Research on the effect and mechanism of the CXCR-4-overexpressing BMSCs combined with SDF-1α for the cure of acute SCI in rats

B.-W. SUN¹, H.-M. SHEN², B.-C. LIU¹, H.-L. FANG³

¹Department of Orthopaedics, The Second People’s Hospital of Jining, Jining, China
²Department of Neurosurgery, The Second People’s Hospital of Jining, Jining, China
³Department of Emergency Trauma Surgery, The First People’s Hospital of Jining, Jining, China

Abstract. – OBJECTIVE: To evaluate the effect and mechanism of bone marrow stem cells (BMSCs) modified with CXCR-4 gene combined with stromal derived factor-1α (SDF-1α) in the treatment of acute spinal cord injury (SCI) in rats.

MATERIALS AND METHODS: CXCR-4 gene was transfected by a virus. Spinal cord injury rats were randomly divided into four groups: control group, SDF-1α group, CXCR-4/BMSC group and combined group. The motor function was evaluated with Blood Brain Barrier (BBB) score and the RNA expression of CXCR-4 were measured by PCR. Apoptosis of spinal cord was measured by TUNEL kit (Hu Bei, China). The protein level of Bcl-2 and Bax were measured by Western-blot. The BBB scores, mRNA CXCR-4 expression, and apoptosis rate were compared between four groups at 1d, 3d, 7d, 14d, 21d after the operation.

RESULTS: The exercise ability in combined group restored in early and late periods of SCI. The apoptosis rates in the combined group are less than other three groups; the difference was statistically significant (p < 0.05). Bcl-2 in combined group is higher than the other 3 groups and Bax is less than the other 3 groups, the difference is statistically significant (p < 0.05).

CONCLUSIONS: The neurological function of rats with a spinal cord can be improved by BMSCs modified with CXCR-4 combined with SDF-1α. The main mechanism may improve the expression of SDF-1α and decrease the apoptosis of the spinal cord.

Key Words:
Bone marrow stem cells, CXCR-4, Spinal cord injury, Repair, Stromal-derived factor-1.

Introduction

As a common traumatic disease in the central nervous system, spinal cord injury (SCI) is characterized by high mortality and morbidity. Currently, all the treatment methods for SCI have been designed to reduce the injury of the spinal cord caused by mechanical compression, only acquiring poor clinical efficacy ¹. In recent years, with the development of the research on the pathogenesis of SCI, stem cell transplantation has become a breakthrough in the therapy of SCI. Some studies ²,³ reported that permeable efficacy was acquired from the application of migration and directed differentiation ability of bone marrow stroma stem cells (BMSCs) to the therapy of SCI. Affected by the microenvironment of the spinal cord, BSMSCs are differentiated to the neuron axon to reconstruct part of the neurological function ⁴. However, there remain some problems, e.g. poor directional homing ability of BMSCs. Stromal-derived factor-1 (SDF-1) is one of the members of the CXC chemokine family, including two subtypes, SDF-1α and SDF-1β ⁵, the former superior to the latter in terms of the chemotactic ability to the BMSCs. In some researches, SDF-1α was injected at the site of SCI, improved the recovery ability of spinal cord and enhanced the regeneration ability of axons. Besides, SDF-1α, through the combination with its receptor, CXCR-4, can promote the homing and differentiation of BMSCs ⁶,⁷. Thus, in this research, we applied the SDF-1α as a chemokine combined with the CXCR-4-overexpressing BMSCs to the synergistic therapy for SCI, and the results could be served as the reference for developing a new and effective treatment method of SCI in clinical practice.

Materials and Methods

Experiment Materials and Instruments

In a total of 85 adult rats provided by Beijing Laboratory Animal Research Center, 80 were for experiment and 5 for extraction of BMSCs. The
weight of these rats ranged from 180 to 200 g with an average of (192 ± 7) g. 293 cells were regularly cultured in the laboratory of cell center. Adenovirus expressing GFP (Ad-GFP) plasmid (Invitrogen, Carlsbad, CA, USA); CO2 cell-culture incubator (Heraeus, Hanau, Germany); inverted phase contrast microscope CX23 (Olympus, Shinjuku, Tokyo, Japan); fluorescence microscope IX81 (Olympus, Shinjuku, Tokyo, Japan); separation liquid of rat bone marrow stroma stem cells (Tianjin Hao Yang Biological Manufacture Co., Ltd, Tianjin, China); TUNEL apoptosis detection kit (Bioss, Shanghai, China); Human recombinant SDF-1α (Peptotech, Rocky Hill, NJ, USA); β-actin, Bcl-2, Bax and the relevant antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); ReverTra Ace qPCR reverse transcription and quantitative detection kit (Takara, Otsu, Shiga, Japan).

**Isolation and Culture of BMSCs**

The bilateral femoral shafts were isolated on the clean bench. After the metaphysis had been removed, they were flushed in 5 mL of serum-free DMEM medium with high glucose extracted by injector until the color of shaft turned into white. Then, the single-cell suspension was prepared by blowing up and beating upon the shaft filtrated through a 200-mesh cell sieve. The separation was performed by centrifugation using the separation liquid of rat bone marrow stroma stem cells. The isolated cells were inoculated in the little flasks, and the procedures of passaging, isolation, amplification and purification were sequentially carried out on the cells spread over the flask with EDTA and pancreatic enzymes.

**Transfection of BMSCs with the Recombinant Ad-GFP Vector Containing CXCR-4 Gene**

The linearized plasmid of Ad-GFP by Pac I was used for transfection of 293 cells through Lipofectamine™2000. In 7 to 10 d, the original virus was acquired from the collected transfected cells that were repeatedly frozen and thawed. Simultaneously, massive amplification was performed using the same method for the virus purified by cesium chloride, and the virus titer (plague-forming unit, PFU) was assayed using the double dilution method. The primer was designed, synthesized and amplified according to the sequence of mRNA on the gene of CXCR-4 in the Genebank, followed by the construction of pDC-CXCR-4-GFP. The successfully constructed adenovirus vector of pAdEasy-GFPCRCRGFP CXCR-4 was then packed and purified using AdMax packing system for determining whether the target gene carried by the recombinant virus vector was correct and detecting the titer of purified virus. The cultured virus solution of pAdEasy-GFPCRCRGFP CXCR-4 (the experiment group) and the virus solution of pAdEasy-GFP (the control group) were used respectively to prepare the virus solutions in 6 different titers, i.e. $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$, $1 \times 10^8$ and $1 \times 10^{10}$ PFU/mL. The virus solution of each titer was added into 4 wells, and the rest 4 wells were set as control. The virus solution was then inoculated on the BMSCs which would be cultured for 48h at 37°C in the CO2 (5%) cell-culture incubator. Regular detection of transfection rate was conducted to identify the virus titer with the best performance. In this experiment, the virus titer with the best performance was $2.45 \times 10^{10}$PFU/mL.

**Constructing and Grouping the SCI Models**

Anesthetized by chloral hydrate, adult male SD rats that were fixed in prone position were used for the construction of models. Then the spinal cord was exposed layer by layer after being disinfected by iodine, and subsequently, the SCI model was established using the spinal cord impactor in accordance with the procedure mentioned in literature8. The successful criteria for SCI models were set as follow: bleeding and edema in the spinal cord; posterior limbs with flaccid paralysis; tail with spastic swing movements. The rats were divided into four groups with a density of BMSCs of $1 \times 10^6$/mL using a random number table: for the combination group (CXCR-4-hUC-BMSCs combined with SDF-1α, n = 20), the rats, after the onset of SCI, were given SDF-1α (4 µg/4 µl) and 5 µl of CXCR-4 transfected BMSCs (50 µL/min) was injected through the caudal vein; for the SDF-1α group (SDF-1α group, n = 20), SDF-1α (4 µg/4 µl) was administrated by the rats before the onset of SCI and the same volume of cell-free medium was injected into the spinal cord after the onset of SCI; for the group of BMSCs simply transfected with CXCR-4 gene (CXCR-4/BMSC group, n = 20), 5 µl of CXCR-4 transfected BMSCs (50 µL/min) was injected through the caudal vein after the onset of SCI; for the control group (NS group, n = 20), no treatment was conducted for the rats after the onset of SCI. This study had been approved by the Ethic Committee of the College.
Assessment of Psychomotor Function (BBB scale)
At the 1st, 3rd, 7th, 14th, and 21st day after the transplantation, we assessed the psychomotor function of the rats in four groups using the BBB scale (Basso, Beattie & Bresnahan locomotor rating scale) in which the condition of psychomotor function was divided into 21 grades. Between 10:00 to 12:00 a.m., the urine of rats was evacuated by compressing the abdomen of rats, and then the rats were placed on the smooth plane with a diameter of 1 m for 5 min of free activity. During this period, according to the criteria set in the scale, two observers were arranged to independently score by observing the activity of the bilateral posterior limbs and the coordination of gaits of the rats and calculated the average; rats with higher scores indicated a better recovery of the psychomotor function.

Expression of CXCR-4 mRNA
After the psychomotor function assessment, 4 rats selected in each group were executed by injection of an excessive amount of chloral hydrate to acquire the spinal cord through anatomy, in which we extracted the total RNA using Trizol. Then the cDNA was prepared by reverse transcription of the total RNA using ReverTra Ace qPCR reverse transcription and quantitative detection kit strictly under the instruction of the kit. The primer of target molecules was detected as follow: for β-actin, upstream 5'-AGATCTGTGAC- CGAGCTG-GGC-3' and downstream 5'-CCA GGAGAAGAGTGCG-3'; for CXCR-4, upstream 5' CAA ACT GCT AAA TGA CGA GG 3' and downstream 5'-GGG AAA GGT TGT GTA GGTC-3'. QRT-PCR was performed using SYBR® Green Real-time PCR Master Mix quantitative detection kit (Thermo Fisher Scientific, Waltham, MA, USA). The semi-quantitative calculation was carried out in the formula of 2-∆∆Ct with the β-actin as an internal reference.

Detecting the Apoptosis of Spinal Cord Cells Using TUNEL
In the 4 rats selected from each group, the spinal cord tissues 1 cm above and below the site of SCI were fixed in the 4% paraformaldehyde for 3 d, followed by dehydration using graded phosphate buffer saline (PBS) containing 20% and 30% of sucrose, and then placed on the freezing microtome at -20°C for serial section. The specific procedures of TUNEL staining were in accordance with the instruction of detection kit. Brown was presented in the nucleus in the cells with positive staining results using TUNEL apoptosis detection kit. The apoptosis rate of spinal cord was analyzed by IPP software. Cell apoptosis rate (%) = the quantity of apoptosis cells (%) / the cell count under the microscope x100.

Detecting the Relative Expression of Bcl-2 and Bax using Western-blot
Total protein of spinal cord was extracted using Radio-Immunoprecipitation Assay (RIPA) protein extract (Beyotime, Jiangsu, China) for detecting the expression of Bcl-2 and Bax, and the extracted protein was quantified using bicinchoninic acid (BCA) protein quantitative detection kit (Boster, Wuhan, Hubei, China). The same amount of total protein in each group was electrohoresed on the SDS-PAGE and, then, transferred to the nitrocellulose membrane. After being blocked by 5% skimmed milk powder for 2h, the membrane was incubated at 4°C overnight with the primary antibodies, i.e. the β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 and Bax (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, the membrane was incubated at the room temperature for 2h with the HRP-conjugated secondary antibody, and then developed and exposed using Electro-Chemi-Luminescence (ECL) contrast agent (Boster, Wuhan, Hubei, China).

Statistical Analysis
Statistical analysis was performed using SPSS 20.0 (SPSS Inc. Chicago, IL, USA), in which the data were all the observation materials at multiple time point among groups. Bivariate variance analysis of repeated measurement data was performed for the integral analysis and was calibrated at HF according to the results of Mauchly’s test of sphericity. Independent sample t-test (among groups) and different t-test (among different time points) were performed in pairwise comparisons and the overall significant level was set as α = 0.05, while the significant level in the pairwise comparison was adjusted using Bonferroni correction.

Results
Comparison of BBB Scores
Materials of BBB scale were listed in Table I. The results of integral analysis showed (bivariate variance analysis of repeated measurement data)
that since the reciprocal actions among groups, among different time points between the grouping and time, were found with remarkable significances \((p < 0.05)\), thus we continued to perform fine pairwise comparisons among the groups and the different time points, and the detailed results were shown in Table I. From the data, we found that: at the 1\textsuperscript{st}, 3\textsuperscript{rd}, 7\textsuperscript{th}, 14\textsuperscript{th} and 21\textsuperscript{st} day after surgery, the BBB scores of the combination group was higher than that of the control group with a statistically significant difference \((p < 0.013)\), while the comparisons at the 1\textsuperscript{st} day and 3\textsuperscript{rd} day among the control group, CXCR-4/BMSC group and SDF-1\(\alpha\) group showed no statistically significant difference. At the 1\textsuperscript{st}, 14\textsuperscript{th} and 21\textsuperscript{st} day after surgery, the BBB score of the CXCR-4/BMSC group was higher than that of the SDF-1\(\alpha\) group with a statistically significant difference \((p < 0.013)\).

### Expression of CXCR-4 mRNA

Materials of expression of CXCR-4 mRNA were listed in Table II. The results of integral analysis showed that since the reciprocal actions among groups, among different time points and between the grouping and time, were found with remarkable significances \((p < 0.013)\), thus we continued to perform pairwise comparisons, and the detailed results were shown in Table II. From the data, we found that: at the 1\textsuperscript{st} day after surgery, the CXCR-4 mRNA level of the combination group was higher than those of the other groups, and the difference in comparison between the combination group and the control group was statistically significant \((p < 0.013)\); at the 3\textsuperscript{rd} day the content of gene in the combination group and CXCR-4/BMSC group was higher than those in the control group and SDF-1\(\alpha\) group with statistically significantly differences

---

### Table I. Comparison of BBB scores among groups after the onset of SCI (\(n = 20\)).

<table>
<thead>
<tr>
<th></th>
<th>A: Control Group</th>
<th>B: CXCR-4/BMSC group</th>
<th>C: SDF-1(\alpha) group</th>
<th>D: Combined group</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1: 1d</td>
<td>3.18 ± 0.37</td>
<td>3.55 ± 0.42*</td>
<td>3.39 ± 0.25*</td>
<td>5.55 ± 0.72*</td>
</tr>
<tr>
<td>T2: 3d</td>
<td>4.31 ± 0.66*</td>
<td>4.75 ± 0.85*</td>
<td>4.41 ± 0.38*</td>
<td>7.36 ± 0.42*</td>
</tr>
<tr>
<td>T3: 7d</td>
<td>6.22 ± 0.25*</td>
<td>7.62 ± 1.09*</td>
<td>6.35 ± 0.51*</td>
<td>8.74 ± 1.23*</td>
</tr>
<tr>
<td>T4: 14d</td>
<td>7.31 ± 0.89*</td>
<td>12.14 ± 1.08*</td>
<td>7.38 ± 0.85*</td>
<td>15.26 ± 1.16*</td>
</tr>
<tr>
<td>T5: 21d</td>
<td>8.39 ± 1.08*</td>
<td>13.25 ± 1.13*</td>
<td>10.47 ± 1.49*</td>
<td>17.25 ± 1.03*</td>
</tr>
</tbody>
</table>

Integral analysis. \(F, p\) (Mauchly HF coefficient: 0.2943)

Comparison among groups \(F = 200.769; 0.000\)

Comparison between time points \(F = 872.352; 0.000\)

Group*time comparison \(F = 278.095; 0.000\)

Notes: As shown in the table, the significant differences marked with a, b and c indicated \(p < \alpha'\) in the pairwise comparison among Group A, Group B and Group C; \(t\) referred to \(p < \alpha'\) in comparison between T1 and other time points in each group; \(\alpha' = 0.013\) was calculated by the Bonferroni correction.

### Table II. Comparison of the expression level of CXCR-4 mRNA after surgery (\(n = 20\)).

<table>
<thead>
<tr>
<th></th>
<th>A: Control Group</th>
<th>B: CXCR-4/BMSC group</th>
<th>C: SDF-1(\alpha) group</th>
<th>D: Combined group</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1: 1d</td>
<td>1.04 ± 0.07</td>
<td>1.05 ± 0.04</td>
<td>0.97 ± 0.06*</td>
<td>1.51 ± 0.17*</td>
</tr>
<tr>
<td>T2: 3d</td>
<td>0.56 ± 0.07*</td>
<td>1.74 ± 0.96*</td>
<td>0.59 ± 0.26*</td>
<td>3.28 ± 0.55*</td>
</tr>
<tr>
<td>T3: 7d</td>
<td>0.39 ± 0.17*</td>
<td>3.29 ± 1.26*</td>
<td>1.34 ± 0.63*</td>
<td>5.64 ± 1.14*</td>
</tr>
<tr>
<td>T4: 14d</td>
<td>0.33 ± 0.05*</td>
<td>3.54 ± 1.25*</td>
<td>1.65 ± 0.69*</td>
<td>8.24 ± 1.08*</td>
</tr>
<tr>
<td>T5: 21d</td>
<td>0.28 ± 0.05*</td>
<td>5.11 ± 0.53*</td>
<td>2.52 ± 0.45*</td>
<td>9.16 ± 1.66*</td>
</tr>
</tbody>
</table>

Integral analysis. \(F, p\) (Mauchly HF coefficient: 0.3027)

Comparison among groups \(F = 108.625; 0.000\)

Comparison between time points \(F = 498.263; 0.000\)

Group*time comparison \(F = 191.685; 0.000\)

Notes: As shown in the table, the significant differences marked with a, b and c indicated \(p < \alpha'\) in the pairwise comparison among Group A, Group B and Group C; \(t\) referred to \(p < \alpha'\) in comparison between T1 and other time points in each group; \(\alpha' = 0.013\) was calculated by the Bonferroni correction.
At the 7th, 14th and 21st days after surgery, the content of gene in the CXCR-4/BMSC group, SDF-1α group and combination group was higher than that in the control group with statistically significant differences ($p < 0.013$).

Comparison of Apoptosis Rates Among Groups

Materials of the apoptosis rate of spinal cord cells were listed in Table III. The results of the integral analysis showed that since the reciprocal actions among groups, among different time points and between the grouping and time, were found with remarkable significances ($p < 0.05$), thus we also continued to perform pairwise comparisons, and the detailed results were shown in Table III. From the data, we found that: at the 7th day after surgery, the apoptosis rate of each group reached the peak, while at the 1st day after surgery, we found no statistically significant difference in comparison of the apoptosis rate of spinal cord cells among the four groups ($p > 0.05$); at the 3rd day after surgery, the apoptosis rate of the combination group was obviously lower than the other groups with statistically significant differences ($p < 0.05$); there was no statistically significant difference lying between the NS group and the CXCR-4/BMSC group ($p > 0.05$); the apoptosis rate in the EPO group was lower than that of the NS group with a statistically significant difference ($p < 0.05$). The apoptosis rates in each group at the 7th, 14th and 21st days after surgery were listed in Table III. The typically stained section for the detection of apoptosis rate at the 21st day after surgery was shown in Figure 1.

Comparison of the Expression of Apoptosis Protein and Anti-Apoptosis Protein Among Groups

The expression of Bax and Bcl-2 was assayed using Western-blot in each group. As shown in Figure 2, the expression of Bcl-2 in the combination group was higher than that in the control group, CXCR-4/BMSC group and BMSCs group, and the statistical analysis showed that the relative expression of Bcl-2 in the combination group was higher than that in the control group.
with a statistically significant difference ($p < 0.05$; see Figure 3). The expression of Bax in the combination group was lower than that in the control group, CXCR-4/BMSC group and BMSCs group, and the statistical analysis showed that the relative expression of Bax in the combination group was lower than that in the control group with a statistically significant difference ($p < 0.05$; see Figure 3).

**Discussion**

In this research, BBB scores in the combination group were found to be higher than those in the other groups at the 1st, 3rd, 7th, 14th and 21st day after the onset of SCI with statistically significant differences ($p < 0.05$). The BBB scores in the SDF-1α group and BMSCs group was all higher than those in the control group with statistically significant differences. Such results indicated that simple application of SDF-1α or CXCR-4 overexpressing BMSCs could efficiently improve the psychomotor functions after the onset of SCI. We also found that the BBB scores in the SDF-1α combined with CXCR-4/BMSCs were higher than those in other groups with statistically significant differences, suggesting that the SDF-1α combined with the CXCR-4/BMSCs can increase the psychomotor ability in the early and advanced stage of SCI, which, therefore, can be served as an efficient treatment method for SCI. Besides, it was confirmed through the apoptosis of spinal cord cells that SDF-1α combined with CXCR-4/BMSCs could protect the spinal cord by decreasing the apoptosis rate of spinal cord cells. SDF-1α-CXCR-4 axis plays an important role in promoting the homing and migration processes of stem cells to the injury site. The rupture of local vessels in the injured site of spinal cord causes the endothelial cells to excrete more SDF-1α, which will be further diffused into the blood after being locally accumulated to accelerate the mobilization of the stem cells in the spinal cord. Also, at the site of injury, the activated receptors of SDF-1α and CXCR-4 can further activate the protein kinase pathway to promote the differentiation of BMSCs. Meanwhile, the microenvironment of the injured spinal cord also facilitates the differentiation of BMSCs to the neurons for repair of the neuron. BMSCs, in the differentiation, can persistently express the nerve...
Conclusions

The CXCR-4 genetic modified BMSCs combined with SDF-1α could maximally inhibit the apoptosis rate of the nerve cells after the onset of SCI and preserve the functions of residual neurons.

In this study we applied the CXCR-4/BMSCs combined with SDF-1α to the therapy of SCI for the first time and preliminarily investigated the therapeutic mechanism, proved that CXCR-4/BMSCs combined with SDF-1α could enhance the expression of SDF-1α and inhibit the apoptosis of spinal cord neurons to protect the functions of neurons, and confirmed the significant efficacy in the early and advanced stages of SCI. However, this study is still limited to the basic experiment, and more efforts are expected for the clinical application.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


