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Original Article

The transplantation of human urine stem cells combined with chondroitinase ABC promotes brain-derived neurotrophic factor and nerve growth factor following spinal cord injury in rats

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Abstract: Cells based on therapies are currently gaining momentum in neural tissue engineering to treat spinal cord injury (SCI). The present study aimed to evaluate the effects of the concomitant use of human urine stem cells (hUSCs) and chondroitinase ABC (ChABC) on functional improvement and to explore the expressions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). The SCI model was induced by a falling heavy object. hUSCs were cultured and transplanted into the impaired spinal cord with ChABC administration. The Basso, Beattie and Bresnahan (BBB) scores were valued, and real time PCR, immunofluorescence and Western blot were used to detect the expression of BDNF and NGF. We found that rats receiving both hUSCs and ChABC treatment demonstrated the best functional recovery. In addition, the mRNA and protein expressions of the BDNF and NGF expressions were found to be effectively higher in the combined treatment group than these in the other groups. In conclusion, hUSCs transplantation combined with ChABC administration promotes motor functional recovery in SCI rats, which may be associated with BDNF and NGF regulation.

Keywords: Human urine stem cells, chondroitinase ABC, spinal cord injury, brain-derived neurotrophic factor, nerve growth factor

Introduction

Spinal cord injuries (SCI), including contusion and compression, may interrupt physiological communication between nerve centers and the body leading to severe functional deficit of limbs below the injury segment [1]. Demyelination refers to the destruction of myelin sheath after the formation of the myelin sheath, which may be caused by primary mechanical trauma, followed by secondary damage [2]. In the early stages of SCI, neurons in the injured spinal cord are largely lost, and the endogenous neurotrophic factors, such as NGF and BDNF are markedly inhibited, resulting in the suppression of axons regeneration. Cell-based therapy has been considered to be effective in promoting myelin regeneration and the recovery of spinal function [3]. The use of transplanted cells, such as embryonic stem cells, bone marrow stromal cells, oligodendrocyte precursor cells, and olfactory glial cells has been reported for the treatment of SCI in a previous study [4]. Cheng et al. [5] isolated a kind of mesenchymal stem cell from human urine, which are called human urine-derived stem cells (hUSCs). Schosserer et al. [6] pointed out that hUSCs could be induced into vascular endothelial cells, adipocytes, chondrocytes and nerve cells in vitro. Mesenchymal lineages, such as hUSCs, have been reported to be effective for the treatment of dystrophic epidermolysis bullosa, which could be associated with autoimmune disorders [7, 8]. Furthermore, the isolation of hUSCs from urine is a noninvasive measure, which may avoid iatrogenic trauma and pain for patients with SCI.

Chondroitinase ABC (ChABC) is a bacterial enzyme, which could promote axonal regeneration and reduce the inhibitory effects on neurite growth via decomposing chondroitin sulfate

proteoglycan [9]. Moreover, previous studies revealed that the combined usage of mesenchymal stem cells and ChABC was shown to have a better therapeutic effect in SCI rats [10].

In this study, we first transplanted hUSCs combined with ChABC into impaired spinal cords to evaluate the functional injuries of the lower extremities and to explore the mechanisms by which hUSCs and ChABC influence SCI.

Materials and methods

Induction of spinal cord injury

SPF grade adult male Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center of Anhui Medical University The rats were clean and healthy, male, and with body weights 290~340 g. All the rats were cared for in strict accordance with the Animal Care and Use Guidelines and all were adaptively fed for one week.

The SCI model was performed as previously described [11]. In brief, the rats were anesthetized intraperitoneally with 3.6% chloral hydrate (1.0 ml/100 g, Sigma-Aldrich, St. Louis, MO, USA). A midline incision was made, and skin and muscle were separated and then the spinal cord was injured at the T9 following an established spinal cord compression model. Rats in the control group were subjected to the same laminectomy without the compression. All rats were intraperitoneally injected with penicillin (1×10^5 U/d, Sangon Biotech Co., Ltd.) for 3 continuous days post-operation. The Basso, Beattie, and Bresnahan (BBB) locomotor rating scale of lower extremity motor function of the rats in each group was recorded for 14 consecutive days starting from day 0, according to the previous study [11].

Preparation of hUSCs

Human urine samples were freshly obtained through the donor, who provided a written, informed consent. Briefly, mononuclear cells were isolated from the low-density mononuclear fraction via using a Ficoll-Paque Plus kit (Item: 17-1440-02, Sigma-Aldrich, St. Louis, MO, USA). Total mononuclear cells were cultured in DMEM low glucose culture medium (U.S. Gibco Company), which contains basal fibroblast growth factor (bFGF; 10 ng/mL), 1,000 U streptomycin (Item: 15140122, U.S. Gibco Company), 2 mM L-glutamine (U.S. Thermo Fisher Scientific Company), stem cell factor (SCF; 10 ng/mL, Item: DS-PB-01871, Ray & Biotech Company) and 20% fetal bovine serum (FBS; U.S. Gibco Company). Total mononuclear cells were transplanted into a culture flask and were cultured at 37°C in a humidified atmosphere. The medium was replaced twice a week, and the hUSCs were passaged using 0.25% trypsin (U.S. Gibco Company).

Injection of hUSCs and ChABC into the injured sites

The injection of PBS, hUSCs and ChABC was performed at 3 days after the completion of SCI model. All the rats were anesthetized with the methods described [11]. For the control group, the lamina of the vertebra was cut without SCI, a 30-gauge needle was used, and 10 μL of PBS was injected into the T9 spinal cord at 3 locations (the upper, middle, and lower parts of the injured area) at a depth of 3 mm. A total of 60 rats were randomly assigned to 5 groups before treatment. For the control group (10 μL of PBS), hUSCs group (1×10^5 cells suspended in 10 μL of PBS), ChABC group (10 μL of 5 U/mL ChABC) and hUSCs+ChABC group (1×10^5 cells suspended in 10 μL of ChABC) were transplanted at the SCI site in the same way as for the control group. The above injection process was completed within 3 min.

RT-PCR

RT-PCR was used to determine the expression level of NGF and BDNF in the injured spinal cord. BDNF mRNA was amplified by using the primer, designated as upstream primer 5'-GAAGAGCTGTTGGACGAGGA-3', and downstream primer 5'-GAGAAGAGGAGGCTCCAAAG-3', 119 bp. NGF, 5'-CTCCGTCAACAGGACTCACA-3', and downstream primer 5'-ATCACCTCCTTGCCCTTGATG-3', 141 bp. GAPDH, 5'-GAACTCAGTGGGTCTGGAATG-3', and downstream primer 5'-GTACCCAGGATGCCTTTGAG-3', 72 bp. Total RNA was extracted from spinal cord tissues using TRIzol (U.S. Thermo Fisher Scientific Company) according to the manufacturer's protocol. The RNA was transcribed to cDNA using an RT kit and then was amplified by PCR (Shanghai Sangon Bioengineering Co., Ltd.). Glyseraldehyde-3-phosphate dehydrogenase (GAPDH, U.S. Thermo Fisher Scientific Company) was used as a refer-

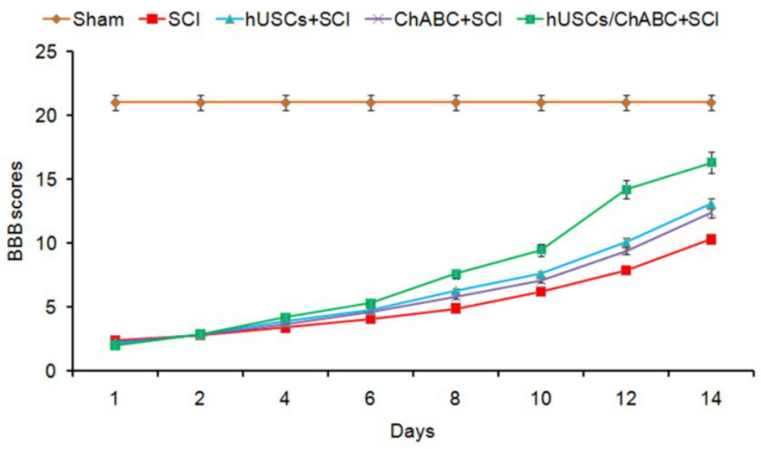

Figure 1. The recovery of motor dysfunction of rats after hUSCs and ChABC transplantation in 14 days. The nerve functions of the rats from each group were evaluated from the baseline to day 14 using the BBB system (n=12 each). SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, BBB: Basso, Beattie and Bresnahan.

ence control. RotorGene software was used for quantitative analysis.

Immunofluorescence analysis

40 g/L of polyoxymethylene was injected into the heart to fix the specimen for 30 min. The T9 spinal cord was exposed and 2 cm of the T5-L2 segment of the spinal cord was extracted. Then the samples were fixed with 40 g/L paraformaldehyde and then were paraffin embedded, microtome sectioned and processed for immunofluorescence analysis. Sections were blocked in 5% PBS for 30 min, then primary antibodies, anti-BDNF (1:750, MAB, Sigma-Aldrich, USA), anti-NGF (1:100, MAB Invitrogen Life Technologies, USA) were added at 4°C overnight. Sections were incubated with secondary antibodies, CyTM3-conjugated AffiniPure goat-anti-mouse IgG (1500, item: 101887, Jackson ImmunoResearch Europe Ltd) and CyTM3-conjugated AffiniPure goat-anti-rabbit IgG (1:1000, item: 111-095-003, Jackson ImmunoResearch Europe Ltd) in darkness at 4°C for 3 h. The hUSCs were counterstained with DAPI Dye (1:5000, Sigma-Aldrich) for 5 min, then washed 3 times with PBS and then mounted with Aqua-Poly/Mount (item: 18606-100, Polysciences, Hirschberg, Germany). The fluorescence intensity and the cells were detected using AxioVision software on the basis of Zeiss AxioPlan 2 imaging MOT fluorescent microscope.

Western immunoblot analysis

The protein in each group was extracted and evaluated using BCA protein quantitative series kits (Thermo Scientific Company). Then the proteins were transplanted to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and subjected to polyvinylidene fluoride (PVDF) membranes. Then, PVDF membranes were blocked with Tris-buffered saline (TBS) or 5% nonfat milk for 3 h. Then, the PVDF membranes were incubated with rabbit polyclonal anti-BDNF (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-NGF (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Secondary antibodies of Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies (1:800 and 1:1000 each; Santa Cruz Biotechnology, Santa Cruz, CA) were used with primary antibodies. A densitometric analysis was performed on the basis of Quantity One software (Bio-Rad Laboratories).

Statistical analysis

SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All measurements were performed in triplicate and the data were presented as the mean ± standard deviation. Comparisons of overall difference between the groups were made by one-way analysis of variance followed by Dunnett's test. P values < 0.05 were considered statistically significant.

Results

BBB value of motor dysfunction

The BBB value is shown in **Figure 1**, and it reveals that the BBB scores decreased significantly after SCI, and the BBB scores increased with a trend for spontaneous recovery over time. It was remarkable that the BBB scores in the hUSCs/ChABC+SCI group began to increase significantly when compared with the hUSCs+SCI group or the ChABC+SCI group at day 8,

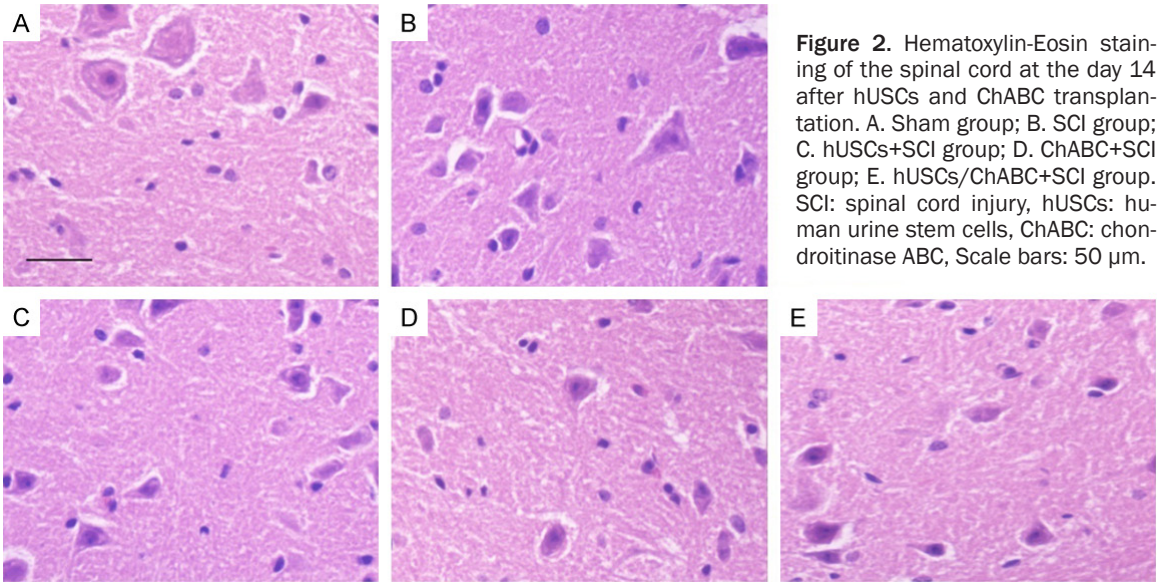

Figure 2. Hematoxylin-Eosin staining of the spinal cord at the day 14 after hUSCs and ChABC transplantation. A. Sham group; B. SCI group; C. hUSCs+SCI group; D. ChABC+SCI group; E. hUSCs/ChABC+SCI group. SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, Scale bars: 50 μm.

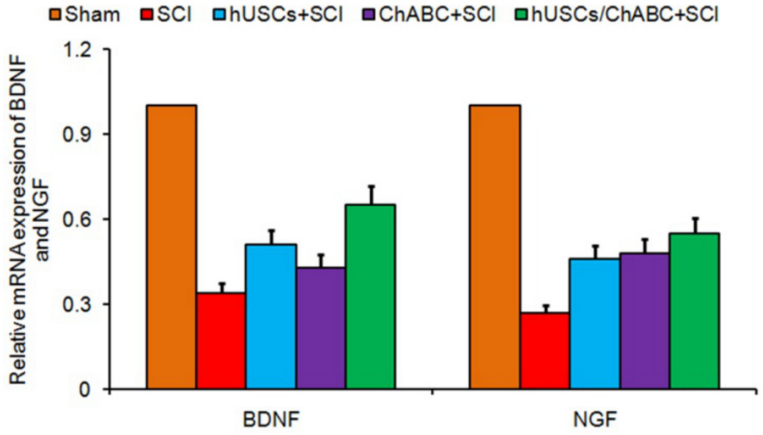

Figure 3. mRNA levels of BDNF and NGF of each group were determined by real-time PCR at day 14 after hUSCs and ChABC transplantation. SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor.

and they remained statistically significant in the following 6 days.

Pathological observation of spinal cord

Pathological changes were observed at day 14 after SCI as shown in **Figure 2**. HE staining revealed that normal spinal cord structure was demonstrated in the sham group. Increased edema, inflammatory cell infiltration, diffuse hemorrhage and congestion with the most obviously degenerated neurons were observed in the SCI group. Besides, mild edema, partial neuron denaturation and less degenerated neurons were found in the hUSCs+SCI, ChABC+SCI and hUSCs/ChABC+SCI groups. In the hUSCs/ChABC+SCI group, slight edema and inflammatory cell infiltration were observed, and nerve corpuscles were relatively complete and clear, and neuranagenesis was very clear.

mRNA levels of BDNF and NGF

Real-time PCR indicated that the mRNA expressions of the BDNF and NGF in each group decreased significantly in the SCI group when compared to the sham group ($P < 0.05$). The hUSCs+SCI, ChABC+SCI and hUSCs/ChABC+SCI groups had an increased mRNA expression of BDNF and NGF, as compared with the SCI group ($P < 0.05$). However, no significant difference was found between the hUSCs+SCI and ChABC+SCI groups ($P > 0.05$). Compared to the hUSCs+SCI and ChABC+SCI groups, the hUSCs/ChABC+SCI group revealed markedly increased BDNF and NGF mRNA expressions ($P < 0.05$), as shown in **Figure 3**.

Immunofluorescence of BDNF and NGF

To confirm the expressions of BDNF and NGF in the spinal cord after cell transplantation, the immunofluorescence of BDNF and NGF in the

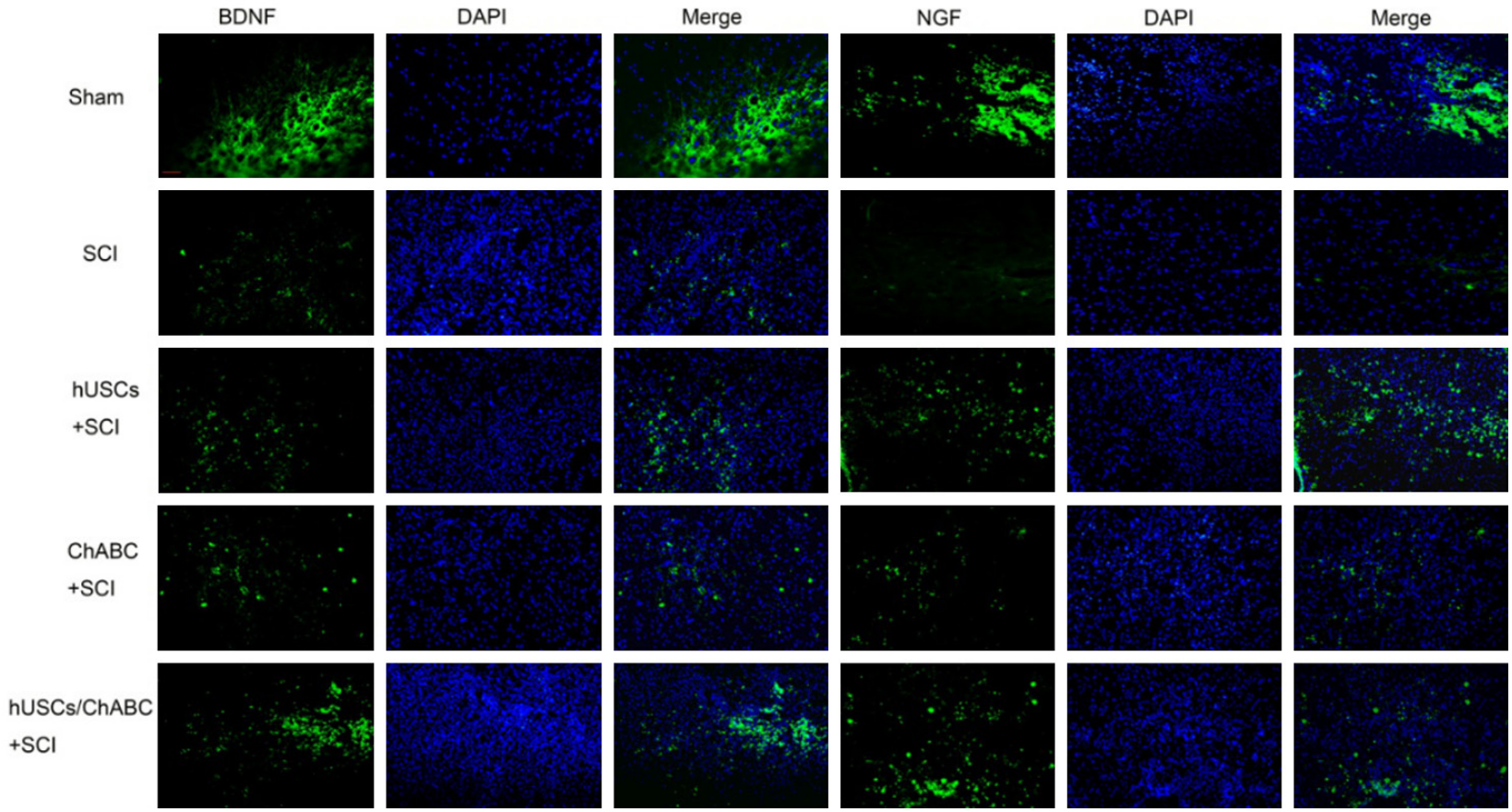

Figure 4. The immunofluorescence of BDNF and NGF in injured spinal cord at the day 14 after hUSCs and ChABC transplantation. SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor, Scale bars: 50 μm.

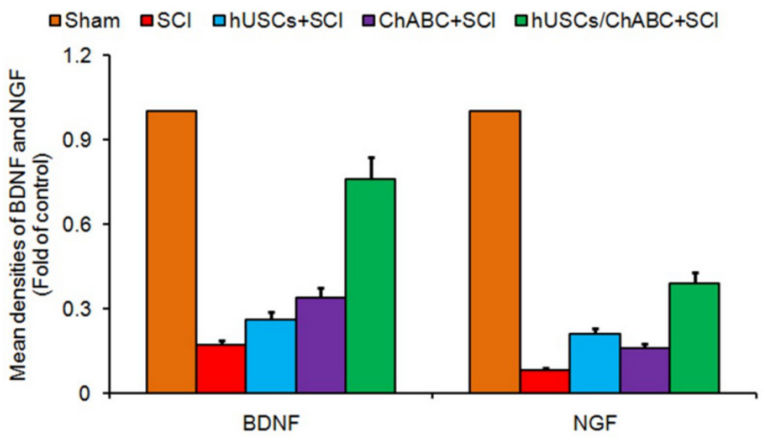

Figure 5. Mean densities of BDNF and NGF in each group. SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor.

spinal cord was performed in each group at day 14 after transplantation ($P < 0.01$). The expressions of BDNF and NGF were significantly higher in the Sham group than in the other 4 groups ($P < 0.05$). Moreover, the expressions of BDNF and NGF in the hUSCs+SCI and the ChABC+SCI groups were markedly higher than that in the SCI group ($P < 0.05$). However, no significant difference was found between the hUSCs+SCI and the ChABC+SCI group ($P > 0.05$). Meanwhile, the expressions of BDNF and NGF in the hUSCs/ChABC+SCI group were significantly higher than those in the hUSCs+SCI and ChABC+SCI groups ($P < 0.05$), as shown in **Figures 4**, **5**.

Protein expression of BDNF and NGF

The western blot analysis was used to assess the expressions of BDNF and NGF protein in the spinal cords of all groups. The results demonstrated that the protein expression of BDNF and NGF in the spinal cords was performed in each group at day 14 after transplantation ($P < 0.01$). The expressions of BDNF and NGF were significantly higher in the Sham group than the other 4 groups ($P < 0.05$). Also, the expressions of BDNF and NGF in the hUSCs+SCI and the ChABC+SCI groups were markedly higher than that in the SCI group ($P < 0.05$). However, no significant difference was found between the hUSCs+SCI and the ChABC+SCI group ($P > 0.05$). Meanwhile, the expressions of BDNF and NGF in the hUSCs/ChABC+SCI group were significantly higher than that in the hUSCs+SCI and the ChABC+SCI group ($P < 0.05$), as **Figure 6** shows.

Discussion

Previous studies have demonstrated that pathologic changes after SCI-induced primary damage are a complicated process, including spinal cord hemorrhage, cell apoptosis and neuron necrosis. Further, secondary impairment, such as spinal cord ischemia, internal environment imbalance, active oxygen radicals, and lipid peroxidation injury, inflammatory response and excitatory toxicity are the of main causes of functional disorders [1, 12]. A glial scar can be induced after SCI, which may suppress the axonal regeneration [13]. Zhu et al. [14] pointed out that ChABC might be the main substance in the scars that was found to inhibit axon regeneration. Therefore, the improvement of the scar microenvironment may contribute to the recovery of spinal cord function.

hUSCs displayed similar characteristics to adipose mesenchymal stem cells (AMSCs), such as antigen and multipotential differentiation [15]. hUSCs could be induced into vascular endothelial cells, adipocytes, chondrocytes and nerve cells in vitro. Moreover, the hUSCs excreted some cytokines, such as mast cell growth factor, which might regulate the microenvironment and provide favorable conditions for stem cells [16]. Studies have demonstrated that ChABC could promote axonal regeneration by relieving scar formation [17]. Karus et al. [18] argued that ChABC might be degraded in the lesion area following intrathecal injection in the injured spinal cord. Hunanyan et al. [19] held that ChABC application might improve neuronal fiber regeneration in the impaired spinal cord but could not promote budding and regeneration of the injured neurons in the integrated spinal cord. In the present study, we transferred the hUSCs combined with ChABC into injured spinal cords successfully. We found that a separate application of hUSCs or ChABC could effectively improve the recovery of hindlimb locomotor function. Besides, the joint use of ChABC with hUSCs transplantation presented better

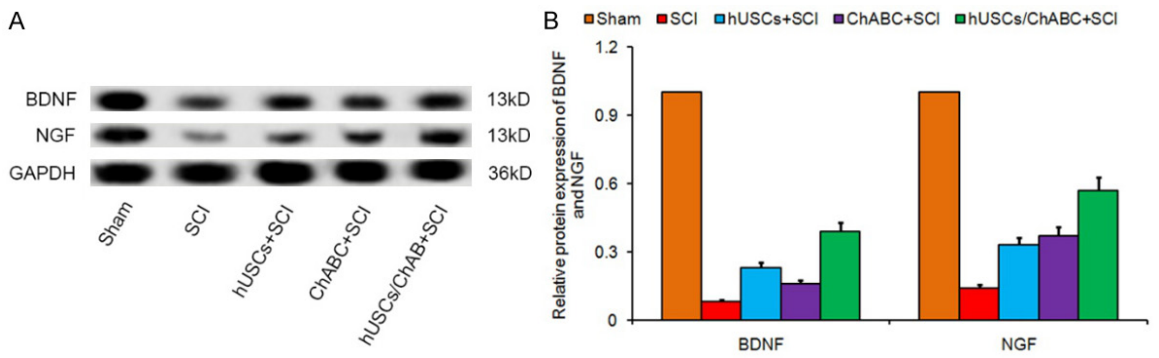

Figure 6. The western blot assay of BDNF and NGF in injured spinal cord at the day 14 after hUSCs and ChABC transplantation. SCI: spinal cord injury, hUSCs: human urine stem cells, ChABC: chondroitinase ABC, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor.

hindlimb locomotor function and histological examination when compared with the separate use of hUSCs and ChABC, an indication that combined treatment may lead to better therapeutic effects.

To investigate further, the effects of chABC and hUSCs transplantation were evaluated in this study from the morphological, genetic and protein aspects using real time PCR, Western blot and immunofluorescence. In the present study, there were significant increases of BDNF and NGF after hUSCs or ChABC transplantation was confirmed. However, the joint usage of ChABC with hUSCs transplantation demonstrated significantly higher expression of BDNF and NGF when compared with the separate usage of hUSCs and ChABC. Previous studies have revealed that neurotrophic factors play a key role in regeneration following SCI [20]. BDNF not only promotes the survival of spinal cord neurons and the prolongation of nerve fibers, it also inhibits the atrophy and apoptosis of neurons [21]. Xiong et al. [22] transfected BNDF into bone marrow mesenchymal stem cells (BMSC), and they found that BMSC-BNDF treatment revealed better functional recovery and axonal regeneration [23]. The fact that stem cells secrete nutritional factors was demonstrated both in vitro and in vivo [24, 25]. Besides, remyelination and axons sprouting could be enhanced by BDNF in the spinal cord [26]. Fournier claimed that BDNF application could promote the growth-associated protein expression, such as T-alpha-1-tubulin and growth associated protein-43 (GAP-43) in neurons [27]. The protection mechanism of BDNF against SCI-induced neurotoxicity is that the Tropomyosin receptor kinase B (TrkB) receptor could be activated by BDNF via TrkB/CREB/BDNF pathway, as TrkB receptors were widely distributed in the spinal cord [28]. Traumatic oxidative stress-induced nerve damage could raise robust spouting of nociceptive axons, and inhibitors to Tropomyosin receptor kinase A (TrkA) or NGF could suppress the axonal sprouting of nociceptive axons [29, 30]. Local injection of NGF could accelerate the sprouting of the corticospinal tract after adult rat spinal cord transection, which indicated that NGF might act via sensory neurons and the motor neurons of the anterior horn [20]. NGF could also inhibit the release of toxic amino acids and inhibit calcium overload following SCI [31, 32].

Conclusion

In this study, we first conducted SCI treatment using hUSCs, and the results revealed that hUSCs could markedly improve motor function. However, the combined use of hUSCs and the administration of ChABC presented the best curative effects, and the mechanism might be associated with increased levels of BDNF and NGF.

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Disclosure of conflict of interest

None.

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