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Methylprednisolone inhibits Nogo-A protein expression after acute spinal cord injury☆

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Abstract

Oligodendrocyte-produced Nogo-A has been shown to inhibit axonal regeneration. Methylprednisolone plays an effective role in treating spinal cord injury, but the effect of methylprednisolone on Nogo-A in the injured spinal cord remains unknown. The present study established a rat model of acute spinal cord injury by the weight-drop method. Results showed that after injury, the motor behavior ability of rats was reduced and necrotic injury appeared in spinal cord tissues, which was accompanied by increased Nogo-A expression in these tissues. After intravenous injection of high-dose methylprednisolone, although the pathology of spinal cord tissue remained unchanged, Nogo-A expression was reduced, but the level was still higher than normal. These findings implicate that methylprednisolone could inhibit Nogo-A expression, which could be a mechanism by which early high dose methylprednisolone infusion helps preserve spinal cord function after spinal cord injury.

Key Words

neural regeneration; spinal cord injury; methylprednisolone; Nogo-A; oligodendrocyte; spinal cord pathology; myelonecrosis; weight-drop contusion; photographs-containing paper; neuroregeneration

Research Highlights

(1) Oligodendrocyte-produced Nogo-A can inhibit axonal regeneration.
(2) Methylprednisolone plays a therapeutic role in acute spinal cord injury by decreasing Nogo-A expression in the injured spinal cord.

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INTRODUCTION

More cases of acute spinal cord injury are caused by road traffic and construction accidents. Following injury, spinal cord injury can result in loss of sensory function, dyskinesia and even paraplegia[1]. At present, surgical operation and non-surgical treatments are the main methods for managing acute spinal cord injury. Surgery can provide the necessary external environment for the functional recovery of damaged spinal cord by relieving compression on the injured segment and reconstructing spinal stability[2-3]. For non-surgical treatments, drugs are usually used to reduce or inhibit secondary injury of the spinal cord. Nogo-A secreted by oligodendrocyte of the spinal cord can strongly inhibit the regeneration of damaged neurons[4]. Some studies suggested that in different periods of acute spinal cord injury, the expression of Nogo-A in the spinal cord were significant different from normal conditions[5-6]. A number of studies also show that inhibiting Nogo-A protein expression benefits the repair and Previous studies have suggested that the role of regeneration of spinal cord injury[7-9].

Methylprednisolone in the treatment of spinal cord injury is to inhibit peroxidation and the anti-inflammatory response, improve microcirculation, reduce cell calcium influx, maintain neuronal excitability and inhibit apoptosis[10]. Recently, methylprednisolone has been shown to play a role in the regulation of a number of nerve-cell proteins[11-12]. However, the effects of methylprednisolone on Nogo-A expression remain poorly understood.

The present study used spinal cord injury models to evaluate the effects of high-dose intravenous methylprednisolone on Nogo-A expression in the spinal cord of rats.

RESULTS

Quantitative analysis of experimental animals

A total of 54 rats were equally and randomly assigned to control, spinal cord injury and methylprednisolone groups. Spinal cord injury models were established in rats in spinal cord injury and methylprednisolone groups, with rats in methylprednisolone group receiving intravenous injection of methylprednisolone sodium succinate. All rats were included in the final analysis and observed at 3, 7 and 14 days after model establishment.

Motor impairment in rats after spinal cord injury

Basso, Beattie, and Bresnahan scale scores of the spinal cord injury and methylprednisolone groups were increased with prolonged time and reached the highest value at 14 days. The Basso, Beattie, and Bresnahan scale score was significantly higher in the control group compared with the other groups ($P < 0.05$; Figure 1).

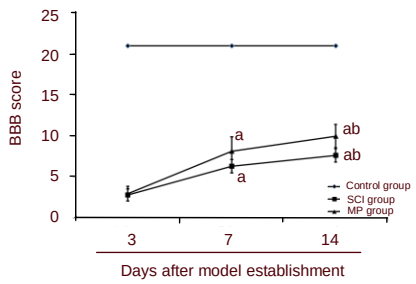


Figure 1 Motor impairment in SCI rats.

The motion ability of limbs was evaluated with BBB scores. Higher score represents better motor function of the limbs. [a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. 3, 7 days (Dunnett's test).

SCI: Spinal cord injury; MP: methylprednisolone; BBB: Basso, Beattie, and Bresnahan scale.

For spinal cord injury and methylprednisolone groups, the Basso, Beattie, and Bresnahan scale score reached the highest level at 14 days after model establishment and the lowest score was observed in the two groups on day 3.

Effect of methylprednisolone on the histology of the spinal cord in the spinal cord injury rats

The structure of the normal rat spinal cord is clear and a large number of band-shaped nerve fibers and oligodendrocyte nuclei can be seen (Figure 2A). The spinal cord injury and methylprednisolone groups presented with congestive spinal cord early after model establishment (day 3), with gradual necrosis of spinal cord nerve fibers, accompanied by a significant increase in the number of fractured and dead nerve fibers in injured tissue, as well as vacuolated changes in the spinal cord (Figures 2B and C). Subsequently, collagen fibers (scar tissue) and small, dense vacuoles appeared in the spinal cord.

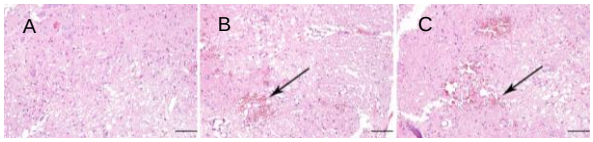


Figure 2 Histology of the rat spinal cord (hematoxylin-eosin staining, × 200, light microscope).

(A) Control group; (B) 3 days after modeling in the spinal cord injury group; (C) 3 days after modeling in methylprednisolone groups. Scale bars: 100 μm. Arrows refer to the blood and dense vacuoles in the spinal cord tissue.

Effect of methylprednisolone on Nogo-A expression in the spinal cord of spinal cord injury rats

Immunohistochemistry showed that little Nogo-A was expressed in the the myelin and cytoplasm of oligodendrocytes in the control group, while a large amount of Nogo-A was detected in the spinal cord injury and methylprednisolone groups at 7 and 14 days (Figure 3). Although methylprednisolone sodium succinat e was used to attenuate acute spinal cord injury in the methylprednisolone group, the level of Nogo-A was lower than the spinal cord injury group, but still appeared higher level than that in the control group.

Western blot analysis indicated that Nogo-A expression in the control group was significantly lower than that in the spinal cord injury or methylprednisolone groups at 3, 7 and 14 days ($P < 0.01$). Although the level of Nogo-A in the methylprednisolone group appeared higher than that in the control group ($P < 0.05$) at 7 and 14 days, the production of Nogo-A significantly decreased compared

with the spinal cord injury group ($P < 0.01$; Figure 4).

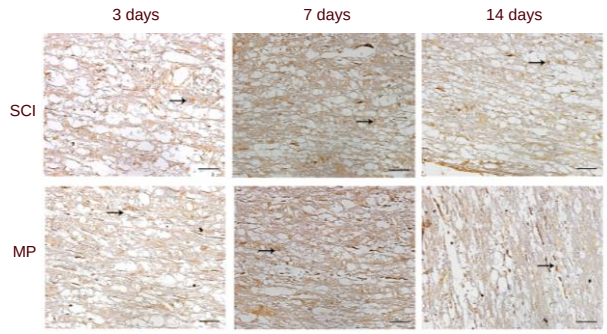


Figure 3 Immunohistochemical analysis of Nogo-A in the injured spinal cord of rats (light microscope).

Samples were collected from rats in the control, SCI and MP groups. Nogo-A was stained dark brown in the spinal cord tissue from the three groups. Scale bars: 50 μm. Arrows refer to the positive expression of Nogo-A. Representative staining in the control group is reported in supplementary Figure 1 online.

SCI: Spinal cord injury; MP: methylprednisolone.

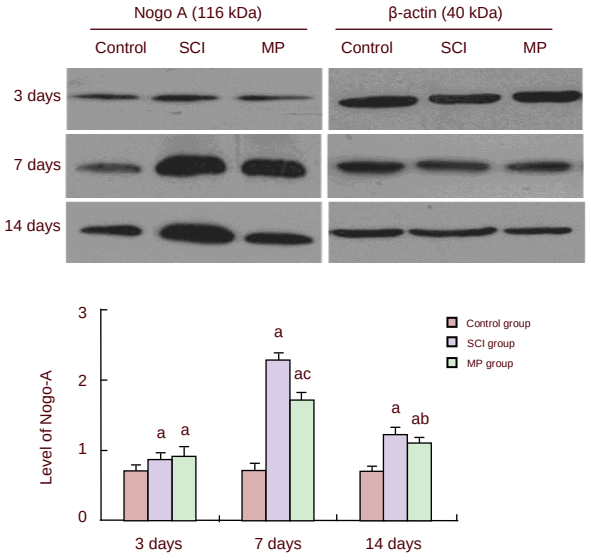


Figure 4 Nogo-A protein expression in the injured spinal cord of rats (western blot analysis).

The absorbance ratio between Nogo-A protein and standard protein (β-actin) represents the relative levels of Nogo-A protein. $^{a}P < 0.01$, *vs*. control group; $^{b}P < 0.05$, $^{c}P < 0.01$, *vs*. SCI group (least significant difference *t*-test).

SCI: Spinal cord injury; MP: methylprednisolone.

DISCUSSION

In the present study, bleeding spots were observed in injured spinal cord after model establishment. Results suggested that Nogo-A, produced by oligodendrocytes in the spinal cord tissue, was mainly located around spinal cord nerve fibers, where oligodendrocytes ensheath nerve fibers and produce myelin. Nogo-A decreased in the spinal cord after methylprednisolone treatment. The animals' motion was evaluated by Basso, Beattie, and Bresnahan scale score. In the absence of methylprednisolone, Nogo-A decreased after 14 days of injury, which was consistent with the methylprednisolone group.

Central nervous regeneration is inhibited by Nogo-A after spinal cord injury. David *et al* [13] showed that some mature central nervous axons can grow into peripheral nerve grafts. It was believed that a lack of neurotropic factors in the microenvironment of the central nervous system obstructs regeneration. However, axons barely regenerate in the presence of neurotrophic factors and axons and fibroblasts can grow through a mature layer of oligodendrocytes in cultivation[14]. In co-culture experiments of neurons and oligodendrocytes, the axons of neurons and growth cones are contact-inhibited by oligodendrocytes[15], which suggests that oligodendrocytes or the formation of a myelin membrane has a capital role in inhibiting the growth of axons[16]. Myelin-associated glycoprotein was the first inhibitor isolated and identified from the myelin[17-18]. Nogo-A has also been considered as another inhibitor of axonal growth[4, 19].

This study suggests that Nogo-A expression is decreased by methylprednisolone. Nogo-66 on the surface of oligodendrocytes was associated with the Nogo receptor of damaged neurons. Binding of Nogo-66 to Nogo receptor is known to inhibit neurite outgrowth. The amino terminus of Nogo-A (amino-Nogo) is released from damaged oligodendroctyes, where it binds Nogo receptor and inhibits the growth of the vegetative cone by secondary signals and activation of GTPases, such as Rho and Cdc42[20-21]. The combined actions of amino-Nogo and Nogo-66 on Nogo receptor has an increased inhibitory role to neural regeneration. Methylprednisolone can enhance membrane stability, maintain neuron independence and integrity, and enhance the excitability and conduction of spinal cord neurons[22-23]. The mechanism is mainly owing to methylprednisolone binding the glucocorticoid receptor in the cytoplasm, which translocates into the nucleus and induces the expression of different genes[24]. Studies have shown that early high-dose (30 mg/kg) methylprednisolone application can increase expression of Bcl-2 protein in rat nerve cells after acute spinal cord injury and reduce apoptosis in spinal nerve

cells[11]. Furthermore, high-dose methylprednisolone enhances the expression of growth-associated protein 43 mRNA in acute spinal cord injury in rats, which may promote spontaneous recovery and restructuring of the nerve system and the normal germination response[12].

The decrease in Nogo-A following methylprednisolone treatment may be related to a reduction in microglial apoptosis. Naso *et al* [25] showed that the preventive use of methylprednisolone in the spinal cord hemisection injury model can inhibit secondary injury of nerve cells. Nogo-A protein is largely expressed and released by oligodendrocytes to inhibit the regeneration of nerve cells and outgrowth of axons after spinal cord injury, and a large number of oligodendrocytes die. In this study, Nogo-A expression in the spinal cord tissues of spinal cord injury rats treated with high-dose methylprednisolone pulse therapy was significantly lower than that in control group. This indicates that the mechanism of action of methylprednisolone may result in an inhibition of Nogo-A protein expression, which may also reduce oligodendrocyte apoptosis. Thus, reducing Nogo-A protein expression may be effective in the treatment of acute spinal cord injury.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The study was performed at the Nanfang Hospital Affiliated to Southern Medical University, China from April 2009 to June 2010.

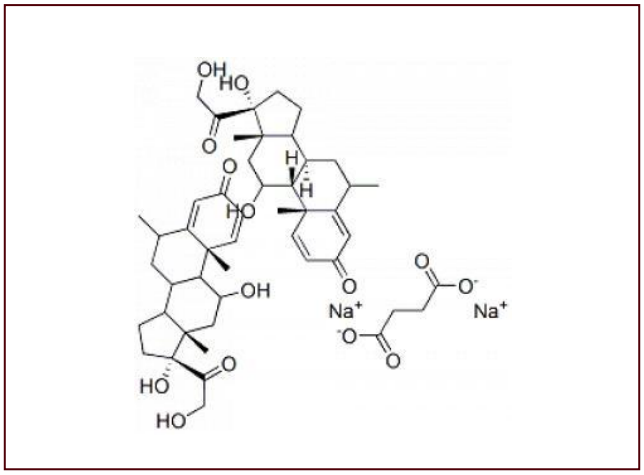
Materials

Animals

A total of 54 adult Sprague-Dawley rats, of specific-pathogen free grade, weighing 250–300 g, male or female, were purchased from the Southern Medical University Animal Center (license No. SCXK (Yue) 20060015). Rats were housed at a constant temperature 23 ± 2°C and 12/12-hour light-dark cycles. The animal experiments were performed in accordance with the guidelines of Animal Ethics Committee of Nanfang Hospital.

Drugs

Methylprednisolone sodium succinate (No. H20080284) was purchased from Pharmacia & Upjohn, Belgium, Pfizer. Chemical structural formula of methylprednisolone sodium succinate is as follows:



Methods

Establishment of spinal cord injury models in rats

Rats were anesthetized by intraperitoneal injection of 15% chloral hydrate (3–4 mL/kg). Vertebrae $T_{8–10}$ and their spinal meninges were exposed after carefully removing the lamina and pedicles. Steel weights were used to induce contusion models of spinal cord injury at $T_{8–10}$ spinal segments, which were guided by a 10-cm long glass catheter according to an improved Allen's method[26], following which the skin was sutured layer by layer. Rats in the control group only received surgery to expose the spinal cord without inducing any injury.

Drug intervention

The methylprednisolone group was slowly injected with methylprednisolone sodium succinate (30 mg/kg) for 15 minutes *via* the tail vein immediately after the model was established, which was performed three times over the following 24 hours. The spinal cord injury group and control group received an equal volume of physical saline (0.5 mL). Three days after the operation, all the animals were intraperitoneally injected with physical saline 10 mL/time, twice daily as well as sodium penicillin 400 000 U/day to maintain water and electrolyte balance and prevent infection. Rats in the spinal cord injury and methylprednisolone groups had their bladders squeezed twice a day to help release urine.

Behavioral examinations

Motor function was evaluated according to the Basso, Beattie, and Bresnahan scale scores[27-30]. The Basso, Beattie, and Bresnahan scale score (ranging from 0 to 21) represents the mobility of four limbs. Higher scores mean better motor function of limbs.

Histological examinations

After 3 days, rats were anesthetized with chloraldurat, following which they were perfused with 100 mL

physical saline and 4% paraformaldehyde though a syringe needle inserted into the aorta (20 drips per minute for 3 hours). Then, a 3-cm incision was made around the back segments of the $T_{8–10}$ layers and on each of the floor muscles. A 2-cm segment of the spine was collected from the injured segments by cutting both ends and a piece of complete spinal cord tissue was harvested after the lamina. The vertebral body and the surrounding scar tissue were carefully removed using a sclerectomy cutting device. After removal, the tissue was washed with 1 × Tris-buffered saline and placed in 4% paraformaldehyde. The spinal cord tissue was conventionally embedded in paraffin and cut into slices of 5 μm thickness for hematoxylin-eosin staining and immunohistochemistry analysis. For hematoxylin-eosin staining, eosin-methylene blue was used to differentiate the cytochylema and nucleus in the spinal cord tissue. Rabbit anti-rat Nogo-A antibody (1:400; Boster, Wuhan, China) was used as the primary antibody to mark the target protein at 4°C overnight. The samples were washed with PBS for 15 minutes and nonspecific antigens were blocked with 30% H_2O_2 for 5 minutes. Goat anti-rabbit IgG (1:200; Boster) was used as the secondary antibody and incubated at 37°C for 30 minutes. After incubation with diaminobenzidine kit (Boster), Nogo-A staining was seen as brown-yellow. The tissue was photographed using Olympus DP71 Image System (Olympus, Tokyo, Japan).

Nogo-A protein detection by western blot assay

After 3, 7 and 14 days, T_{8-10} spinal cord segments of the rats in all three groups were harvested and stored at –70°C. 40 mg tissue of each sample of spinal cord was grinded into cell lysate in 30 μL and homogenized in an ice bath. The supernatant was centrifuged at 7 500 r/min after boiling and mixed with an equal volume of 2 × sodium dodecyl sulfate sample buffer to obtain the total protein extract. The protein was transferred to polyvinylidene fluoride membrane (4°C, 2.5 hours, 50 V) after electropheresis on a 10% sodium dodecyl sulfate polyacrylamide gel. The membrane washed slowly with Tris-buffered saline solution three times for 5 minutes each, blocked in a solution of 1% bovine serum albumin and 0.02% Tween 20 in Tris-buffered saline, at 4°C for 6 hours. The membrane was then incubated with the primary antibodies, which were polyclonal rabbit anti-Nogo-A (1:400; Boster) and anti-β-actin antibodies, in blocking solution at 4°C overnight. Then the membrane was washed with Tris-buffered saline three times for 5 minutes, washed with 5 mL blocking solution, washed with Tris-buffered saline three times for 5 minutes, and incubated with goat anti-rabbit IgG (1:400; Boster) secondary antibody, followed by detection of the chemiluminescent signal (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Images were digitized and analyzed using a computerized image analysis software system (Image-Pro Plus6.0; Media cybernetics Inc., Silver Spring, MD, USA). The absorbance ratio of Nogo-A protein to standard protein (β-actin) represenst the relative levels of Nogo-A protein.

Statistical analysis

Data were expressed as mean ± SD. SPSS 13.0 (SPSS, Chicago, IL, USA) was used to analyze the data. Basso, Beattie, and Bresnahan scale scores and the expression of Nogo-A among the three groups were compared by one-way analysis of variance. One-way analysis of variance and least significant difference *t*-test (or Dunnett's test) for multiple comparisons were adopted to have a separate analysis of effects. The results were considered statistically significant with a *P* level of 0.05.

Author contributions: Zhaozong Fu participated in the manuscript writing and data collection. Hai Lu completed the protein quantitation. Jianming Jiang was responsible for study design, study supervision, and manuscript instruction. Hui Jiang participated in the animal modeling. Zhaofei Zhang participated in the immunohistochemistry. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The study was approved by Animal Ethics Committee of Nanfang Hospital, 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.

Supplementary information: Supplementary data associated with this article can be found in the online version by visiting www.nrronline.org.

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