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ScienceDirect
Journal of Clinical Neuroscience 14 (2007) 1089–1098
Journal of Clinical Neuroscience
www.elsevier.com/locate/jocn

Laboratory Study

Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells

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Received 5 April 2006; accepted 26 August 2006

Abstract

Amniotic fluid mesenchymal stem cells have the ability to secrete neurotrophic factors that are able to promote neuron survival *in vitro*. The purpose of this study was to evaluate the effects of neurotrophic factors secreted by rat amniotic fluid mesenchymal stem cells on regeneration of sciatic nerve after crush injury. Fifty Sprague–Dawley rats weighing 250–300 g were used. The left sciatic nerve was crushed with a vessel clamp. Rat amniotic fluid mesenchymal stem cells embedded in fibrin glue were delivered to the injured nerve. Enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry were used to detect neurotrophic factors secreted by the amniotic fluid mesenchymal stem cells. Nerve regeneration was assessed by motor function, electrophysiology, histology, and immunocytochemistry studies. Positive CD29/44, and negative CD11b/45, as well as high levels of expression of brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor, ciliary neurotrophic factor (CNTF), nerve growth factor, and neurotrophin-3 (NT-3) were demonstrated in amniotic fluid mesenchymal stem cells. Motor function recovery, the compound muscle action potential, and nerve conduction latency showed significant improvement in rats treated with amniotic fluid mesenchymal stem cells. ELISA measurement in retrieved nerves displayed statistically significant elevation of CNTF and NT-3. The immunocytochemical studies demonstrated positive staining for NT-3 and CNTF in transplanted cells. The histology and immunocytochemistry studies revealed less fibrosis and a high level of expression of S-100 and glial fibrillary acid protein at the crush site. Rat amniotic fluid mesenchymal stem cells may facilitate regeneration in the sciatic nerve after crush injury. The increased nerve regeneration found in this study may be due to the neurotrophic factors secreted by amniotic fluid mesenchymal stem cells.

Keywords: Amniotic fluid mesenchymal stem cells; Sciatic nerve crush injury; Neurotrophic factors

1. Introduction

The process of peripheral nerve regeneration involves interaction of the extracellular matrix, neurotrophic factors, and cellular components.[1–6] Peripheral nerves are composed primarily of two kinds of cells: neurons and Schwann cells. Schwann cells promote peripheral nerve regeneration by increasing the synthesis of cell adhesion molecules (CAMs), by elaborating basement membrane that contains many extracellular matrix proteins (also called neurite outgrowth promoting factors), and by producing neurotrophic factors and their receptors. Neurite outgrowth promoting factors, either on the cell surface (cell adhesion molecules and recognition molecules) or in the extracellular matrix, promote extension of the axon by providing appropriate adhesiveness in the substrate, which

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doi:10.1016/j.jocn.2006.08.008

facilitates axon-to-axon and axon-to-Schwann cell attachment.[7,8] Recent advances in the understanding of the molecular pathways and their physiological roles have demonstrated that neurotrophic factors play an important part in the development, maintenance and regeneration of the nervous system. Neurotrophic factors are a family of polypeptides that are necessary for survival of a discrete neuronal population, and they are divided into three major families. (1) Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), are small basic polypeptides that act at tyrosine kinase receptors of the trk family (trkA, trkB, and trkC), and probably share a low-affinity NGF receptor (LNGFR). (2) Neurokines, including ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), act at receptors that have both a common (gp130, LIFR-β) and a specific (CNTFR-α) transmembrane subunit. (3) The transforming growth factor (TGF)-β family, which includes TGF-β1, TGF-β2, TGF-β3, and their distant relative glial cell line-derived neurotrophic factor (GDNF), whose receptor is composed of a ligand-binding subunit called GDNF receptor alpha (GDNFR-α), and a signaling tyrosine kinase subunit, Ret.[9]

Due to accelerated effects of neurotrophic factors in nerve regeneration, the broad applications of neurotrophic factors including GDNF, BDNF, CNTF, and NT-3 have been investigated and facilitated nerve regeneration has been reported.[10–14] However, in general, the neurological outcome in these studies did not reach the pre-injury level. The use of cell therapy with Schwann cells, neuronal stem cells, mesenchymal stem cells (MSCs) or derived Schwann-like cells has been investigated and enhanced regeneration has also been reported.[15–17] Nevertheless, no single treatment strategy has achieved successful nerve regeneration. Cell transplants with a targeted gene secreting neurotrophic factors, which supply the injured cell and secrete vital trophic factors necessary for cell growth, have been considered as treatment alternatives.[18,19] MSCs that have been isolated from amniotic fluid possess the ability to proliferate and differentiate into neural tissue, bone, or cartilage.[20,21] From a conditioned medium, Saiko et al. identified multiple neurotrophic factors that were secreted by amniotic epithelial cells, including BDNF, NT-3 and NGF, which increased neuron survival *in vitro*.[22] Due to the characteristics of cell supplementation and secretion of neurotrophic factors in amniotic fluid stem cells, they may be another option for treatment of nerve injury.

Our previous study demonstrated that human amniotic fluid stem cells could augment nerve regeneration across a sciatic nerve gap, but the mechanism of this effect was not fully understood.[23] In this study, we delivered rat amniotic fluid MSCs around the crushed sciatic nerve to evaluate nerve regeneration, behavior of the transplanted cells, and expression of neurotrophic factors, to better understand the role of amniotic fluid MSCs in peripheral nerve regeneration.

2. Materials and methods

2.1. Animal model

Fifty Sprague–Dawley rats weighing 250–300 g were used in this study; permission for their use was obtained from the Ethics Committee of the Taichung Veterans General Hospital. The rats were anesthetized with 4% isofluorane for induction followed by a maintenance dose (1–2%). The left sciatic nerve was exposed under the microscope using the gluteal muscle-splitting method. A vessel clamp (B-3, pressure 1.5 g/mm^2, S&T Marketing Ltd, Neuhausen, Switzerland) was applied 10 mm from the internal obturator canal for 20 min. The sciatic function index (SFI) of a nerve crushed for 20 min revealed a statistically significant difference compared with that of a sciatic nerve crushed for < 20 min (data not shown). The crush site was sutured with 9-0 nylon over the epineuria as a mark. The animals were categorized into two groups. In group I ($n = 20$), the crushed nerve was wrapped with fibrin glue and woven Surgicel (Johnson & Johnson, Warren, NJ, USA) as a control. In group II ($n = 30$), the crushed nerve was wrapped with rat amniotic fluid MSCs, woven Surgicel, and fibrin glue. The wounds were closed in layers and the animals were allowed to recover.

2.2. Preparation and culture of amniotic mesenchymal stem cells[20]

For culturing MSCs, non-adhering amniotic fluid cells in the supernatant medium were collected from rats on the 14th day of pregnancy. The cells were centrifuged and plated in 5 mL of β-minimum essential medium (β-MEM; Gibco-BRL, Grand Island, NJ, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 4 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA) in a 25-cm flask and incubated at 37 °C with 5% humidified CO_2 for MSC culture.

2.3. Flow cytometry analysis

The specific surface antigen profile of rat amniotic fluid cells in culture at passage 2–3 was characterized by flow cytometry analysis. The cells were trypsinized and stained with fluorescein (FITC), or phycoerythrin (PE)-conjugated antibodies against CD11b (BD PharMingen, San Diego, CA, USA), CD45, CD29, and CD44 (Eurolonepero, Italy). Thereafter, the cells were analyzed using a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.4. RT-PCR in the analysis of neurotrophic factors in amniotic fluid mesenchymal stem cells

Total RNAs were extracted from the cultured cells by using Tir Reagent (MRC Inc. Cincinnati, OH, USA)

according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a One Step RT-PCR kit (QIAGEN Inc., Valencia, CA, USA) with specific DNA primers as follows:

β-actin (185bp) sense 5′-TCCTGTGGCATCCACGAAACT-3′ and antisense 5′-GGAGCAATGATCCTGATCTTC-3′;
NT-3(387bp) sense 5′-GCTGATCCAGGCGGATATCT-3′ and antisense 5′-ATGGCTGAGGACTTGTCGGT-3′;
NGF (403bp) sense 5′-CCAAGGACGCAGCTTTCTAT-3′ and antisense 5′-CTCCGGTGAGTCCTGTTGAA-3′;
GDNF (639bp) sense 5′-GATGAAGTTATGGGATGTCGTG-3′ and antisense 5′-GGTCAGATACATCCACACCG-3′;
BDNF (751bp) sense 5′-GATGACCATCCTTTTCCTTACTATGG-3′ and antisense 5′-ACTATCTTCCCCTTTTAATGGTCAG-3′;
CNTF (560bp) sense 5′-GATGGCTTTCGCAGAGCAAACAC -3′ and antisense 5′-GCTACATCTGCTTATCTTTGGC-3′.

RT-PCR was performed initially at 50 °C for 30 min and at 95 °C for 15 min for reverse transcription, followed by 35 cycles, with each cycle consisting of denaturing at 94 °C for 1 min, annealing at 57 °C for 1 min, elongation at 72 °C for 1 min, and the final extension at 72 °C for 10 min. The amplified DNA fragments were visualized by 2% agarose gel electrophoresis, then stained and photographed under UV light.

2.5. Grafting procedure

The rat amniotic fluid MSCs were labeled with BrdU (10 μmol, Roche, Mannheim, Germany) 72 h before grafting. A volume of 50 μL of MSCs with a density of 3×10^5 cell/mL was suspended in 100 μL of fibrin glue (Aventis Behring, Hattersheim am Main, Germany) containing woven Surgicel for the graft.

Some of the rat amniotic fluid MSCs labeled with the nucleic acid stain Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) were used for the tracing, evaluation of viability, and immunocytochemistry at the transplanted site. Both the cell density and graft procedure were as described above.

2.6. Analysis of functional recovery

A technical assistant who was blinded to treatment allocation evaluated sciatic nerve function at 4 weeks after the surgery. The evaluation method included ankle kinematics[24] and sciatic function index (SFI).[25] In the sagittal plane analysis, the following formula was used in the mechanical analysis of the angle of the rat ankle: θ ankle $= \theta$ foot-leg 90. Several measurements were taken from the footprint: (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. The SFI was calculated according to the equation:

$$\mathrm{SFI} = -38.3(\mathrm{EPL} - \mathrm{NPL}/\mathrm{NPL}) + 109.5(\mathrm{ETS} - \mathrm{NTS}/\mathrm{NTS}) + 13.3(\mathrm{EITS} - \mathrm{NITS}/\mathrm{NITS}) - 8.8$$

The SFI oscillates around zero for normal nerve function, whereas an SFI of around –100 represents total dysfunction.

2.7. Electrophysiology study

The left sciatic nerve from 10 rats in each group were exposed at 4 weeks after the operation. Electric stimulation was applied to the proximal side of the injured site; the conduction latency, and the compound muscle action potential were recorded with an active electrode needle 10 mm below the tibial tubercle and a reference needle 20 mm from the active electrode. The stimulation intensity and filtration range were 5 mA and 20–2000 Hz, respectively. The compound muscle action potential data were converted to ratios of the injured side divided by the normal side to adjust for the effect of anesthesia.

2.8. Quantification by enzyme-linked immunosorbent assay of neurotrophic factors in retrieved nerve

Six nerves in each group were removed at 10 days after the operation, the regenerating tissues (10 mm in length) were retrieved, and the samples were stored at −80 °C. Subsequently, each tissue sample was homogenized in homogenate buffer (400 mmol NaCl, 0.1% Triton X-100, 2.0 mmol ethylendiaminetetraacetic acid, 0.1 mmol benzthonium chloride, 2.0 mmol benzamidine, 0.1 mmol phenylmethyl-sulfonyl fluoride, 10 μg/mL leupeptin, 0.5% bovine serum albumin (BSA), and 0.1 mol phosphate buffer, pH 7.4). The homogenate was centrifuged for 10 minutes at 12 000 *g* at 4 °C. The tissue homogenate, 100 μL in triplicate, was applied to a microtiter plate and allowed to adhere overnight at 4 °C. The microtiter plates were washed with phosphate-buffered saline (PBS), Tween-20 and blocked with 1% BSA in PBS (200 μL) for 1 h. The plates were then treated with primary antibodies and allowed to react for 4–6 h at 37 °C. The respective polyclonal antibodies (100 μL) against NGF, GDNF, CNTF (R&D System, Inc, Minneapolis, MN, USA), and NT-3 (Chemicon, Inc., Pittsburgh, PA, USA) were applied overnight to the microtiter plates. After further washing in PBS containing Tween-20, the plates were incubated for 1 h with 100 μL of the respective second antibody conjugated to alkaline phosphatase. The reaction was developed using 3 mmol disodium *p*-nitrophenyl phosphate in carbonate buffer, pH 9.6 (100 mmol Na_2CO_3 and 5 mmol $MgCl_2$) (150

μL), and the reaction was terminated after 30 min using 0.5 mol NaOH (50 μL). The absorbance of the colored product at 405 nm was measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The relative amount of antigen present was measured from the densitometric readings against a standard curve.

2.9. Examination of regenerated nerves

Ten days after grafting with Hoechst 33342-labeled stem cells, eight crushed nerves were removed and permeabilized with 0.3% Triton X-100 in PBS for 2 h and then incubated for 24 h at 4 °C with the following antibodies: rabbit polyclonal NGF, NT-3, BDNF, GDNF, and CNTF. Slides were then washed with PBS and incubated with rabbit anti-goat immunoglobulin G (IgG) and goat anti-rabbit IgG secondary antibodies, and conjugated with fluorsecein isothiocynate (Genei, Bangalore, India) at the recommended dilution for 1 h at room temperature. After further washing with PBS, cover-slips were mounted with bicarbonate-buffered glycerol at pH 8.6 and viewed with a microscope with an epifluorescent attachment.

Four resected nerves were also taken 10 days after grafting with Hoechst 33342-labeled stem cells for tracing and evaluation of the viability of transplanted cells. Resected nerves were cut longitudinally into 6-μm thick sections and observed under a light microscope.

At 4 weeks after grafting, 10 sciatic nerves in each group were harvested after the electrophysiological test, and the nerve tissues were allowed to adhere to a plastic plate with a stay suture to keep the nerve straight. Ten nerves in each group were embedded in epoxy resin by standard methods and cut longitudinally into 6-μm thick sections, stained with hematoxylin-eosin (H&E), Sirius red and Fast green. A part of the resected tissue of the regenerated nerve was embedded in paraffin, and a section was prepared for microscopy. These sections were reacted with anti-mouse anti-BrdU antibody (1:10), anti-rabbit anti-S100 antibody (1:200), glia fibrillary acid protein (GFAP) antibody (DAKO, Carpinteria, CA, USA), and then with the secondary antibodies anti-mouse IgG FITC and anti-rabbit (anti-goat) IgG Gy3 according to the manufacturer's instructions. All the histological examinations were performed by a pathologist who was blinded to the treatment method.

The maximum diameter of resected nerve tissue with a crush mark was chosen to be examined. Ten comparative studies with staining by H&E, S-100, GFAP, and Sirius red and Fast green either with or without stem cell treatment were examined blindly. Less vacuole formation in H&E staining was scored as positive and greater vacuole formation was scored as negative. Greater intensity of S-100 and GFAP was scored as positive and lesser intensity was scored as negative. Greater intensity of red stain was assessed as positive, whereas lesser intensity of red stain was assessed as negative.

The values of positive, no difference, and negative were scored 2, 1, and 0, respectively.

2.10. Statistical analysis

Analysis of angle of ankle, SFI, compound muscle action potential, conduction latency, and ELISA results were evaluated by Student's t-test and by analysis of variance (ANOVA). The χ^2 test was used to evaluate the severity of fibrosis tissue, and the staining intensity of neurotrophic factors. The value calculated was mean ± SE (standard error). A p-value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Identification of amniotic fluid mesenchymal stem cells

Flow cytometry analysis demonstrated that rat amniotic fluid stem cells were positive for CD29 and CD44, but negative for CD11b and CD45, which is compatible with previous reports[20] (data not shown). The RT-PCR results showed constitutive expression of BDNF, GDNF, CNTF, NGF, and NT-3, in rat amniotic fluid MSCs. However, no expression of NT-4 was seen (Fig. 1).

3.2. Motor function evaluation

The SFI in the control and amniotic fluid MSCs treatment groups at 1, 2 and 4 weeks follow-up was −100(SE 0)/−78.85(SE 11.4), −82.65(SE 8.91)/−64.89(SE 14.38), and −63.85(SE 14.93)/−11.45(SE 2.44), respectively, which was statistically significant ($p = 0.002$) (Fig. 2A). The angle of ankle in the control ($n = 10$) and amniotic fluid stem cells treatment groups ($n = 10$) at 4 weeks follow-up were 62° (SE 1.55) and 80.7° (SE 3.68), respectively, which was a statistically significant ($p = 0.002$) (Fig. 2B).

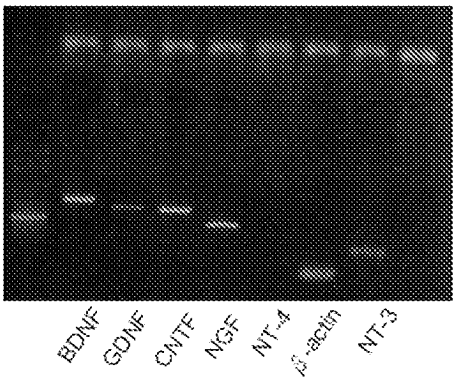

Fig. 1. Reverse transcription polymerase chain reaction in amniotic fluid stem cell analysis revealed strong expression of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), β-actin, and neurotrophin-3 (NT-3), and weak expression of glia cell line-derived neurotrophic factor (GDNF). No expression of neurotrophin-4 (NT-4) was observed. The detection of β-actin was used as an experimental control. This figure is available in colour at www.sciencedirect.com.

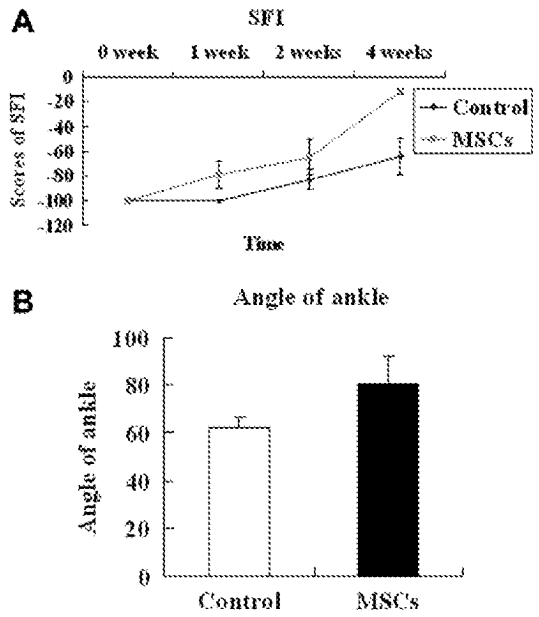

Fig. 2. Motor function evaluation including sciatic nerve function index (SFI) and angle of ankle are illustrated. (A) SFI in the control and amniotic fluid mesenchymal stem cell (MSC) treatment groups at 1, 2, and 4 weeks follow-up were −100(SE 0)/−78.85(SE 11.4), −82.65(SE 8.91)/−64.89(SE 14.38), and −63.85(SE 14.93)/−11.45(SE 2.44), respectively, which were statistically significant differences ($p = 0.002$). (B) The respective angles of ankle in the control ($n = 10$) and amniotic fluid stem cell treatment groups ($n = 10$) at 4 weeks follow-up were 62° (SE 1.55) and 80.7° (SE 3.68). The difference was statistically significant ($p = 0.002$).

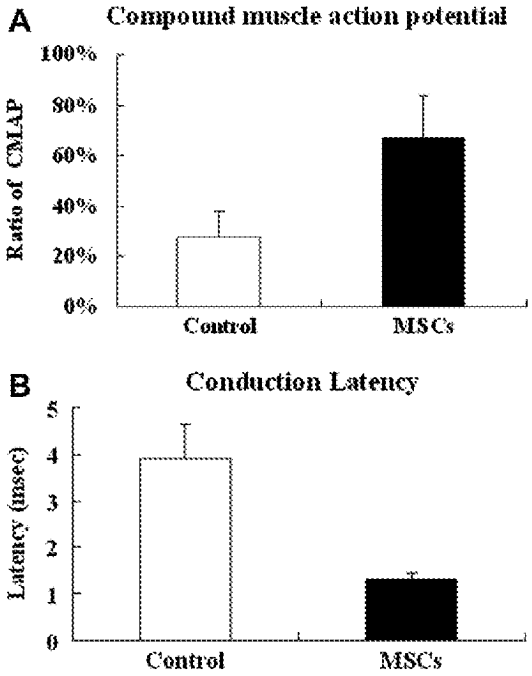

Fig. 3. Compound muscle action potential (CMAP) and conduction latency at 4 weeks follow-up are illustrated. (A) The ratio of compound muscle action potential in the control ($n = 10$) and mesenchymal stem cell (MSC) treated groups ($n = 10$) was 27.80% (SE 4.22) and 67% (SE 6.98), respectively, which was a statistically significant difference ($p < 0.001$). (B) The conduction latency in the control ($n = 10$) and MSC treated groups ($n = 10$) was 3.91 msec (SE 0.303) and 1.33 msec (SE 0.048), respectively ($p < 0.001$).

3.3. Electrophysiology study

The ratio of compound muscle action potential in the control ($n = 10$) and MSCs treated groups ($n = 10$) was 27.80% (SE 4.22%) and 67% (SE 6.98%), respectively, which was a statistically significant difference ($p < 0.001$) (Fig. 3A).The conduction latency in the control ($n = 10$) and the MSCs treated groups ($n = 10$) was 3.91 msec (SE 0.303) and 1.33 msec (SE 0.048), respectively, which was a statistically significant difference ($p < 0.001$) (Fig. 3B).

3.4. ELISA in retrieved nerves

The CNTF level in retrieved nerves without ($n = 6$) and with ($n = 6$) stem cell transplants were 1750.16 (SE 80.84) pg/30 μg protein and 2331.91 (SE 156.82) pg/30 μg protein, respectively, which was a statistically significant difference ($p = 0.008$) (Fig. 4A). The NT-3 level was 0.114 (SE 0.0659) pg/700 μg protein and 2.261 (SE 1.0782) pg/700 μg protein in the control ($n = 6$) and transplanted groups ($n = 6$), respectively ($p = 0.04$) (Fig. 4B).There was no statistical significance in the level of NGF between control ($n = 6$) and transplanted groups ($n = 6$) 23.25 [SE 1.59] pg/700 μg protein and 24.63 [SE 1.76] pg/700 μg protein, respectively). GDNF, BDNF, NT-4, and β-actin were not detected in the retrieved nerves.

3.5. Histology and immunocytochemistry in retrieved nerves

Ten days after the operation, the retrieved nerves showed visible stem cells (labeled with Hoechst 33342) around the crush site but no stem cells penetrating into the nerve (Fig. 5). No expression in transplanted stem cells of GFAP or S100 was observed (data not shown). Positive stains for NT-3 and CNTF were demonstrated in transplanted cells (Fig. 6). However, BDNF and GDNF were not detected in transplanted cells (data not shown).

Four weeks after stem cells transplantation, the comparative studies of crushed nerve injury treated with or without transplanted MSCs stained with H&E, S-100, GFAP, and Sirius red and Fast green are summarized in Table 1. Less vacuole formation was found in 8/10 paired studies, which is a statistically significant difference ($p = 0.007$) (Fig. 7A,B).The compound muscle action potential and SFI were also consistent with the histological findings (Fig. 7C–F). In the histological staining with S-100 and GFAP in the injured nerve, the MSCs transplant group demonstrated strong expression of S-100 and GFAP in 9/10 and 8/10 of comparative studies, respectively, which are statistically significant differences ($p = 0.002$ and $p = 0.007$, respectively) (Fig. 8A–D), but there was no positive staining of GFAP, S-100 or BrdU in the transplant cells (data no shown). The Sirius red and Fast green stain-

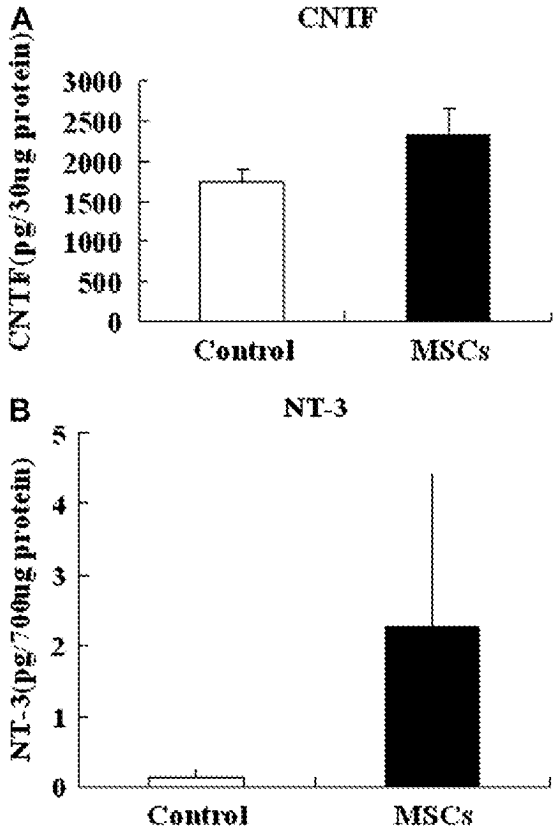

Fig. 4. The ELISAs for ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) in the retrieved nerve (1-cm long) were conducted at 10 days after the injury. (A) The CNTF level in retrieved nerves without ($n = 6$) and with ($n = 6$) stem cell transplants was 1750.16 (SE 80.84) pg/30 μg protein and 2331.91 (SE 156.82) pg/30 μg protein, respectively, which was a statistically significant difference ($p = 0.008$). (B) The NT-3 level was 0.114 (SE 0.0659) pg/700 μg protein and 2.261 (SE 1.0782) pg/700 μg protein in the control ($n = 6$) and transplanted groups ($n = 6$), respectively ($p = 0.04$).

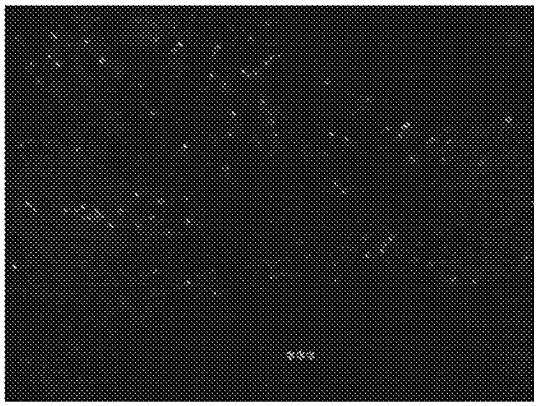

Fig. 5. Retrieved nerve and transplanted cells ($n = 4$) (labeled with Hoechst 33342) show visible stem cells around the injury site but no cell penetration into the injured nerve. *** indicates the sciatic nerve. This figure is available in colour at www.sciencedirect.com.

ing (8/10 comparative studies) displayed strong collagen staining and less fibrosis in the transplant groups, which was a statistically significant difference ($p = 0.007$) (Fig. 8E,F).

4. Discussion

4.1. Strategy of augmented regeneration in peripheral nerve injury

Nerve injury due to either deliberate or inadvertent surgical resection often leaves significant cosmetic and functional deficits in patients. Even though modern repair strategies have reached a point of maximal benefit, the outcomes are often disappointing and any further advance in peripheral nerve regeneration will need to incorporate more than just mechanical manipulation.[26] Peripheral nerve regeneration involves Schwann cells, neurotrophic factors, and inflammatory cells working synergistically to promote and maintain axon sprouting and myelination of axons.[9,27,28] The supplementation of neurotrophic factors or cell therapy with cultured Schwann cells, ensheathing olfactory cells, neuronal stem cells or induced Schwann cells has been fully investigated. It seemed for a time that these strategies had great potential for clinical application.[14–17] However, with the exception of bone marrow stem cells, feasibility and ethical concerns have been major obstacles to initiating mass clinical studies. On the other hand, amniotic fluid MSCs have the characteristic of secreting neurotrophic factors that increase neuron survival and involve no ethical concerns for clinical application.[18,20–22] Our previous study revealed that human amniotic fluid MSCs could promote nerve regeneration across a gap in the rat sciatic nerve but the mechanism was not understood.[23] In this study, we delivered amniotic fluid stem cells around a crushed nerve with preservation of epineurium intact to investigate the paracrine effect of transplanted stem cells on injured nerve tissue. The transplanted cells displayed a high level of expression of neurotrophic factors NT-3 and CNTF adjacent to the crush site and no transplanted cells penetrated the injured nerve. Collectively, secretion of neurotrophic factors is a possible mechanism of augmenting nerve regeneration by rat amniotic fluid MSCs.

4.2. Nerve regeneration related to neurotrophic factors

Neurotrophic factors, soluble substances produced by a peripheral target structure, are taken up by axons and then transported through retrograde axonal flow to the nerve cell body, where they exert trophic effects.[29] Some factors, including members of the neurotrophic family such as CNTF and NT-3, have been investigated for their ability to enhance the regenerative capacity of nerve tissue.[14,30] CNTF is expressed by Schwann cells of the peripheral nervous system and astrocytes of the CNS.[31] In intact peripheral nerves, CNTF is found predominantly in Schwann cells, suggesting a role in the maintenance of differentiated neurons.[32] After axotomy, the levels of CNTF mRNA fall dramatically in the distal stump and do not recover unless axons regenerate.[33] This raises the possibility that exogenous CNTF might play a role in peripheral nerve regener-

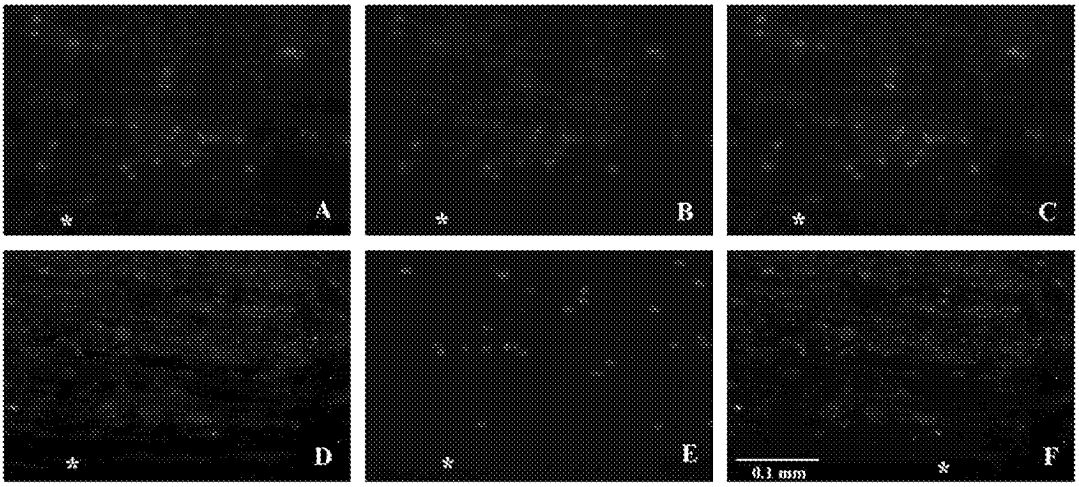

Fig. 6. Expression of neurotrophin-3 (NT-3) and ciliary neurotrophic factor (CNTF) is demonstrated in transplanted cells at 10 days after injury. (A) Positive CNTF; (B) Hoechst 33342 in transplanted cells; (C) fusion of CNTF and Hoechst 3342; (D) positive NT-3; (E) Hoechst 33342 in transplanted cells; (F) fusion of NT-3 and Hoechst 33342. * indicates the sciatic nerve. This figure is available in colour at www.sciencedirect.com.

Table 1
Comparative studies in crushed nerves treated with or without stem cell transplantation

	Vacuoles ($n = 10$)	S-100 ($n = 10$)	GFAP ($n = 10$)	Sirius red and Fast green ($n = 10$)
Positive	8	9	8	8
Negative	0	0	0	0
No difference	2	1	2	2
p value	0.007	0.002	0.007	0.002

GFAP, glial fibrillary acid protein; Positive vacuoles: less vacuole formation in H&E staining in treatment group; Negative vacuoles: more vacuole formation in H&E staining in treatment group; Positive S-100, GFAP, and Sirius red and Fast green: more expression of S-100, GFAP, and Sirius red and Fast green in treatment group; Negative in S-100, GFAP, and Sirius red and Fast green: less expression of S-100, GFAP, and Sirus red and Fast green in treatment group; No difference: no difference in vacuoles formation and intensity of S-100, GFAP, and Sirus red and Fast green between treatment and control group; A p-value less than 0.05 indicates statistical significance.

ation.[14] NT-3 mRNA has been detected clearly in the intact nerve of adult rats, with the level decreasing ninefold in the distal segmental by 6–12 h after nerve resection, then returning progressively to control levels at 2 weeks after the injury.[34] Hence, external application of neurotrophic factors such as CNTF or NT-3 by way of an infusion pump, or embedded in fibrin glue or by liganding to synthetic tubes could increase nerve regeneration in either crush or transection injury.[10–14] Amniotic fluid stem cells enable the secretion of multiple neurotrophic factors, including NGF, BDNF, and NT-3, which increase the survival and proliferation of neuronal stem cells *in vitro*.[22] To the best of our knowledge, there has been no report concerning the effect *in vivo* of neurotrophic factors secreted by rat amniotic fluid stem cells. In this study, we demonstrate nerve regeneration possibly due to the neurotrophic factors secreted by rat amniotic fluid MSCs. The RT-PCR assay in rat amniotic fluid stem cells showed strong elevation of the levels of NGF, NT-3 and BDNF, which is consistent with previous reports.[20–22] The transplanted stem cells at the site of the crushed nerve showed significant elevation of the levels of CNTF, and NT-3 in the ELISA study as compared with those without cell transplants. Therefore, the increased expression in these neurotrophic factors was due to additional transplanted stem cells resulting in augmented nerve regeneration.

In this study, there was an inconsistency between the expression of mRNA in amniotic fluid stem cell culture and ELISA of the retrieved nerves. The retrieved nerve and the transplanted stem cells were homogenised for the ELISA study. The levels of neurotrophic factors, such as BDNF and NGF, expressed by the transplanted cells could be diluted by the retrieved nerve itself. In addition, the decreased levels of CNTF and NT-3 as a response to nerve injury could explain the greater detection of neurotrophic factors secreted by the transplanted cells.[33,34]

4.3. Fate of transplanted amniotic fluid mesenchymal stem cells

Transplanted stem cells in the injured tissue could become part of the restored tissue, secrete trophic factors, mediate immunomodulation, or increase the repair ability of host tissue.[17,18,35] The ideal transplant cell should be able to self-renew, to give rise to anticipated cells, to repopulate a damaged region and to eliminate the need for immunosuppression.[36,37] The final destination of transplanted stem cells may allow them to be integrated into

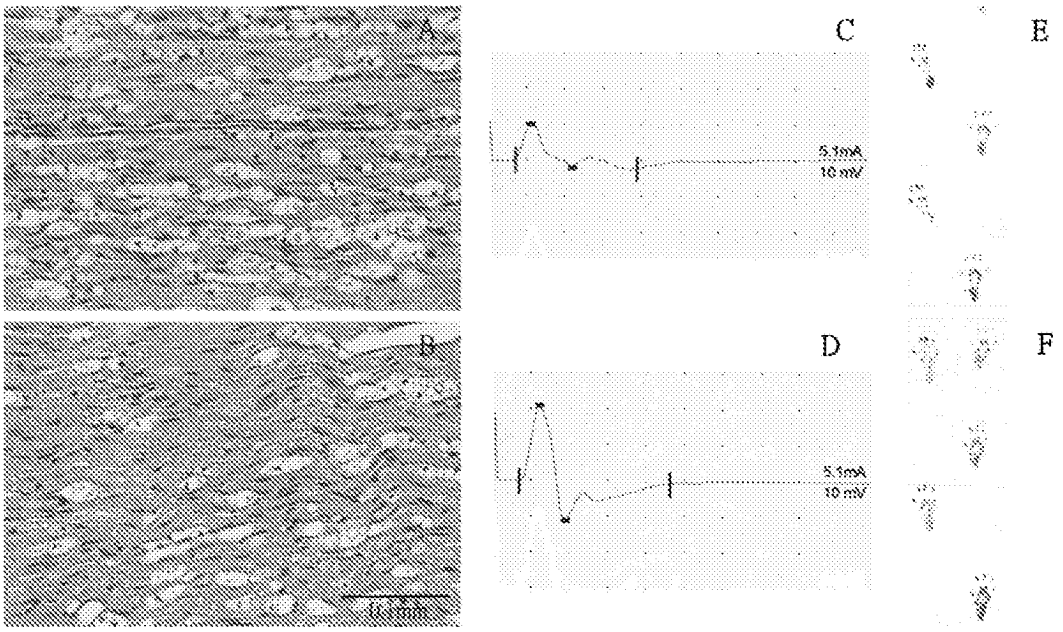

Fig. 7. The histology, electrophysiology and sciatic nerve function index (SFI) at 1 month after crush injury treated with ($n = 10$) or without ($n = 10$) stem cells. (A) H&E staining at the injury site without stem cell transplantation displays more vacuole formation. (B) The nerves retrieved from the stem cell transplant group show less vacuole formation. (C, E) The compound muscle action potential and SFI at the crush site without cell transplantation display low amplitude and low SFI scores. (D, F) The compound muscle action potential and SFI at the crush site with stem cells transplantation reveal high amplitude and high SFI scores. A, C and E indicate injured nerves without cell transplantation. B, D and F show injured nerves with stem cell transplantation.

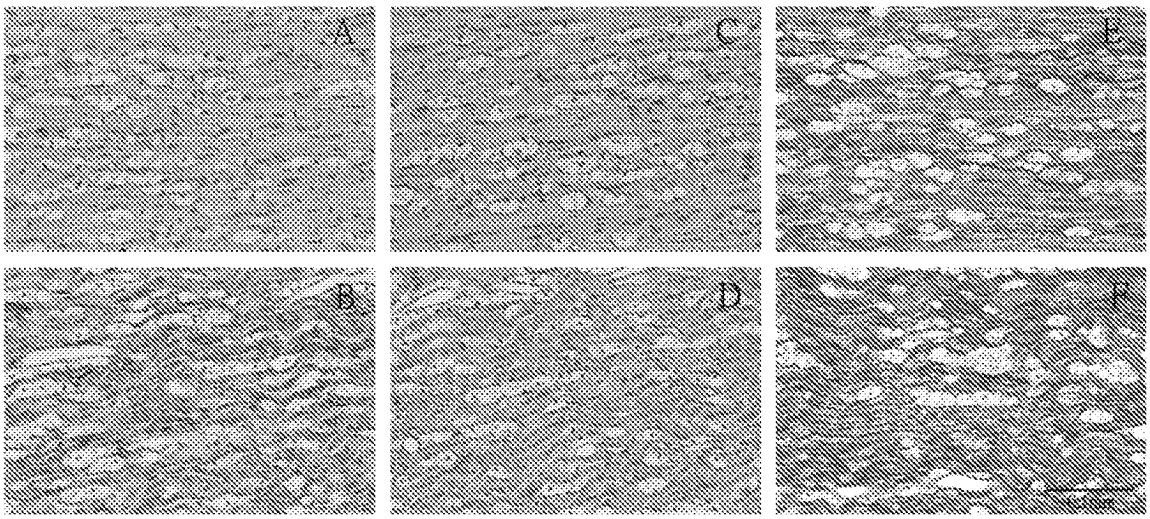

Fig. 8. Staining for S-100, glial fibrillary acid protein (GFAP), and Sirius red and Fast green was obtained in the retrieved nerve at 1 month after crush injury treated without ($n = 10$) or with ($n = 10$) stem cell transplantations. (A) and (B) Staining with S-100 shows strong expression in retrieved nerve treated with stem cells as compared with those without cell transplantation. (C, D) Staining with GFAP reveals strong expression at the injury site treated with stem cells. (E, F) Staining with Sirus red and Fast green shows less fibrosis and increased collagen formation at the injury site. A, C and E indicate injured nerves without cell transplantation. B, D and F show injured nerves with stem cell transplantation. This figure is available in colour at www.sciencedirect.com.

the host tissue or disappear due to unfavourable or inappropriate niches or rejection by immune cells. Amniotic fluid MSCs have shown low levels of expression of MHC class I, which is a major rejection mediator in non-hematogenous stem cells.[38] Even though MHC-I molecules were up-regulated by interferons (INFs), MHC-II molecules were absent from both undifferentiated and differentiated stem cells in the presence of INFs.[39] In our study, the transplanted amniotic fluid stem cells were observed only in a short-term follow-up, but there was no evidence of cells

surviving more than one month. Furthermore, there was no penetration of stem cells at the crush site into the regenerated nerve. Although there was no definite survival of transplanted cells, increased nerve regeneration was demonstrated by the increased motor function, the improvement in electrophysiology study results, and high levels of expression of S-100 and GFAP, which indicated increased myelination. Increased collagen synthesis and less fibrotic reaction were observed as well. The decreased fibrosis reaction was consistent with a previous report on the use of amniotic fluid in the repair of sciatic nerve injury, which showed less fibrosis. This phenomenon was suggested to be due to the secretion of neurotrophic factors.[40] Although the supportive effect of the transplanted cells was demonstrated, the question of whether cell death was due to an immune reaction or to inappropriate niches was not answered. This phenomenon needs to be investigated further.

Murakami et al. reported that neuronal stem cells that impacted into a collagen tube supported nerve regeneration across a nerve gap and could differentiate into Schwann-like cells and survive for up to 2 months.[15] In contrast, Aquino et al. reported that boundary cap neuronal crest stem cells did not differentiate into Schwann cells *in vivo* due to insufficient signal transduction in the milieu of the peripheral nerve.[41] Our study revealed that no transplanted stem cell differentiated into Schwann or Schwann-like cells, which was consistent with the result of the study by Aquino et al. The short life-span and lack of potential of differentiation in transplanted cells inter that pre-transplant induction may be another option in stem cell transplantation.

5. Further studies

Increased nerve regeneration at short-term follow-up was demonstrated in this study with the secretion of neurotrophic factors by the transplanted stem cells. However, long-term follow-up studies are needed. The reason for only short-term survival of transplanted cells was not investigated fully in this study. Further studies of the immune reaction, including staining for macrophages, B cells, and MHC-I need to be conducted to provide a detailed explanation of limited survival in stem cells. Furthermore, filling a collagen tube with amniotic fluid stem cells as a substitute for a nerve graft in nerve repair requires additional study for possible clinical applications.

6. Conclusion

This study demonstrated that nerve regeneration is augmented possibly by neurotrophic factors secreted by rat amniotic fluid MSCs. However, maintenance and differentiation of transplanted stem cells in the injured site needs further investigation. This study paves the way for clinical application in peripheral nerve injury, as there is no ethical barrier to obtaining human amniotic fluid for harvest of the MSCs.

Acknowledgement

This study is supported from Natïonal Science Council, R.O.C.

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