

Effects of gangliosides on expressions of caspase-3 and NGF in rats with acute spinal cord injury

B. YUAN, S. PAN, W.-W. ZHANG

Department of Spine Surgery, Xinchang Hospital Affiliated to Wenzhou Medical University, Shaoxing, China

Abstract. – **OBJECTIVE:** To investigate the effects of ganglioside [monostalotetra-hexosylganglioside (GM1)] on the expressions of caspase-3 and nerve growth factor (NGF) in rats with acute spinal cord injury (SCI).

MATERIALS AND METHODS: Male Sprague-Dawley (SD) rats were selected and randomly divided into Sham group, SCI group and GM1 administration group. The rats in Sham group, SCI group and GM1 group were subjected to behavioral examinations of Basso Beattie Bresnahan (BBB) and oblique-plate test at 1, 7 and 14 d after operation. The content of methylene dioxymphetamine (MDA) and the activity of superoxide dismutase (SOD) of every rat in each group were measured by enzyme-linked immunosorbent assay (ELISA). Immunofluorescence staining assay was used to detect the expression levels of caspase-3 and NGF of rats in each group. The messenger ribonucleic acid (mRNA) and protein expressions of caspase-3 and NGF of rats in Sham group, SCI group and GM1 group were detected using reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting assay.

RESULTS: The BBB scores and the results of oblique-plate test in Sham group, SCI group and GM1 group at 1, 7 and 14 d showed that the BBB scores and the results of oblique-plate test of rats in each group were significantly decreased at 1 d after SCI, and had different degrees of recovery at 7 and 14 d after injury. The results of ELISA detection revealed that SCI group had increased content of MDA and clearly decreased activity of SOD in comparison with Sham group; at the same time, MDA content in GM1 group was overtly lower than that in SCI group, while SOD activity was enhanced evidently in GM1 group compared with that in SCI group. According to immunofluorescence assay, significantly increased expression of caspase-3 and distinctly decreased expression of NGF were found in SCI group. However, this phenomenon was significantly reversed by GM1. RT-PCR and Western blotting assay severally proved that the mRNA and protein expressions of caspase-3 were raised in SCI group and decreased clearly after the administration of GM1; while the mRNA

and protein expressions of NGF was significantly reduced in SCI group and overtly elevated after the administration of GM1. ANOVA showed that there were statistically significant differences in expressions of caspase-3 and NGF among Sham group, SCI group and GM1 group ($p < 0.05$).

CONCLUSIONS: GM1 has an evident effect on the expressions of caspase-3 and NGF in rats with acute SCI, and is able to down-regulate the expression of caspase-3 and up-regulate the expression of NGF, so as to achieve its therapeutic effect on SCI.

Key Words

Ganglioside, Acute spinal cord injury, caspase-3, NGF.

Introduction

Acute spinal cord injury (SCI) is the most severe complication of spinal injury and often results in severe dysfunction of the limbs below injured segments¹. With the accelerating pace of life and a tremendous change in the way of life in recent years, the incidence rate of acute SCI is increasing year by year, which seriously affects and threatens the quality of life^{2,3}. Therefore, it is eager to find effective methods and drugs for the treatment of acute SCI. Gangliosides are drugs that are currently known and play great roles in the acceleration of nerve regeneration. Furthermore, gangliosides can also be used to treat nerve cell damage, and have the function of repairing nerve damage; however, the roles of gangliosides in SCI and their molecular mechanisms are not yet clear^{4,5}. Therefore, this study established rat models of acute SCI and given gangliosides, to observe the effects of gangliosides on rats with acute SCI, and further studied the expressions of caspase-3 and nerve growth factor (NGF) to deeply discuss the mechanisms of action of gangliosides.

Materials and Methods

Experimental Animals and Grouping

A total of 63 male Sprague-Dawley (SD) rats purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were selected and randomly divided into Sham group, SCI group (modeling via Nystrom method) and monostalotetra-hexosylgangliside (GM1) group (30 mg/kg per day for continuous 7 days). Each group had 21 rats. In both SCI group and GM1 group, rats were further subdivided into 1 d group, 7 d group and 14 d group according to different time points after SCI, with 7 rats in each group. This investigation was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

Main Reagents

Gangliosides (GM1) (Qilu Pharmaceutical Co., Ltd., Jinan, China); methylene dioxyamphetamine (MDA) and superoxide dismutase (SOD) enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA); bicinchoninic acid (BCA) protein quantitation kits (Beyotime, Shanghai, China); TRIzol total ribonucleic acid (RNA) extraction kits (Tiangen, Beijing, China); reverse transcription-polymerase chain reaction (RT-PCR) kits (Tiangen, Beijing, China); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-caspase-3, anti-NGF monoclonal antibodies and secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Behavioral Observation

Rats in Sham, SCI and GM1 groups were subjected to Basso Beattie Bresnahan (BBB) behavioral examination and oblique-plate test at 1, 7 and 14 d after operation. Each measurement was performed for three times, and data statistics and results analyses were carried out.

Detections of MDA Content and SOD Activity Via ELISA

The serum was severally collected from Sham group, SCI group and GM1 group, and the content of MDA and the activity of SOD in the serum were detected according to the instructions of ELISA kits; statistical analyses were performed.

Immunofluorescence Staining

The spinal cord tissues were respectively taken from rats in Sham, SCI and GM1 groups, and then fixed with 10% formalin for 48 h, conven-

tionally embedded with paraffin, and finally made into sections in a thickness of 5 μ m. The paraffin sections were taken, deparaffinized with xylene, dehydrated with alcohol with gradient concentration, and subjected to antigen retrieval. Next, sections were rinsed with 0.01 M phosphate-buffered saline (PBS) (pH 7.4) for three times (5 min/time), and blocked in a 10% bovine serum albumin (BSA) wet box for 30 min (at 37°C). After that, sections were added with appropriately diluted fluorescently labeled antibodies (diluted at 1:70) dropwise, placed in the wet box and incubated overnight at 4°C. After rinsing with PBS (pH 7.4) for three times (5 min/time), sections were added with fluorescent secondary antibodies (diluted at 1:100) dropwise in a dark place, and placed in the wet box at 37°C for 2 h of incubation. Last, sections were mounted with buffered glycerol, observed and photographed under an upright fluorescence microscope.

RT-PCR Analysis

Moderate amount of spinal cord tissues of rats in Sham group, SCI group and GM1 group were rapidly transferred into 1 mL TRIzol reagent and fully ground into homogenate. The homogenate was stood still at room temperature for 5 min, and the sample was completely lysed. Then, centrifugation at 12000 g for 5 min at 4°C was performed, and the supernatant was carefully collected, added with chloroform, mixed evenly, and placed still at room temperature for 5 min, followed by centrifugation at 12000 g for 5 min at 4°C. After that, the supernatant was carefully collected, added with a same volume of isopropanol, placed still at room temperature for 10 min, and centrifuged at 12000 g for 10 min at 4°C. The precipitate was taken, added with 75% ethanol and mixed evenly, to wash the RNA precipitation. Later, ribonuclease free (RNase-free) water was added to completely dissolve it. After that, the ratio of the optical density at 260 and 280 nm (OD_{260}/OD_{280}) and the RNA concentration were measured. Last, according to the instructions, stepwise amplification was done based on the primer sequence templates shown in Table I, and the reaction product was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Western Blotting Analysis

Spinal cord tissues of rats in each group were collected and washed twice with frozen saline, respectively. Operations were carried out according to the instructions of the total protein extraction kit.

Table I. RT-PCR primer sequences of caspase-3, NGF and β -actin messenger ribonucleic acid (mRNA).

Gene name	Primer sequence
Caspase-3	5'-3'CATTGAGACAGACAGTGGTGTG 3'-5'GGTCTATTTGATGGAAGGAGTA
NGF	5'-3'AATCAACTCCTGCTTGCC 3'-5'GTATTAGCCCCCTCCTCC
β -actin	5'-3'GAGCCGGGAAATCGTGCGT 3'-5'GGAAGGAAGGCTGGAAGATG

Tissues were added with lysis buffer, homogenized for 1 min by a homogenizer and centrifuged at 12000 g for 10 min at 4°C. Then, the supernatant, i.e. total protein in liver tissue, was collected. The protein concentration was determined by the BCA protein concentration kits and the supernatant was sub-packaged and stored at -80°C until use. The total protein extract was mixed evenly with 2× loading buffer based on a volume ratio of 1:1, heated in boiling water for 5 min, cooled naturally, and stored in a refrigerator at 4°C until use. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation gel in an appropriate proportion was prepared according to the molecular weight of target protein, followed by a coagulation of about 1 h. Then, 5% SDS-PAGE spacer gel was prepared and coagulated for about 0.5 h. After that, electrophoresis buffer was added, and the denatured protein samples were loaded onto sample loading wells according to the protein concentration, so that the total protein content per well was the same. Electrophoresis was performed at a constant voltage of 220 V and stopped after bromophenol blue reached the bottom of the gel. Then, the gel was cut based on the molecular weight of target protein, and put into transfer buffer. 1 layer of polyvinylidene difluoride (PVDF) membrane and 6 layers of filter paper were cut according to the size of the gel. The PVDF membrane was firstly immersed in methanol for 10 s, and then put into the transfer buffer together with the filter paper. They were put into a transfer unit in the sequence of positive electrode – three layers of filter paper – the PVDF membrane – gel – three layers of filter paper – negative electrode, with attention paid to edge alignment so as to prevent blistering. The membrane transfer was last for 2 h at a constant voltage of 110 V. After that, the PVDF membrane containing proteins was placed and blocked in 5% skimmed milk on a shaker at room temperature for 2 h. The blocked membrane was washed with Tris-buffered saline and Tween 20 (TBST) for 5

min, placed into the primary antibody in corresponding proportion, and incubated overnight at 4°C. The membrane was washed with TBST for 3 times (10 min/time), put into the corresponding secondary antibody, incubated on the shaker at room temperature for 3 h, and rinsed with TBST for 3 times (10 min/time). A gel imager was started, and preheated for 30 min. Reagent A and Reagent B in the electrochemical luminescence (ECL) kits were mixed evenly at the same volume, added dropwise onto the PVDF membrane, with full contact, kept in dark place and subjected to color development for 1 min. The filter paper was used to blot up excess liquid around the membrane, and then the membrane was placed into the gel imager. Last, dynamic integration mode was applied to take pictures, and the results were observed. Image analysis software was employed for image analysis.

Statistical Analysis

Experimental data were expressed as mean $\pm$ standard error of mean (mean $\pm$ SEM). Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. *t*-test was employed for mean comparison between two groups. One-way analysis of variance (ANOVA) was utilized for comparison of sample average among multiple groups. *P*-test was used for pairwise comparison. *p*<0.05 suggested that the difference was statistically significant.

Results

Results of Behavioral Observation

The BBB scores and the results of oblique-plate test in Sham group, SCI group and GM1 group were observed at 1, 7 and 14 d, respectively. It was found that at 1 d after SCI, the BBB scores and oblique-plate test results were significantly reduced in animals in all groups; at 7 and 14 d after injury, they were recovered in varying degrees (Table II and Table III).

Table II. BBB scores in Sham group, SCI group and GM1 group.

Group	1 d	7 d	14 d
Sham group	19.98 $\pm$ 0.75	20.03 $\pm$ 0.65	20.25 $\pm$ 0.51
SCI group	2.87 $\pm$ 2.40 [#]	8.92 $\pm$ 2.47 [#]	14.33 $\pm$ 4.24 [#]
GM1 group	5.52 $\pm$ 1.66 [*]	14.34 $\pm$ 4.24 [*]	18.96 $\pm$ 5.61 [*]

In comparison with Sham group, [#]*p*<0.05; compared with SCI group, ^{*}*p*<0.01.

Table III. Results of oblique-plate test in Sham group, SCI group and GM1 group.

Group	1 d	7 d	14 d
Sham group	62.17±6.46	62.62±6.81	63.22±7.46
SCI group	25.12±4.31#	32.31±7.16#	39.58±7.28#
GM1 group	31.26±3.66*	39.25±5.18*	49.27±7.29*

In comparison with Sham group, # $p<0.05$; compared with SCI group, * $p<0.01$.

Results of ELISA Detection of MDA Content and SOD Activity in the Serum

ELISA was used to detect MDA content and SOD activity in the serum of rats in Sham group, SCI group and GM1 group, and the results revealed that in comparison with Sham group, SCI group had significantly increased MDA content and overtly decreased SOD activity; at the same time, the content of MDA in GM1 group was clearly lower than that in SCI group, while the activity of SOD was higher in GM1 group compared with that in SCI group (Figure 1).

Immunofluorescence Staining Results

The expressions of caspase-3 and NGF in spinal cord tissues of rats in Sham group, SCI group and GM1 group were measured by immunofluorescence assay, and the results indicated that evidently raised caspase-3 expression and distinctly decreased NGF expression were found in SCI

group in comparison with Sham group. Meanwhile, the expression of caspase-3 in GM1 group was overtly reduced compared with that in SCI group, and the expression of NGF was significantly higher in GM1 group than that in SCI group (Figure 2).

Results of RT-PCR of caspase-3 and NGF mRNA Expressions

Total RNA was extracted from the spinal cord tissue samples of rats in Sham group, SCI group and GM1 group, respectively, and subjected to RT-PCR, and it was detected that there were increased mRNA expression of caspase-3 and overtly decreased mRNA expression of NGF in SCI group in comparison with Sham group, which was effectively reversed after the administration of GM1 (Figure 3).

Western Blotting Results of Protein Expressions of caspase-3 and NGF

Protein was extracted from the spinal cord tissue samples of rats in Sham group, SCI group and GM1 group, respectively, and subjected to Western blotting. The results revealed that compared with those in Sham group, distinctly increased protein expression of caspase-3 and clearly lowered protein expression of NGF were found in SCI group; after the administration of GM1, caspase-3 protein expression was lowered, and NGF protein expression was significantly enhanced compared with those in SCI group (Figure 4).

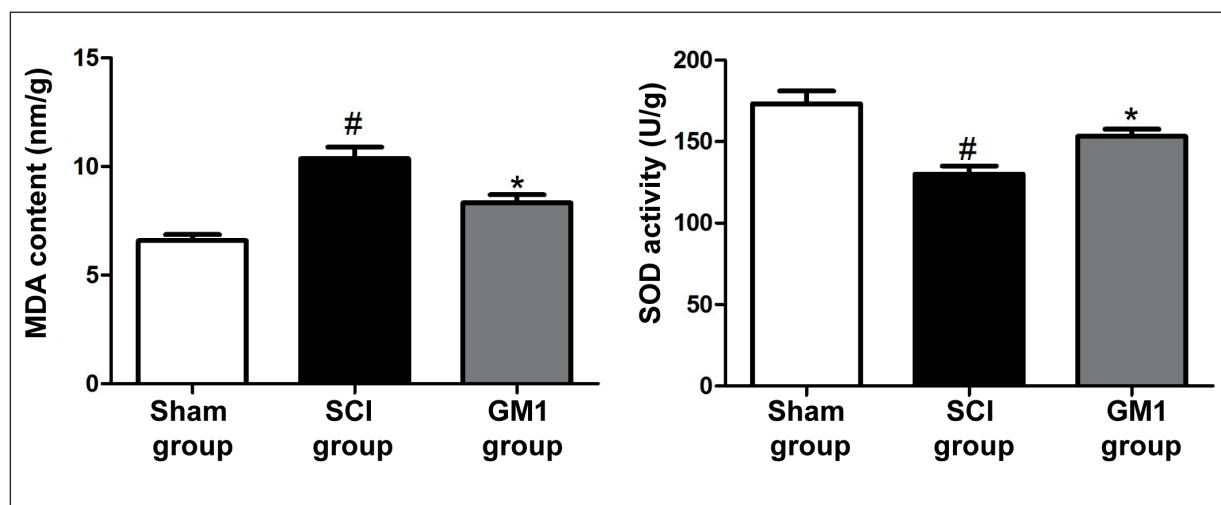


Figure 1. MDA content and SOD activity in the serum detected via ELISA. Compared with Sham group, # $p<0.05$; in comparison with SCI group, * $p<0.05$.

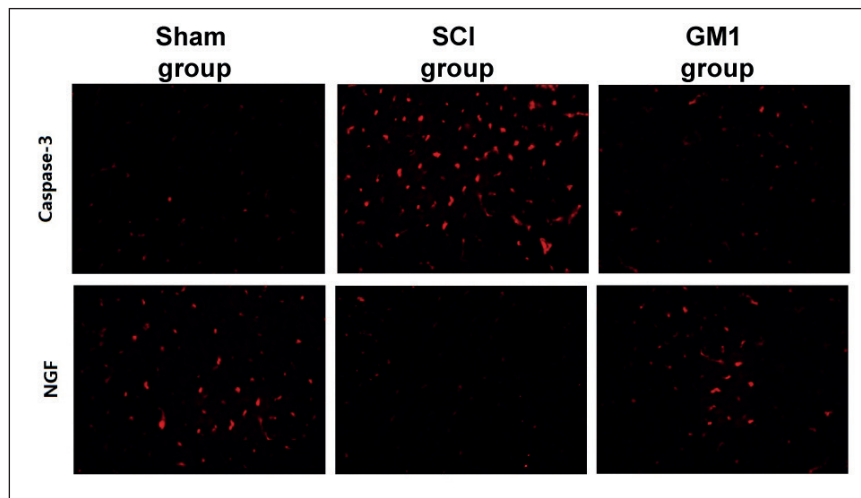


Figure 2. Expressions of caspase-3 and NGF in spinal cord tissues of rats in Sham group, SCI group and GM1 group measured by immunofluorescence assay ($\times 200$).

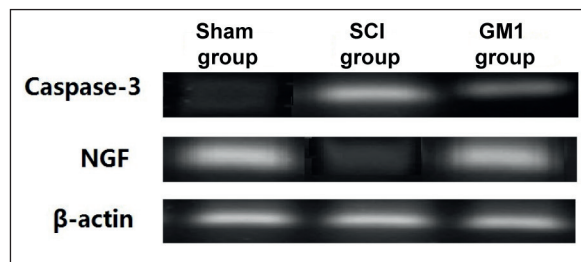


Figure 3. mRNA expressions of caspase-3 and NGF in spinal cord tissues of rats in Sham group, SCI group and GM1 group. Compared with Sham group, $\#p < 0.05$; in comparison with SCI group, $*p < 0.05$.

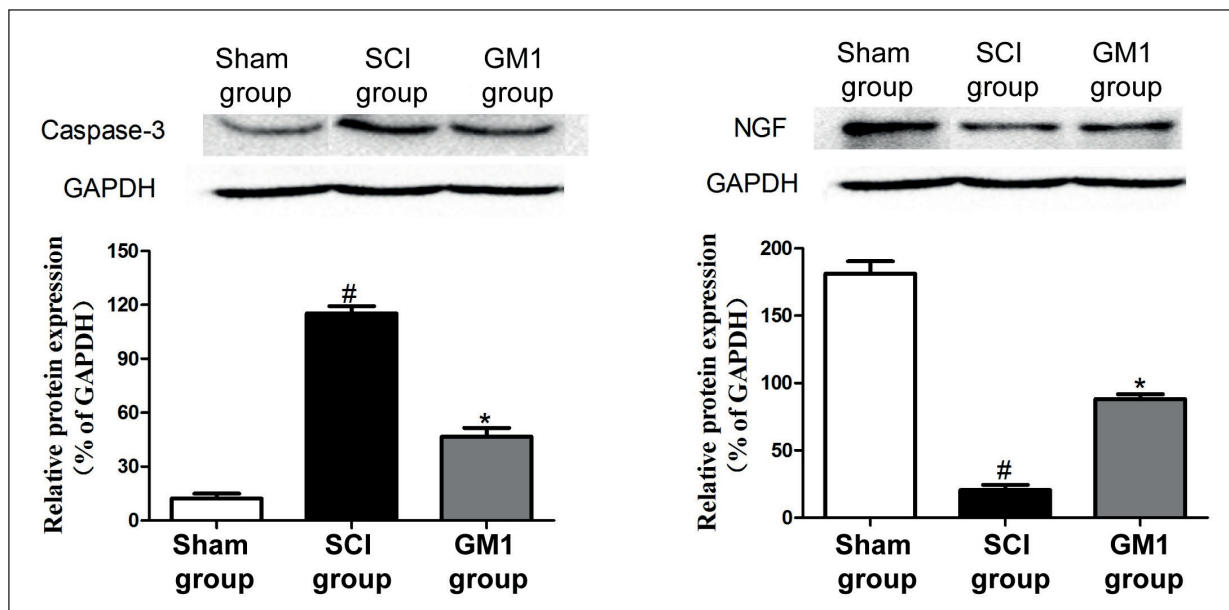


Figure 4. Protein expressions of caspase-3 and NGF in spinal cord tissues of rats in Sham group, SCI group and GM1 group. Compared with Sham group, $\#p < 0.05$; in comparison with SCI group, $*p < 0.05$.

Discussion

With the improvement of living standards, the incidence rate of acute SCI shows an upward trend year by year. Acute SCI is the most serious complication of spinal injury, often leading to severe dysfunction of the limbs below injured segments⁶⁻⁸. SCI not only brings serious physical and psychological harm to the patient, but also places a huge economic burden on the whole society^{9,10}. The social and economic losses caused by SCI, and the prevention, treatment and rehabilitation of SCI, have become key issues in the medical field¹¹. Therefore, the development of new drugs with special effects is very necessary. However, new drugs in research and development are mainly chemical synthetic drugs, with high development costs, great risks, long cycles, and low success rates, so the annual number of new drugs in research and development is extremely limited¹².

In recent years, a large number of papers have reported that gangliosides have the functions of neuroprotection and promotion of nerve injury repair. Nerve injury can produce many inflammatory and apoptotic factors^{13,14}. Caspase-3 is an apoptotic factor, NGF is a nerve growth factor, and both caspase-3 and NGF play important roles in various diseases¹⁵⁻¹⁷. Gangliosides are glycosphingolipids containing sialic acid. Studies have reported¹⁸⁻²⁰ that gangliosides have anti-inflammatory and anti-apoptotic effects. However, whether gangliosides have significant functions in anti-acute SCI and their specific molecular mechanisms are unknown. Therefore, in-depth studies of these unknown areas were carried out. In this work, male SD rats were chosen and randomly divided into Sham group, SCI group and GM1 group. Rats in Sham group, SCI group and GM1 administration group were subjected to behavioral examination of BBB and oblique-plate test at 1, 7 and 14 d after operation, respectively. The BBB scores and oblique-plate test results in the three groups at 1, 7 and 14 d showed that in the three groups, the BBB scores and oblique-plate test results were significantly reduced at 1 d after SCI, and elevated in different ranges at 7 and 14 d after injury. ELISA was adopted to detect the content of MDA and the activity of SOD of every rat in each group, and the results revealed that GM1 group had significantly decreased content of MDA and increased activity of SOD in comparison with SCI group. In accordance with immunofluorescence assay, overtly increased expression of caspase-3 and evidently reduced expression

of NGF were found in SCI group. However, this phenomenon was reversed by GM1. RT-PCR and Western blotting assay severally proved that the mRNA and protein expressions of caspase-3 were significantly higher in SCI group than those in GM1 group, while the mRNA and protein expressions of NGF in SCI group were significantly decreased compared with those in GM1 group.

Conclusions

This study demonstrated that GM1 has significant effects on the expressions of caspase-3 and NGF in rats with acute SCI, and can reduce the expression of caspase-3 and enhance the expression of NGF, so as to realize its curative effect on SCI.

Conflict of Interest:

The authors declared no conflict of interest.

References

- 1) MCKINLEY W, MEADE MA, KIRSHBLUM S, BARNARD B. Outcomes of early surgical management versus late or no surgical intervention after acute spinal cord injury. *Arch Phys Med Rehabil* 2004; 85: 1818-1825.
- 2) PAPADOPOULOS SM, SELDEN NR, QUINT DJ, PATEL N, GILLESPIE B, GRUBE S. Immediate spinal cord decompression for cervical spinal cord injury: feasibility and outcome. *J Trauma* 2002; 52: 323-332.
- 3) GELB DE, AARABI B, DHALL SS, HURLBERT RJ, ROZZELLE CJ, RYKEN TC, THEODORE N, WALTERS BC, HADLEY MN. Treatment of subaxial cervical spinal injuries. *Neurosurgery* 2013; 72: 187-194.
- 4) KIRSHBLUM SC, BURNS SP, BIERING-SORENSEN F, DONOVAN W, GRAVES DE, JHA A, JOHANSEN M, JONES L, KRASSIOUKOV A, MULCAHEY MJ, SCHMIDT-READ M, WARING W. International standards for neurological classification of spinal cord injury (revised 2011). *J Spinal Cord Med* 2011; 34: 535-546.
- 5) DEVIVO MJ. Epidemiology of traumatic spinal cord injury: trends and future implications. *Spinal Cord* 2012; 50: 365-372.
- 6) ROWLAND JW, HAWRYLUK GW, KWON B, FEHLINGS MG. Current status of acute spinal cord injury pathophysiology and emerging therapies: promise on the horizon. *Neurosurg Focus* 2008; 25: E2.
- 7) SEKHON LH, FEHLINGS MG. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine (Phila Pa 1976)* 2001; 26: S2-S12.
- 8) SUN BW, SHEN HM, LIU BC, FANG HL. Research on the effect and mechanism of the CXCR-4-overexpressing BMSCs combined with SDF-1α for the cure of acute SCI in rats. *Eur Rev Med Pharmacol Sci* 2017; 21: 167-174.

- 9) YASHON D, BINGHAM WJ, FADDOUL EM, HUNT WE. Edema of the spinal cord following experimental impact trauma. *J Neurosurg* 1973; 38: 693-697.
- 10) LA ROSA G, CONTI A, CARDALI S, CACCIOLA F, TOMASELLO F. Does early decompression improve neurological outcome of spinal cord injured patients? Appraisal of the literature using a meta-analytical approach. *Spinal Cord* 2004; 42: 503-512.
- 11) FEHLINGS MG, VACCARO A, WILSON JR, SINGH A, W CD, HARROP JS, AARABI B, SHAFFREY C, DVORAK M, FISHER C, ARNOLD P, MASSICOTTE EM, LEWIS S, RAMPERSAUD R. Early versus delayed decompression for traumatic cervical spinal cord injury: results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS). *PLoS One* 2012; 7: e32037.
- 12) WILSON JR, SINGH A, CRAVEN C, VERRIER MC, DREW B, AHN H, FORD M, FEHLINGS MG. Early versus late surgery for traumatic spinal cord injury: the results of a prospective Canadian cohort study. *Spinal Cord* 2012; 50: 840-843.
- 13) GELMAN S. Venous function and central venous pressure: a physiologic story. *Anesthesiology* 2008; 108: 735-748.
- 14) FURLAN JC, FEHLINGS MG. Cardiovascular complications after acute spinal cord injury: Pathophysiology, diagnosis, and management. *Neurosurg Focus* 2008; 25: E13.
- 15) AMAR AP, LEVY ML. Pathogenesis and pharmacological strategies for mitigating secondary damage in acute spinal cord injury. *Neurosurgery* 1999; 44: 1027-1039.
- 16) DOLAN EJ, TATOR CH. The effect of blood transfusion, dopamine, and gamma hydroxybutyrate on posttraumatic ischemia of the spinal cord. *J Neurosurg* 1982; 56: 350-358.
- 17) SANDLER AN, TATOR CH. Effect of acute spinal cord compression injury on regional spinal cord blood flow in primates. *J Neurosurg* 1976; 45: 660-676.
- 18) RYKEN TC, HURLBERT RJ, HADLEY MN, AARABI B, DHALL SS, GELB DE, ROZZELLE CJ, THEODORE N, WALTERS BC. The acute cardiopulmonary management of patients with cervical spinal cord injuries. *Neurosurgery* 2013; 72 Suppl 2: 84-92.
- 19) LEVI L, WOLF A, BELZBERG H. Hemodynamic parameters in patients with acute cervical cord trauma: description, intervention, and prediction of outcome. *Neurosurgery* 1993; 33: 1007-1016.
- 20) VALE FL, BURNS J, JACKSON AB, HADLEY MN. Combined medical and surgical treatment after acute spinal cord injury: results of a prospective pilot study to assess the merits of aggressive medical resuscitation and blood pressure management. *J Neurosurg* 1997; 87: 239-246.