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Effect of mesenchymal stem cells transplantation combining with hyperbaric oxygen therapy on rehabilitation of rat spinal cord injury

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ABSTRACT

Objective: To investigate the effect of BMSCs transplantation plus hyperbaric oxygen (HBO) on repair of rat SCI.

Methods: Seventy five male rats were divided randomly into five groups: sham, vehicle, BMSCs transplantation group, combination group, 15 rats in each group. Every week after the SCI onset, all animals were evaluated for behavior outcome by Basso–Beattle–Bresnahan (BBB) score and inclined plane test. Axon recovery was examined with focal spinal cord tissue by electron microscope at 6 weeks after the SCI onset. HE staining and BrdU staining were performed to examine the BMSCs and lesion post injury. Somatosensory evoked potential (SEP) testing was performed to detect the recovery of neural conduction.

Results: Results from the behavior tests from combination group were significant higher than rats which received only transplantation or HBO treatment. Results from histopathology showed favorable recovery from combination group than other treatment groups. The number of $BrdU^+$ in combination group were measureable more than transplantation group ($P < 0.05$). The greatest decrease in TNF-α, IL-1β, IL-6, IFN-α determined by Elisa assay in combination group were evident too.

Conclusions: BMSCs transplantation can promote the functional recovery of rat hind limbs after SCI, and its combination with HBO has a synergistic effect.

1. Introduction

Spinal cord injury (SCI) is the most serious complication in spinal injury which caused hemiparalysis or general paralysis and jeopardize the living quality of patients. SCI usually causes neuron death and axonal damage resulting in dyskinesia or somatosensory loss. The adverse microenvironment in focal area such as ischemia, neuroinflammation and glial scarring which made the neural system regeneration even more difficult [1,2]. Neuroinflammation is a pathological process principally involving activation of microglia and astrocytes by inflammatory mediators in various CNS disorders, including brain trauma, ischemia, and SCI [3,4]. To date, there is still lack of effective therapy to treat traumatic SCI.

Stem cells transplant is a promising therapy for the repair of damaged nervous system [5–7]. Bone marrow derived mesenchymal stem cells (BMSCs) are one of the most commonly used cell to treat injured spinal cord. The transplants of BMSCs in neural systems are considered to repair the injury because of the various immunoregulatory macromolecules secreted by BMSCs that contributing to structure regenerative microenvironments in fields of injury [8–10]. However, these effects and axonal regeneration are limited due to the damage from cytokines and immunological rejection. Hyperbaric oxygen (HBO) is occasionally used as a prognosis therapy for the treatment of SCI. The beneficial effects of HBO in SCI

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stem from lower endothelial adhesion molecule expression and decreased neutrophil activation [11]. Moreover, HBO stabilizes metabolism, improves angiogenesis and collateral circulation which enhance the focal area recovery [12,13]. Base on the above reasons, the purpose of this experimental study was to investigate the effectiveness of HBO either alone or in combine with BMSCs transplant on the recovery of locomotive in a SCI model in rat.

2. Materials and methods

2.1. Experimental animals

The experimental animals were purchased from Institute of Laboratory Animal Science, license NO: SCXK20060023. The experimental procedures were in according with the "Opinion on the treatment of experimental animal" published by Chinese National Technical Department, 2006.

2.2. Experimental reagents & instruments

Dulbecco's modified Eagle's medium was purchased from Life Technology, USA. BrdU immunohistochemistry kit was purchased from Biyuntian biotechnology Co.

2.3. Preparation of acute SCI model

Rats were anesthetized by ip injection of pentobarbital sodium (20 mg/kg). Total laminectomy was performed at T9 spinous. The interscapular distance incision was acquired through the skin and subcutaneous tissues, via a 2-cm incision at the level of T8–T9 and dura mater was exposed by Rats vertebral wrench. Separated the dura mater and the spinal cord were half-cutted. After washing the wound by Penicillin, skin tissues were primarily sutured according to anatomic layers. Artificial urination was proceeded twice a day until micturition reflex recovery. The right hindlimb paralysis was considered as the model succeed.

2.4. BMSCs culture and transplantation

BMSCs were isolated from the bone marrow of tibias and femurs of 8-week old Sprague Dawley rats. Cell were harvested by trypsinisation and plated in six-well plates in 2 mL DMEM with high glucose, 10% FBS. The medium was changed after 24 h and cells were passaged for another 48 h. Fresh medium was changed every 48 h. BMSCs of passage 4 were used for transplantation.

Cell transplantation was proceeded 6 h post SCI. 10 μM BrdU were added into the BMSCs culture to mark the cell. The focal area was exposed before the cell transplantation. A microsyringe contained 10 μL cells (1×10^{10}/L) was lowered into the upper region central of the injured site. Needle retention should be operated for at least 5 min after the injection which was finished within 3 min.

2.5. Hyperbaric oxygen treatment procedure

Animals received HBO therapy in an animal monoplace chamber. Before pressurization, 100% medical oxygen was flushed through the chamber for 10 min to displace ambient air. The oxygen pressure was then increased slowly and reached 2.5 atm. in 5 min. The chamber was ventilated during HBO therapy to keep the oxygen volume fraction around 70%. The chamber was decompressed to normal atmospheric pressure in 10 min. The rats in HBO group received 4 times of treatment a day, 7 d in total.

2.6. Study groups

The experimental adult male Sprague–Dawley (SD) rats weighing 220–250 g were randomly allocated into five study groups (28 rats in each group). The HBO group received a single session of HBO treatment, the BMSC group received cell transplantation therapy; the HBO + BMSC group received both HBO and cell transplantation therapy; the vehicle group received only 0.9% saline and the fifth group was the sham group received only laminectomy.

2.7. Behavioral assessment

The Basso, Beattie, and Bresnahan (BBB) locomotor rating scale was used to assess the overground walking ability of the rats (n = 10/group) [14,15]. This scale measures hindlimb movements starting with a score of 0, indicating no observable movement. Increasing scores are given for movement of individual joints, limb coordination, weight-supported plantar stepping, *etc*., up to a maximum score of 21, which indicates normal movement. Rats were tested by double-blind assessment. Rats were familiarized with the open field and baseline values were determined before surgery. Locomotor functions were scored weekly from the 1st to the 6th week after SCI. The score was obtained by averaging the value of all limbs.

In the inclined plane test, the rats were put on a rubber tray parallel (8 mm thickness) to a flat surface. The unstable tray edge was raised so that the inclined level is increased. The highest angle during which the rats could stay stable for 5 s was the inclined plane angle. Angle was recorded for three times and the mean value was the final stay-time of one rat. Inclined plane test were scored weekly from the 1st to the 6th week after SCI.

2.8. Electron microscopy

6 weeks after the SCI surgery, randomly sacrificed 3 rats by heart perfusion with 25 g/L glutaraldehyde from each experimental group. Then the tissue were fixed in glutaraldehyde overnight and the spinal cord segment from 1 cm rostral to 1 cm caudal of the injury epicenter (2 cm total length) was harvested and post-fixed in the same fixative solution for 2 h. Then the samples were subjected to gradient dehydration by acetone and dyed by uranyl acetate for 4 h in 4 °C. The samples were embedded in epoxy resin 618 and observed using transmission electron microscopy.

2.9. HE staining and immunohistochemistry

6 weeks after the SCI surgery, randomly sacrificed 3 rats to determine the focal injury by immunohistochemistry. HE staining and BrdU antibody were used on tissue slice. Randomly chose 10 fields of vision under 200 × lens. Calculate $BrduU^+$ cell from every fields. The mean value was the final $BrduU^+$ cell number of one rat.

2.10. Somatosensory evoked potentials (SEP) assessment

6 weeks after the SCI surgery, randomly chose 6 rats to determine the SEP. To observe the recovery of nerve conduction, the KEYPOINT 4 evoked potential instrument was used to record the latency and the onset-to-peak amplitude. Rats were anesthetized by *ip.* injection of pentobarbital sodium (20 mg/kg). The stimulating electrode was placed on the hindlimbs. The recording electrode was embedded under the skin of anterior fontanelle. The reference electrode was embedded 5 mm below the anterior fontanelle. 5–15 mA direct current square wave of 5–15 mA, 0.2 ms wide, 3 Hz and 50–60 times overlaps was given to the stimulating electrode. The latency and the onset-to-peak amplitude were recorded in accordance with the method of literature [16].

2.11. ELISA assays

At the end of 1, 3 and 6 weeks after SCI surgery, randomly chose 3 rats from each group to investigate the inflammatory factor from the focal area by ELISA assays. Tissue samples were suspended in lysis buffer contained PMSF, then incubated on ice, sonicated and cleared by centrifugation. Supernatants were collected and their protein content was determined. TNF-α, IL-1β, IL-6, IFN-α were measured using respective ELISA systems according to the manufacturer's instructions.

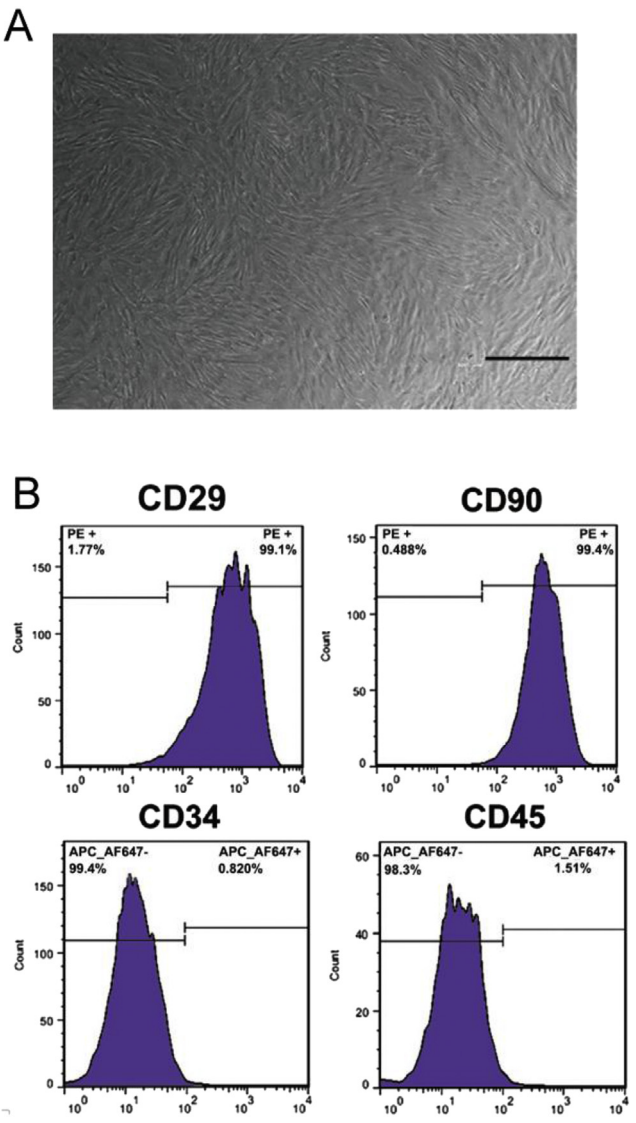

Figure 1. Characteristics of BMSCs.
A: BMSCs cultured under conditions described above were anatomically homogeneous in size and morphology. Scale bar, 500 μm. B: The cell surface markers were analysed by flow cytometric analysis.

2.12. Statistical analysis

The statistical analysis was calculated using SPSS 19.0 software. $^{*}P < 0.05$ was considered significantly different. All values are expressed as the mean ± SEM. The results were analyzed by variance (ANOVA) test. The one-way ANOVA test was followed by *post hoc* Dunnett' s test.

3. Results

3.1. Characteristics of BMSCs

BMSCs cultured under conditions described above were anatomically homogeneous at passage 4 in size and morphology (Figure 1A). More than 98% of the BMSCs were characterized by $CD29^{+}$, $CD90^{+}$, and $CD34^{-}$, $CD45^{-}$ which suggested that the cell population is phenotypically homogenous (Figure 1B).

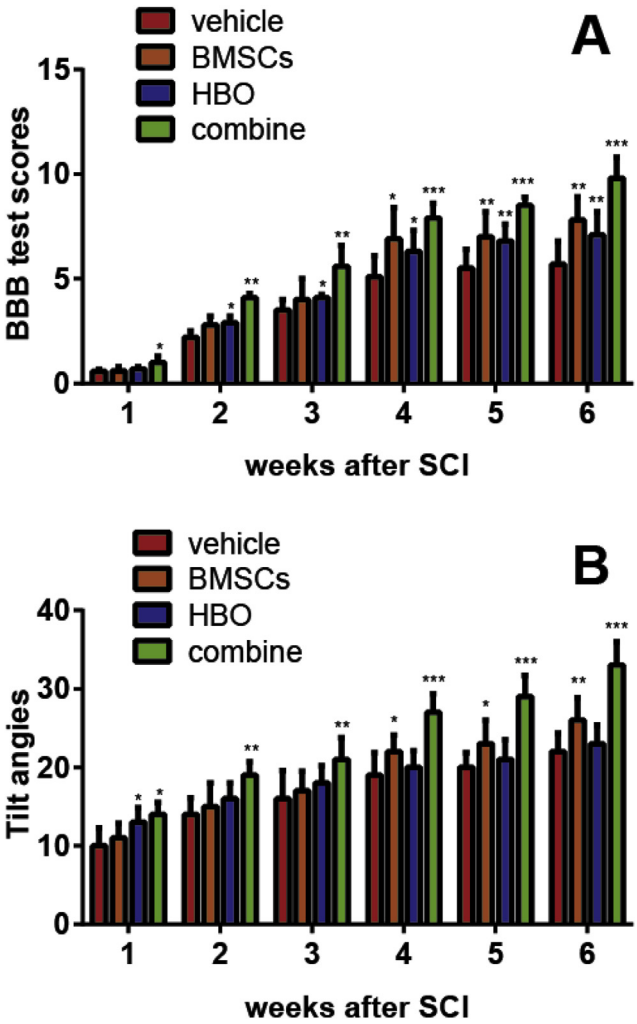

Figure 2. Neurological function outcome ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* vehicle, ANOVA).

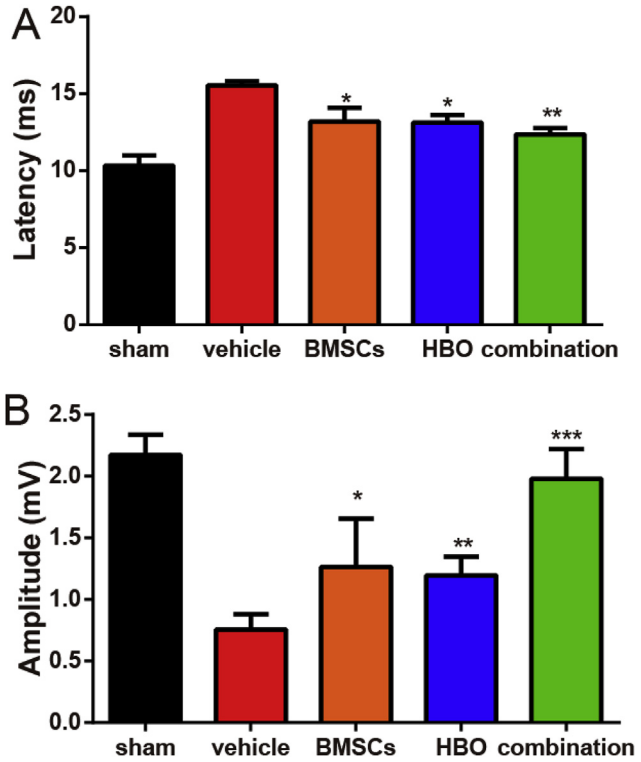

Figure 3. SEP test.
A: The SEP latent period of rats at 6 weeks after SCI. B: The SEP amplitude during 1–6 weeks after SCI (n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. vehicle, ANOVA).

3.2. Neurological function outcome

The BBB scores in sham group were 21 point. All rats that underwent SCI lost hindlimbs function immediately after injury characterized by complete paraplegia with micturition disability but not cacation disability. Although the rats from all groups exhibited a gradual recovery over time in 6 weeks, the locomotor function of the HBO-treated rats and combine-treated rats significantly continued to increase their behavior scores compared with the SCI control rats. The combine-treated rats showed even better recovery than the single HBO-treated rats (Figure 2A–B).

3.3. SEP test

There were no tracings of induced electric potential right after SCI surgery. As was demonstrated in Figure 3A–B, a limit recovery of induced electric potential can be observed in vehicle group. Both the latency and amplitude of the rats in HBO-treated group and combine-treated group showed significantly improved. The combine-treated rats showed the most improvement than other rats, suggesting that combine treatment has a better effect on recovering the electrophysiological abnormalities from hindlimbs to head.

3.4. Histopathology examination

As was shown in Figure 4A–B, the injured spinal cords exhibited obvious cavitary lesions at 6 weeks after SCI in the vehicle group characterized by H&E staining (Figure 4A).

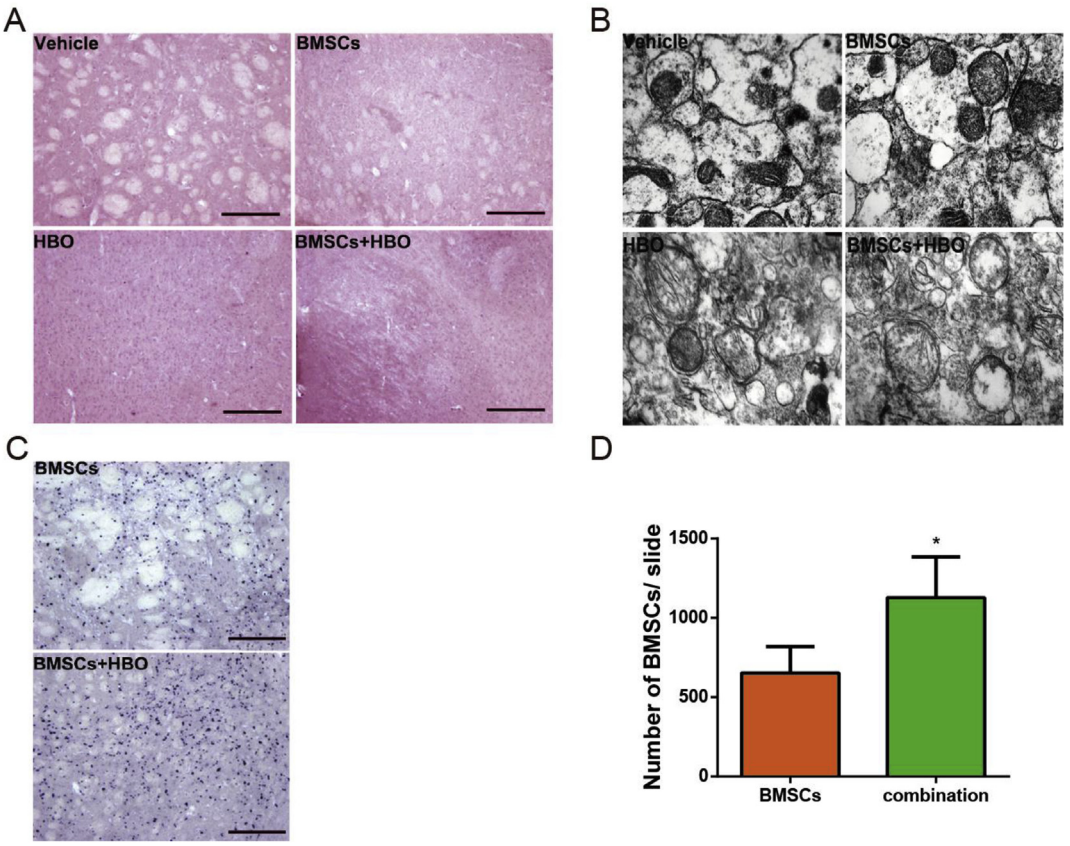

Figure 4. Histopathology examination.
A: The H&E staining of the injury site at 6 weeks after SCI. B: The ultrastructure of the neuron in the injury site detected by transmission electron microscope at 6 weeks after SCI. C–D: The BrdU^{+} cell were observed by immunohistochemisty staining of the injury site at 6 weeks after SCI ($^{*}P < 0.05$).

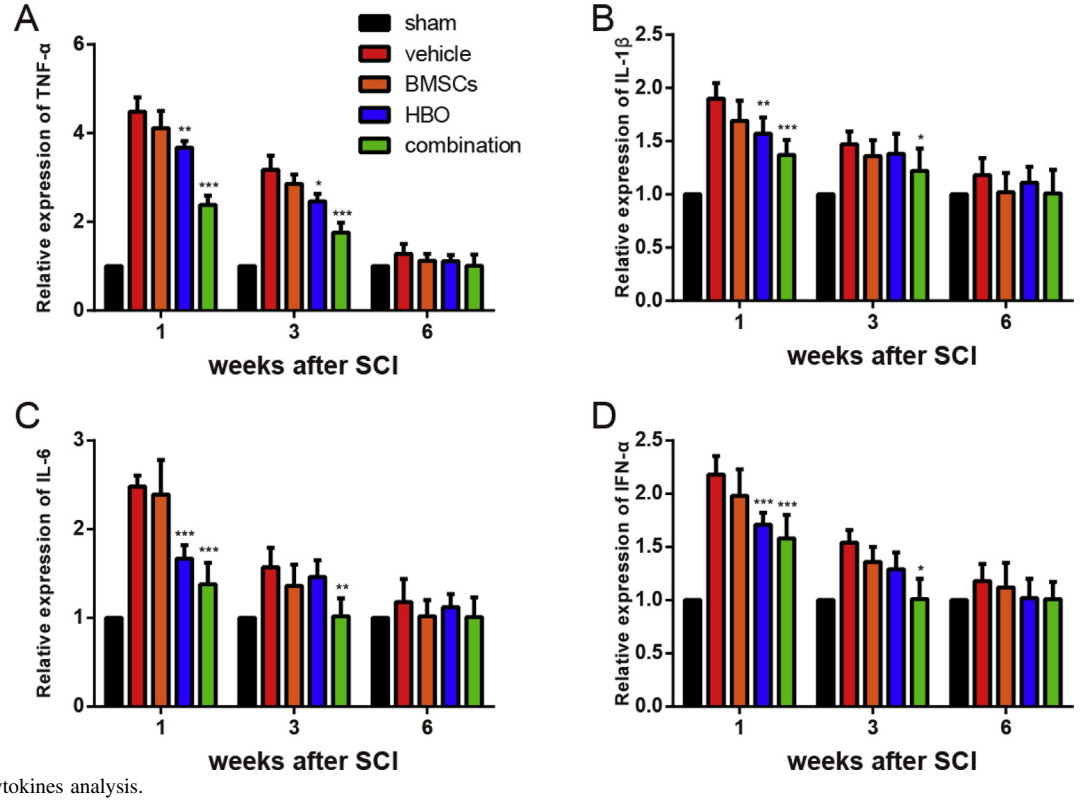

Figure 5. Cytokines analysis.
A–D: The concentration of the cytokines TNF-α, IL-1β, IL-6 and IFN-α in focal spinal cord tissue was analyses and quantified by ELISA assay at 1, 3 and 6 weeks after SCI ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* vehicle, ANOVA).

Dropsy and cavity in neuron from focal area can also be observed by a transmission electron microscope (Figure 4B). This phenomenon showed significantly release in HBO-treated group and combine-treated group. Although the lesion of the rats from all groups exhibited a gradual recovery over time, the combine-treatment showed the most improvement than other single treatments. Moreover, the number of $BrdU^{+}$ cell in combine-treatment group was measurable outnumber than BMSC group (Figure 4C).

3.5. Cytokines analysis

The concentration of the cytokines TNF-α, IL-1β, IL-6 and IFN-α in focal spinal cord tissue was analyzed and quantified by ELISA assay at 1, 3 and 6 weeks after SCI surgery. The HBO-treated group and combine-treated group efficiently attenuated the quantity of all four cytokines in the traumatic spinal cord compared with the BMSC group and vehicle group, suggesting anti-inflammation role of HBO treatment (Figure 5A–D).

4. Discussion

Severe spinal cord injury leads to the loss of locomotor, sensorial, and autonomic functions below the site of trauma [17]. After SCI, secondary damage aggravate the condition which is caused by neuroinflammation that is mediated by the activation of glial cells that produce immunomodulatory molecules such as TNF-α, IL-1β, IL-6, IFN-α [18]. Stem cells transplant has been a research hotspot for repair of the damaged nervous system. However, the clinical application of stem cell transplantation have been encountered several difficulties, such as the poor rate of survival post-transplant or the risk of tumorigenesis. The obstacle list above impede the application of stem cell transplantation therapy [19].

The HBO therapy increased tissue oxygen and improved collagen synthesis, angiogenesis and epithelization. HBO treatment attenuated the focal inflammatory reaction for the reason that pure oxygen as a nature broad-spectrum antibiotic [20]. In the present study, we have investigated the combine use of BMSCs transplants and HBO treatment after SCI and found the prominent improvement on the locomotive outcome. The cavitary lesions and neural fibers damage after SCI were limited in a minor volume by combination therapy compared with single treatment of HBO or BMSCs transplantation. More importantly, the post-transplant BMSCs retain a better microenvironment to survive due to the measurable decline of the pro-inflammatory cytokines production and the increase of the $BrdU^{+}$ cell after HBO treatment which gave BMSCs transplantation a better therapeutic potential.

Collectively, the combination therapy of HBO and BMSCs transplantation on SCI rat provide a beneficial microenvironment for the survival of BMSCs, contributing to the functional recovery of the neurological function, which provided new thinking and methods for the clinical application.

Conflict of interest statement

We declare that we have no conflict of interest.

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