rehabilitation of traumatic injury of the sciatic nerve to stimulate nerve regeneration and improve functional recovery (2,3). Physical exercise can lead to persistent alterations in neuronal activity in both the peripheral and the central nervous system (4,5). Exercise has been found to increase axonal regeneration from sensory neurons of the dorsal root ganglion, and to improve functional recovery as well as motor nerve conduction velocity after peripheral nerve injury (3,6-8). It has also been shown that improved axonal regeneration in peripheral nerves, as a result of exercise, correlates with increased levels of neurotrophic factors that act as molecular mediators for increased neuronal plasticity (5,6,9,10). Swimming and treadmill running are two forced and voluntary exercise paradigms commonly used in the exercise training of animals. Swimming is a naturally-occurring behavior in rats that shares some important features with ambulation (11), such as right/left alternation of limb flexion and extension. Swimming significantly reduces loading and forcibly promotes physical activity of the paralyzed hindlimb in the peripheral nerve transected animal (11). Forced hyperactivity of rat denervated hindlimb muscles by immobilization of the contralateral hindlimb led to an increase in the mean diameter of regenerating myelinated nerve fibers compared with control animals with sciatic nerve injury (12).

Although physical exercise regimens can elicit some functional recovery following peripheral nerve injury, the degree of improvement is nevertheless limited after such a lesion. Cell implantation has recently emerged as the most widely used technique for the reconstruction of peripheral nerve gaps. The graft contains progenitor cells, such as embryonic, neural and mesenchymal stem cells (MSCs), which act as a nerve guide and direct the outgrowing nerve fibers towards the distal nerve stump; MSCs have also been proposed to exert beneficial effects on peripheral nerve regeneration (13-15). Therefore, in the current study we investigated the use of MSCs in a combined approach for enhancing the outcome of exercise therapy on nerve repair. Numerous therapeutic interventions, mostly pharmacotherapeutic, have been tested to evaluate their effects on functional recovery of peripheral nerve injuries, in combination with either stem cell transplantation therapy (15-17) or exercise therapy (18). Conversely, few investigations have explored the possibility of applying physical exercise combined with stem cell therapy in peripheral nerve lesions.

The main objective of this study was to examine whether transplantation of MSCs in combination with exercise would have synergistic effects leading to recovery of function that is greater than that produced by exercise.
alone after sciatic nerve transection. We hypothesised that supplementary MSCs combined with exercise therapy would yield a greater improvement in function than exercise treatment alone.

Materials and methods

General design

To observe the synergistic effects of MSC transplantation and exercise on functional recovery of transected sciatic nerve in exercise-treated model rats, all rats (n=32) received unilateral sciatic nerve transection and were then randomly divided into four groups. One group received MSC transplantation applied to the transected nerve (MSC group, n=8), whereas a second group was submitted to MSC transplantation and a swimming exercise regimen (30 minutes/day for seven days) beginning 12 hours after surgery (MSC+Ex group, n=8). Another group received non-MSC culture media (control group, n=8). The fourth group was made up of control animals submitted to the same swimming exercise regimen (control+Ex group, n=8). Assessments of functional recovery included serial changes in sciatic function index (SFI), vertical locomotor activity (VA), and ankle angle (AA) measured pre-surgery, post-surgery and at four post-swimming treatment timepoints (weeks 1, 2, 3 and 4). Electrophysiological evaluations were performed after surgery, and at weeks 1 (immediately after exercise) and 4 (end of the experiment). The rats were sacrificed after completion of electrophysiological studies on the final day of the four-week period for analysis of nerve morphology. The experimental design and experimental groups are shown in figure 1.

Animal model

Adult male Sprague-Dawley rats (CD®(SD) IGS BR, purchased from BioLASCO Taiwan Co., Ltd, Taipei) weighing from 250 to 300 g were individually housed in a 12/12 h light/dark vivarium with food and water available ad libitum. All experimental procedures were approved by the China Medical University Committee on Animal Care and Use.

The animals were anesthetized with 4% isoflurane (Aertrane, Baxter Healthcare of Puerto Rico, PR, USA) in induction followed by a 1-2% maintenance dose. Body temperature during anesthesia was stabilized by placing the rats on an electric warming pad. All rats received unilateral sciatic nerve transection. The hindlimbs of anesthetized rats were shaved and cleaned with povidone-iodine solution. Using a double-headed operating microscope, the sciatic nerve on one randomly selected side was exposed by skin incision along the femur and separation of biceps femoris and superficial gluteal muscles. Nerves were transected at mid-thigh level. A 2-mm-long nerve segment was removed, and the nerve endings were sutured with two stitches using 9-0 Ethilon®, Ethicon, Somerville, USA) inside an 8-mm-long silicone tube (2-mm outer diameter and 1-mm internal diameter, Kunii, Japan), leaving a 2-mm gap between the two ends (Fig. 2). Then the nerve-transected rats were randomly assigned to the MSC treatment group (MSC group) or the non-MSC treatment group (control group). Rats in the experimental group and control group received an injection of MSCs and culture medium only, respectively, delivered by microsyringe into the gap inside the tube. Finally, muscle layers (4/0 resorbable suture, Ethicon) and skin (3/0 non-resorbable suture, Ethicon) were sutured.

Preparation and graft of rat mesenchymal stem cells

Mesenchymal stem cells isolated from rat bone marrow and cryopreserved at second passage were purchased from Cell Applications Inc. (San Diego, Salisbury, CA, USA). The cells were then centrifuged and plated in rat MSC growth medium (RMSCGM, R419-500, Cell Applications Inc., USA) supplemented with 10% fetal bovine serum (FBS, GIBCO®, Invitrogen Corporation, New Zealand), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO®). The cells were passed or were supplied with differentiation medium containing 1.5% of dimethyl sulfoxide and 2.5% of FBS once they reached 60% confluence, mostly 24 hours after plating (and before the surgery on the rats). A volume of 1x10^5 MSCs was placed in 0.2 ml of collagen gel medium not containing bFGF. The cells were suspended evenly by repeatedly flushing using a micropipette. In the MSC-treated animals (MSC and MSC+Ex groups), the MSCs embedded in collagen gel were infused into the silicone tubes using a micropipette. In the remaining animals, which served as controls (control and control+Ex groups), 0.2 ml of culture medium alone was infused into the silicone tubes.

Figure 1 - MSC and swimming exercise treatments and assessments conducted in the course of the experiment.
Abbreviations: SFI= sciatic function index; VA= vertical locomotor activity; AA= ankle angle; CMAP= compound muscle action potential
Swimming exercise protocol

One week prior to the surgery, animals were exposed to the swimming exercise apparatus for 1-3 minutes per day. The swimming exercise apparatus consisted of a plastic container (80 cm in height x 60 cm in diameter). The apparatus was filled to an approximate depth of 65 cm with tap water maintained at room temperature (~30°C) for each swimming session and was thoroughly cleaned daily. Twelve hours after surgery, rats were randomly assigned to swimming exercise training. Each rat was carefully transferred from its housing cage and placed individually into the exercise apparatus. A drop of soap was added to reduce surface tension: this reduced the frequency of “floating” behavior. In the rare instances of such behavior, animals were gently stimulated to swim by nudging the nape with a pen. This ensured a full session of exercise conditioning. Each animal had one 30-minute swimming session per day, in the afternoon (5:00 pm). After each swimming exercise session, animals were gently dried with a cloth towel. Non-swimming treated rats (those in the MSC and control groups) were also placed in the same swimming exercise apparatus, but without water in the container, for sham swimming treatment.

Assessments of functional recovery

For each rat, a series of functional assessments including SFI, VA and AA were performed to evaluate the animal’s motor functional recovery. A technical assistant who was blinded to treatment allocation evaluated sciatic nerve function one week after surgery.

Sciatic function index

The degree of recovery was monitored by evaluating the rats’ walking patterns, in order to obtain an SFI according to the method described by de Medinaceli et al. (19). Before the recording, a few conditioning trials were performed to accustom the animals to the track. All animals underwent preoperative walking-track analysis. Briefly, the plantar surfaces of both hind paws were wetted with red ink in order to obtain clear footprints, and they were allowed to walk along a specially designed alley (84 cm length x 8.5 cm width) lined with scaled paper. Recordings continued until five measurable footprints had been collected. The data used for calculations were taken from the footprints as follows: i) distance from the heel to the third toe, the print length (PL); ii) distance from the first to fifth toe, the toe spread (TS); and iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. Prints were then calculated using the formula described by Bain et al. (20): SFI= -38.3 ([EPL - NPL]/NPL) + 109.5 ([ETS - NTS]/NTS) + 13.3 ([EIT - NIT]/NIT) -8.8. An SFI equal to -100 indicates total impairment, such as would result from a complete transection of the sciatic nerve, whereas an SFI oscillating around 0 is considered to reflect normal function.

Vertical locomotor activity

The automated Digiscan Animal Activity Monitor System (model RXYZCM 16, Omnitech Electronics, Columbus, OH, USA) was applied to monitor the rats’ locomotion by using a procedure previously established in our laboratory (21). Animals were placed singly into a transparent acrylic activity monitoring cage (40 cm width x 40 cm depth x 30.5 cm height). Infrared monitoring sensors, at a height of 4.5 cm from the floor of the cage, were located at 2.54-cm intervals around the perimeter (16 infrared beams on each side) while two additional sets of 16 sensors were located 10.5 cm above floor level on opposite sides. Data were collected and analyzed by a Digiscan Analyzer (Accuscan Model CDA-8, Omnitech Electronics, Columbus, OH, USA), which in turn sent information to a computer where it was stored for future analysis. One of the activity variables, VA, calculated directly by the Digiscan Analyzer, was selected for examination. The rats were not previously habituated to the cage in order to elicit more vertical exploratory locomotion after repeated exposures.
ANKLE ANGLE

Animals walked on a wooden track (20 cm width x 150 cm length x 15 cm height). Before the data collection, several trial “walks” were conducted to habituate the rats to the testing environment, and then 10 successful walks were collected for analysis.

To ensure locomotion in a straight line, the width of the apparatus was adjusted to the size of the rats during the experiments, and a darkened cage was connected to the end of the track to attract the animals. Also, food was placed at the other end of the track to further encourage walking.

The Expert Vision HiRes motion analysis system (Motion Analysis Corporation, CA, USA) equipped with five CCD cameras was used at 60 Hz sampling rate to capture the rat’s gait motion. A workstation (Dell Precision Workstation 650, Dell Inc., Texas, USA) was used to help with the data collection. Reflective skin markers were tattooed at three anatomical landmarks: at the proximal edge of the tibia, in the lateral malleolus and, in the fifth metatarsal head (Fig. 3).

The AA was defined by the intersection of the lines extending from the knee to the ankle joint and from the ankle joint to the metatarsal head. The angles were expressed as degrees.

The computer software “EVA 4.2” (Motion Analysis Corporation, CA, USA) was used to analyze the AA at terminal stance phase (the last moment at which the foot is in contact with the ground). In the sagittal plane analysis, the following formula was used in the mechanical analysis of the rat ankle: \( \theta_{\text{ankle}} = \theta_{\text{foot}} - \theta_{\text{leg}} \). If \( \theta_{\text{ankle}} \) was above 90°, the foot was considered to be in plantarflexion; if it was below 90°, the foot was considered to be in dorsiflexion (Fig. 3).

Electrophysiological studies

The electrophysiological recordings were made with two-channel portable digital miniature EMG equipment (Neuro-EMG-Micro®, Neurosoft, Ivanovo, Russia). The nerve was administered a single electrical pulse of 50-µs duration up to supramaximal stimulus intensity through a pair of needle electrodes directly applied to the proximal side of the injured site. Compound muscle action potential (CMAP) amplitudes and latencies were recorded with an active needle electrode placed 10 mm below the tibia tubercle and a reference needle electrode placed 20 mm from the active electrode. We used 37-mm, disposable, monopolar, Teflon-coated EMG needle electrodes. The measurements included the amplitude and the onset latency of the CMAP in the sciatic nerve. The latency (in ms) was measured from stimulus to the takeoff of the first negative deflection. The amplitude (in mV) and the area under the CMAP curve from baseline to the maximal negative peak were measured. The stimulation intensity and filtration ranges were 10-20 mA and 20-2000 Hz, respectively. For comparisons, the ratio between the transected and intact side values (transected side/intact side) of amplitude and onset latency, expressed as a percentage, was calculated (% amplitude and % latency of CMAP) to adjust for the effect of anesthesia.

Morphological examinations

Four weeks after the transection, the rats were sacrificed and the sciatic nerves of the operated parts were harvested from the animals while completing the final recording of the electrophysiological study. Morphology was performed blindly. All nerves were fixed in 10% neutral formalin, and routine tissue processing for light microscopy was performed. Tissues were embedded in paraffin. Sections were cut longitudinally into 5-µm-thick slices by microtome and were examined using Masson’s trichrome and hematoxylin and eosin (H&E) stain to determine nerve continuity and myelination. Ten consecutive longitudinal resections contiguous to a maximum diameter were chosen for data collection and subsequent comparisons. Of the vacuoles formed proximal to the nerve stumps, the one with the greatest diameter was identified. Greater vacuole formation on H&E staining on the injured versus the normal side was scored as “positive” and lesser vacuole formation was scored as “negative”. Slides were examined by a light microscope and photographed using the Automatic Photomicrographic System PM10SP (Olympus, PA, USA).

Statistical analysis

All SFI, VA, AA values and CMAP amplitudes and onset latencies were expressed as mean values ± standard error mean (SEM). To analyze between-group differences and serial within-group changes, the results of these parameters were compared using repeated measures ANOVA. Post hoc analysis was conducted using the Bonferroni post hoc test. The morphological studies for assessing vacuole formation were tested by sign test. The level of statistical significance for all the tests was set at p<0.05 for all comparisons. All statistical analyses were performed using the Statistical Package for the Social Sciences Version 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Figure 3 - The ankle angle (\( \theta_{\text{ankle}} \)) is defined by two rigid segments: foot and leg (A). Measurement of ankle angle at the terminal stance phase of the gait cycle (B).
Results

Assessments of functional recovery

SCIATIC FUNCTION INDEX

Pre- and post-surgery changes. Calculation of the SFI, a measure of general use of the paw, from the pre- and post-surgery footprints indicated a normal and a complete loss of paw function. The return of motor function was tested postoperatively at weeks 1, 2, 3 and 4, and SFI values were calculated. After transection, SFI values showed a significant difference compared with pre-surgery levels (repeated measures ANOVA, p<.005), indicating the effectiveness of the transection model.

Serial changes within each group. The serial changes in SFI from post-surgery to the end of the experiment showed significant improvements in the MSC+Ex and control+Ex groups (repeated measures ANOVA, p<.05), but no differences in the MSC and control groups (repeated measures ANOVA, p>.05). Comparing the post-surgery data, there were no significant changes after the one-week swimming treatment at weeks 1 and 2 in the MSC+Ex and control+Ex groups (Bonferroni test; week 1: p>.05; week 2: p>.05 for both groups), but significant improvements were recorded at weeks 3 and 4 (Bonferroni test; MSC+Ex group: week 3 vs post-surgery, p<.05; week 4 vs post-surgery, p<.01; control+Ex groups: week 3 vs post-surgery, p<.05; week 4 vs post-surgery, p<.05).

Between-group differences. Significant differences emerged between the four groups at weeks 3 and 4 (repeated measures ANOVA, week 3: p<.05; week 4: p<.05). The SFI was significantly increased at weeks 3 and 4 in the MSC+Ex and control+Ex groups compared with the MSC and control groups, respectively (Bonferroni test; at both weeks, MSC+Ex vs MSC: p<.05; control+Ex vs control, p<.05, Fig. 4A); but there was no difference between the MSC+Ex and control+Ex groups (Bonferroni test; p>.05), or between the MSC and control groups (Bonferroni test; p>.05).

VERTICAL LOCOMOTOR ACTIVITY

Pre- and post-surgery changes. VA development was monitored in all the sciatic nerve transected animals submitted to MSC/non-MSC transplantation and swimming/non-swimming exercise treatment. No significant differences in VA levels were found between the four groups at the post-surgery assessment (ANOVA, p>.05), although post-surgery VA levels were significantly decreased compared with the pre-surgery values in all four groups (pre- vs post-surgery in all groups: p<.001).

Serial changes within each group.

Comparing serial changes in VA from post-surgery to the end of the experiment, there emerged significant differences in all four groups (repeated measures ANOVA, p<.05). Swimming exercise treatment immediately after nerve transection, both in combination with MSC (MSC+Ex group) and in the non-MSC therapy animals (control+Ex group), resulted in significantly decreased hindlimb activities at week 1 compared with post-surgery levels (Bonferroni test, MSC+Ex group: p<.05; control+Ex groups: p<.01), but significant recovery started at week 2 (Bonferroni test, MSC+Ex group: p<.001; control+Ex group: p<.001). Non-swimming treatment (in both the MSC and the control groups) was instead associated with a significant increase in VA at week 1 compared with post-surgery levels (Bonferroni test, MSC group: p<.05; control group: p>.05), but there was no significant recovery at weeks 2 to 4 when compared with week 1 (Bonferroni test, week 1 vs weeks 2, 3, 4, all p>.05).

Differences between groups. Significant differences were found between the four groups at weeks 1, 2, 3 and 4 (ANOVA, week 1: p<.001; week 2: p<.05; week 3: p<.001; week 4: p<.001; Fig. 4B). There was a significant decrease at week 1 (Bonferroni test, MSC vs MSC+Ex: p<.01; control vs control+Ex: p<.01), but a significant increase in VA at weeks 2 to 4 in the MSC+Ex and in the control+Ex groups compared with the MSC and the control groups, respectively (Bonferroni test, MSC vs MSC+Ex: p<.05; control vs control+Ex: p<.05). A signifi-
cant increase was found in VA following MSC treatment compared with the control group at week 4 (Bonferroni test, p<.05), but no difference between the MSC+Ex and control+Ex groups (Bonferroni test; p>.05).

**ANKLE ANGLE**

Pre- and post-surgery changes. In normal animals at terminal stance phase the foot-ankle was in plantarflexion. The AA decreased significantly immediately after sciatic nerve injury compared with pre-injury data in all groups (p<.001).

Serial changes within each group. Comparing serial changes in AA from post-surgery to the end of the experiment, there emerged significant differences in all four groups (repeated measures ANOVA, p<.05). As with the VA data at week 1, swimming exercise treatment immediately after nerve transection, regardless of whether or not it was combined with MSC transplantation (i.e., MSC+Ex and control+Ex groups), resulted in significantly decreased AA when compared with post-surgery levels (Bonferroni test, MSC+Ex group: p<.01; control+Ex group: p<.001), but a significant improvement was recorded at week 4 (Bonferroni test, MSC+Ex group: p<.01; control+Ex group: p<.01). Non-swimming treatment (in both MSC and control groups) was instead associated with a significant increase in AA at week 1 compared with post-surgery levels (Bonferroni test, MSC group: p<.05; control group: p<.05), but no significant recovery was recorded at weeks 2 to 4 compared with post-surgery level (Bonferroni test, post-surgery vs weeks 2, 3, 4, all p>.05).

**Differences between groups.** Significant differences were recorded between the four groups at weeks 1 and 4 (ANOVA, week 1: p<.05; week 4: p<.001; Fig. 4C). The acute effect of one week of swimming exercise on AA (i.e. at week 1) was significantly lower values in the MSC+Ex and control+Ex groups compared with those obtained in the MSC and control groups, respectively (Bonferroni test; MSC vs MSC+Ex: p<.01; control vs control+Ex: p<.01); a significant increase in AA was instead recorded at week 4 (Bonferroni test, MSC+Ex vs MSC, p<.05; control+Ex vs control, p<.01). However, a significant increase in AA was found in the MSC group compared with the control group at week 4 (Bonferroni test, p<.05), but no difference between the MSC+Ex and control+Ex groups (Bonferroni test; p>.05).

**Electrophysiological studies**

Pre- and post-surgery changes. Before surgery, CMAP amplitude and onset latency data did not differ between sides in any of the experimental animals (data not shown). The CMAP waveforms after sciatic nerve transection were characterized by an initial negative peak with markedly diminished amplitude and prolonged onset latency in all four groups. There were no significant differences between the four groups in the ratios of injured/intact side CMAP amplitudes and onset latencies recorded immediately after surgery (p>.05, Fig. 5). The changes in CMAP morphology before, immediately after surgery and after treatment (at week 4) are shown in figure 6.

Figure 5 - The effects of MSC and swimming exercise treatments on electrophysiological studies including ratios of intact/injured CMAP amplitudes (A) and onset latencies (B) recorded post-surgery and post-swimming therapy (at weeks 1 and 4) in the four groups. * p<.05 tested by the Bonferroni post hoc test.

Figure 6 - Representative waveforms of CMAPs recorded pre-surgery, post-surgery and post-swimming (at week 4) in the MSC (A), MSC+Ex (B), control (C) and control+Ex (D) groups.
Immediately after swimming exercise. After one week of swimming exercise training the average CMAP amplitude ratios (Fig. 5A) recorded in the four groups at week 1 were 25.00±11.2% (control group), 40.92±10.20% (control+Ex group), 28.58±6.92% (MSC group), and 42.12±12.44% (MSC+Ex group). The average CMAP onset latency ratios (Fig. 5B) were 123.00±8.96% (control group), 129.12±10.25% (control+Ex group), 121.75±12.82% (MSC group), and 127.45±13.6% (MSC+Ex group). As regards both CMAP amplitude and latency values, significant differences emerged between the four groups (ANOVA, p<.05): significant differences between the control and control+Ex group (Bonferroni test; p<.05); and with the MSC+Ex group (Bonferroni test; p<.05), with the control+Ex group (Bonferroni test; p<.05), with the control group (Bonferroni test; p<.05), and with the MSC group (Bonferroni test; p<.05). However, there were no significant differences between the control and MSC groups (Bonferroni test; p>.05) or the MSC and MSC+Ex group (Bonferroni test; p>.05). The average CMAP amplitude ratios (Fig. 5A) were 15.95±8.60% (control group), 30.10±9.20% (control+Ex group), 25.93±9.40% (MSC group), and 32.31±6.76% (MSC+Ex group). The average CMAP onset latency ratios (Fig. 5B) were 118.24±7.88% (control group), 116.30±8.50% (control+Ex group), 112.52±6.90% (MSC group), and 114.64±6.92% (MSC+Ex group).

As regards both the amplitude and the latency values, significant differences emerged when the control group was compared with the MSC group (Bonferroni test; p<.05), with the control+Ex group (Bonferroni test; p<.05), and with the MSC+Ex group (Bonferroni test; p<.05). However, there were no significant differences between the MSC+Ex vs control+Ex groups (Bonferroni test; p>.05).

Discussion

Despite recent advances in physical medicine and a better understanding of nerve regeneration, recovery from a severe nerve transection remains difficult and the results are far from satisfactory. In order to develop strategies to accelerate nerve regeneration and functional recovery, the rat sciatic nerve has been used as an experimental model of peripheral nerve injuries and extensive research has been done to understand and develop strategies to promote this regeneration process. Our results demonstrated that both transplantation of MSCs and a swimming exercise regimen can elicit incremental but limited improvements in function, which was consistent with the findings of previous studies (14,22). However, the experiments presented here also suggest that the transected sciatic nerves that were treated with swimming exercise combined with MSC therapy did not show significant differences in functional recovery compared with those treated with swimming exercise alone. The MSC therapy failed to augment the effect of exercise intervention on recovery of sciatic nerve transection. This finding, therefore, did not confirm our hypothesis that additionally beneficial effects would be observed. The results revealed that any synergistic effect of MSC transplantation on functional recovery of transected nerves treated with exercise training that may have existed was negligible. This result was similar to the findings of a previous study which indicated that combining the beneficial effects of rat MSC transplantation and exercise was not sufficient to bring about a greater behavioral recovery after spinal cord contusion injuries in rats (23).

The methods most widely used for evaluating sciatic nerve injury models are SFI, locomotor activity and AA (24-28). Serial (weekly) changes in these parameters, which represent various responses of injured animals during recovery, were monitored in this study. The muscle function related to the SFI is that of the intrinsic muscles of the feet; indeed, parameters contributing to the SFI include PI, TS and ITS. The SFI scores in the transection models did not improve, indicating that the animals were almost completely disabled and near total paralysis. As indicated by the SFI score, our transection model represented a true neurotmesis. At weeks 1 and 2 after nerve injury, the absence of a dramatic improvement in the SFI implied that reinnervation of the intrinsic muscles of the feet had not been established due to the long distance from the injured site to the target muscle. However, the SFI showed a significant increase starting at week 3 in the swimming-treated animals, regardless of whether or not swimming was combined with MSC transplantation. These results revealed that the swimming exercise significantly improved the function of the denervated intrinsic muscles. However, evaluation of the SFI did not reveal satisfactory functional motor recovery in the animals treated with MSC transplantation alone. MSC therapy is considered to have a weak effect on improvement of intrinsic muscle function in nerve transection studies (14,28,29). This probably indicates that recovery from a severe nerve transection unlikely to be obtained through exogenous MSC supplementation. Therefore, the SFI results presented here suggest that the transected sciatic nerves treated with swimming exercise combined with MSC therapy did not show more significant improvement than those treated with swimming exercise alone.

In normal gait the AA is highly consistent at terminal stance phase, when it reaches maximal plantarflexion; therefore, any decrease of the AA under pathological conditions is best reflected at this phase. The muscle function related to the AA is that of the larger muscle groups of the lower extremity, i.e., the plantarflexors. In sciatic nerve injury, the loss of ankle plantarflexor function results in a dorsiflexion deformity that can be clearly seen at terminal stance. Dorsiflexion deformities after sciatic nerve injury have been found to be fully devel-
Figure 7 - (A). Masson’s trichrome staining (40 x magnification) of longitudinal sections through the transected transplant sites in representative MSC (a), MSC+Ex (b), control (c) and control+Ex (d) animals. The images show the gap between the two stumps of the sciatic nerve one month after transection. White dotted lines outline the nerve stumps. (B). H&E staining (200 x magnification) of the longitudinal cuts of transected nerves shows myelin-digestion chambers in representative MSC (a), MSC+Ex (b), control (c) and control+Ex (d) groups. The arrows indicate myelin-digestion chambers (vacuole formation).
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oped as early as two weeks after surgery (30). In the present study, improvements of the AA in the non-swimming groups (MSC and control groups) after sciatic nerve transection were limited, probably indicating the presence of fixed contractures. But a significant increase in AA was found following MSC treatment compared with animals that did not receive MSC treatment, probably indicating that MSCs can promote the reinnervation of the gastrocnemius muscle, which is consistent with the finding of a previous study (14). Conversely, the dorsiflexor contracture of the ankle detected in the swimming-treated groups (MSC+Ex and control+Ex groups) progressed slowly over a period of four weeks. The AA results also suggest that the transected sciatic nerves treated with swimming exercise combined with MSC therapy did not show more significant improvement than those treated with swimming exercise alone. It is worth noting that there was a significant decrease in the AA in the swimming-treated groups immediately after one week of swimming training, probably due to deleterious effects of early forced exercise on functional recovery or regeneration (31,32). The stress effect induced by this exercise probably affected the MSC differentiation in the early stage and, because of this, the synergistic effect of MSC transplantation on functional recovery of transected nerve treated with exercise training was negligible.

The measurement of VA can provide information about an animal's rearing behavior and is important for proper indexing of the behavioral effects of various experimental manipulations (21,33). Injury-evoked paralysis can result in decreased rearing behavior following sciatic nerve transection in rats. Therefore, the VA value, like the AA value, is related to the function of larger muscle groups of the lower extremity. Indeed, in the present study, the serial (weekly) alterations of VA paralleled those of AA. The VA levels after sciatic nerve transection were below those observed before treatment but recovered progressively in all animals in the course of the experiment. A more marked recovery in VA was observed after swimming treatment than in the non-swimming animals. Significant increases in VA following MSC treatment, compared with the values of animals that did not receive MSC treatment, probably indicate more successful nerve reinnervation of the gastrocnemius muscle in the groups that received the treatment with MSCs. This would suggest that the increase in rearing behavior is due to increased activity of the rat's hind limb and that the paralytic limb has gained sufficient strength to bear the animal's body weight as it explores the unfamiliar environment. But the effects of swimming treatment combined with MSC transplantation were similar to those of swimming treatment alone. It was found that VA, like AA, decreased immediately after one week of swimming treatment in the swimming-treated groups, and was significantly lower than the values recorded in the non-swimming groups. These findings confirmed the supposition that early forced swimming exercise was probably a stressor that resulted in worse survival of MSCs, or impaired nerve regeneration. Thus, again, there was no enhancement of treatment effect using a combination of MSC transplantation with exercise treatments.

Results of electrophysiological studies have been found to correlate well with severity of nerve injuries (20). The CMAP amplitude reflected the number of muscle fibers that depolarized (34). Low CMAP amplitudes most often result from loss of axons and from conduction block. Therefore, a significant improvement (increase in CMAP amplitude ratio) was found in the swimming-treated groups (control+Ex and MSC+Ex groups), regardless of whether or not swimming was combined with MSC transplantation, at weeks 1 (immediately after one week of swimming training) and 4 (the end of the experiment). This means that the number of depolarized muscle fibers was augmented probably by the effect of exercise alone. The CMAP onset latency reflects the muscle's initial response to the first arriving action potentials of the fastest-conducting nerve fibers (35,36). The persistent prolongation of onset latency found in our transected nerve rat model was probably due to demyelination that causes slowing of nerve conduction. This conjecture was supported by our morphological studies that showed severe axonal degeneration after transection injury in all the animals. As shown in our study, the conduction latency in the MSC, MSC+Ex and control+Ex groups was shorter than that recorded in the control group. But there was no significant difference in onset latency between the MSC, MSC+Ex and control+Ex groups. This indicates that both combined and individual use of MSC and exercise treatments were insufficient to improve conduction time in such a severely transected nerve.

Supplementary MSCs, which may be an alternative source of Schwann cells, potentially provide a practical strategy for the management of peripheral nerve injury. With appropriate stimuli and environmental conditions, MSCs can differentiate into myelinating cells of the peripheral nerve (37-39). But the exact fate of transplanted MSCs and the environmental mechanisms that control MSC differentiation in vivo, specifically differentiation towards Schwann cell lineage, remain poorly defined. Mesenchymal stem cells are known to secrete neurotrophins and angiogenic growth factors (40,41), and transplanted MSCs may therefore provide molecular reservoirs of neurotrophic factors within the neuroregenerative field. This, together with the fact that transplantation of MSCs transduced with neurotrophic factor genes is more efficient than primary transplantation of MSCs in inducing functional improvement, highlights the neuroprotective role of neurotrophic factors (42,43). Many animal studies have demonstrated that transplantation of stem cells combined with numerous therapeutically interventions designed to boost neuroprotection, i.e., G-CSF administration, hyperbaric oxygen and electroacupuncture, has the potential to serve as an adjunct therapy to provide greater effects on repair of injured peripheral nerve (16,17) and spinal cord (44,45). However, in the present study the combination of MSC and swimming therapies did not elicit greater functional recovery than either approach used alone. Because there were different mechanisms involved in the improvement of motor function of the injured nerve between exercise and MSC transplantation, a beneficial synergistic effect of both of them was lacking. Forced swimming exercise tends to have a detrimental effect, especially applied in reinnervating muscle. MSC transplantation may exert its influence on axonal outgrowth and nerve maturation following nerve injury. Therefore, the synergistic effect of combining these strategies was negligible.
In the current study, when control rats (without MSC therapy) were trained in a swimming exercise regimen for one week after the injury, functional recovery, including SFI, VA, AA values and CMAP amplitudes, was improved. These findings are in agreement with previous studies that showed that exercise could improve functional recovery after nerve crush and section lesions in rats, and that such improvements could be maintained in the late phase of peripheral nerve recovery (3,6-8). However, the swimming regimen in this study seemed to affect the MSC action and showed deleterious effects on MSC therapy for enhancing the functional recovery after nerve transection. It is likely that the training pattern, time of intervention, and intensity of the training may have affected the action of the MSCs on nerve regeneration and functional recovery. Swimming undoubtedly increases physical activity, but might represent physical overload: when placed in water, rats initially “swim” energetically for 5 to 10 minutes but this activity may also induce stress, even in a single session, resulting in behavioral and physiological alterations (46,47). Moreover, swimming training was started immediately after the MSC transplantation, and this too may have led to an exaggerated stressful effect, affecting the survival of MSCs and thereby contributing to reduced nerve recovery. Stress-related changes might impede functional recovery after a sciatic nerve lesion (3). A limitation of the present study was the fact that the fate of the MSCs could not be tracked throughout the four-week observation in all the transplanted rats. But in other data we have (unpublished), bromodeoxyuridine (BrdU)-positive cells were few immediately after swimming exercise and had disappeared seven days after exercise. Therefore, the swimming exercise protocol with early and forced intervention may not promote grafted MSC survival and differentiation: MSCs failed to provide a supplementary effect on improvement of nerve repair by exercise. In conclusion, animal studies have demonstrated that both stem cell transplantation and exercise therapy, individually, can potentially serve as an adjunct therapy to promote repair of injured peripheral nerves (7,13,29,38,48). Our study, in addition to confirming the beneficial effects of swimming exercise on injured nerves, also showed that MSC transplantation contributed to promoting functional recovery after a period of four weeks. However, our findings demonstrated that MSC transplantation combined with swimming exercise did not elicit greater functional recovery of transected nerve than swimming treatment alone, as shown by the lower levels of functional recovery, and the results of the electrophysiological and morphological examinations when compared with those of the groups treated with exercise alone. Thus, there was no synergistic effect of these strategies on functional recovery and the exercise regimen might even have tended to exert a detrimental influence on proper maintenance of the transplanted cells. Future research on the synergistic effect of MSC therapy should be conducted with longer observation periods as well as different rehabilitation exercise regimens (49), including unforced exercise and delayed intervention.

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