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Chondroitinase ABC plus bone marrow mesenchymal stem cells for repair of spinal cord injury☆

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Abstract

As chondroitinase ABC can improve the hostile microenvironment and cell transplantation is proven to be effective after spinal cord injury, we hypothesized that their combination would be a more effective treatment option. At 5 days after T_8 spinal cord crush injury, rats were injected with bone marrow mesenchymal stem cell suspension or chondroitinase ABC 1 mm from the edge of spinal cord damage zone. Chondroitinase ABC was first injected, and bone marrow mesenchymal stem cell suspension was injected on the next day in the combination group. At 14 days, the mean Basso, Beattie and Bresnahan score of the rats in the combination group was higher than other groups. Hematoxylin-eosin staining showed that the necrotic area was significantly reduced in the combination group compared with other groups. Glial fibrillary acidic protein-chondroitin sulfate proteoglycan double staining showed that the damage zone of astrocytic scars was significantly reduced without the cavity in the combination group. Glial fibrillary acidic protein/growth associated protein-43 double immunostaining revealed that positive fibers traversed the damage zone in the combination group. These results suggest that the combination of chondroitinase ABC and bone marrow mesenchymal stem cell transplantation contributes to the repair of spinal cord injury.

Key Words

neural regeneration; spinal cord injury; stem cells; chondroitin sulfate proteoglycans; astrocytes; glial scar; chondroitinase ABC; bone marrow mesenchymal stem cells; transplantation; chemical barrier; neuroregeneration

Research Highlights

(1) After spinal cord injury, a large number of inhibitory factors are generated in the microenvironment of the damage zone, such as chondroitin sulfate proteoglycan.
(2) To avoid the acute-stage complex pathological conditions, and to improve the effect of cell transplantation, chondroitinase ABC is used to overcome the damaging chemical barrier. This study paid attention to the improvement of microenvironment in the damage region, which is a novel idea for breaking conventional transplantation.

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INTRODUCTION

Spinal cord injury often leads to tragic results, which include primary injury and secondary injury stages. The primary injury is very serious and difficult to control.

Another important feature of spinal cord injury is the secondary damage of neurons. In this period, the pathology of spinal injury is dictated not only by the initial mechanical insult, but also by secondary processes that include inflammatory ischemia and cell death[1], and eventually results in cavity

formation and enlargement of the injury zone. Cytokines, free radicals and nitric oxide generated by microglia stimulate reactive astrocytes to secrete proteoglycans and to form the glial scar[2-3]. In addition, retrograde degeneration occurs in the lesioned spinal tracts[4]. Severed axons are limited by the inhibitory nature of myelin and the glial scar, and are only capable of sprouting with little functional recovery[5-7].

The limited repair capacity of axons is a challenge for clinical treatment. Studies suggest that after spinal cord injury, the mechanical barrier formed by the glial scar and the chemicals secreted are important factors that hinder the growth of central nervous system axons. The glial scar is intertwined[8], which is not enough to hinder the growth of nerve fibers[9-10]. Also, the chemical barrier plays a very prominent role in axonal growth, which determines the microenvironment for the growth of nerve fibers. The glial scar is composed of several components, of which chondroitin sulfate proteoglycans are very important. Chondroitin sulfate proteoglycans are the main extracellular matrix proteins secreted by astrocytes, and neuron-glial antigen 2 is the main component of chondroitin sulfate proteoglycans[11]. Early suppression of chondroitin sulfate proteoglycan secretion from astrocytes can reduce the inhibitory effect of regenerated fibers[6]. You *et al* [8] found that in complete rat spinal cord injury model, astrocyte proliferation occurs in the surrounding tissue of the injured central area of the spinal cord, and part of the cell protrusions are intertwined to form a loose network structure. It is not enough to interfere with nerve fiber *via* the mechanical barrier[12-14]. It is generally accepted that inhibiting factors, including Nogo-A, MAG and extracellular matrix, noticeably affect the regeneration and repair of the spinal cord. Chondroitin sulfate proteoglycans, keratan sulfate proteoglycan and tenascin-C are the predominant molecules. Among them, chondroitin sulfate proteoglycans are the most typical and representative[15-16]. The expression of specific chondroitin sulfate proteoglycans after spinal cord injury, such as neuron-glial antigen 2, versican, neurocan, brevican and phosphacan, is not consistent[16]. Several chemical substances have already been isolated to inhibit axonal outgrowth. These inhibitory molecules can form a chemical barrier to hinder the growth of central nervous axons. Among them, the action of chondroitin sulfate proteoglycans caused by the molecular chemical barrier is outstanding. During central nervous system development, chondroitin sulfate proteoglycans can adjust nerve hyperplasia, migration, differentiation, axonal growth, the direction of growth and the formation and maturation of synapses. After spinal cord injury, chondroitin sulfate proteoglycans show powerful depressant effects[17-19]. Because of massive secretion of chondroitin sulfate proteoglycans by hyperplastic astrocytes, other studies show that nervous process growth may associate with chondroitin sulfate proteoglycans, thus chondroitin sulfate proteoglycans play a leading role in axonal growth[20-21].

It has been confirmed that bone marrow mesenchymal stem cells can survive and differentiate in the spinal cord injury region[22]. They can also survive long term in the host brain tissue, with an absence of immune activity or gene therapy, and can support the survival of neurons[23-24]. Therefore, bone marrow mesenchymal stem cells are an ideal source of cells[25-28]. Cell transplantation has been a hot topic of spinal cord injury in clinical treatment[29]. Cell transplantation holds the potential for repair and functional plasticity following spinal cord injury. Stem and progenitor cells are capable of modifying the lesion environment and providing structural support, myelination, increasing neurotrophic factors for neuroprotection and endogenous activation. Cellular therapies for dysmyelination include transplantation of neural stem/progenitor cells, oligodendrocyte precursors or Schwann cells to directly promote remyelination of axons. The injection of bone marrow mesenchymal stem cells and growth factors can upregulate the survival and activity of myelinating cells[30-31]. Bone marrow mesenchymal stem cells can be conveniently harvested, easily isolated, can be used for autologous transplantation without ethical problems[32], are unlikely to cause allograft rejection, and have the advantage of multipotential differentiation[33-37]. Thus, bone marrow mesenchymal stem cells may act as transplanted cells for treatment of spinal cord injury.

This study used chondroitinase ABC to inhibit neuron-glial antigen 2 in the early injury stage, and observed the repair of spinal cord injury by combination with bone marrow mesenchymal stem cells in rat models of spinal cord crush injury. It provides an experimental basis for new methods of treatment of clinical spinal cord injury.

RESULTS

Quantitative analysis of experimental animals

A total of 24 Sprague-Dawley rats were included in the experiment. Animals were equally and randomly divided into the model group, bone marrow mesenchymal stem cell group, chondroitinase ABC group and combination

group. At 5 days after spinal cord injury, bone mesenchymal stem cell suspension or chondroitinase ABC were injected 1 mm from the edge of the spinal cord damage zone in the bone marrow mesenchymal stem cell group and chondroitinase ABC group, respectively. Chondroitinase ABC was first injected, and bone marrow mesenchymal stem cell suspension was injected on the following day in the combination group. All 24 rats were included in the final analysis.

Effects of chondroitinase ABC combined with bone marrow mesenchymal stem cells on motor function and necrotic areas in rats with spinal cord injury

After spinal cord compression injury, the rats initially manifested complete functional loss of hindlimbs, and their Basso, Beattie and Bresnahan score was 0. Hindlimb motor function began to recover after 3 days. At 7 days after spinal cord injury, the rats had recovered movement of three joints of the hindlimbs, showing rhythmic movement. No significant differences were found between groups. At 14 days after spinal cord injury, rat hindlimbs in the model group, bone marrow mesenchymal stem cell group and chondroitinase ABC group dragged and there was no weight loading of the three joints, but there was no significant difference. In the combination group, the rats only put the plantar surface of their foot in the weight-bearing position, with occasional weight-bearing walking being seen only on the dorsum of the foot. The Basso, Beattie and Bresnahan scores in the combination group were higher than the other three groups ($P < 0.05$; Table 1).

Table 1 Change of Basso, Beattie and Bresnahan scores and necrotic area of spinal cord injury zone in rats following treatment with chondroitinase ABC plus bone marrow mesenchymal stem cells

Group	Basso, Beattie and Bresnahan scores		Area of necrosis (mm^2, hematoxylin-eosin staining)
	7 d	14 d	
Model	4.21±1.88	7.22±2.42[a]	4.18±1.03[a]
Bone marrow mesenchymal stem cell	4.58±1.67	7.61±2.59[a]	4.04±0.93[a]
Chondroitinase ABC	4.48±1.58	7.75±2.32[a]	3.82±0.85[a]
Combination	4.55±1.74	9.36±3.58	2.81±0.87

Higher Basso, Beattie and Bresnahan scores indicate improved motor function. At 14 days after injury, the actual area of necrosis was evaluated by hematoxylin-eosin staining. [a]$P < 0.05$, *vs*. combination group. Data are expressed as mean ± SD with six rats in each group. Intergroup comparisons were conducted by one-way analysis of variance followed by *post hoc* test.

At 14 days after spinal cord injury, hematoxylin-eosin staining showed the cavity of the injury zone. There were a large number of macrophages in and surrounding the cavity, which was wrapped by scar tissue. There was a distinct boundary formed with the normal spinal cord tissue, and it was distinct from the damage zone. The damage area of the combination group was significantly smaller than the other three groups ($P < 0.05$; Figure 1, Table 1).

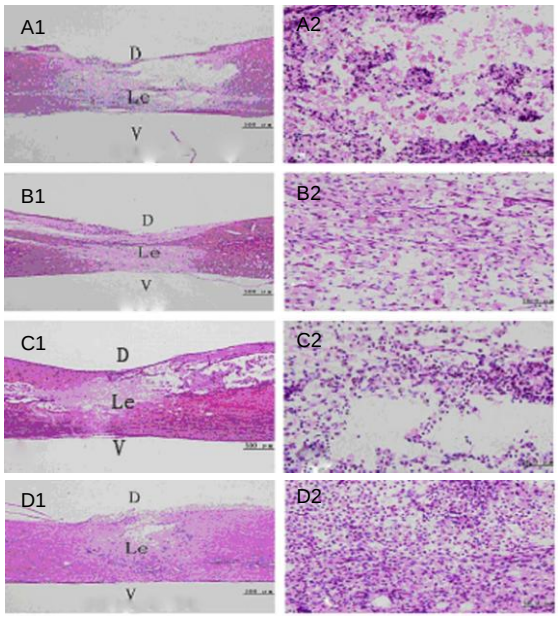

Figure 1 Hematoxylin-eosin staining in the spinal cord damage zone following treatment with chondroitinase ABC plus bone marrow mesenchymal stem cells.

At 14 days after injury, hematoxylin-eosin staining was carried out to evaluate the actual area of necrosis. (A1, A2) Model group (× 4, × 40). (B1, B2) Bone marrow mesenchymal stem cell group (× 4, × 40). (C1, C2) Chondroitinase ABC group (× 4, × 40). (D1, D2) Combination group (× 4, × 40).

D: Dorsal; Le: lesion damage zone; V: ventral. The cavity in the bone marrow mesenchymal stem cell group, chondroitinase ABC group and combination group was significantly reduced.

Effects of chondroitinase ABC combined with bone marrow mesenchymal stem cells on glial fibrillary acidic protein/chondroitin sulfate proteoglycans double immunostaining in rats with spinal cord injury

CS56 is a specific marker for chondroitin sulfate proteoglycans. Double immunostaining showed that glial fibrillary acidic protein-positive astrocytes uniformly distributed in the gray matter and white matter of the staining in the normal rat spinal cord. There were seldom positive CS56 or CS56-glial fibrillary acidic protein double staining (data not shown). After spinal cord injury, glial fibrillary acidic protein expression was enhanced and a few double-labeled CS56 and glial fibrillary acidic

protein expressers were found. Glial fibrillary acidic protein expression was also enhanced at 14 days. Glial fibrillary acidic protein expression was significantly reduced in the combination group compared with the model, chondroitinase ABC and bone marrow mesenchymal stem cell groups. At 14 days, glial fibrillary acidic protein expression was mainly concentrated at the edge of the damage zone in the model group. The astrocytic scars and their limiting membrane were formed and a visible cavity was observed. Chondroitin sulfate proteoglycans were expressed in the center of the injury and were concentrated in the glial limiting membrane. In the bone marrow mesenchymal stem cell group, bone marrow mesenchymal stem cells filled the damage zone and no obvious cavity was formed. In the chondroitinase ABC group, the expression of chondroitin sulfate proteoglycans in scar limiting membrane of astrocytes was significantly reduced. In the combination group, astrocytic scars were significantly reduced, without cavity formation (Figure 2).

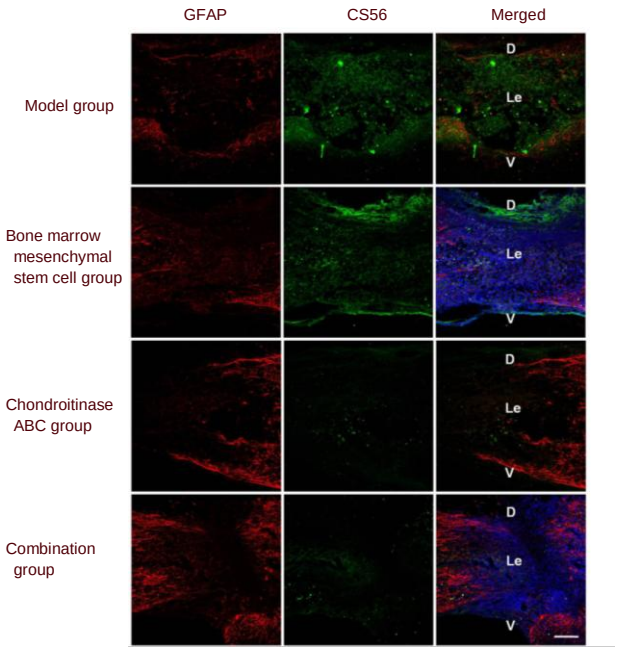

Figure 2 Double immunostaining for glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs) of injured spinal cord treated with chondroitinase ABC plus bone marrow mesenchymal stem cells under a fluorescence microscope.

At 14 days after spinal cord injury, GFAP/CSPGs double labeling was performed to evaluate the expression of CSPGs. D: Dorsal; Le: lesion damage zone; V: ventral. Scale bar: 200 μm. CS56, specific marker of CSPGs, is green; GFAP is red for labeled glial scar. Blue means the transplanted bone marrow mesenchymal stem cells. The expression of GFAP in the combination group was less than the other groups.

CS56 fluorescence relative intensity results showed that chondroitinase ABC significantly inhibited chondroitin sulfate proteoglycan formation. In the model group, the relative intensity of CS56 fluorescence was 1 ± 0.05, while it was 0.95 ± 0.08 in the bone marrow mesenchymal stem cell group. The relative intensities of CS56 fluorescence in the chondroitinase ABC group (0.24 ± 0.06) and combination group (0.2 ± 0.04) were significantly lower than the model group and bone marrow mesenchymal stem cell group ($P < 0.05$). No significant difference was detectable between the bone marrow mesenchymal stem cell group and the model group ($P > 0.05$).

Effects of chondroitinase ABC combined with bone marrow mesenchymal stem cells on glial fibrillary acidic protein/growth-associated protein-43 double immunostaining in rats with spinal cord injury

At 14 days, glial fibrillary acidic protein expression was mainly detected in the damage zone in the model group with the formation of glial scar, where the glial limiting membrane boundary was clear at the edge of the damage zone and the cavity formed in the damage zone. A small amount of growth-associated protein-43 expression was observed within the damage zone.

In the bone marrow mesenchymal stem cell group, the damage zone was full of bone marrow mesenchymal stem cells, without cavity formation. Growth-associated protein-43 expression showed a punctuate distribution in the damage zone. In the chondroitinase ABC group, a small amount of growth-associated protein-43 positive fibers were arranged in vertical distribution within the damage zone. In the combination group, growth-associated protein-43-positive fibers traversed the damage zone (Figure 3).

Growth-associated protein-43 staining showed that combined transplantation significantly promoted axonal growth. Chondroitinase ABC combined with bone marrow mesenchymal stem cells had better results compared with bone marrow mesenchymal stem cells or chondroitinase ABC alone, where the relative intensity of growth-associated protein-43 fluorescence was 3 ± 0.08 *vs*. 2.10 ± 0.07 and 1.50 ± 0.06 ($P < 0.05$), respectively. The relative intensities of growth-associated protein-43 fluorescence in the bone marrow mesenchymal stem cell group and chondroitinase ABC group were each higher than the model group (1.00 ± 0.04; $P < 0.05$). There was no significant difference in the bone marrow mesenchymal stem cell group compared with the chondroitinase ABC group ($P > 0.05$).

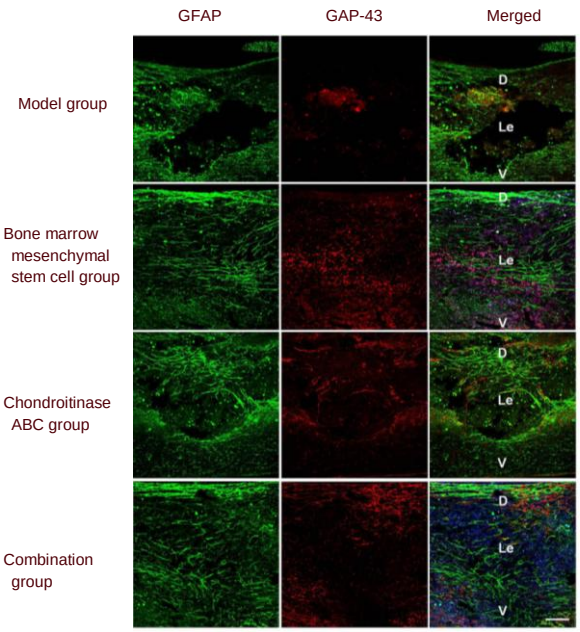

Figure 3 Double immunostaining for glial fibrillary acidic protein (GFAP) and growth-associated protein-43 (GAP-43) of injured spinal cord treated with chondroitinase ABC plus bone marrow mesenchymal stem cells under a fluorescence microscope.

At 14 days after spinal cord injury, GFAP/GAP-43 double immunofluorescence staining was performed to evaluate the regeneration of nerve fibers. D: Dorsal; Le: lesion damage zone; V: ventral. Scale bar: 200 μm. GFAP is green for labeling the glial scar; GAP-43 is red for labeled neurons. Blue represents the transplanted bone marrow mesenchymal stem cells. The expression of GAP-43 in the combination group was more obvious than other groups.

DISCUSSION

Some other techniques have been used to establish spinal cord transection models, which is detected through a number of methods to ensure complete spinal cord transection[38]. However, the damaged zone of completely paralyzed patients has remaining neural tissue in clinical practice. Long-term research has found that spinal cord injury models can simulate pathological changes in the process of clinical spinal cord injury[39], so the rat spinal cord crush injury was selected in this experiment.

Even though specific implementation of this intervention strategy was performed, there are two problematic factors that must be taken into account. First, the injury time window is a critical determinant factor, which is due to changes in the structure of the damaged neurons and in the local microenvironment over time. After the acute stage of injury, some inhibitory factors appear, such as high expression of chondroitin sulfate proteoglycans, which disappear in the chronic stage of injury. This indicated to us that the ideal state of axonal regeneration is time-dependent. Second, the measures to stimulate axonal growth are selective. For example, some interventions are chosen to weaken the role of chondroitin sulfate proteoglycans so that glial scar formation may be inhibited, which leads to a wide range of downstream axonal regeneration and causes incomplete reinnervation into host tissue.

Cell therapy is a promising approach to restore locomotor or sensory function, and mesenchymal stem cells have been investigated extensively as a promising candidate for cell therapy[40-42]. Hofstetter *et al* [43] suggested that bone marrow mesenchymal stem cells are mainly concentrated in the outer periphery of the damage zone immediately after injection in the adjacent zone of the injury site. However, in their study, bone marrow mesenchymal stem cells were injected at 7 days after spinal cord injury. We performed cell transplantation and microinjection of chondroitinase ABC, which can prevent the acute immune inflammation stage and remove the pathological effects of the chemical barrier[44-45]. Bone marrow mesenchymal stem cells mainly gathered in the injured spinal cord, where lower extremity functional recovery was more apparent. Because of spinal cord injury, these unfavorable factors, such as ischemia, hypoxia, spinal cord tissue necrosis, the release of oxygen free radicals, the aggregation of a variety of inflammatory cells and various cytotoxic chemical factors, commonly become harmful and are not conducive to the survival of transplanted cells[46-47]. After bone marrow mesenchymal stem cell transplantation, growth-associated protein-43 expression in the damage zone was accelerated. In particular, the application of chondroitinase ABC combined with bone marrow mesenchymal stem cells into the spinal cord injury stump reduced glial fibrillary acidic protein expression in the damage zone and scar tissue was less. Bradbury[48] confirmed that about 20% of the transected corticospinal tract axons may be able to regenerate through injured segments injected with chondroitinase ABC to C_4 injured segments by cephalad intrathecal injection at 14 days after spinal cord injury. This regeneration was seen around the cavity and the growth may extend to injured distal white matter and gray matter of a few millimeters. At 14 days after spinal cord injury, results showed that growth-associated protein-43 expression was stronger in the chondroitinase ABC group and combination group than in the model group. The necrotic area and diameter of the combination group and chondroitinase ABC group were much smaller than in the model group. Basso, Beattie and Bresnahan scores manifested that combined

transplantation might have some therapeutic effect in this model of spinal cord injury. In the combination group, chondroitinase ABC and bone marrow mesenchymal stem cells were injected into the injury site resulting in decreased glial fibrillary acidic protein expression in the injured area. When combined transplantation was selected, the glial scar was digested by chondroitinase ABC, CS56 immune intensity was remarkably decreased, growth-associated protein-43 expression increased, and functional recovery was promoted, thus indicating that combined transplantation is better than cell transplantation alone.

Chondroitin sulfate proteoglycans secreted by astrocytes and oligodendrocytes gradually increased at the site of injury. In addition to microglia, the other cell components in the glial scar can produce chondroitin sulfate proteoglycans[49-50]. There are many types of chondroitin sulfate proteoglycans, which vary in specific molecule expression. Nevertheless, GAG chain is a common chemical structure of chondroitin sulfate proteoglycans, and a critical molecular structure that hinders the growth of axons[51-52]. The chondroitin sulfate proteoglycan mechanism of inhibiting axonal growth is very complex. Chondroitinase (chondroitinase ABC) is a soluble enzyme that can digest the GAG side chains of the chondroitin sulfate proteoglycans[48, 53-54]. In the model group, glial fibrillary acidic protein expression was elevated after 14 days of spinal cord injury, and CS56 expression was significantly high. At 14 days of spinal cord injury, after application of chondroitinase ABC, glial fibrillary acidic protein expression was significantly reduced compared with the model group, and CS56 expression showed a relatively reduced trend. These results confirm that chondroitinase ABC inhibited reactive hyperplasia of glial cells. Thus, the effect of chondroitinase ABC may create a way for the regenerated nerve fibers to traverse the damage zone.

Astrocytes affect the synthesis and release of various neurotrophic factors, such as cholinergic neurotrophic factor[55], neurotrophin-3 and brain-derived neurotrophic factor[56]. Leme *et al* [57] have shown that the glial cell response is enhanced after spinal cord injury. Glial fibrillary acidic protein expression is a marker of normal and reactive astrocytes. The level of glial fibrillary acidic protein expression may indirectly reflect astrocyte proliferation, hypertrophy and migration[10-11]. After spinal cord injury, neuronal degeneration and necrosis were found in the damage area[45]. Neuronal damage and cell disintegration lead to a loss in the ability to respond to instant trauma. Under traumatic stress stimulation and a variety of induced factors, nerve cells from the adjacent damaged area can manifest reactive hyperplasia to adapt to the needs of nerve regeneration.

This study observed that in 1 week after spinal cord injury, a layer of astrocytes form a barrier around the damage area, which has adverse effects on axonal regeneration. To date, the most common treatment for spinal cord injury is the transplantation of single cells, but the microenvironment is not considered. This may be the reason for poor therapeutic outcomes. We believe that both aspects must be emphasized and combined[58] by adjusting the microenvironment in the injury site and performing stem cell transplantation. Chondroitinase ABC can degrade the glial scar[59] and specifically degrade chondroitin sulfate proteoglycans, thus improving the bad microenvironment. This treatment opens a window of opportunity for task-specific rehabilitation. Furthermore, cell transplantation has been shown to be a good choice for combating cell loss in the damage zone[60]. Thus, therapies for spinal cord injury include many aspects to promote remyelination, such as scar-degrading enzymes, trophic support, and cell replacement[61-62]. We hypothesized that injecting chondroitinase ABC into the spinal cord injury site might digest the glial scar. Under normal circumstances, astrocytes play a role in support and nutrition for neurons. After spinal cord injury, astrocytes become inhibitory, they are replaced by the excessive proliferation of glial cells and result in the formation of the cavity. A chemical barrier forms and inhibits nerve fiber regeneration. We applied chondroitinase ABC in the subacute stage of spinal cord injury, aiming to address this dual role of astrocytes and inhibit its disadvantages. According to these strategies, we hoped to provide a suitable microenvironment for the repair of spinal cord injury. From the expression of growth-associated protein-43, we found that application of chondroitinase ABC may significantly inhibit chondroitin sulfate proteoglycans and this may be considered as an adjuvant method before cell transplantation. Thus, this study provided a theoretical basis for its clinical use, but its spinal cord repair mechanisms need to be further studied.

MATERIALS AND METHODS

Design

A randomized, controlled animal study.

Time and setting

This experiment was performed from January to

December 2007 in the Neuroscience Institute of the Fourth Military Medical University, China.

Materials

A total of 24 adult male Sprague-Dawley rats aged 10–12 weeks, weighing 200–230 g, were offered by the Experimental Animal Center of Xi'an Jiaotong University, China (license No. SCXK 2012-003). Animal care and experimental procedures were approved by the Committee of Xi'an Jiaotong University in China for Ethics in Animal Experiments. Rats were allowed free access to food and water and maintained on a 12-hour light/dark cycle. After surgery, rats were placed in temperature- and humidity-controlled incubation chambers until they awoke. They were housed in cages, and bladder evacuation was applied daily until the return of reflexive bladder control. Animal procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[63].

Methods

Culture of bone marrow mesenchymal stem cells

The medulla was removed from newborn rat femurs and tibias. Bone marrow was anticoagulated and diluted by low-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 1 000 mg/L D-glucose, 584 mg/L L-glutamine and 125 mg/L pyruvic acid sodium (Invitrogen, Carlsbad, CA, USA), which was equally divided into two parts and separated by Percoll (Invitrogen) and Ficoll (Invitrogen), respectively. Percoll separation: specific gravity of 1.073 g/mL Percoll was placed in the bottom of the tube, and then the bone marrow was dropped slowly and diluted in a mixture of Percoll and marrow (1:1) suspension, followed by centrifugation at 1 000 r/min for 30 minutes. Ficoll (1.077 g/mL) separation: Ficoll and bone marrow suspension ratio was 1:2, followed by centrifugation at 800 r/min for 30 minutes. Separated mononuclear cells were harvested, rinsed with DMEM twice, and seeded in 24-well plates at a concentration of $2.0 \times 10^5/cm^2$. Low-glucose DMEM containing fetal bovine serum of five different batches and two concentrations (10% and 20%) was added to each well. After 48 hours, non-adherent cells were removed and the culture medium was replaced. Cells were incubated at 37°C in 5% CO_2 until the cells reached 90% confluence, followed by 0.05% trypsin (Invitrogen) digestion and subculturing for further use.

Animal model of spinal cord injury

Rats were intraperitoneally anesthetized with 1% sodium pentobarbital (50 mg/kg). In prone position, T_8 lamina was opened to expose the spinal cord. Spinal dura mater was intact. Under guidance of the SR-6 stereotaxic apparatus (Narishige, Tokyo, Japan), a spinal cord injury impact device (the Fourth Military University Institute of Neuroscience Institute in China) was applied by squeezing at a weight of about 20 g and vertical speed of 0.5 mm/min for 5 minutes. The extrusion pressure was removed within 1 minute. Muscle and skin were sutured. Postoperatively, the rats were assisted to urinate twice daily. At 5 days after spinal cord injury, 2 μL of bone marrow mesenchymal stem cell (2.0×10^5/μL) suspension or 2 μL of chondroitinase ABC (Sigma, St. Louis, MO, USA) was injected respectively 1 mm from the edge of the spinal cord damage zone in the bone marrow mesenchymal stem cell group and chondroitinase ABC group with a syringe (Narishige, Tokyo, Japan). The lyophilized chondroitinase ABC powder was reconstituted with 0.01% fetal bovine serum, and then diluted into 50 mM Tris, 60 mM acetate (pH 8.0) and 0.02% fetal calf serum. The concentration of selected chondroitinase ABC is 1 unit/mL. Chondroitinase ABC was first injected, and bone marrow mesenchymal stem cell suspension was injected in the combination group on the next day.

Motor function assessment

Motor function tests were performed before transplantation and weekly after transplantation. Locomotor activity was evaluated using the Basso, Beattie and Bresnahan locomotor rating scale[20, 27-28]. A high score indicated good motor function. Paralysis is 0 points and normal is 21 point. Two independent blinded examiners observed hindlimb movements and assessed the animal's locomotor function. The score was obtained by averaging the values of both limbs.

Histological observation and quantitative analysis

Rats were intraperitoneally over-anesthetized with sodium pentobarbital, perfused with Tyrode's solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). In the damage zone, 2 cm of spinal cord was placed in 0.1 mM PBS with 25% sucrose (4°C, pH 7.4). Consecutive sagittal slices of 20 μm thickness were made using a CM1900 constant cold box slicer (Leica, Germany). Hematoxylin-eosin staining: eosin alcohol solution concentration was 0.5–1%. Hematoxylin dye bath formulation (hematoxylin was dissolved in anhydrous ethanol, and then potassium aluminum sulfate was dissolved in distilled water, and mixed with glycerol, and finally glacial acetic acid and sodium iodide was added) was separated with 1% hydrochloric acid alcohol. Under a BX-51 microscope (Leica, Wetzlar,

Germany), the sagittal sections were selected, including the center of the spinal cord injury zone. Under the brightfield of an Olympus BX-60 microscope (Olympus, Tokyo, Japan), the damage zone was observed and digital images were collected under a 4 × magnification. The outline of the visible necrosis and scale amplification of the same slice was portrayed on A4 paper with microscopic tracers (Camera lucida, Germany). Photoshop 7.0 image processing software (Adobe, San Jose, CA, USA) was used to obtain the pixel values of the chosen area and to measure the necrotic areas within the injured spinal cord[64].

Immunofluorescence staining

Consecutive sagittal slices of 20 μm thickness were obtained in a CM1900 constant cold box slicer (Leica, Germany). Each set of slices were glial fibrillary acidic protein/chondroitin sulfate proteoglycans, and glial fibrillary acidic protein/growth-associated protein-43 double immunostaining. The specific method is as follows: slices were dried at room temperature, rinsed with 0.01 M PBS for 30 minutes, and blocked with diluent containing 1% (v/v) bovine serum albumin and 0.3% (w/v) Triton X-100 antibody at room temperature for 1 hour. Rabbit anti-glial fibrillary acidic protein polyclonal antibody (1:1 000; Sigma) and mouse anti-rat chondroitin sulfate proteoglycan monoclonal antibody (1:100; Sigma) were mixed at room temperature overnight. Fluorescein isothiocyanate-labeled donkey anti-mouse IgG (1:200; Sigma) and Texas Red-labeled donkey anti-rabbit IgG (1:200; Sigma) were incubated at room temperature for 2 hours, followed by a wash with 0.01 M PBS. The specimens were mounted in Fluoromount G, and observed under a fluorescence microscope (BX-51; Leica). Glial fibrillary acidic protein/ GAP-43 staining was performed as per the above method. Primary antibody was a mixture of rabbit anti-rat glial fibrillary acidic protein antibody (1:1 000; Sigma) and mouse anti-rat GAP-43 monoclonal antibody (1:200; Sigma). The nuclei were stained by Hoechst 33258. The relative intensity of CS56 and GAP-43 was calculated as follows: the fluorescence intensity value of each group was measured with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). The value of the control group was selected as a reference and the fluorescence intensity of the other group was compared to derive the relative fluorescence intensity.

Statistical analysis

Data were expressed as mean ± SD and processed with SPSS 12.0 software (SPSS, Chicago, IL, USA). Intergroup comparisons were conducted by one-way analysis of variance, followed by *post hoc* test analysis. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Xijing He and Haopeng Li guided the experiment. Chun Zhang completed and analyzed the data. Guoyu Wang collected the data. Xijing He revised the data. Chun Zhang was responsible for this article. All authors have read and approved the final version of the manuscript submitted.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Biomedical Sciences Research Ethics Committee of the Medical College of Xi'an Jiaotong University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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