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# Hyperbaric oxygen therapy combined with Schwann cell transplantation promotes spinal cord injury recovery

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## Abstract

Schwann cell transplantation and hyperbaric oxygen therapy each promote recovery from spinal cord injury, but it remains unclear whether their combination improves therapeutic results more than monotherapy. To investigate this, we used Schwann cell transplantation *via* the tail vein, hyperbaric oxygen therapy, or their combination, in rat models of spinal cord contusion injury. The combined treatment was more effective in improving hindlimb motor function than either treatment alone; injured spinal tissue showed a greater number of neurite-like structures in the injured spinal tissue, somatosensory and motor evoked potential latencies were notably shorter, and their amplitudes greater, after combination therapy than after monotherapy. These findings indicate that Schwann cell transplantation combined with hyperbaric oxygen therapy is more effective than either treatment alone in promoting the recovery of spinal cord in rats after injury.

***Key Words:*** *nerve regeneration; spinal cord injury; Schwann cells; hyperbaric oxygen therapy; rats; spinal cord injury; transplantation; motor function; repair; central nervous system; electrophysiology; neural regeneration*

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## Introduction

It is conventionally considered that the central nervous system possesses no regenerative capacity. However, a recent study demonstrated that changes in the local environment after spinal cord surgery enables damaged neurites to regenerate (Duchossoy et al., 2011). Schwann cells play an important role in peripheral nerve injury, regeneration and repair. They are the basic units of peripheral nerves, and the main glia of the peripheral nervous system (De Vries et al., 2008; Fehlings and Sekhon, 2011). After peripheral nerve surgery, neuronal regeneration depends on neurotrophic factors secreted by Schwann cells at the injury site, which promote axon maturation and reinnervation, contributing to the recovery of the injured nerve.

Several studies have demonstrated that hyperbaric oxygen therapy can increase the partial oxygen pressure in arterial blood and blood oxygen content in the nervous tissue, promoting aerobic metabolism. Strong evidence exists that hyperbaric oxygen therapy can markedly improve the microenvironment in the region of spinal cord injury, protect its cells and tissue structure, and promote regeneration of nerve fibers, thereby contributing to the recovery of the injured nerve (Huang et al., 2013; Long et al., 2014).

The purpose of the present study was to investigate whether Schwann cell transplantation combined with hyperbaric oxygen therapy results in better recovery of spinal cord injury than either treatment alone.

## Materials and Methods

### Animals

Eight healthy male Sprague-Dawley rats were used for Schwann cell culture, and 80 healthy female rats of the same strain were used to establish animal models of spinal cord injury. All rats (weighing 200–250 g; certification No. SCXK (Jin) 20050076) were purchased from the Laboratory Animal Room of the Chinese Academy of Medical Sciences, China. The experimental protocol was approved by the Animal Ethics Committee of Jilin University, China.

### *In vitro* culture, purification and identification of Schwann cells

Sciatic nerves were stripped under aseptic conditions, as described previously (Wan et al., 2003). They were digested with 0.25% trypsin / 0.2% collagenase for 40 minutes, centrifuged at 300 × *g* for 5 minutes, and incubated with DMEM/F12 medium containing 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) at 37°C and 5% $CO_2$ (v/v). After 30 minutes, fibroblasts were discarded using the differential adherence method. After 24 hours, 100 μL Ara-C (10−5 mM; Hisun Pharmacology, Taizhou, Zhejiang

Province, China) was added to remove the remaining fibroblasts. Passage 4 Schwann cells were cultured for 48 hours on coverslips, washed three times with PBS, fixed with 4% paraformaldehyde (pH 7.4) for 20 minutes at room temperature. They were then washed three times with PBS, and incubated overnight at 4°C with rabbit anti-MBP antibody (1:800 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three further PBS washes, the cells were incubated with goat anti-rabbit IgG (1:700; Santa Cruz Biotechnology) at 37°C for 2 hours and then with 4′,6-diamidino-2-phenylindole (DAPI) for 10 hours. Following three final PBS washes, the cells were mounted and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Spinal cord injury model**

The 80 female rats were anesthetized with 2.5% ketamine (20 mg/kg, i.p.) and placed in the supine position on a surgery table. A median incision was made, with the $T_{8–9}$ spinous processes at the center, to expose the spinous processes and vertebral plate at $T_{7–10}$. The $T_{8–9}$ spinous processes and part of the vertebral plate were resected to expose the spinal cord tissue. According to the method described by Young (2002), a 10 g weight was dropped from 2.5 cm to directly impact the exposed rat spinal cord tissue. Tail spasm followed by paralysis of both lower limbs indicated success of the spinal cord injury model. The incision was rinsed using saline containing penicillin, and sutured layer by layer. Manual extrusion of the bladder was performed in the model rats by abdominal massage twice per day (morning and afternoon).

**Experimental grouping and treatment**

Six hours after surgery, the spinal cord injury models were randomly and equally allocated to four groups: (1) blank control group: Rats received 1 mL DMEM/F12 medium containing 10% fetal bovine serum (Gibco BRL) through the tail vein. (2) Schwann cell transplantation group: Rats received 1 mL Schwann cell suspension (about $3 \times 10^6$ Schwann cells) though the tail vein. (3) Hyperbaric oxygen therapy group: Rats were placed in a hyperbaric chamber (Yangyuan Hyperbaric Oxygen Chamber Factory of Shanghai 701 Institute, China). The hyperbaric chamber was washed with pure oxygen for 10 minutes, then pressurized to 0.2 MPa at a rate of 0.01 MPa/min, and held at 0.2 MPa for 30 minutes. During this period, pure oxygen was given to maintain ≥ 70% oxygen. Thereafter, the pressure was gradually decreased to atmospheric pressure over 10 minutes. This procedure was performed four times daily, for 7 consecutive days. (4) Schwann cell transplantation + hyperbaric oxygen therapy group (combination group): Rats received 1 mL Schwann cell suspension ($3 \times 10^6$ cells) immediately before the first session in the hyperbaric chamber through the tail vein and were simultaneously subjected to hyperbaric oxygen therapy.

**Evaluation of hindlimb motor function**

Before surgery, and 1, 2, 4, 6, and 8 weeks postoperatively, six rats from each group were randomly selected for evaluation of hindlimb motor function (Pearse et al., 2007; Haku et al., 2008) using the inclined plane test (Young et al., 2002), modified Tarlov scoring system (Finch et al., 1999), and Basso, Beattie and Bresnahan (BBB) locomotor scale (Pallini et al., 2005; Albin et al., 2006). The tests were started at 8:00 a.m. and were performed by two investigators blind to experimental grouping.

(1) Inclined plane test (Young et al., 2002; Wan et al., 2009): Rats were placed on a smooth wooden platform. Every 5 seconds, one side of the platform was elevated by 5°. The maximum angle at which a rat was able to stay on the platform for 5 seconds was taken as the value for hindlimb motor function of that rat.

(2) Modified Tarlov scoring system (Pearse et al., 2007; Haku et al., 2008). Locomotion of the rats was scored as follows: 0, no movement, cannot load; 1, slight movement, cannot load; 2, frequent movement or powerful, cannot load; 3, can support body weight and able to walk one or two steps; 4, able to walk with assistance; 5, normal gait.

(3) BBB locomotor scale (Papastefanaki et al., 2007; Pearse et al., 2007): a score of 0 indicated complete paralysis and 21 represented normal function. Observation indices included range of motion, number of joints involved in motion, degree of loading, forelimb and hindlimb concordance, and movement of front and hind paws and tail.

**Hematoxylin-eosin staining**

Four weeks after surgery, four rats from each group were selected at random to receive an overdose of anesthesia (2% sodium pentobarbital, i.v.), and intubated from the left ventricle to the ascending aorta through a chest incision. The right atrium was cut open and 200 mL of iced physiological saline was perfused at a constant rate using a perfusion pump. When the fluid flowing from the right atrium became colorless, a 1.0 cm length of spinal cord containing the injured tissue was harvested. Half of this was used for hematoxylin-eosin staining to determine the degree of injury, and the remaining half was frozen at −80°C for sectioning and viewing under an optical microscope (Olympus, Tokyo, Japan).

**PCR for detection of sex-determining region of Y-chromosome (SRY)**

Four weeks after surgery, 1.0 cm of injured spinal cord tissue was harvested from each of five rats using the same method as that used for hematoxylin-eosin staining. DNA was extracted and preserved using a kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China), according to the manufacturer's instructions. SRY gene primer sequences were as follows: forward, 5′-CTG GCT CTG CTC CTA CCT-3′; reverse, 5′-GCT GTT TGC TGC CTT TGA-3′. The PCR reaction mixture contained 1.0 μL DNA, 0.2 mM dNTP, 22.0 mM $MgCl_2$, 3.0 μL 10× buffer, 1 μL Taq enzyme, 0.5 μM of each primer, and deionized water per 30 μL sample. PCR amplification was carried out as follows: 32 cycles of predenaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 54°C for 45 seconds, and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 7 minutes. The resulting products were

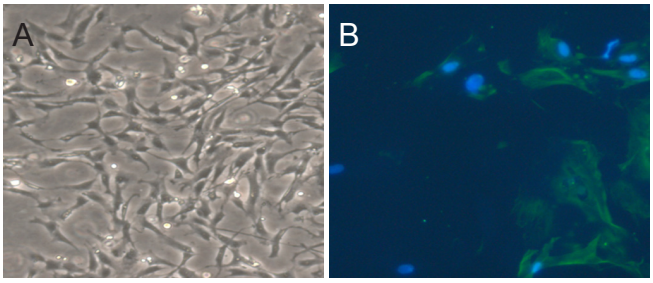


**Figure 1 Morphology of cultured Schwann cells (× 40).**
(A) Inverted phase contrast microscope: Schwann cells were long, shuttle-shaped, and tightly arranged. (B) Fluorescence microscope: Schwann cell soma and processes showed green fluorescence after 6 days of culture, and immunofluorescence staining of myelin basic protein.


**Figure 3 SRY mRNA expression in the injured spinal cord tissue of rats.**
SRY mRNA expression was detected in the injured spinal cord tissue of rats in the Schwann cell transplantation and combination groups, indicating that the transplanted Schwann cells survived. 1: Blank control group; 2: Schwann cell transplantation group; 3: hyperbaric oxygen therapy group; 4: combination group (Schwann cell transplantation + hyperbaric oxygen therapy).

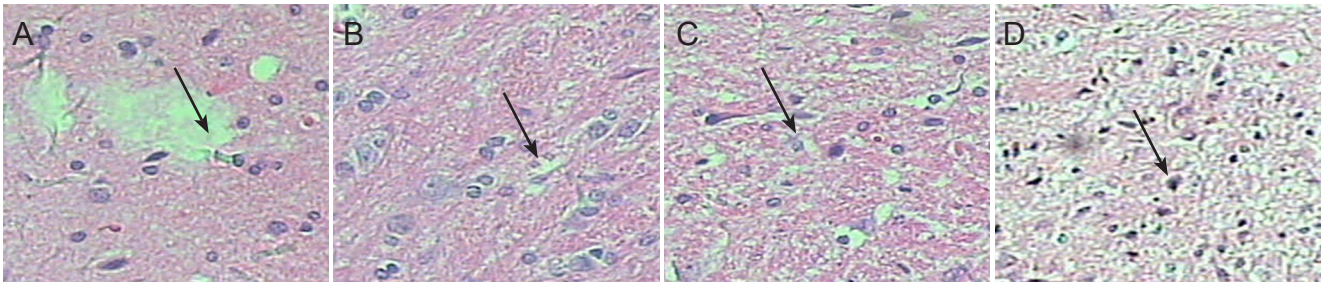


**Figure 2 Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on cell morphology in the injured rat spinal cord (hematoxylin-eosin staining, optical microscope, × 100).**
(A) Blank control group, showing damaged spinal cord tissue with empty cavities. (B, C) Empty cavities were smaller in the Schwann cell transplantation group (B) and hyperbaric oxygen therapy group (C) than in the blank control group (A) (Schwann cell transplantation + hyperbaric oxygen therapy). (D) Combination group, showing a scar at the margin of injury, with no empty cavities. Arrows indicate empty cavities.

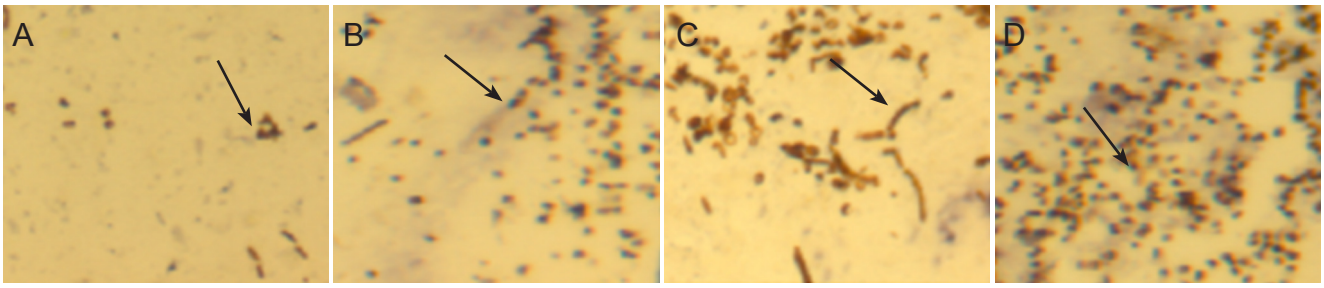


**Figure 4 Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on number of nerve fibers in injured rat spinal cord (DAB staining, optical microscope, × 200).**
Horseradish peroxidase retrograde labeling shows that at 8 weeks after injury, the number of horseradish peroxidase-positive nerve fibers (arrows) was lowest in the blank control group (A), followed by the hyperbaric oxygen therapy group (B) and the Schwann cell transplantation group (C) and highest in the combination group (Schwann cell transplantation + hyperbaric oxygen therapy; D).

electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed using a Gel2Doc200 gel imaging analysis system (Beijing Saibaiao Science & Technology Co., Ltd., Beijing, China). The product size was 325 bp.

### Detection of somatosensory (SEP) and motor evoked potentials (MEP)

Eight weeks after surgery, eight rats from each group were randomly selected for determination of SEP and MEP (Albin et al., 2006) using EMG equipment (Keypoint 4, Medtronic Inc., Minneapolis, MN, USA) and anesthetized by intraperitoneal injection of 10% chloral hydrate. The rats were placed on the table and stimulating electrodes were fixed to the hindlimbs. For SEP measurement, the recording electrode was positioned beneath the scalp at the convergence of the coronal and sagittal sutures (*i.e.*, hindlimb cortical sensory area). The reference electrode was placed 0.5 cm posterior to the recording electrode. Stimulation using direct-current square wave electrical pulses was applied until the hindlimbs twitched slightly. Current intensity was 5–15 mA, pulse width 0.2 ms, frequency 3 Hz, and 50–60 superimposed traces were recorded. The latency and amplitude of SEP were noted. For MEP detection, the stimulating needle was positioned beneath the scalp, 2 mm anterior to the coronal suture and 2 mm lateral to the sagittal suture (*i.e.*, cerebral cortex motor area) with a current amplitude of 40 mA, pulse width of 0.1 ms, frequency of 1 Hz, a scanning speed of 5 ms/D and sensitivity of 5 μV/D. The latency and amplitude

**Table 1 Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on recovery of hindlimb motor function in rats with spinal cord injury**

| | | Time after surgery (week) | | | | |
|---|---|---|---|---|---|---|
| | Before injury | 1 | 2 | 4 | 6 | 8 |
| **BBB scores** | | | | | | |
| Blank control | 21.00±0.00 | 11.00±0.30 | 12.30±0.40 | 13.90±0.70 | 14.50±1.60 | 17.30±1.00 |
| Schwann cell transplantation | 21.00±0.00 | 11.30±0.40* | 12.50±0.50* | 15.70±1.10* | 16.30±1.30* | 19.10±1.10* |
| Hyperbaric oxygen therapy | 21.00±0.00 | 11.30±0.50* | 13.70±0.60* | 15.90±1.00* | 16.70±1.20* | 19.30±1.50* |
| Combination | 21.00±0.00 | 11.40±0.50*# | 14.70±0.70*# | 17.10±1.00*# | 18.70±1.20*# | 20.58±0.50*# |
| **Inclined plate test** (°) | | | | | | |
| Blank control | 42.50±2.46 | 16.46±0.75 | 18.13±0.78 | 20.13±1.45 | 22.92±2.13 | 26.22±2.34 |
| Schwann cell transplantation | 42.50±1.62 | 18.74±0.64* | 21.24±1.21* | 24.64±2.13* | 30.21±2.31* | 34.67±2.08* |
| Hyperbaric oxygen therapy | 42.50±2.24 | 19.38±0.70* | 21.75±1.37* | 24.73±1.44* | 30.30±2.11* | 34.32±2.14* |
| Combination | 42.51±2.63 | 21.97±0.70*# | 23.75±1.37*# | 27.32±2.11*# | 33.50±2.42*# | 38.46±2.64*# |
| **Modified Tarlov scoring system** | | | | | | |
| Blank control | 5.00±0.00 | 0.82±0.20 | 1.64±0.32 | 2.16±0.21 | 2.65±0.54 | 2.95±0.54 |
| Schwann cell transplantation | 5.00±0.00 | 1.58±0.21* | 2.79±0.32* | 3.60±0.20* | 3.47±0.32* | 3.87±0.32* |
| Hyperbaric oxygen therapy | 5.00±0.00 | 1.60±0.20* | 2.80±0.53* | 3.61±0.19* | 3.46±0.29* | 3.86±0.29* |
| Combination | 5.00±0.00 | 1.83±0.13*# | 3.02±0.34*# | 3.43±0.21*# | 3.84±0.23*# | 4.44±0.23*# |

At all time points examined, hindlimb motor function scores were highest in the combination group (Schwann cell transplantation + hyperbaric oxygen therapy), followed by the Schwann cell transplantation and hyperbaric oxygen therapy groups, and lowest in the blank control group. *$P$ < 0.05, *vs.* blank control group; #$P$ < 0.05, *vs.* Schwann cell transplantation group or hyperbaric oxygen therapy group (repeated measures analysis of variance). All measurement data are expressed as the mean ± SD ($n$ = 6). BBB: Basso, Beattie and Bresnahan (BBB) locomotor scale.

**Table 2 SEP and MEP in injured rat spinal cord 8 weeks after surgery**

| | SEP | | MEP | |
|---|---|---|---|---|
| Group | Latency (ms) | Amplitude (μV) | Latency (ms) | Amplitude (μV) |
| Blank control | 35.123±1.234 | 1.352±0.123 | 15.734±0.342 | 1.623±0.114 |
| Schwann cell transplantation | 26.367±0.745* | 1.745±0.167* | 11.931±0.315* | 2.442±0.246* |
| Hyperbaric oxygen therapy | 26.276±0.876* | 1.746±0.102* | 11.927±0.323* | 2.460±0.264* |
| Combination | 15.215±0.645*# | 2.208±0.123*# | 7.843±0.126*# | 4.153±0.276*# |

*$P$ < 0.05, *vs.* blank control group; #$P$ < 0.05, *vs.* Schwann cell transplantation group and hyperbaric oxygen therapy group (repeated measures analysis of variance). All measurement data are expressed as the mean ± SD ($n$ = 6). SEP: Somatosensory evoked potential; MEP: motor evoked potential.

of MEP were taken, and 300–500 superimposed traces were recorded.

### Horseradish peroxidase (HRP) retrograde labeling

HRP was dissolved in physiological saline to 50%. Eight weeks after surgery, three rats from each group were randomly selected and anesthetized for HRP retrograde labeling. The spinal cord was exposed, a needle was inserted 1 mm lateral to the median vein, at a depth of 1.5 mm. HRP (1 μL) was injected at a rate of 0.1 μL/10 minutes, and the needle was left in place for 15 minutes. The wound was sutured and the rats were allowed to recover for 3 days. Under chloral hydrate anesthesia, the heart was perfused with 4% paraformaldehyde, and the spinal cord ($T_{3–11}$) was harvested and immersed in 30% sucrose solution at 4°C. Twenty hours later, frozen sections (thickness, 5 μm) were prepared, stained with 3,3′-diaminobenzidine (DAB), and viewed under an optical microscope (Olympus). HRP-positive nerve fibers were counted in ten sections at 8 weeks, selected at random in each group.

### Statistical analysis

All measurement data were statistically analyzed using SAS 8.0 software (SAS Institute Inc., Cary, NC, USA) and are expressed as the mean ± SD. Repeated measures analysis of variance was used for comparison between groups. $P$ < 0.05 was considered statistically significant.

## Results

### Morphology of cultured Schwann cells

Viewed under an inverted phase contrast microscope, Schwann cells were observed to cover the whole floor of the flask after 5–6 days of culture. The majority of cells were Schwann cells, with some fibroblasts. After purification, over 95% of cells were Schwann cells. These cells were identified by their long and shuttle-shaped appearance, with small nuclei, and with a large amount of secretions around the cells (**Figure 1A**). Green fluorescence was observed in Schwann cell soma and neurites after immunofluorescent staining of myelin basic protein (MBP). Nuclei of Schwann cells and fibroblasts appeared blue after 4′,6-diamidino-2-phenylindole (DAPI)

staining, whereas fibroblast cytoplasm was not stained (**Figure 1B**).

**Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on recovery of hindlimb motor function in rats with spinal cord injury**

At all time points tested, the scores from the BBB locomotor scale, inclined plate test and modified Tarlov scale were highest in the combination group, followed by the Schwann cell transplantation group, hyperbaric oxygen therapy group, and lowest in the blank control group ($P < 0.05$) (**Table 1**).

**Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on injured rat spinal cord morphology**

Hematoxylin-eosin staining showed that 4 weeks after surgery, in the blank control group, spinal cord tissue at the injury site was broken and showed a scar-like, disordered structure containing empty cavities (**Figure 2A**). In the Schwann cell transplantation and hyperbaric oxygen therapy groups, scars had formed in the margin of the injury zone, and the empty tissue cavities were smaller than those in the blank control group, but larger than those in the combination group (**Figure 2B** and **C**). In the combination group, scars were also noted at the edge of the injured spinal cord; some cells were long and shuttle-shaped, and the lattice between neurites was dense, with no empty cavities (**Figure 2D**).

**Survival of Schwann cells**

As expected, no SRY gene expression was detected in the injured spinal cord tissue of rats in the blank control or hyperbaric oxygen therapy groups. However, SRY expression was detected in the Schwann cell transplantation and combination groups 4 weeks after injury, indicating that the transplanted Schwann cells had survived and were involved in the repair of spinal cord injury (**Figure 3**).

**Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on number of nerve fibers in injured rat spinal cord**

Eight weeks after injury, the HRP-DAB reaction revealed a region at the injection site with a darkly stained center surrounded by a lightly stained area. In the blank control group, 2 days after injection, HRP was reversely transported to the segments above $T_8$, and a few HRP-positive cells were seen (**Figure 4A**). In the Schwann cell transplantation and hyperbaric oxygen therapy groups, the number of HRP-positive nerve fibers was smaller than that in the blank control group (**Figure 4B** and **C**). In the combination group, a large number of HRP-positive nerve fibers were observed in the injured spinal cord tissue (**Figure 4D**). The number of HRP-positive nerve fibers was highest in the combination group ($13.2 \pm 2.22$), followed by the Schwann cell transplantation group ($19.8 \pm 2.34$) and hyperbaric oxygen therapy group ($20.3 \pm 1.82$) and lowest in the blank control group ($13.2 \pm 2.22$) ($P < 0.01$).

**Effects of Schwann cell transplantation combined with hyperbaric oxygen therapy on recovery of electrophysiological function of injured rat spinal cord**

Immediately after surgery, neither SEP nor MEP were detectable in the injured spinal cord, in any group. At 8 weeks postoperatively, both measures showed some recovery in the blank control group, and a greater degree of recovery in the monotherapy groups. In the combination group, SEP and MEP latencies were significantly shorter and amplitudes significantly greater than those in the control and monotherapy groups ($P < 0.05$). These findings suggest that the electric signal conduction time from hindlimb to scalp in the combination group was shorter than that in the other groups, indicating that conduction pathway had been unblocked and the cord tissue had recovered better in this group than in the others (**Table 2**).

## Discussion

Schwann cells can secrete a variety of neurotrophic, cell matrix and cell adhesion factors, which promote nerve injury repair and neurite growth in the central nervous system. There is strong evidence that Schwann cells can accelerate the recovery of spinal cord histomorphology and function, contribute to axon growth and myelination, and show targeted induction effects, when used in the treatment of spinal cord injury or demyelinating diseases (Finch et al., 1999; Albin and Mink, 2006; Lepore et al., 2006; Saberi et al., 2008; Ban et al., 2009; Chi et al., 2010).

Neurological lesions after spinal cord damage are caused by primary and secondary injury (Crowe et al., 1997; Liu et al., 1997; Amar and Levy, 1999; Masahiro et al., 2005; Ohta et al., 2005). During secondary injury, a series of oxidative reactions occur, producing a large number of free radicals and leading to nervous tissue damage and associated functional deficits. Neuronal apoptosis plays an important role in secondary spinal cord injury; the majority of apoptotic cells are oligodendrocytes, but gray matter neurons do not undergo apoptosis (Crowe et al., 1997; Liu et al., 1997; Masahiro et al., 2005).

Hyperbaric oxygen therapy is known to alleviate the inflammatory response. Therefore, in the present study, we used Schwann cell transplantation combined with hyperbaric oxygen therapy to treat spinal cord injury in rats. Our results show that Schwann cell transplantation combined with hyperbaric oxygen therapy exhibits superior therapeutic effects to either treatment alone, from histological combined with functional perspectives. Hyperbaric oxygen improves the environment in the injury zone *via* the following pathways (Huang et al., 2013; Long et al., 2014; Wang et al., 2014; Liang et al., 2015): (1) The central nervous system is extremely sensitive to oxygen concentration. When spinal cord injury occurs, local tissue becomes markedly hypoxic. Hyperbaric oxygen increases blood oxygen concentration, enlarging the diffusion distance of oxygen and reducing hypoxia-induced apoptosis at the injury site. (2) Hyperbaric oxygen increases the deformability of red blood cells, decreases the permeability of blood capillaries and strengthens

the oxygen and energy supply to local tissues, in particular to the region of injury. (3) Blood flow in the injured vessels is improved by hyperbaric oxygen therapy, reducing platelet aggregation, maintaining neuronal energy metabolism, and improving functional recovery.

Our results demonstrate that under hyperbaric oxygen conditions, transplanted Schwann cells can survive, proliferate, differentiate and migrate to the region of damage, and that the use of both hyperbaric oxygen and Schwann cell transplantation promotes better recovery of neurological function in rats after spinal cord injury than either treatment alone. The combination of Schwann cell transplantation and hyperbaric oxygen therapy is a promising alternative solution for the clinical treatment of spinal cord injury.

**Author contributions:** *CGP was responsible for data collection and integration, conceived and designed this study, and wrote this paper. SQZ and RG analyzed the data. QY authorized the paper. YL performed statistical analysis. MFW was in charge of fundraising, providing suggestions in technique or material use. DKW guided the study. All authors approved the final version of this paper.*
**Conflicts of interest:** *None declared.*

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