

# Anti-inflammatory effect of Mongolian drug Naru-3 on traumatic spinal cord injury and its mechanism of action

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## Abstract

**Objective:** This study was performed to confirm the anti-inflammatory effect of the Mongolian drug Naru-3 on traumatic spinal cord injury (TSCI) and its possible mechanism of action.

**Methods:** We prepared a TSCI model using Sprague–Dawley rats. The rats were divided into a Naru-3 group and a methylprednisolone group. Real-time polymerase chain reaction and western blotting were performed to measure the expression levels of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, and IL-1 $\beta$ . Enzyme-linked immunosorbent assay kits were employed to detect serum inflammatory cytokine levels. The localization and expression of monocyte chemoattractant protein-1 (MCP-1) in spinal cord tissue was determined by immunohistochemical analysis. Flow cytometry was performed to analyze the ratio of M1- and M2-phenotype macrophages. The locomotor function recovery was evaluated by the Basso, Beattie, and Bresnahan score.

**Results:** Naru-3 significantly inhibited the inflammatory response and reduced the expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in both spinal cord and blood in a time- and concentration-dependent manner. Immunohistochemical analysis indicated that Naru-3 significantly reduced MCP-1 expression in spinal cord and promoted M2-phenotype macrophage differentiation.

**Conclusions:** Naru-3 is an effective treatment for impact-induced TSCI in rats. Naru-3 treatment affects inflammatory cytokine levels and macrophage differentiation, which play a role in TSCI remission.

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## Keywords

Naru-3, traumatic spinal cord injury, inflammatory cytokines, macrophages, methylprednisolone, monocyte chemoattractant protein-1

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## Introduction

Traumatic spinal cord injury (TSCI) is usually a serious condition that can lead to paraplegia or quadriplegia. Globally, the morbidity of TSCI ranges from 10.4 to 83.0 per million people<sup>1</sup> and differs substantially among different countries and regions. In China, the incidence of TSCI steadily increased to 23.7 per million people per year from 2004 to 2008.<sup>2</sup> TSCI in developing and developed countries primarily affects men aged 18 to 32 years.<sup>3</sup> Because no fully restorative therapies for TSCI have yet been established, TSCI strongly impacts patients' life expectancy, quality of life, and family economic burden.

Mechanical trauma of the spinal cord initiates the formation of the primary injury, and a secondary lesion can lead to craniocaudal and protracted lesion expansion after TSCI.<sup>4</sup> This primary injury of the spinal cord is considered to be irreversible; however, the secondary injury is believed to be amenable.<sup>5,6</sup> TSCI activates the immune system, and the inflammatory response may be a major mechanism underlying the development of secondary lesions.<sup>7</sup> Invading and resident inflammatory cells (including T cells, microglia, and macrophages) can have a series of destructive and reparative effects.<sup>8</sup> Bethea et al.<sup>9</sup> noted that interleukin (IL)-10 (an anti-inflammatory cytokine) has neuroprotective properties and promotes functional recovery following SCI by inducing activated macrophages to reduce tumor necrosis factor (TNF)- $\alpha$  synthesis.

Although no complete restorative therapies for TSCI have been established, various rehabilitative, cellular, and molecular treatments have been applied in animal models. Can et al.<sup>10</sup> found that dexmedetomidine could decrease the levels of TNF- $\alpha$  and IL-6 in serum and that the anti-inflammatory effects of dexmedetomidine and methylprednisolone (MP) were equivalent. Bradbury et al.<sup>11</sup> reported that chondroitinase ABC upregulates a regeneration-associated protein, restores post-synaptic activity, and promotes regeneration of both descending corticospinal tract axons and ascending sensory projections, thus promoting functional recovery after human SCI. The glucocorticoid MP is currently the only clinical treatment widely used for human TSCI.<sup>8</sup>

The Mongolian drug Naru-3 has been used in traditional Chinese medicine for at least 1,000 years. This drug is widely in the treatment of various inflammatory diseases in China and Mongolia. Clinical studies have shown that Naru-3 has anti-inflammatory, analgesic, and antibacterial effects.<sup>12</sup> However, as a traditional Chinese drug, the underlying mechanisms by which Naru-3 affects TSCI are unclear. In this study, we prepared an in vivo TSCI model and confirmed the anti-inflammatory effect of Naru-3 as well as its possible mechanism of action.

The study was approved by the ethics committee at the Second Affiliated Hospital of Guangzhou Medical University. All surgical procedures were performed under 10%

(0.3 mL/100 g) chloral hydrate anesthesia, and all efforts were made to minimize suffering.

## Materials and methods

### *Preparation of drugs and animals*

Naru-3 pills (National medicine permission number: Z20050034) were purchased from Inner Mongolia Mongolian Medicine Co., Ltd. (Tongliao, China), and MP (Approval number: H20130301) was purchased from Pfizer Manufacturing Belgium NV (Puurs, Belgium).

The adult Sprague–Dawley (SD) rats (age, 9 weeks; weight, 200–250 g) used in these experiments were obtained from Southern Medical University's Experimental Animal Center (Guangzhou, China). The study protocol and care of rats in this project were performed in accordance with the Helsinki Declaration Accords. All rats were housed in a standard plastic, transparent cage under a stable 12-/12-hour light/dark cycle at a constant room temperature of  $21^{\circ}\text{C} \pm 2^{\circ}\text{C}$  by following the animal care rules and laboratory animal guidelines of Southern Medical University.

### *TSCI model preparation*

All SD rats were intraperitoneally injected with 10% chloral hydrate (0.3 mL/100 g) for anesthesia. The following operations were then performed under an aseptic environment. Once the rats had been completely anesthetized, their backs were shaved and a 2-cm midline incision was made over the T10–L1 spinous processes. The superficial fascia was separated, and the deep fascia and muscle of T8–T11 were cut on both sides of the spinous process. The spinous process was exposed, and the T9–T10 spinous process and lamina were removed to expose the spinal canal. The spinal cord was then fully exposed. A homemade modified

weight drop device (20-g weight dropped along a plastic sleeve from a 5-cm height, which had a damage energy of 100 g/cm) was aligned to T9–T10 and then dropped to hit the spinal cord, the surface of which was covered with a  $3 \times 7$ -mm metal arc. The impact area was  $3 \times 7$  mm. Following injury, the musculature was sutured and the skin stapled; the animals were then allowed to recover from anesthesia. The signs of successful model establishment were a spastic swinging gait, retraction-like flutter in the hind limbs and torso, and bilateral hind limb flaccid paralysis. The rats were fed in the sub-cages after modeling, and their bladders were manually emptied three to four times daily until automatic urination was restored.

### *Grouping and intervention treatment*

The SD rats were randomly divided into four groups: a sham group (sham surgery without treatment), a model group (TSCI modeling without other treatment), an MP group (TSCI modeling followed by injecting MP (30 mg/kg/day) into the tail vein), and a Naru-3 group. The Naru-3 group was divided into subgroups by dose (10, 20, 50, and 100 mg/kg gavage groups) and time of administration (8-, 16-, 32-, and 48-day administration groups); each subgroup contained six rats. At the end of the experiment, the SD rats were killed by intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g). Blood specimens and a 1.2-cm-long section of spinal cord tissue were then collected.

### *Extraction of total RNA and quantitative real-time polymerase chain reaction*

Total RNA was first extracted from the spinal cord using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA was performed using a Takara cDNA Synthesis Kit (Takara Co.,

Ltd., Dalian, China). Quantitative real-time polymerase chain reaction was then performed to detect the expression levels of mRNA using a LightCycler 480 detection system (Roche Diagnostics, Indianapolis, IN, USA) and the interaction dye SYBR Green. The expression levels of  $\beta$ -actin mRNA were used for normalization. The polymerase chain reaction results were analyzed as relative mRNA levels of the Ct values, which were then converted into the fold change.

### **Western blot assays**

The spinal cord was lysed in radioimmunoprecipitation assay buffer (Youdi Biotechnology, Guangzhou, China). A BCA Protein Assay Kit (Pierce/Thermo Fisher Scientific, Waltham, MA, USA) was employed to measure the concentration of total protein lysates, and 50  $\mu$ g of total protein lysates per lane was then resolved on 10%(w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Immunoblot detection and visualization were then performed (Pierce/Thermo Fisher Scientific). Immunoblotting was performed with anti-TNF- $\alpha$ , IL-6, and IL-1 $\beta$  and anti-monocyte chemoattractant protein-1 (MCP-1) antibodies (Cell Signaling Technology, Danvers, MA, USA). The protein bands were quantified with the ChemiDoc image analysis system (BioRad Laboratories, Hercules, CA, USA).

### **Quantitative detection of TNF- $\alpha$ , IL-6, and IL-1 $\beta$ in serum**

Blood was centrifuged and the serum collected. The IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels in serum were assessed by enzyme-linked immunosorbent assay (ELISA) kits. A Rat TNF-alpha Quantikine ELISA Kit (RTA00; R&D Systems, Minneapolis, MN, USA), Rat IL-6 Quantikine ELISA

Kit (R6000B; R&D Systems), and Rat IL-1 beta/IL-1F2 Quantikine ELISA Kit (RLB00; R&D Systems) were purchased to determine supernatant concentrations. The absorbance (optical density) at 450 nm was measured with an ELISA reader (BioTek Instruments, Winooski, VT, USA).

### **Immunohistochemical analysis**

The spinal cord was sliced into 5- $\mu$ m frozen sections. Following deparaffinization, hydration, and blocking with serum, the slides were treated with peroxide (Invitrogen). The anti-MCP-1 antibodies were incubated overnight. After washing three times and incubating with the secondary antibodies for 30 minutes at 37°C, the slides were placed in 3,3'-diaminobenzidine solution. Finally, the slides were lightly counterstained, dehydrated, and mounted. Positive staining of MCP-1 in the spinal cord was determined by counting five random visual fields with a 400 $\times$  magnification microscope.

### **Flow cytometric analysis of macrophages**

Fresh spinal cord tissue was ground into a single cell suspension and filtered through a 45- $\mu$ m nylon mesh filter. The macrophages were then separated by density gradient centrifugation. Macrophages were identified by flow cytometry using fluorescent-labeled antibodies. Isolated macrophages were collected in phosphate-buffered saline (PBS) at pH 7.4. After being washed by PBS several times, the cells were resuspended using permeabilization solution (0.1% saponin in PBS). The cells were then reacted with FITC anti-CD86 antibodies (BD Pharmingen; BD Biosciences, San Jose, CA, USA) and anti-CD163 antibodies (GeneTex, Irvine, CA, USA). After being washed by PBS, these cells were analyzed

on a FACSCalibur flow cytometer (Accuri C6; BD Biosciences).

### Evaluation of locomotor function

The Basso, Beattie, and Bresnahan (BBB) score in each group was calculated to evaluate the locomotor function, which was categorized into combinations of rat hind limb movements, toe clearance, stepping, trunk position and stability, coordination, paw placement, and tail position; 0 represented “no locomotion” and 21 “normal motor function.”<sup>13</sup> These item scores were finally summed into a total score ranging from 0 to 21.

### Statistical methods

Statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Student’s t-test or one-way analysis of variance was used to determine statistical significance. The measurement data are presented as mean  $\pm$  standard deviation. A *P* value of  $<0.05$  was considered statistically significant.

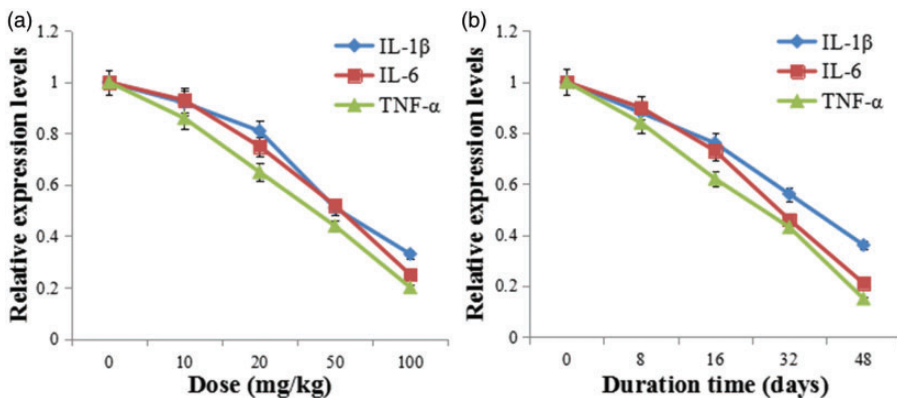
## Results

### Dose and time effects of Naru-3 on spinal cord injury in vivo

The three targets (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) are primary inflammatory cytokines that can reflect the host’s immune status. With extended Naru-3 treatment, the expression levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  were significantly downregulated ( $P < 0.05$ ) (Figure 1(a)). As indicated in Figure 1(b), the expression levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  significantly decreased after Naru-3 treatment for 8 days ( $P < 0.05$ ), followed by a gradual decrease upon prolonged exposure to Naru-3. Thus, the Naru-3-induced anti-inflammatory effect increases in a time- and concentration-dependent manner.

### Expression of Naru-3 regulates TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1 in the spinal cord

We determined the effect of Naru-3 on TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1 expression in the spinal cord after the rats had been exposed to Naru-3 at different doses and



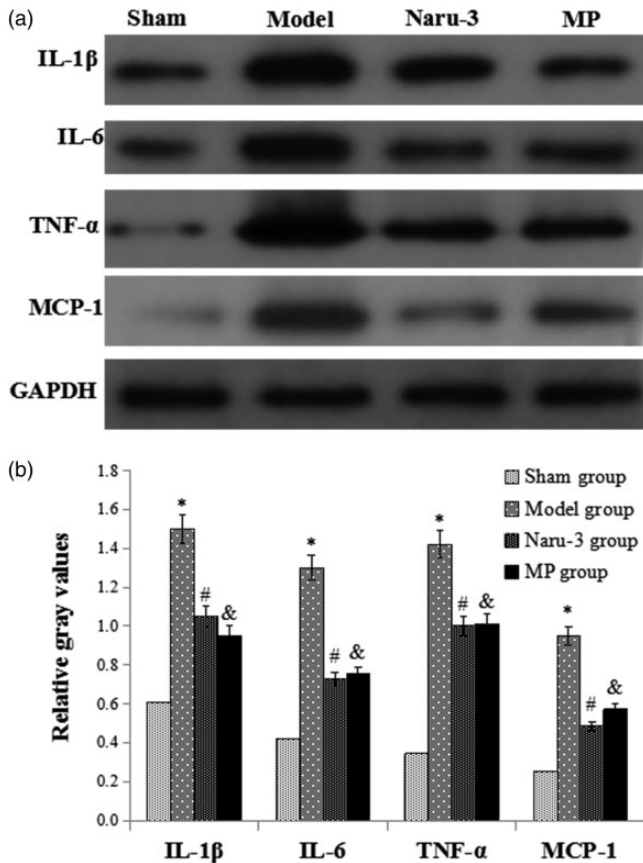
**Figure 1.** Relative expression levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  mRNA in (a) different dose groups and (b) different administration time groups. Data are presented as the mean  $\pm$  standard deviation from three independent experiments. IL, interleukin; TNF, tumor necrosis factor.

for different lengths of time. As shown in Figure 2, after 16 days of continuous gavage of Naru-3 at 20 mg/kg per day, the TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1 levels significantly decreased in the Naru-3 group ( $P < 0.05$ ). There was no significant difference between the positive control group (MP group) and Naru-3 group. Moreover, the other Naru-3 treatment subgroups showed decreases in their TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1 levels to vary degrees (not shown). These results indicate that Naru-3 can regulate the expression of

TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1 in the spinal cord in a time- and concentration-dependent manner.

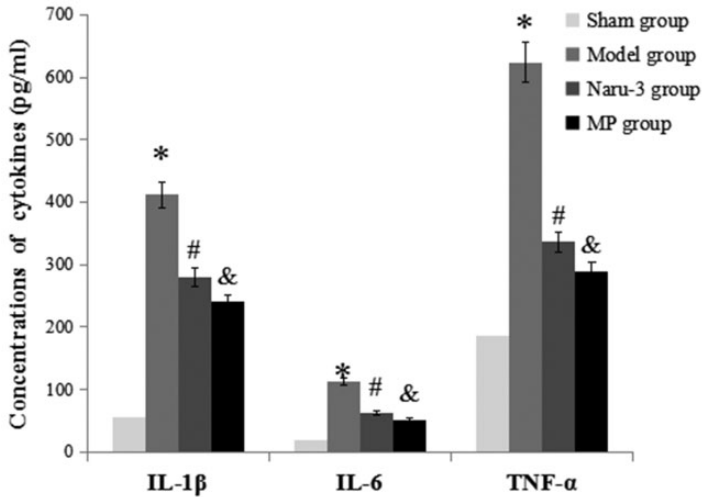
### Naru-3 regulates the expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$ in the serum

ELISA was employed to measure the concentrations of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in blood. As shown in Figure 5, after 16 days of continuous gavage at 20 mg/kg per day, the levels of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  significantly decreased in the Naru-3



**Figure 2.** Western blot detected the expression of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and MCP-1 in each group. (a) Schematic image of western blotting. (b) Statistical results of relative gray values in each group. GAPDH was used as a loading control. Data are presented as the mean  $\pm$  standard deviation from three independent experiments. # $P < 0.05$  compared with \*; & $P < 0.05$  compared with \*; & $P > 0.05$  compared with #. IL, interleukin; TNF, tumor necrosis factor; MCP-1, monocyte chemoattractant protein-1; MP, methylprednisolone.





**Figure 3.** Concentrations of serum proinflammatory cytokines in each group. Data are presented as the mean  $\pm$  standard deviation from three independent experiments. # $P < 0.05$  compared with \*; & $P < 0.05$  compared with \*; & $P > 0.05$  compared with #. IL, interleukin; TNF, tumor necrosis factor; MP, methylprednisolone.

group ( $P < 0.05$ ) (Figure 3); the same effect was observed in the MP group. In addition, the other Naru-3 treatment subgroups showed decreased concentrations of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in serum to vary degrees (data not shown). These results indicate that Naru-3 regulates TNF- $\alpha$ , IL-6, and IL-1 $\beta$  levels in the serum in a time- and concentration-dependent manner.

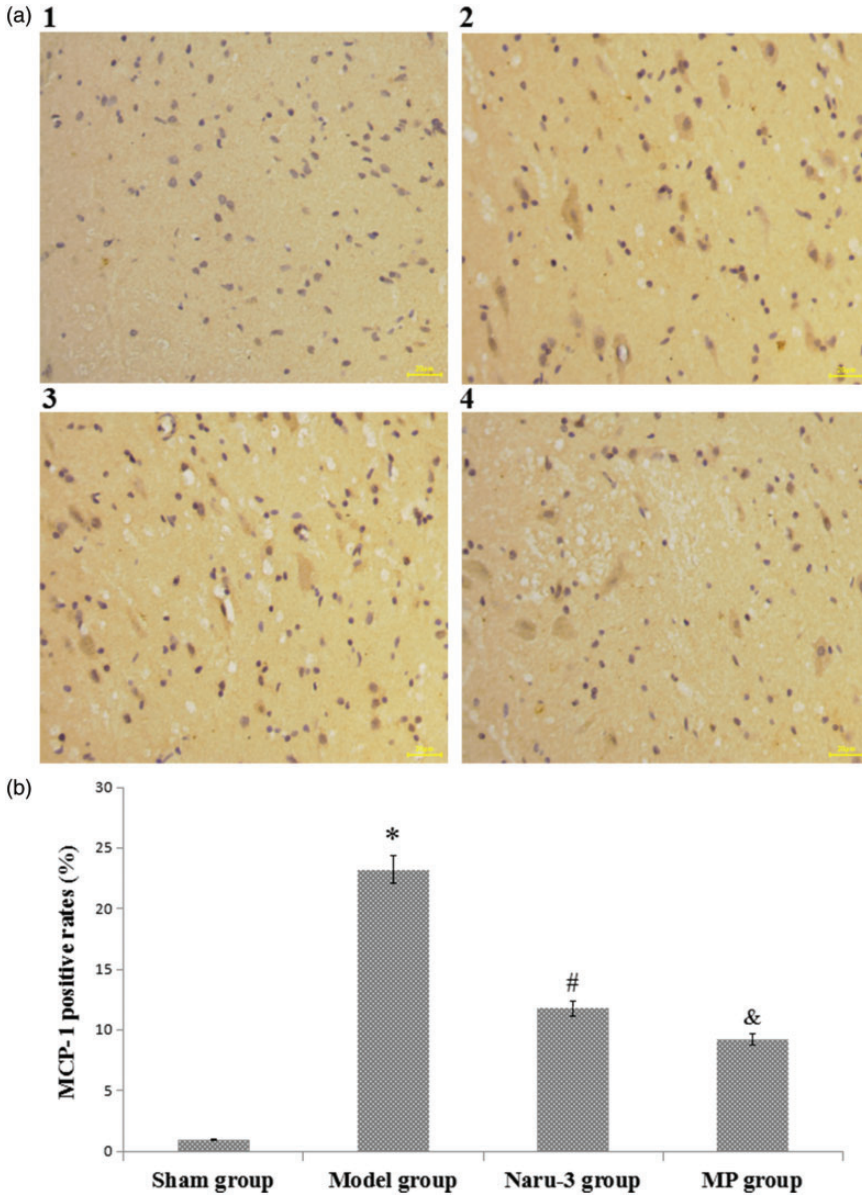
#### *Naru-3 regulates MCP-1 expression in the spinal cord*

Immunohistochemical staining showed that MCP-1 was expressed in the cytoplasm (Figure 4). Additionally, TSCI modeling significantly increased the rate of MCP-1 expression from 1.09% in the sham group to 23.23% in the TSCI model group ( $P < 0.05$ ). After 16 days of continuous gavage at 20 mg/kg per day, Naru-3 significantly decreased the rate of MCP-1 expression compared with the TSCI model group ( $P < 0.05$ ); the same effect was seen in the MP group. The other Naru-3 treatment

subgroups showed decreased MCP-1 expression to varying degrees (data not shown).

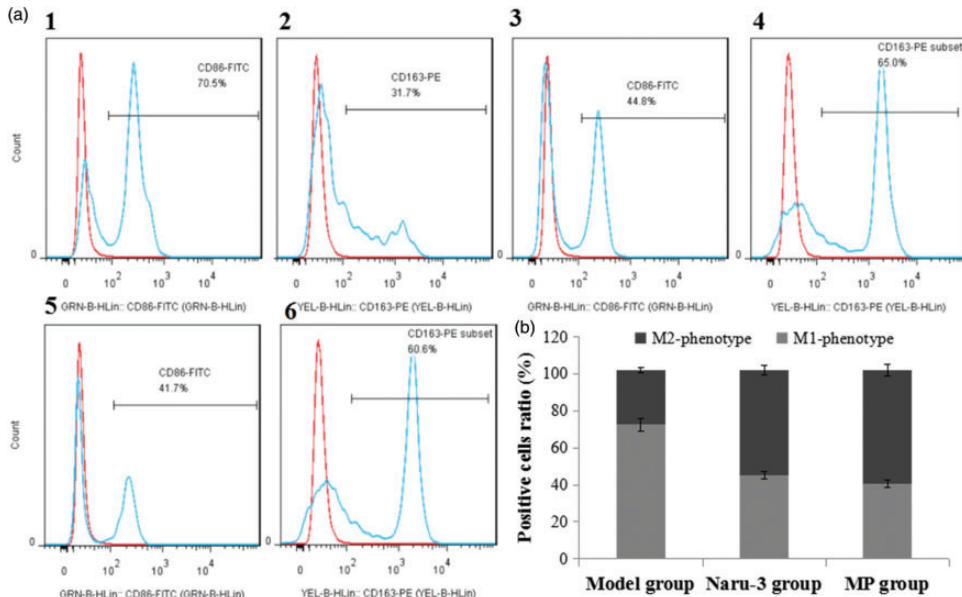
#### *Naru-3 regulates macrophage differentiation*

CD86, an M1-phenotype macrophage-specific marker, and CD163, an M2-phenotype macrophage-specific biomarker, were used for macrophage identification in TSCI. We evaluated the M1- to M2-phenotype ratio using a flow cytometer (Figure 5). In TSCI model rats, the M1-phenotype macrophage ratio (CD86-FITC positive) was 72.5%  $\pm$  5.8%, whereas the proportion of M2-phenotype macrophages (CD163-PE positive) was only 29.7%  $\pm$  3.2%. After 16 days of Naru-3 treatment at 20 mg/kg per day, the M1-phenotype macrophage ratio decreased to 45.2%  $\pm$  2.6%, whereas the M2-phenotype macrophage ratio increased to 56.8%  $\pm$  4.4%. Compared with the model group, there was a significant difference ( $P < 0.05$ ). In the MP group, the



**Figure 4.** Immunohistochemical analysis of MCP-1 in the spinal cord. (a) Schematic image of immunohistochemical staining. (1) Sham group. (2) Model group. (3) Naru-3 group. (4) MP group. (b) MCP-1-positive rates in each group. Data are presented as the mean  $\pm$  standard deviation from three independent experiments.  $^{\#}P < 0.05$  compared with \*;  $^{\&}P < 0.05$  compared with \*;  $^{\&}P > 0.05$  compared with #. MCP-1, monocyte chemoattractant protein-1; MP, methylprednisolone;





**Figure 5.** Flow cytometric analysis of M1- and M2-phenotypes. (a) M1-phenotype and M2-phenotype macrophages measured using flow cytometry. (1) M1-phenotype-positive macrophages of the TSCI model group. (2) M2-phenotype-positive macrophages of the TSCI model group. (3) M1-phenotype-positive macrophages of the Naru-3 group. (4) M2-phenotype-positive macrophages of the Naru-3 group. (5) M1-phenotype-positive macrophages of the MP group. (6) M2-phenotype-positive macrophages of the MP group. The red line in (1) to (6) represents the sham group, and the blue line represents the corresponding group. (b) The M1- to M2-phenotype macrophage ratio in each group. Values are presented as mean  $\pm$  standard deviation;  $n = 3$ . \* $P < 0.05$  compared with sham group; # $P < 0.05$  compared with \*. TSCI, traumatic spinal cord injury; MP, methylprednisolone.

M1-phenotype macrophage ratio was  $40.6\% \pm 4.7\%$ , and the proportion of M2-phenotype macrophages was only  $61.5\% \pm 5.2\%$ . These results indicate that both Naru-3 and MP can promote M2-phenotype macrophage differentiation.

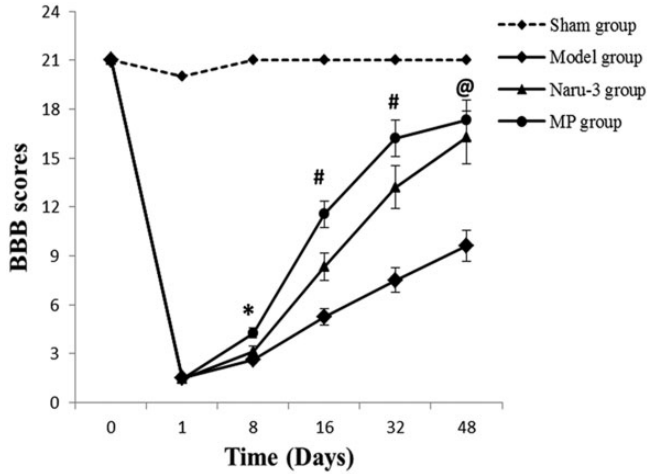
### *Naru-3 promotes locomotor functional recovery after TSCI*

As shown in Figure 6, no functional deficits (BBB score of 21) were observed in the rats tested prior to surgery. However, flaccid hind limb paralysis (BBB score of 0) was present at 24 hours in all rats in the TSCI group. Eight days after TSCI, all rats began to recover as indicated by one or two slight isolated hind limb joint movements with

small variations among the Naru-3, MP, and model groups, although there was no statistically significant difference. The gradual recovery of hind limb locomotion was investigated in these rats during the following 40 days. From 16 to 32 days, the MP group showed significantly greater recovery of locomotor function than the Naru-3 group ( $P < 0.05$ ). The locomotor recovery of the Naru-3 group ( $16.25 \pm 1.65$ ) was close to that of the MP group ( $17.34 \pm 1.21$ ) at 48 days, although the difference was not significant.

## **Discussion**

Cytokines have important effects and functions in the signaling network underlying



**Figure 6.** Locomotor recovery of hind limb function after TSCI in each group during 48 days. Values are presented as mean  $\pm$  standard deviation;  $n = 6$ . There were no significant differences in the BBB scores among the model group, Naru-3 group, and MP group 8 days after TSCI ( $*P > 0.05$ ). Rats in the Naru-3 and MP groups showed significantly ( $\#P < 0.05$ ) greater locomotor recovery at 16, 32, and 48 days after TSCI when compared with the sham group. There was no significant difference between the Naru-3 and MP groups at 48 days ( $@P < 0.05$ ). TSCI, traumatic spinal cord injury; BBB, Basso, Beattie, and Bresnahan; MP, methylprednisolone.

inflammatory responses. A robust immune response is triggered by SCI and is characterized by the synthesis of chemokines and cytokines and coordinated infiltration of peripheral leukocytes at the site of damage.<sup>14</sup> Although the contusion induces the initial damage, the final histopathological damage is much greater than initially recognized after injury.<sup>15</sup> Thus, regulation of the secondary injury cascade is very important for functional recovery. Several laboratories have shown that therapeutic approaches may be effective by interfering with the acute central nervous system inflammatory cascade.<sup>16,17</sup> Brambilla et al.<sup>18</sup> studied transgenic mice and concluded that selective NF- $\kappa$ B signaling inhibition in astrocytes results in protective effects and that this NF- $\kappa$ B pathway could be a new biomarker for the development of therapeutic strategies in the treatment of SCI. Naru-3, an ancient anti-inflammatory drug, decreased the expression levels of

proinflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) in spinal cord tissue and blood. These findings indicate that Naru-3 treatment effectively inhibits the inflammatory response and secondary injury cascade.

The release of proinflammatory cytokines (IL-1 and TNF- $\alpha$ ) stimulates the synthesis of adhesion molecules (intercellular adhesion molecule-1) and chemotactic factors (IL-8 and monocyte chemotactic protein-1 (MCP-1)).<sup>19</sup> MCP-1 expression has been found in immune-mediated inflammation, and some studies have reported a close correlation between elevated levels of MCP-1 and macrophage infiltration.<sup>20</sup> Studies also have shown that MCP-1 can activate macrophages and is involved in the secondary inflammatory response-induced lesion in SCI.<sup>21</sup> When the rats in the present study were subjected to TSCI, the expression of MCP-1 was upregulated, which may have been induced by increases in proinflammatory cytokines

(IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ). Moreover, as the inflammatory cytokines decreased and M2-phenotype macrophages increased, MCP-1 expression in the Naru-3 group was down-regulated in the spinal cord. Future studies should examine the regulatory relationship among the inflammatory cytokines, MCP-1 expression, and macrophages in greater depth.

Macrophages have functions of damage and repair, and these different effects may be caused by different macrophage subsets: “classic activation” of proinflammatory (M1) cells and “alternative activation” of anti-inflammatory (M2) cells.<sup>22</sup> The differentiation of “classically activated” M1 macrophages is promoted by various injuries, and these cells also produce high levels of proinflammatory cytokines and oxidative metabolites (e.g., superoxide and nitric oxide).<sup>23</sup> In the presence of cytokines, the activating macrophages increase their “alternatively activated” M2 phenotype, which promotes matrix remodeling and angiogenesis while suppressing destructive immunity.<sup>24</sup> In the present study, the levels of inflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) and the proportion of M1-phenotype macrophages in the TSCI model group were much higher than those in the sham group, demonstrating that TSCI can induce a proinflammatory response and that M1 macrophages play a role in injury. Additionally, after Naru-3 treatment, the inflammatory cytokine levels and proportion of M1-phenotype macrophages in the TSCI model group decreased while the M2 macrophages increased to play a repair role.

Despite the widespread acceptance of MP as a standard therapy for TSCI in the clinical setting, some studies have suggested that MP has little or even no therapeutic value and has clear adverse effects.<sup>25</sup> MP may even damage the reparative functions of post-traumatic inflammation.<sup>26</sup> Therefore, it is necessary to establish new

treatments for TSCI. Moreover, BBB scoring revealed that both MP and Naru-3 can promote locomotor function recovery after TSCI and that Naru-3 has more effective promotion than MP in long-term medication.

In this study, we found that Naru-3 has an anti-inflammatory effect on TSCI. The most important findings is that after 16 days of administration, Naru-3 showed the same anti-inflammatory effect as MP without obvious adverse effects, indicating its superiority over MP administration. Further studies are needed to evaluate the effect of Naru-3 on TSCI in human patients and to deeply explore some other related molecular mechanisms such as those mediated by neutrophils and other inflammation-related signaling pathways.

#### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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