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Original Article

Repair of spinal cord injury in rats by subarachnoid transplantation of genetically modified schwann cells

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Abstract: Objective: We discussed the repair of spinal cord injury (SCI) in rats by subarachnoid transplantation of Schwann cells (SCs) transfected with bcl-2 gene. Method: Rat spinal cord injury (SCI) were cultured in vitro and transfected with B-cell lymphoma 2 gene (bcl-2 gene) using Ad-EGFP (Enhanced Green Fluorescent Protein) vector. Three groups were set up: control group, negative transfection group and bcl-2 gene transfection group. The bcl-2 gene expression at 3 d and 14 d after transfection was detected by using Western Blot. Among 83 adult female SD (Sprague Dawley) rats, modeling was successful in 72 rats. These rats were randomly divided into control group, Schwann cells (SCs) group and bcl-2-Schwann cells (SCs) group, with 24 rats in each group. Schwann cells (SCs) model was built by modified Allen's method. Basso Beattie Bresnahan (BBB) scoring and inclined plate test were performed before modeling and at 1 d, 3 d, 1 week, 2 weeks, 3 weeks and 4 weeks after modeling, respectively. The gene and protein expressions of glial fibrillary acidic protein (GFAP) and neurofilament heavy (NF-200, neurofilament-200) in the region of spinal cord injury (SCI) were detected at 7 d after modeling by using RT-PCR and Western Blot, respectively; the nerve cell apoptosis was detected by TUNEL assay. The tissues were harvested at 4 weeks after modeling and made into pathological sections for (HE, hematoxylin-eosin staining) and fluorescence microscopy of survival and distribution of EGFP (Enhanced Green Fluorescent Protein)-labeled Schwann cells (SCs). HRP (Horseradish Peroxidase) retrograde tracing was adopted to visualize the nerve repair. The electrophysiology of the nerve injury was observed by somatosensory evoked potential (SEP) and motor evoked potential (MEP) monitoring. Results: Western Blot showed that the bcl-2 gene transfection of Schwann cells (SCs) using Ad-EGFP (Enhanced Green Fluorescent Protein) vector led to stable expression of bcl-2. The bcl-2-Schwann cells (SCs) group had improved lower limb motor function compared with the Schwann cells (SCs) group, and the lower limb motor function was the worst in the control group. At 72 h after modeling, the number of apoptotic cells in the bcl-2-Schwann cells (SCs) group was significantly lower than that of the other two groups ($P<0.05$). At 7 d, the gene and protein expressions of glial fibrillary acidic protein (GFAP) and (NF-200, neurofilament-200) in the bcl-2-Schwann cells (SCs) group were significantly upregulated compared with the control group and the Schwann cells (SCs) group ($P<0.05$). At 4 weeks, (HE, hematoxylin-eosin staining) revealed the spinal cord defect and the forming of cavity in the spinal cord in the control group with the absence of neural axis. A few neural axes were seen in the injury region of the Schwann cells (SCs) group with cavity of smaller size. More neural axes were observed in the bcl-2-Schwann cells (SCs) group and no cavity was found. Moreover, the bcl-2-Schwann cells (SCs)group had greater number of EGFP-positive cells than the Schwann cells (SCs) group, and the control group had the least positive cells, showing significant difference ($P<0.05$). The bcl-2-Schwann cells (SCs) group had the largest number of HRP-positive nerve fibers, while the control group had the least, and the differences between the groups were of statistical significance ($P<0.05$). At 4 weeks after modeling, the SEP and MEP latencies were significantly shorter in the bcl-2-SCs group, and the Schwann cells (SCs) group had the second shortest SEP and MEP latencies ($P<0.05$); the wave amplitude was significantly higher in the bcl-2-Schwann cells (SCs) group than in the Schwann cells (SCs) group and the control group ($P<0.05$). Conclusion: Subarachnoid transplantation of genetically modified Schwann cells (SCs) promoted the regeneration of the injured synapses. The gene and protein expressions of GFAP and NF-200 in the injury region were upregulated in rats, accompanied by decreased apoptosis of the nerve cells and improved limb motor function and electrophysiological result.

Keywords: Subarachnoid, bcl-2 gene, spinal cord injury, schwann cell

Introduction

Spinal cord injury (SCI) is very difficult to repair clinically in spite of many techniques trialed, including promotion of axon regeneration, reduction of inhibitory factors, peripheral nerve transplantation and reflex arc reconstruction [1]. The major reasons for difficult nerve repair include the loss of nerve cells in the spinal cord, reduced secretion of neurotrophic factors and the presence of factors inhibitory to axon regeneration [2]. Secondary injuries usually ensue after Spinal cord injury (SCI), also leading to poor nerve regeneration. Nerve cell apoptosis is one of the most devastating secondary injuries [2]. Schwann cells (SCs) play an important role in the repair of injured central nervous system [3, 4] by secreting neurotrophic factors and removing the scar tissues and local necrotic tissues [5]. The role of Schwann cells (SCs) in the repair of Spinal cord injury (SCI) has been particularly studied in recent years [6, 7]. Programmed cell death is another factor affecting the repair of Spinal cord injury (SCI). Pro-apoptotic genes and anti-apoptotic genes are two major regulators of cell apoptosis [8]. High expression of bcl-2 can prevent cell apoptosis [8, 9], such as the liver cancer cells [10]. Along with the development in genetic engineering, gene therapies are now more commonly applied to the treatment of Spinal cord injury (SCI). The key to the success lies in the introduction of relevant genes into the host cells and the high, stable expression of the introduced genes [2]. However, the feasibility of bcl-2 gene transfection of Schwann cells (SCs) to the treatment of Spinal cord injury (SCI) has not been fully elucidated.

Intraspinal transplantation of the cells is more common clinically [11] with the insertion of the needle directly into the spinal cord. But this procedure requires the opening of the vertebral plates, causing secondary injury to the spinal cord and the release of inflammatory mediators, which will reduce the survival rate of seed cells and then impede nerve repair [12]. Besides, intraspinal transplantation is not fit for multi-lesion treatment [13] or repeated surgeries. Subarachnoid transplantation can overcome this defect and exhibits higher applicability.

We performed subarachnoid transplantation of Schwann cells (SCs) transfected with bcl-2 gene. The Basso Beattie Bresnahan (BBB) scores were obtained to characterize the outcome of nerve repair. The gene and protein expressions of glial fibrillary acidic protein (GFAP) and neurofilament heavy (NF-200) in the injury region were detected along with cell apoptosis and electrophysiology of the injured nerves. Based on these detections, we discussed the treatment effect of subarachnoid transplantation of genetically modified Schwann cells (SCs) in Spinal cord injury (SCI).

Materials and method

The randomized controlled animal experiment was designed

Experimental animals: Two healthy male newborn Wistar rats were provided by Laboratory Animal Center of Neurosurgical Institute of Zhengzhou University. In the meantime, 86 healthy female SD (Sprague Dawley) rats (provided by Laboratory Animal Center of Zhengzhou University) weighing 250-300 g were randomly divided into the following groups: control group, negative transfection group (transplantation of Schwann cells (SCs)), and bcl-2 gene transfection group (transplantation of Schwann cells (SCs) transfected with bcl-2 gene). The rats were reared at the constant temperature of 23-27°C and 50-70% humidity. The rats were allowed free access to conventional granular feed and sterilized water, and they were fasted from food and water at 1 d before experiment.

Reagents and equipments: D-Hank's solution (Hyclone, USA), trypsin (Sigma, USA), collagenase (type XI, Sigma), fetal bovine serum (Gibco, USA), DMEM (Hyclone), bacterial incubator (Heraeus, Germany), poly-L-lysine (Sigma), cytarabine (Sigma), 100 U/ml penicillin (Hyclone), streptomycin (Hyclone), forskolin and bFGF (Sigma); disodium hydrogen phosphate, monosodium phosphate, RIPA lysis buffer, and BCA protein assay kit (Beyotime), Bcl-2 IgG antibody (Santa Cruz, USA), biotinylated goat anti-rabbit IgG (1:500, CUSABIO Biotech Co., Ltd.), microtome (Leica CM 1900, Germany), mouse anti-NF20 IgG1 (Santa Cruz, USA), rabbit anti-GFAP IgG (Santa Cruz, USA), acid guanidinium thiocyanate-phenol-chloroform (Sigma), (electromyography, EMG) & evoked potential response unit equipment (Keypoint).

Method

In vitro culture, purification and identification of Schwann cells (SCs): Referring to literature [2], the sciatic nerve was detached under sterile condition with the assistance of microscope. Schwann cells (SCs) were digested with 0.25% trypsin/0.2% collagenase for 40 min and centrifuged at 10004/min for 5 min. Then the cells were added with DMEM/F12 medium (containing 10% FBS, fetal bovine serum) and cultured at 37°C in a 5% CO_2 incubator for 30 min. The fibroblasts were removed by differential adherence, and after 24 h, 100 μL l cytarabine (Ara-C, 10-5 mmol/L) was added to further eradicate the fibroblasts. SCs of the fourth generation were grown on the coverslip for 48 h, washed with PBS three times and fixed in 4% paraformaldehyde (pH=7.4) at room temperature for 20 min. The cells were washed again three times in PBS and cultured with primary antibodies to (myelin basic protein, MBP) at 4°C overnight using a wet box. After washing with PBS three times, secondary antibodies were added to culture the cells at 37°C in an incubator for 2 h. Next the cells were stained with DAPI (4',6-diamidino-2-phenylindole) for 10 min and washed with PBS three times. The cells were sealed with mounting medium. Following in vitro culture, the Schwann cells (SCs) were transfected with bcl-2 gene using Ad- EGFP (Enhanced Green Fluorescent Protein) vector. Three groups were set up: control group, negative transfection group (SCs group) and bcl-2 gene transfection group (bcl-2-SCs group). At 2 days before transplantation, the log phase Schwann cells (SCs) were harvested, centrifuged and added with the culture medium. The cells were repeatedly blown to form a single-cell suspension. The cells were counted with cell density adjusted to $1*10^6$/ml. In the culture flask the stocking solution containing bcl-2-Ad-EGFP particles was added with multiplicity of infection (MOI) of 200PFU per cell. The solution was cultured in an incubator for 48 h and centrifuged with supernatant discarded. Then the cells were blown again to form a single-cell suspension and counted. With the cell density adjusted to $2*10^7$/ml, the cells were placed on ice. In the meantime, Schwann cells (SCs) suspension ($2*10^5$/ul) was prepared and placed on ice. Trypan blue staining was performed over the remaining cell suspension after transplantation so as to determine the cell survival rate.

Detection of bcl-2 protein expression at 3 d and 14 d after transfection by Western blot: The cells were harvested into the culture flask at 3 d and 14 d after transfection. After lysis, total protein extraction was performed, and the protein was assayed by plotting the standard curve. The proteins were then analyzed by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) electrophoresis and transferred to PVDF (Polyvinylidene Fluoride) membranes. The primary antibody was rabbit anti-bcl-2 IgG and the secondary antibody was biotinylated goat anti-rabbit IgG. The X-ray films were scanned and analyzed by Quantity One software. The optical density of each band was measured and expressed as absolute optical density [14].

Establishment of rat spinal cord injury (SCI) model by modified Allen's method: The rats of all three groups were anesthetized by intraperitoneal injection of 10% (v/v) chloral hydrate, disinfected and immobilized in prone position. With T_{11} as the center, a midline incision was made on the back to expose a region of 30 mm×4 mm in the spinal cord, where a 3 mm^2 knock pad was placed. The dura mater was struck by Allen's falling strike method using Allen's tube with the force of 40 g×1 cm. The hindlimbs of the rats would twitch with flicking of tail and then the whole body was relaxed. The position at 1.5 cm at the lower end of the dura mater was injured by striking, and the dura mater was punctured. A silicone tube connected with a guide wire at one end was inserted into the arachnoid membrane along the dura mater in the middle and upper part of the injured region. After that, the guide wire was withdrawn, and the modeling was considered successful when the cerebrospinal fluid flowed into the silicone tube. The tube was tightly fixed and sealed by burning with flame, and the incision was sutured layer by layer. During surgery, adrenaline was administered and the incision was washed with normal saline containing gentamicin. Within 4 weeks after surgery, urination was forced in rats three times daily and the rats were cleaned [15, 16]. For the rats in the Schwann cells (SCs) group and the bcl-2-SCs group, 10 μl of Schwann cells (SCs) transfected and not transfected with bcl-2 gene was injected into the injured dura mater, respectively. For the control group, 10 μl of DMEM (Dulbecco Modified Eagle Medium) was injected [17].

Motor function evaluation: Basso Beattie Bresnahan (BBB) scores were obtained at different time points after Spinal cord injury (SCI) in three groups. Motor functions were evaluated by inclined plate test. Motor function of the hindlimbs in rats was divided into 22 grades [10], corresponding to Basso Beattie Bresnahan (BBB) scores of 0 to 21 points. 0 point indicated complete paralysis of the hindlimbs; 21 points indicated normal function. The observation indicators were number of movements of joint, load and scope of movement, and coordination between forelimbs, hindlimbs, front paws, hind paws and tail. For inclined plate test, the rats were placed on the smooth plate with body axis being perpendicular to the vertical axis of the plate. The angle increment was 5° every time. The motor functions of rats were considered as effective if the rats stayed on the plate for 5 s under the maximum angle of inclination [11].

(HE, hematoxylin-eosin) staining of pathological sections at 4 weeks after modeling and fluorescence microscopy of EGFP (nhanced green fluorescent protein)-labeled SCs: Three rats were randomly selected from each group at 4 weeks after surgery and received intraperitoneal injection of 10% chloral hydrate. Myocardial perfusion was performed using 80 ml of normal saline. After fixed in 200 ml of 4% paraformaldehyde, the spinal cord tissue segments were cut transversely into thickness of 8 μm. After treatment with *EGFP* (nhanced Green Fluorescent Protein), the sections were adhered to gelatin-embedded slide and preserved at -20°C [18]. (HE, hematoxylin-eosin) staining was performed as follows: xylene was added dropwise twice to immerse the cells for 5 min, followed by immersion with anhydrous enthanol for 3 min, 95% ethanol for 3 min, 80% ethanol for 10 min, and distilled water for 1 min. Hematoxylin staining was performed for 20 min, and the slide was washed with distilled water and 1% hydrochloric acid in ethanol for 3 s, respectively. This was followed by washing with water for 30 s, Scott's solution for 30 s, running water for 15 min and distilled water for 2 s. After staining with 0.5% eosin for 3 min, the slide was washed with distilled water for 2 s, and immersed in 80% ethanol for 2 s, 95% ethanol for 8 s, anhydrous ethanol for 10 min, carboxylol for 5 min, xylene for 2 min, three times for the removal of carbolic acid. After complete dehydration and transparentization, the slide was sealed with neutral balsam. The distribution of Schwann cells (SCs) and the forming of cavity in the spinal cord were observed under the fluorescence microscope (×400). The images were stored and analyzed with Image-Pro Plus 6.0 software. The positive area was compared [18].

Observation of nerve repair by HRP (horseradish peroxidase) retrograde tracing: Before surgery, 3 rats were randomly selected from each group and anesthetized by intraperitoneal injection of 10% chloral hydrate. The rats were immobilized in the back. The hairs on the hind legs were removed and the skin was disinfected with iodine and alcohol. An incision was made in the hind leg. The muscles were detached and the sciatic nerve was gently elevated with ophthalmic forceps. At 30 h after injection of 0.5 μl of 30% *HRP* (Horseradish Peroxidase), the spinal cord tissue was harvested near the upper end of the transverse injury. The tissue was fixed in 4% paraformaldehyde for 10 h and then refrigerated. HRP-DAB-nickel ammonium sulfate was added for enhanced color development. Under ordinary light microscope, the IMP-positive cells over the transverse section of the spinal cord were counted. The positive cells were counted by randomly selecting five slides at each time point in each group [19].

Detection of gene and protein expression of GFAP (glial fibrillary acidic portein) and NF-200 (NF-200, neurofilament-200) by RT-PCR and Western blot, respectively: Three rats were selected from each group at 7 d post-surgery and sacrificed by cervical dislocation. Spinal cord at segment T_8 was harvested and cryopreserved. For RT-PCR, 15 mg of the tissue was taken and added with 2 ml Trizol. The cells were blown, mixed well and left to stand for 5 min. Then 200 μl of chloroform was added with oscillation for 15 s, and the solution was left to stand for 2-3 min. Refrigerated centrifugation was carried out at 10000 r/min for 20 min. The supernatant was drawn into the centrifuge tube, added with 0.5 ml isopropyl alcohol and left to stand for 10 min. Refrigerated centrifugation was performed again at 10000 r/min for 20 min, with supernatant discarded. With the addition of 75% ethanol, the cells were blown, mixed well and subjected to refrigerated centrifugation at 5000 r/min for 10 min. The supernatant was discarded, and the precipitate was

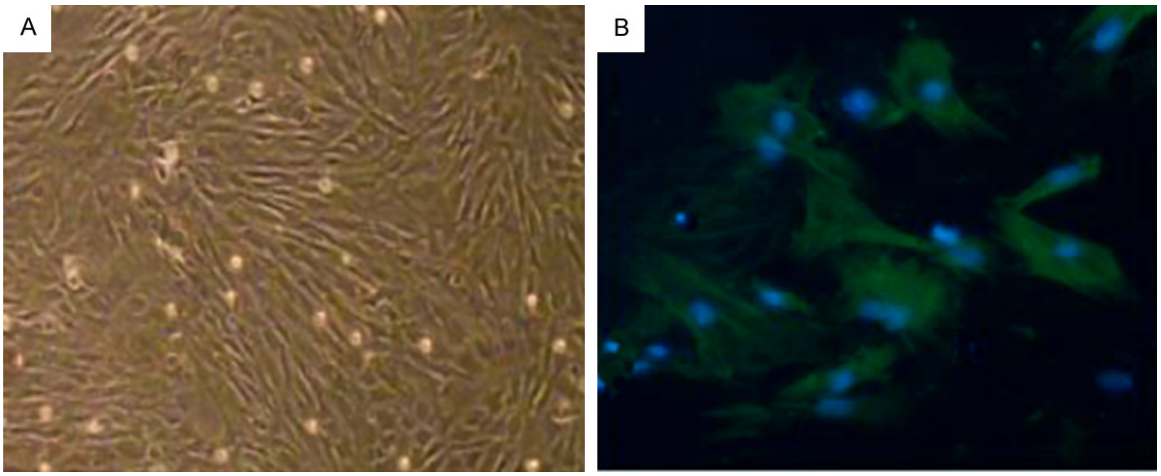

Figure 1. A: Under the inverted phase contrast microscope, SCs were elongated, spindle shaped and in tight arrangement (×40); B: Immunofluorescence staining of Schwann cells (SCs) with (myelin basic protein, *MBP*) (×40).

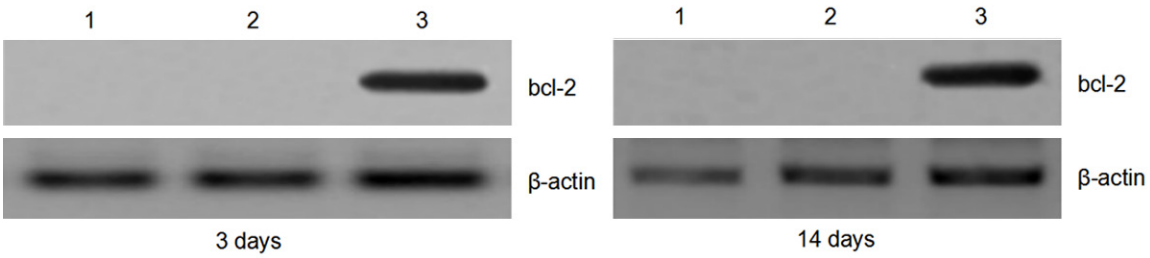

Figure 2. The bcl-2 protein can be stably expressed in Schwann cells in 3 days and 14 days after transfection. Note: 1. control group, 2. negative transfection group, 3. bcl-2 transfection group.

dried and dissolved in 50 μl DEPC (diethyl pyrocarbonate) solution [20]. Upstream primer of *GFAP* (glial fibrillary acidic portein): 5'-TAATGACTATCGCCGCCAACTG-3', downstream primer 5'-TTCGCCCTCCAGCAATTTCC-3'; the length of the amplified fragment was 274 bp. Forward primer of NF-200: 5'-CCGGGAGATCGTGATGAAGT-3', reverse primer 5'-ATCCCAGCCTCCGTTATCCT-3'; the length of the amplified fragment was 5'-ATCCCAGCCTCCGTTATCCT-3'. The upstream and downstream primers of β-actin were 5'-AVGGTCAGGTCAT-CACTATCG-3' and 5'-AGCACTGTGTTGGCATAGAGG-3', respectively. The conditions of RT-PCR were: denaturation at 94°C for 15 s, 55°C for 30 s, 72°C for 30 s, 30 cycles, final extension at 72°C for 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The gel images were analyzed by the image analysis system [20].

The cells were lysed in RIPA buffer and the spinal cord specimen was cryopreserved. Total protein was extracted and assayed by plotting the standard curve using BCA protein assay kit. The proteins were subjected to 8% SDS-PAGE electrophoresis and transferred to the membrane. The primary antibody was rabbit anti-GFAP IgG (1:1000) and mouse anti-NF20 IgG1 (1:400); the secondary antibody was biotinylated goat anti-rabbit IgG (1:500). The optical density of each band was measured and expressed as absolute optical density [14].

Detection of cell apoptosis by TUNEL assay: At 1 week after surgery, 3 rats were randomly selected from each group and anesthetized by intraperitoneal injection of 10% chloral hydrate. The spinal cord at segment T_8 was harvested and acid guanidinium thiocyanate-phenol-chloroform extraction was performed to obtain RNA. Dot blot hybridization was performed using digoxigenin-labeled probe according to the manufacturer's instruction for the dUTP Mix. GS-9000 scanner was used for image analysis. Apoptotic index (AI) was calcu-

lated as the ratio of the number of apoptotic nuclei to the total number of nuclei based on the number of apoptotic motor neurons in the ventral horn of the spinal cord [21].

Somatosensory evoked potential (SEP) and motor evoked potential (MEP) detection at 4 weeks after surgery: After 4 weeks after surgery, 3 rats were randomly selected from each group for the detection of SEP and MEP. SEP recording electrode was placed between the two ears, with reference electrode over the forehead and stimulating electrode on the ankle of rats and the on the back of mice. The intensity of the electric current was determined when the mild movement of the fingers was evoked. For MEP monitoring, an incision was made near the sagittal suture to expose the dura mater. The stimulating electrode was positioned outside the dura mater, and the reference electron beneath the scalp, with an interval of 1 cm. The recording electrode was inserted into the gastrocnemius muscle, and the grounding electrode was connected to the tail. The direct current square wave pulse was administered with the intensity of 10 mA, wave width of 0.1 ms, voltage of 2 μV and frequency of 5 Hz. The collected signals were amplified, filtered, superimposed, averaged and analyzed by the computer [17].

Statistical analysis

Statistical analysis was performed using SPSS 11 software. All measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± s). Analysis of variance and t-test were used to compare the differences between the groups. $P<0.05$ indicated significant difference.

Results

In vitro culture, identification of Schwann cells (SCs)

Under the inverted phase contrast microscope: The cells grew to confluence in the flask after 1 week. Most were Schwann cells (SCs), and a few were fibroblasts. After purification, Schwann cells (SCs) accounted for over 96%. The cells were narrow, elongated and spindle-shaped, with small nuclei and a large amount of secretions near the cells (**Figure 1A**). (Myelin basic protein, MBP) fluorescence: green fluorescence was observed from the cell body and the protuberances of Schwann cells (SCs). The nuclei of Schwann cells (SCs) and fibroblasts emitted blue fluorescence after DAPI staining. However, the cytoplasm of the fibroblasts was not stained, which was transparent. The percentage of Schwann cells (SCs) can be roughly estimated from the figure. As shown in **Figure 1B**, the Schwann cells (SCs) were narrow, elongated and spindle-shaped, with small nuclei.

Bcl-2 gene was successfully introduced into the SCs

At 3 d and 14 d after bcl-2 gene transfection of Schwann cells (SCs) using Ad-EGFP vector, the bcl-2 protein was stably expressed. However, no bcl-2 protein was expressed in the control group and the negative transfection group. This indicated that the bcl-2 gene was successfully introduced into the Schwann cells (SCs) (**Figure 2**).

Lower limb function recovery following spinal cord injury

Before surgery, the rats of the three groups did not differ significantly in BBB scores or inclined plate test scores ($P>0.05$). At 2-4 weeks after transplantation, the two scores were improved significantly in the SCs group and the bcl-2-SCs group compared with the control group ($P<0.05$). Moreover, the increase of the scores was greater in the bcl-2-SCs group at 2-4 weeks after modeling than in the Schwann cells (SCs) group ($P<0.05$) (**Table 1**).

HE staining and fluorescence microscopy

(HE, hematoxylin-eosin staining) staining showed that the spinal cord tissue was fractured in the injury site in the control group at 4 weeks after surgery. The scars were observed with disordered structure and the forming of cavity (**Figure 3A**). In the Schwann cells (SCs) group, typical neuron-like morphology was observed at the transplantation site, with cavities smaller than that of the control group but larger than that of the bcl-2-SCs group (**Figure 3B**). The bcl-2-SCs group showed typical neuron-like morphology and the disappearance of cavity (**Figure 3C**). Green fluorescence was observed from the EGFP-positive cells scatteredly distributed on the slides of Schwann cells (SCs) group and bcl-2-SCs group (**Figure 3D-F**). There were 0 ± 0.00 positive cells per high-power field in the control group, 12.84 ± 3.42 positive cells per high-power field in the SCs group

Table 1. Basso Beattie Bresnahan (BBB) scores and inclined plate test scores at each time point in the three groups (x ± s)

Group	Cases	Before modeling	After modeling					
			1 d	3 d	1 W	2 W	3 W	4 W
BBB score								
Control group	5	21.00 ± 0.00	0.00 ± 0.00	1.44 ± 0.21	2.75 ± 0.89	8.35 ± 1.56#	11.24 ± 2.23#	13.64 ± 1.20#
Schwann cells (SCs) group	5	21.00 ± 0.00	0.00 ± 0.00	2.67 ± 0.26	4.58 ± 0.32#	10.6 ± 2.62#	12.66 ± 1.98#	15.66 ± 1.18#
Bcl-2-SCs group	5	21.00 ± 0.00	0.00 ± 0.00	3.85 ± 0.39	6.98 ± 0.43#,▲	12.82 ± 2.63#,▲	15.62 ± 2.24#,▲	17.56 ± 0.35,▲
Inclined plate test								
Control group	5	42.41 ± 2.27	15.65 ± 2.34	16.8 ± 2.36	20.22 ± 3.46	23.42 ± 4.26	26.40 ± 2.71	28.33 ± 2.47
Schwann cells (SCs) group	5	42.48 ± 3.70	15.72 ± 2.63	19.52 ± 3.12#	25.16 ± 4.80#	30.38 ± 6.34	32.81 ± 3.14#	36.58 ± 2.40#
Bcl-2-SCs group	5	42.50 ± 3.31	15.80 ± 3.22	23.75 ± 3.42#,▲	29.24 ± 6.20#,▲	33.71 ± 5.15#,▲	36.58 ± 4.32▲	40.32 ± 3.39▲

Note: #P<0.05 compared with the control group; ▲P<0.05 compared with Schwann cells (SCs) group.

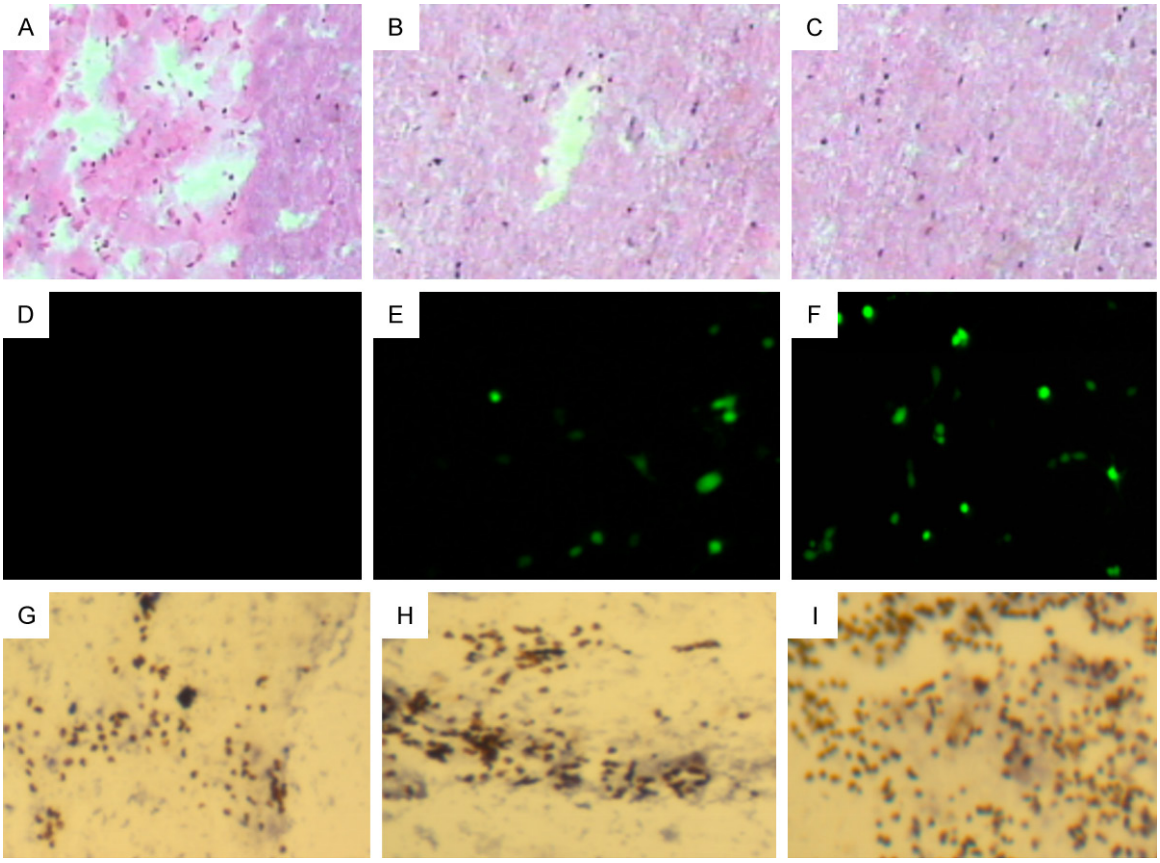

Figure 3. A: HE staining in the control group at 4 weeks (×40); B: HE staining in the SCs group at 4 weeks (×40); C: HE staining in the bcl-2-SCs group at 4 weeks (×40); D: Green fluorescence from EGFP-positive cells in the control group at 4 weeks (×200); E: Green fluorescence from EGFP-positive cells in the SCs group at 4 weeks (×200); F: Green fluorescence from EGFP-positive cells in the bcl-2-SCs group at 4 weeks (×200); G: HRP-positive nerve fibers in the control group at 4 weeks (×200); H: HRP-positive nerve fibers in the SCs group at 4 weeks (×200); I: HRP-positive nerve fibers in the bcl-2-SCs group at 4 weeks (×200).

and 24.62 ± 4.54 positive cells per high-power field in the bcl-2-SCs group, indicating significant differences (P<0.01). At 3 d after injection of *HRP* (Horseradish Peroxidase), into the lumbar enlargement in the control group, retrograde transport of *HRP* (Horseradish Peroxidase), was observed. A few *HRP* (Horseradish Peroxidase), -labeled nerve fibers were seen above segment T_8, as shown in **Figure 3G** (8.26 ± 2.62). More *HRP* (Horseradish Peroxidase), -positive nerve fibers were seen in the Schwann cells (SCs) group than in the control group, but the number of these nerve fibers was still smaller than that of the bcl-2-SCs group (**Figure 3H**, 17.62 ± 3.20). More HRP-positive nerve fibers were seen in the bcl-2-SCs group (**Figure 3I**, 26.34 ± 4.32). The differences in the number of HRP-positive nerve fibers were statistically significant between the bcl-2-SCs group and the control group at 4 weeks after surgery (P<0.01).

GFAP and NF-200 expression in the injured regions of SCI rats after transplantation

Both gene and protein expressions of glial fibrillary acidic protein (GFAP) and (NF-200, neurofilament-200) were upregulated significantly in the bcl-2-SCs group at 7 d after surgery compared with the control group and the Schwann cells (SCs) group (P<0.05). Moreover, the expressions were increased significantly in the Schwann cells (SCs) group compared with the control group (P<0.05) (**Figure 4**).

Apoptosis in the injured region of SCI rats after transfection

Specific brownish yellow particles were seen in the apoptotic nuclei of the neurons. The apoptotic cells were distributed in scatter over the entire injury site under the light microscope. Apoptotic cells were also seen near the edge of

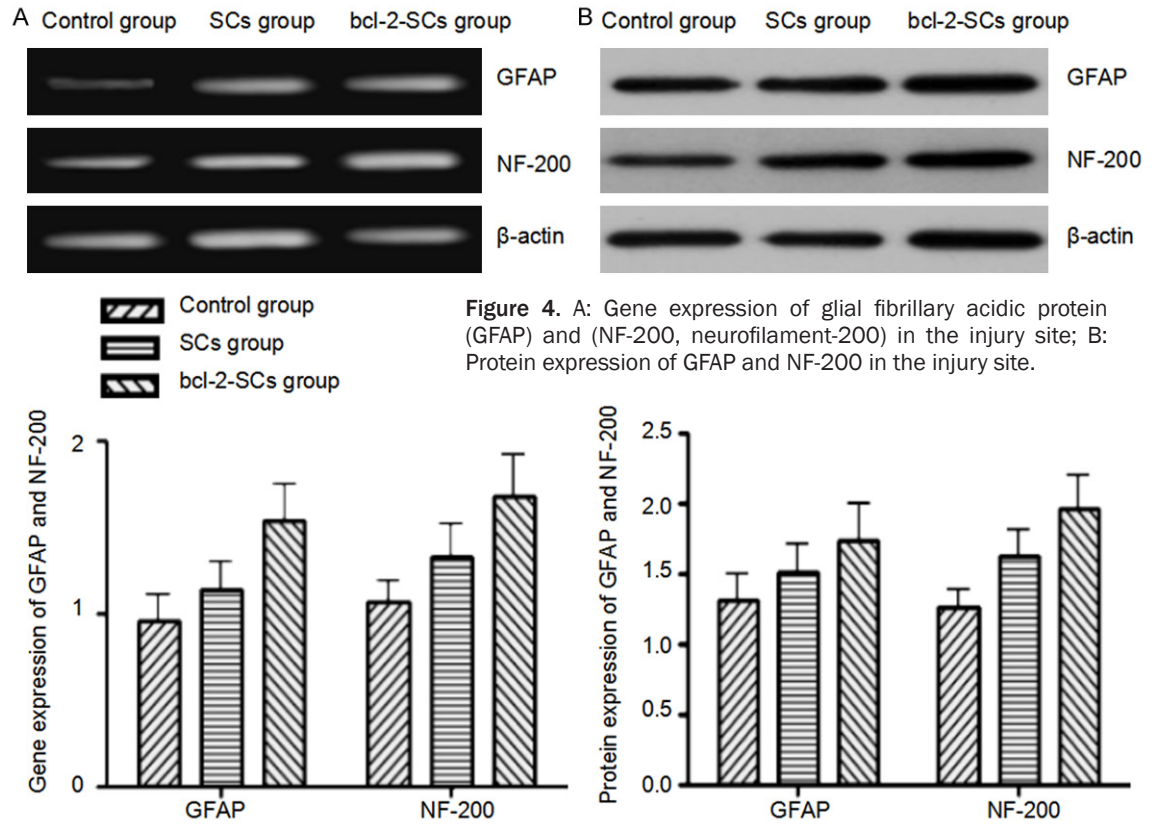

Figure 4. A: Gene expression of glial fibrillary acidic protein (GFAP) and (NF-200, neurofilament-200) in the injury site; B: Protein expression of GFAP and NF-200 in the injury site.

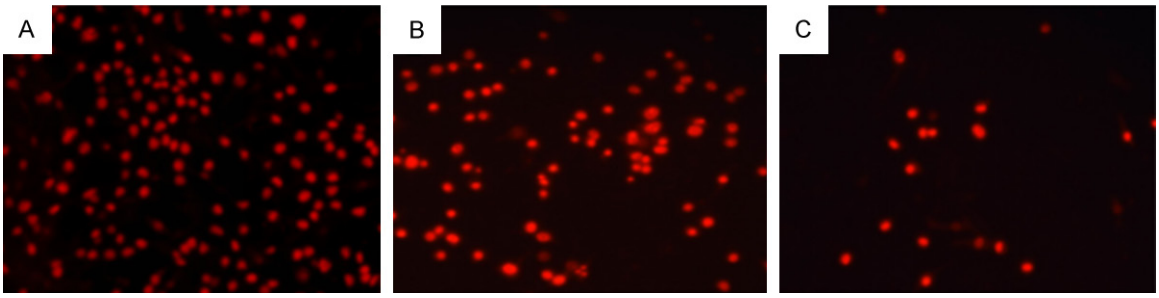

Figure 5. A: Number of apoptotic cells in the control group (29.73 ± 3.74); B: Number of apoptotic cells in the Schwann cells (SCs) group (20.48 ± 3.51); C: Number of apoptotic cells in the bcl-2-SCs group (8.72 ± 2.21).

the injury site. According to TUNEL assay, the number of apoptotic cells in the Schwann cells (SCs) group (20.48 ± 3.51) was obviously smaller than that in the control group (29.73 ± 3.74) ($P<0.05$); the number of apoptotic cells was the smallest in the bcl-2-SCs group (8.72 ± 2.21), as shown in **Figure 5**.

Changes in somatosensory evoked potentials and motor evoked potentials in the rats after transfection

No waveform was detected in all groups upon somatosensory evoked potential (SEP) and motor evoked potential (MEP) monitoring immediately after modeling. At 4 weeks after surgery, somatosensory evoked potential (SEP) and motor evoked potential (MEP) waveforms were slightly restored in the control group. In contrast, the somatosensory evoked potential (SEP) and motor evoked potential (MEP) waveforms were obviously restored in the bcl-2-SCs group with increased wave amplitude. The somatosensory evoked potential (SEP) and motor evoked potential (MEP) latencies and the wave amplitudes in each group are given in **Table 2** and **Figure 6**. The SCs group and the control group showed significant differences in

Table 2. Somatosensory evoked potential (SEP) and motor evoked potential (MEP) monitoring of rats in each group after 4 weeks ($\bar{x}$ ± s, n=6)

Group	Somatosensory evoked potential (SEP)		Motor evoked potential (MEP)	
	Latency (ms)	Wave amplitude (μV)	Latency (ms)	Wave amplitude (mV)
Control group	32.252 ± 2.560	1.231 ± 0.114	16.213 ± 0.340	1.643 ± 0.113
Schwann cells (SCs) group	26.870 ± 2.554#	1.735 ± 0.126#	12.423 ± 0.258#	2.453 ± 0.314
Bcl-2-SCs group	17.245 ± 1.422▲	2.228 ± 0.139▲	8.733 ± 0.323▲	3.742 ± 0.452▲

Note: #P<0.05, ▲P<0.01 compared with the control group; ▲P<0.05 compared with Schwann cells (SCs) group.

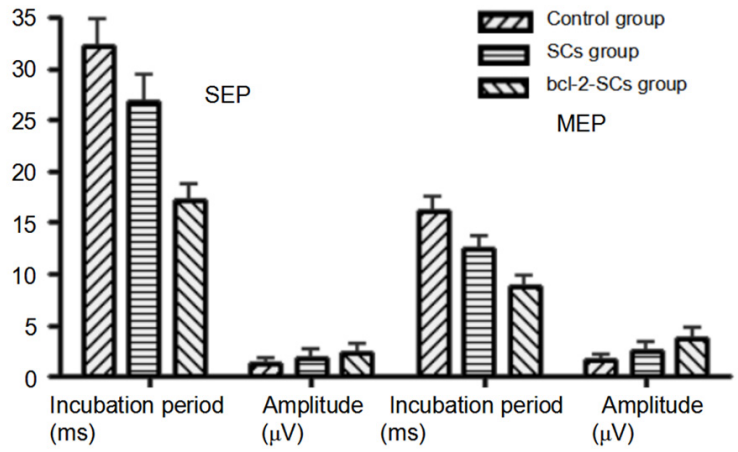

Figure 6. Somatosensory evoked potential (SEP) and motor evoked potential (MEP) monitoring at 4 weeks after transplantation.

the results of somatosensory evoked potential (SEP) and motor evoked potential (MEP) monitoring (P<0.05); the differences between the bcl-2-SCs group and the control group were extremely significant (P<0.01); the differences between the bcl-2-SCs group and the SCs group were significant (P<0.05). It was indicated that the transduction of electric signals took much shorter time from the hind limbs to the scalp in the bcl-2-SCs group. Thus the function of the spinal cord was greatly restored.

Discussion

The repair of Spinal cord injury (SCI) is notoriously difficult. After Spinal cord injury (SCI), the neurons still possess the regenerative potential. It is found that Spinal cord injury (SCI) play an important role in the regeneration of nerve cells after Spinal cord injury (SCI) [7, 22, 23]. Bcl-2 gene is the proto-oncogene isolated from the B lymphocytes of mice. With very low expression or now expression in the mature cells or dying cells, bcl-2 gene can reduce free radicals and lipid peroxidation, while increasing the content of NADPH I, glutathione and the hypoxia tolerance of cells [24]. Thus the Schwann cells (SCs) transfected with bcl-2 gene may display higher potential of nerve cell repair. In this study, Western Blot revealed that the genetically modified SCs had a stably high expression of bcl-2 protein in vitro, thus facilitating the repair of SCI. Schwann cells (SCs) introduced by subarachnoid transplantation can migrate to wider areas of the brain and the spinal cord after penetrating the blood-brain barrier with directed differentiation. Therefore, subarachnoid transplantation is considered ideal for the repair of nervous system injury [25].

We performed subarachnoid transplantation of the genetically modified SCs in this study, with less invasiveness and reduced secondary injury to the rats. As demonstrated by BBB scores, the motor function of the lower limbs of the bcl-2-SCs group was significantly improved compared with the other two groups. In contrast, the motor function was the worst in the control group. This result can be attributed to the repair effect of the Schwann cells (SCs) transfected with bcl-2 gene. At 72 h after modeling, the AI of the bcl-2-SCs group was obviously lower than that of the control group and the Schwann cells (SCs) group, which was in agreement with the above motor function evaluation. Following Spinal cord injury (SCI), the forming of cavity and glial scars in the spinal cord will impede the regeneration and extension of the nerve axons [26]. At 4 weeks after modeling, HE staining and fluorescence microscopy revealed the presence of defects and cavity of the spinal cord in the control group

without neural axes. In the SCs group, fewer neural axes were seen with smaller cavity; in the bcl-2-SCs group, more neural axes were found without cavity. It can be inferred that the genetically modified SCs exerted least impedance to the regeneration and extension of the nerve axons. This may be explained by the formation of direction canal by the axons along the Schwann cells (SCs) or by the promotion of remyelination [27]. GFAP is one of the major cytoskeletal proteins in the glial cells. After Spinal cord injury (SCI), the astrocytes will express a large number of GFAP [28]. Increasing the level of GFAP will promote the mitosis of the glial cells, thus facilitating the differentiation into mature astrocytes [29]. Neurofilament is a neurotrophic factor that maintains the survival of injured neurons in the spinal cord both in vivo and in vitro. By promoting the extension of the neural axons, the neurofilament can facilitate axonal regeneration [30]. As shown by our experiment, the gene and protein expressions of glial fibrillary acidic protein (GFAP) and (NF-200, neurofilament-200) were upregulated significantly in the bcl-2-SCs group compared with the control group and the SCs group. This result was consistent with the number of EGFP-positive cells and HRP-positive cells in the three groups. That is, bcl-2-SCs group had the largest number of EGFP-positive cells and HRP-positive cells, while the control group had the least. At 4 weeks after modeling, the somatosensory evoked potential (SEP) and motor evoked potential (MEP) latencies were the shortest in the bcl-2-SCs group, followed by the Schwann cells (SCs) group and the control group; the bcl-2-SCs group had the highest wave amplitude, and the control group had the lowest amplitude. This proved the protective effect of the genetically modified Schwann cells (SCs) to the nerve cells.

The repair of Spinal cord injury (SCI) is a complex process. We found that the Schwann cells (SCs) transfected with bcl-2 gene promoted the regeneration of injured neurons in the rat model of Spinal cord injury (SCI). The gene and protein expressions of glial fibrillary acidic protein (GFAP) and (NF-200, neurofilament-200) were upregulated in the injury site, with decreased neuron apoptosis and improved motor function and electrophysiology of the limbs. Thus subarachnoid transplantation of the genetically modified Schwann cells (SCs) is suitable for the treatment of Spinal cord injury (SCI).

Disclosure of conflict of interest

None.

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References

[1] Li J. Experimental study of olfactory ensheathing cells subarachnoid transplantation in the treatment of spinal cord injury. Tai Shan Yi Xue Yuan 2001; 7.

[2] Lian XF. Preliminary experimental study of tissue engineering transplant Schwann cells to treat spinal cord injury. Di Er Jun Yi Da Xue 2007; 15.

[3] Papastefanaki F, Chen J, Lavdas AA, Thomaidou D, Schachner M and Matsas R. Grafts of Schwann cells engineered to express PSA-NCAM promote functional recovery after spinal cord injury. Brain 2007; 130: 2159-2174.

[4] Pearse DD, Sanchez AR, Pereira FC, Andrade CM, Puzis R, Pressman Y, Golden K, Kitay BM, Blits B, Wood PM and Bunge MB. Transplantation of Schwann cells and/or olfactory ensheathing gila into the contused spinal cord: Survival, migration, axon association, and functional recovery. Gila 2007; 55: 976-1000.

[5] Ban DX, Kong XH, Feng SQ, Ning GZ, Chen JT and Guo SF. Intraspinal cord graft of autologous activated Schwann cells efficiently promotes axonal regeneration and functional recovery after rat's spinal cord injury. Brain Res 2009; 1256: 149-161.

[6] Moore MJ, Friedman JA, Lewellyn EB, Mantila SM, Krych AJ, Ameenuddin S, Knight AM, Lu L, Currier BL, Spinner RJ, Marsh RW, Windebank AJ and Yaszemski MJ. Multiple-channel scaffolds to promote spinal cord axon regeneration. Biomaterials 2006; 27: 419-429.

[7] Zhang N, Yan HH and Wen XJ. Tissue-engineering approaches for axonal guidance. Brain Res Rev 2005; 49: 48-64.

[8] Middleton G, Cox SW, Korsmeyer S and Davies AM. Differences in bcl-2 and bax-independent function in regulating apoptosis in sensory neuron populations. Eur J Neurosci 2000; 12: 819-827.

[9] Luo C, Lu Y, Jiang J and Zhu C. Changes of bcl-x(L) and bax mRNA expression following trau-

matic brain injury in rats. Chin J Traumatol 2002; 5: 299-302.
[10] Jacysyn JF, Conde-Moscatelli M, Barrichello CR, Silva UR, Macedo MS and Amarante-Mendes GP. Thrmic epithelial cells mediate a Bcl-2-independent protection of single-positive thymocytes from examethasone-induced apoptosis. Exp Cell Res 2002; 272: 119-126.
[11] Li J, Wei KB, Liu H, Liu YL, Wang YH, Zhang WZ, Zhuo F and Wang Q. Different doses of olfactory ensheathing cells transplantation in subarachnoid for repairment of spinal cord injury, an experimental study. Chinese Journal of Spine and Spinal Cord 2011; 21: 929-933.
[12] Han MY, Feng SQ, Li H, Wang CY and Yu TQ. Transplantation of human umbilical cord derived-mesenchymal stem cells via subarachnoid space way for the treatment of spinal cord injury in rats. Zhong Hua Shi Yan Wai Ke Za Zhi 2010; 27: 1645-1647.
[13] Aimone JB, Leasure JL, Perreau VM and Thallmair M. Spatial and temporal gene expression profiling of the contused rat spinal cord. Exp Neurol 2004; 189: 204-221.
[14] Ge CG, Jin FS, Li QS, Jiang J, Li YF, Wang LF and Jin WS. Bcl-2 overexpression protection of Schwann's cells apoptosis. Zhong Guo Kang Fu Yi Xue Za Zhi 2004; 19: 95-97.
[15] Wu JQ, Feng DX, Yang TF and Liu Y. Mesenchymal stem cells subarachnoid spinal cord injury in rats and its influence T cell subsets group of transplantation therapy. Zhong Guo Xiu Fu Chong Jian Wai Ke Za Zhi 2007; 21: 492-496.
[16] Li HY, Wen YM, Chen KM, Lan X, Wang DB, Sun ZG and Hu LY. Repeated transplantation of bone marrow stromal cells into subarachnoid space to promote spinal cord injury repair. Zhong Guo Jiao Xing Wai Ke Za Zhi 2009; 17: 1725-1728.
[17] Xu YQ, Feng SQ, Wang P, Ma XL, Ning GZ, Ban DX and Wang XS. The combined therapy of chondroitinase and Schwann cells transplantation after spinal cord injury in rats. Zhong Hua Shi Yan Wai Ke Za Zhi 2010; 27: 972-975.
[18] Wang L. Wallerian degeneration and oligodendrocyte precursor cells and Schwann cell transplantation. Shang Hai Jiao Tong Da Xue 2008; 16-17.
[19] Li W, Li CR, Cai WQ and Zhou DS. Retrograde HRP tracing after transplantation of BDNF genetically modified NSCs at spinal cord injury in rat. Di San Jun Yi Da Xue Xue Bao 2003; 25: 47-50.
[20] Liu WG, Lin JJ, Wang F and Chen M. The Effect of Basic Fibroblast Growth Factor on the Expression of bcl-2 and bax Gene in Spinal Cord Injury. Fu Jian Yi Ke Da Xue Xue Bao 2005; 39: 281-283.
[21] Wei YK, Ma HL, Liu FH, Tian LG, Yang L and Yu Y. Effects of MP and GM1 on apoptosis and bcl-2 gene expression in spinal neuron of rats after spinal cord injury. Ji Lin Yi Xue 2011; 32: 7428-7430.
[22] Mitsui T, Fischer I, Shumsky JS and Murray M. Transplants of fibroblasts expressing BDNF and NT-3 promote recovery of bladder and hindlimb function following spinal contusion injury in rats. Exp Neurol 2005; 194: 410-431.
[23] Hu Y, Leaver SG, Plant GW, Hendriks WT, Niclou SP, Verhaagen J, Harvey AR and Cui Q. Lentiviral-Mediated Transfer of CNTF to Schwann Cells within Reconstructed Peripheral Nerve Grafts Enhances Adult Retinal Ganglion Cell Survival and Axonal Regeneration. Mol Therapy 2005; 11: 906-915.
[24] Feng SQ, Wang JB, Kong XH, Chen JT, Ji JF, Jin F, Zhou XH and Hou W. Treatment of spinal cord injury by neurobiological membrane guided neural Stem cells combined with schwann cells transplantation. Zhong Hua Shi Yan Wai Ke Za Zhi 2006; 11: 1401-1403.
[25] Li JL, Zhou CC, Yang XH and Li M. Subarachnoid transplantation of bone marrow stromal cells to treat spinal cord injury model of Fas, Fas-L expression. Zhong Guo Shi Yong Shen Jing Ji Bing Za Zhi 2007; 10: 54-57.
[26] Shi E, Kazui T, Jiang X, Washiyama N, Yamashita K, Terada H and Bashar AH. Therapeutic benefit of intrathecal injection of marrow stromal cells on ischemia injured spinal cord. Ann Thorac Surg 2007; 83: 1484-1490.
[27] Bachelin C, Lachapelle F, Girard C, Moissonnier P, Serguera-Lagache C, Mallet J, Fontaine D, Chojnowski A, Le Guern E, Nait-Oumesmar B and Baron-Van Evercooren A. Efficient myelin repair in the macaque spinal cord by autologous grafts of Schwann cells. Brain 2005; 128: 540-549.
[28] Brohlin M, Mahay D, Novikov LN, Terenghi G, Wiberg M, Shawcross SG and Novikova LN. Characterisation of human mesenchymal stem cells following differentiation into Schwann cell-like cells. Neurosci Res 2009; 64: 41-49.
[29] Cassina P, Pehar M, Vargas MR, Castellanos R, Barbeito AG, Estévez AG, Thompson JA, Beckman JS and Barbeito L. Astrocyte activaUon by fibroblast growth factor-1 and motor neuron apoptosis: jmplicstions for amyotrophic lateral sclerosis. Neurochem 2005; 93: 38-46.
[30] Hagg T, Baker KA, Emsley JG and Tetzlaff W. Prolonged local neruotrophin-3 in fusion reduces ipsilateral collateral sprouting of spared cortico-spinal ax ons in adult rates. Neuroacience 2005; 130: 875-887.