

## Original Article

# Protective effects of LM22A-4 on injured spinal cord nerves

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**Abstract:** Objective: The goal of this study was to elucidate the protection by and potential mechanisms of LM22A-4, a specific agonist of tyrosine kinase receptor B, against spinal cord injury (SCI). Methods: Spinal cord trauma was induced by the application of vascular clips to the dura of mice via a four-level T7-T11 laminectomy. Thirty minutes after the injury, an abdominal injection of LM22A-4 (at dosages of 10 mg/kg and 15 mg/kg) or an equal volume of solvent was provided. Twenty-four hours after SCI, a Western blot was performed to examine the expression of p-TrkB, p-Akt, p-ERK, cleaved-caspase-3, and Bcl-2; a TUNEL assay and Nissl staining were performed to study apoptosis and the survival of neurons. In addition, another batch of mice was allowed to live for 14 days after the SCI treatment, during which the LM22A-4 was provided at the same time each day and the neurological function was assessed. Results: Spinal cord injury in mice resulted in severe trauma characterized by tissue damage and apoptosis. Treatment of the mice with LM22A-4 (10 mg/kg) significantly reduced the degree of tissue injury (histological score) and apoptosis (TUNEL staining and caspase-3 and Bcl-2 expression) compared with vehicle treated group ( $P < 0.05$ ). In a separate set of experiments, chronic treatment with LM22A-4 also significantly ameliorated the recovery of limb function ( $P < 0.05$ ). Conclusion: This study provides an experimental evidence that LM22A-4 reduces the development of tissue injury associated with spinal cord trauma, and activation of the activity of TrkB may represent a novel approach for the therapy of spinal cord trauma.

**Keywords:** SCI, LM22A-4

## Introduction

Previous studies have indicated that brain-derived neurotrophic factor (BDNF) can activate the TrkB signaling pathway to exert a protective function against spinal cord trauma. LM22A-4 is a newly developed TrkB-specific agonist that has been demonstrated to play a neuroprotective role in brain injury. However, its role in SCI has not been reported. Hence, the LM22A-mediated protection and potential mechanisms against SCI are examined herein, which might shed light on strategies and methods for clinically treating SCI patients.

## Materials and methods

### Materials

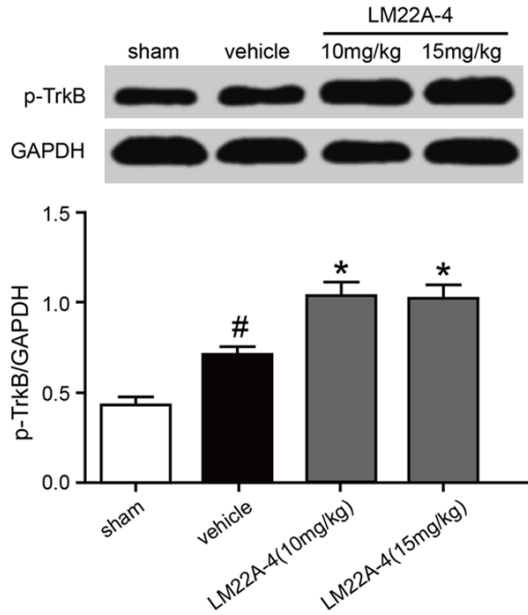
Experimental ICR mice were purchased from the Laboratory Animal Medical Center, X

University. A total protein extraction solution was purchased from Beyotime Technology. Phosphatase inhibitors were purchased from Sigma (USA). Antibodies for p-TrkB, p-Akt, p-ERK, p-CREB, cleaved-caspase-3, and Bcl-2 were purchased from Cell Signaling (USA). Vascular clips for aortic clamping in mice were purchased from Kent Scientific (USA). LM22A-4 was purchased from Sigma and was dissolved in DMSO.

### Animal grouping and preparation of the mouse model of spinal cord injury

ICR mice were randomly divided into five groups: sham treatment, spinal cord injury, spinal cord injury combined with solvent treatment, spinal cord injury combined with LM22A-4 treatment (10 mg/kg), and spinal cord injury combined with LM22A-4 treatment (15 mg/kg), with each group containing 26 animals. Preparation of the

## Protection of SCI by LM22A-4



**Figure 1.** Influences of LM22A-4 treatment on p-TrkB expression of injured spine cord tissue. In comparison with the solvent treatment, LM22A-4 significantly upregulated the expression of p-TrkB. Two doses of 10 mg/kg and 15 mg/kg did not generate an appreciable difference in p-TrkB expression. #  $P < 0.05$  compared with the sham-treatment group.

mouse SCI model was based on a previous study [1, 2] and on a protocol employed by this group. Briefly, a mouse was anesthetized via the administration of chloral hydrate (4 mg/kg) before a 3-cm incision was introduced on its back. T7-T11 vertebrae were exposed under a surgical microscope before the laminae were removed with a vascular clip to fully expose the spinal cord. The spinal cord was clamped with the vascular clip for 1 minute with a force of 10 g. The animal was then subjected to complete staunching of the bleeding, and the incised dorsal muscle and skin were sutured. Mice from the control group underwent laminectomy, full exposure of the spinal cord, and subsequent suturing, but without aortic clamping. After the surgery, the mice were placed on a warm blanket until fully awake and were then housed in cages accommodating a normal diet. After the SCI treatment, 14 mice were sacrificed to enable molecular and histological examinations; the other 14 mice were allowed to live for 20 days for neurological scoring and were sacrificed on day 20 via cervical dislocation.

### Western blot

Twenty-four hours after spinal cord trauma, one of the mice was excessively anesthetized to

allow opening of the thoracic cavity and exposure of the heart. An injection needle was inserted into the left apical position and fixed with a vascular clamp. Subsequently, the right atrial appendage was opened, and venous blood was drawn, followed by rapid infusion of 80 mL saline until clear fluid flowed out from the right atrial appendage. The original dorsal incision was reopened to expose the original dorsal incision to expose vertebrae T5-T9 and to remove the lamina. A 1.5-cm segment of spinal cord tissue flanking the clip mark was retrieved and stocked in liquid nitrogen. Consequently, 50 mg of spinal cord tissue was obtained from each mouse; it was mixed with tissue lysis buffer at a ratio of 1:1000 (W/V), fully ground, and centrifuged at 12000 r/minute to generate a supernatant. One portion of the supernatant was mixed with four parts of 5× loading buffer, which was boiled for 10 minutes. Thirty-five micrograms total protein from each sample was loaded into each well, electrophoresed, and transferred to a membrane. The membrane was blocked with 5% skim milk at room temperature for 10 minutes and incubated with each primary antibody at 4°C. The membrane was then rinsed with TBST buffer, incubated with a secondary antibody at room temperature for 1 hour, and rinsed with TBST buffer. Bands were revealed by incubating the membrane with developer and were quantitatively analyzed with Image J.

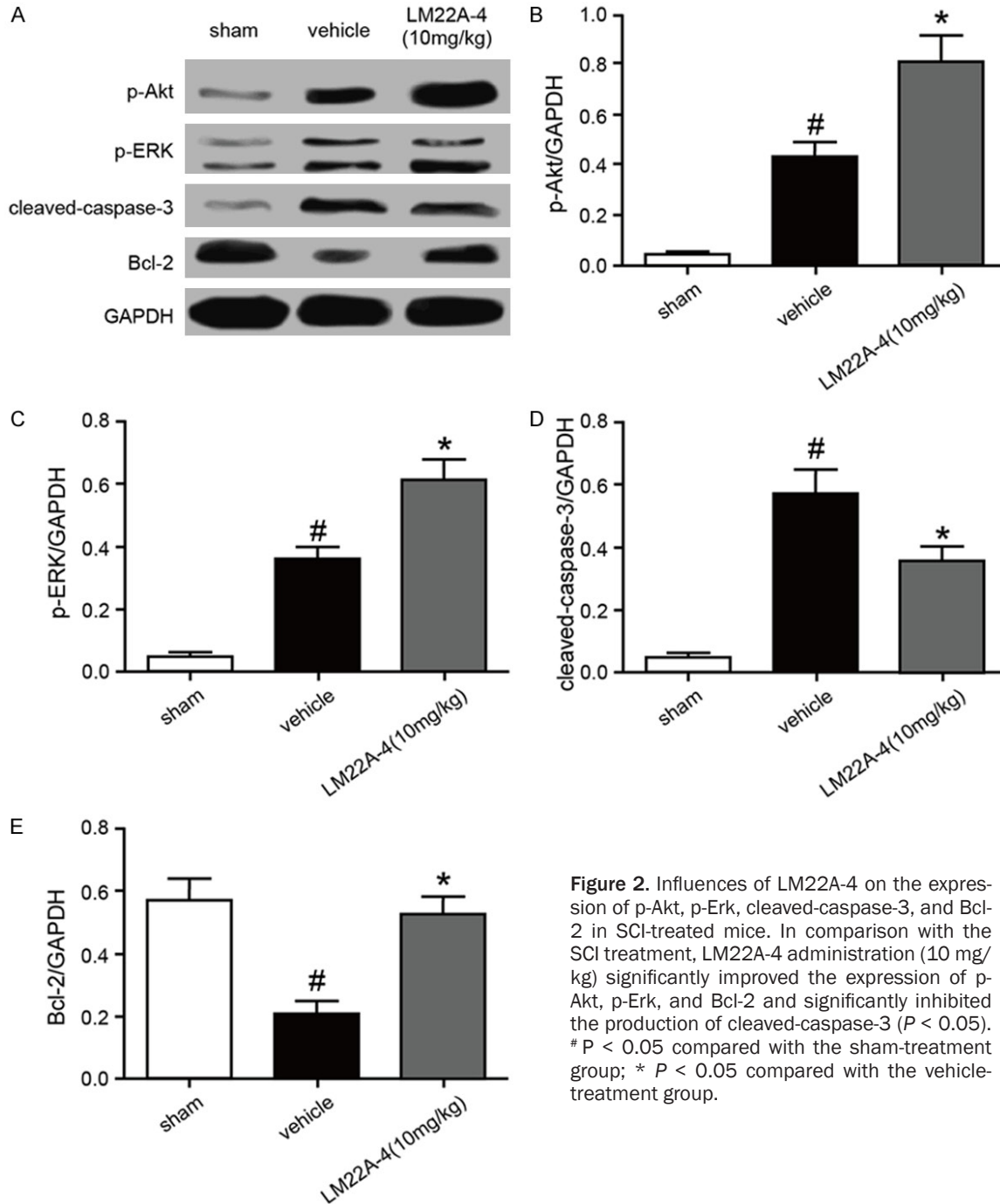
### TUNEL assay and Nissl staining

A group of mice was injected with saline into the left apical position before 80 mL of 4% paraformaldehyde was injected around the spinal cord tissue, which was retrieved and paraffin-embedded. Consecutive sectioning at a thickness of 6 μm was then performed. A TUNEL assay was then conducted to examine neuronal apoptosis, following the manufacturer's instructions. Under 400× magnification, 10 fields were randomly chosen to enumerate positively stained cells. The mean cell counts on each section slide were documented. In addition, four section slides of spinal cord tissue from each mouse were subjected to Nissl staining following standard procedures.

### Motor function scoring

Motor function was assessed via a new neurological scoring system called Basso Mouse Scale for Locomotion (BMS) [3]. Its main con-

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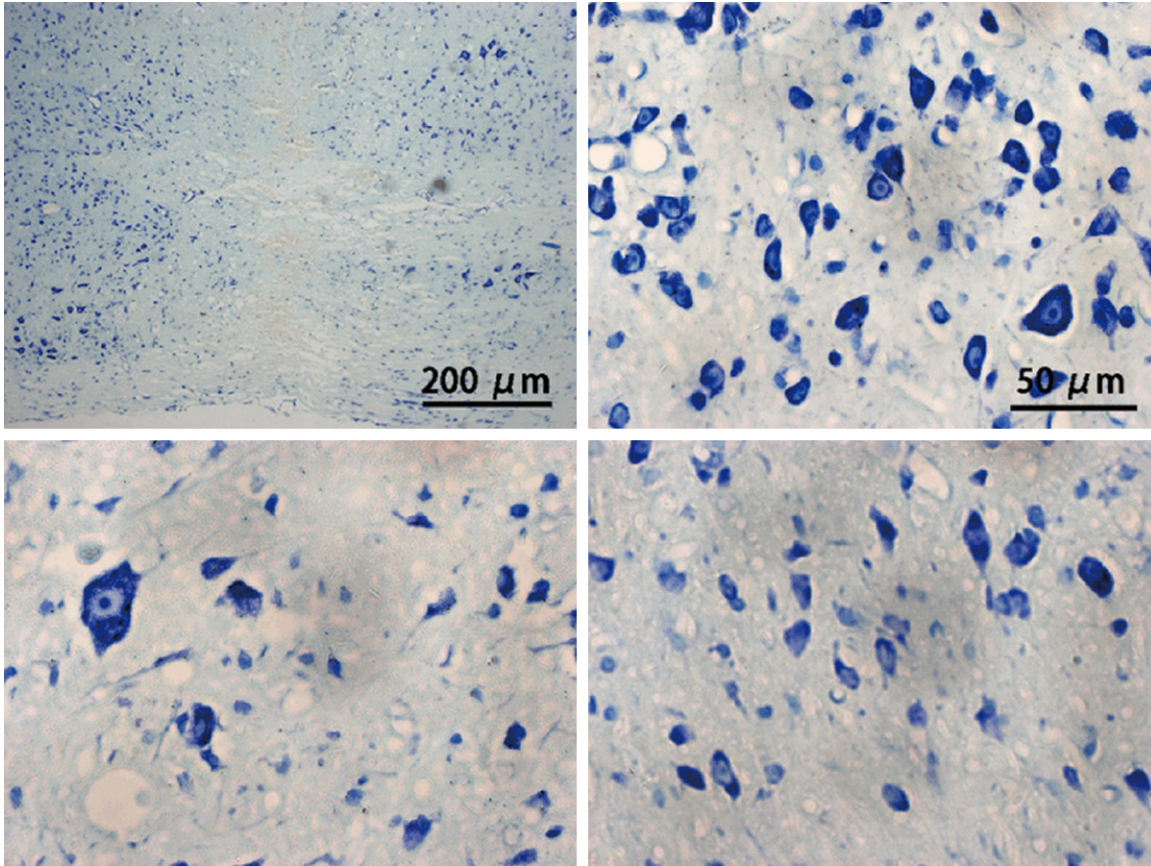
**Figure 2.** Influences of LM22A-4 on the expression of p-Akt, p-Erk, cleaved-caspase-3, and Bcl-2 in SCI-treated mice. In comparison with the SCI treatment, LM22A-4 administration (10 mg/kg) significantly improved the expression of p-Akt, p-Erk, and Bcl-2 and significantly inhibited the production of cleaved-caspase-3 ( $P < 0.05$ ). #  $P < 0.05$  compared with the sham-treatment group; \*  $P < 0.05$  compared with the vehicle-treatment group.

tent monitors the coordination of the forelimbs (FLs) and hindlimbs, consistent position of the paw during stepping, adequate toe clearance, maintenance of a stable trunk, and tail position of SCI-treated mice within 4 minutes. Our assessment included a major scoring system and an auxiliary scoring system. The first assessment was performed 24 hours after the SCI treatment, and the second assessment

was performed 13 days later at the same time point.

### Statistical analysis

The experimental data were analyzed via the SPSS17.0 software. The data were expressed as mean  $\pm$  standard deviation ( $x \pm S$ ). One-way ANOVA was performed to facilitate compari-



**Figure 3.** Nissl staining after LM22A-4 treatment. Nissl staining revealed that SCI-treated mice exhibited neuronal pyknosis and significantly reduced neuronal counts. LM22A-4 (10 mg/kg) treatment resulted in a significant increase in neuron number. #  $P < 0.05$  compared with the sham-treatment group; \*  $P < 0.05$  compared with the vehicle-treatment group.

sons between groups.  $P < 0.05$  was considered statistically significant.

### Results

#### *Influences of LM22A-4 on the expression of p-TrkB, p-Akt, and p-ERK*

The dosage of LM22A-4 used in this study was based on a previous study [1]. Our results indicated that, in comparison with the solvent treatment, administration of LM22A-4 may significantly upregulate p-TrkB expression and that both doses (10 mg/kg and 15 mg/kg) displayed no difference in this upregulation (**Figure 1**). Hence, 10 mg/kg was adopted as the dose in subsequent experiments. In comparison with the sham treatment, exposure to solvent appeared to cause increased expression of p-TrkB, p-Akt, and p-ERK, but the margins were not statistically significant (**Figure 2**,  $P > 0.05$ ). By contrast, the administration of LM22A-4 significantly elevated the expression of the three

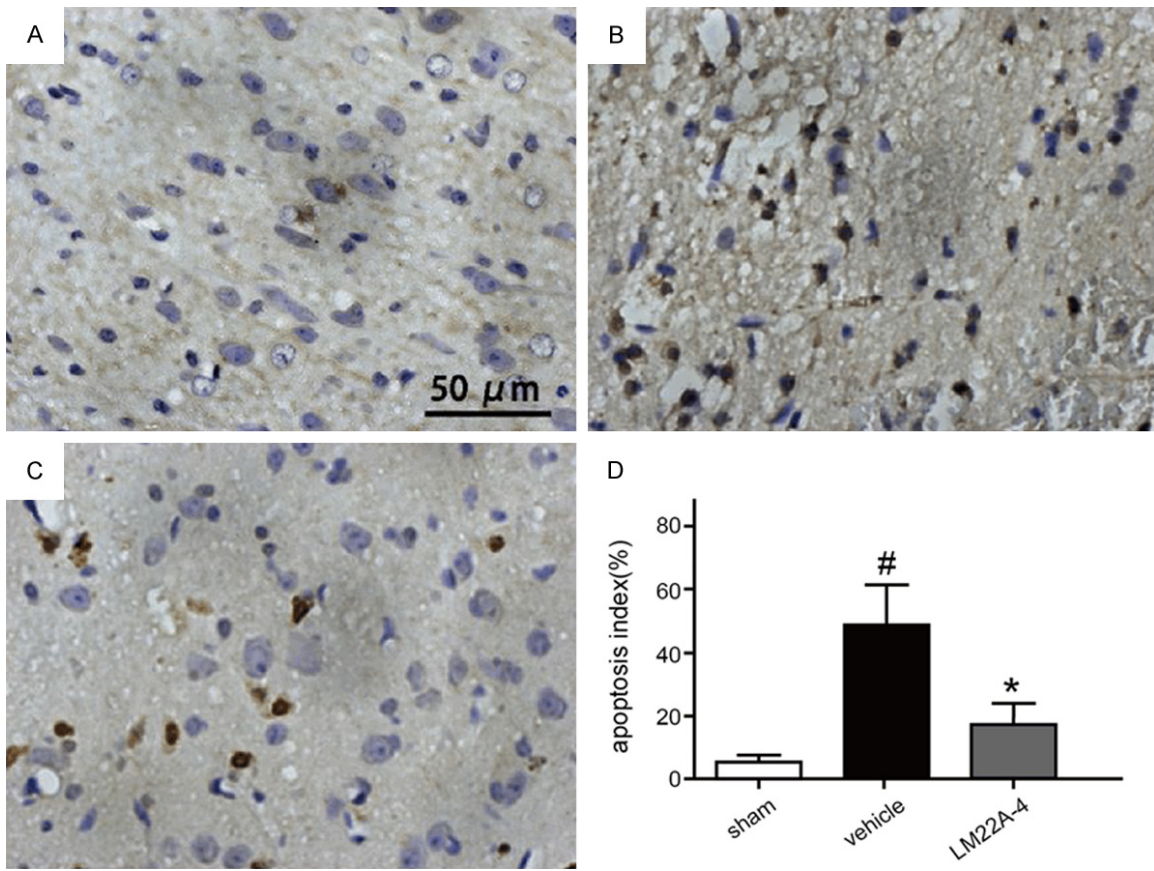
genes compared with those of the solvent-treated mice ( $P < 0.05$ ).

#### *LM22A-4 significantly inhibits the expression of the apoptosis-related protein cleaved-caspase-3 and augments the expression of the anti-apoptotic protein Bcl-2*

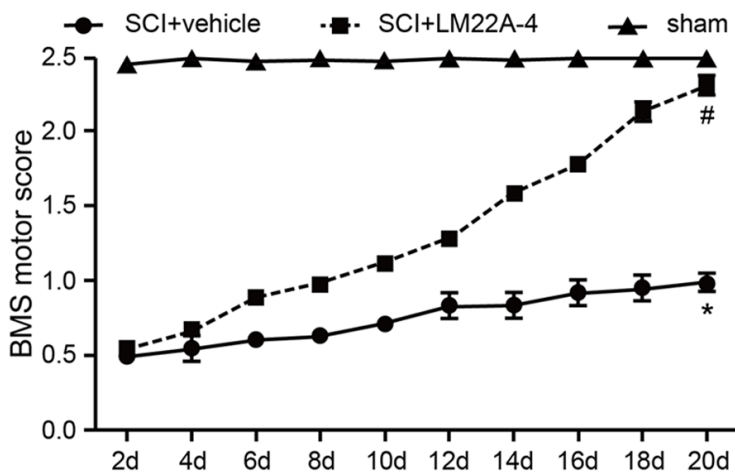
In comparison with the sham-treated animals, SCI treatment caused apparently increased expression of cleaved-caspase-3, which was suppressed by administration of LM22A-4 (10 mg/kg, **Figure 2**,  $P < 0.05$ ). In addition, as shown in **Figure 2**, SCI significantly reduced the expression of the anti-apoptotic protein Bcl-2 ( $P < 0.05$ ), which, however, was clearly increased by the LM22A-4 treatment (10 mg/kg,  $P < 0.05$ ).

#### *Morphological changes in the spinal cord tissue*

As shown in **Figure 3**, Nissl staining revealed that SCI-treated mice exhibited neuronal pyknosis and significantly decreased neuron num-



**Figure 4.** Influence of LM22A-4 treatment on SCI-resulted apoptosis. The apoptosis-positive neurons are revealed by brown nuclear staining. LM22A-4 administration (10 mg/kg) significantly decreased the number of apoptotic neurons in the SCI-treated mice ( $P < 0.05$ ). <sup>#</sup>  $P < 0.05$  compared with the sham-treatment group; <sup>\*</sup>  $P < 0.05$  compared with the vehicle-treatment group.



**Figure 5.** Influence of LM22A-4 administration on the neurological function in SCI-treated mice. Animals in the sham treatment group displayed no apparent change during the observation period. In comparison with the solvent treatment, LM22A-4 (10 mg/kg) administration significantly improved the neurological scores 20 days after the SCI treatment ( $P < 0.05$ ).

bers; whereas animals administered LM22A-4 (10 mg/kg) exhibited significantly increased neuron numbers. In addition, **Figure 4** illustrates the positive nuclear staining of the apoptotic neurons. In comparison with the sham-treated animals, SCI treatment caused a significant elevation in the number of apoptotic neurons ( $P < 0.05$ ), which was apparently decreased by the LM22A-4 administration (10 mg/Kg,  $P < 0.05$ ).

*LM22A-4 significantly ameliorated SCI neurological scores*

As shown in **Figure 5**, the neurological scores of the sham-treated animals did not exhibit

apparent changes during the observation period. By contrast, LM22A-4 administration (10 mg/kg,  $P < 0.05$ ) significantly improved the neurological scores compared with those of the solvent-treated animals ( $P < 0.05$ ).

### Discussion

Despite rapid economic development, spinal cord trauma still has a high incidence worldwide and yields considerable mortality and disability rates. Moreover, the survivors often exhibit severe dysfunctions. Hence, a focus and challenge of global research remains how to improve the prognosis of SCI patients. Although many drugs have been experimentally indicated to ameliorate the SCI prognosis, none has demonstrated reliable efficacy or been clinically applied to treat SCI patients [4]. Hence, it is of vital importance to develop novel and effective anti-SCI drugs that have few side effects. BDNF is a member of the family of endogenous neuroprotective factors. Previous studies have demonstrated that injection of BDNF can generate neuroprotective activities and effectively improve the prognosis of spinal cord trauma. However, it is difficult for BDNF to penetrate the blood-brain barrier, which is compounded by the molecule's short half-life [5]. These issues greatly restrict its clinical applications. BDNF mainly activates the TrkB signaling pathway to exert neuroprotection; therefore, the development of drugs that target TrkB, a BDNF ligand, is currently a research focus. LM22A-4 is a newly designed TrkB-specific agonist that has been demonstrated to play neuroprotective roles in multiple nervous system diseases. In agreement with previous results, our data revealed that LM22A-4 can significantly upregulate the expression of p-TrkB in a dose-dependent manner. Previous studies have indicated that TrkB activation can effectively reduce the expression of caspase-3, which is one of the most important apoptosis effectors and is overexpressed after spinal cord trauma to aggravate the severity of SCI [6]. Correspondingly, inhibition of caspase-3 expression can significantly improve the prognosis of SCI [7]. Our results indicated that LM22A-4 could markedly decrease the expression of apoptotic protein caspase-3 and the number of apoptotic neurons, thereby effectively improving the histological appearance of SCI. As such, our data revealed that LM22A-4 might play a prominent neuroprotective role in SCI.

In light of the above results, we are further exploring the potential neuroprotective mechanisms of LM22A-4, which might provide theoretical guidance for the clinical treatment of SCI. Many previous studies have indicated that TrkB activation may subsequently activate Akt, which is a serine/threonine protein kinase. Akt is composed of 480 amino acids, and its protein structure comprises a PH domain, a catalytic domain, and a C-tail regulatory domain. After its activation, Akt is first recruited to the cell membrane before translocation to the cytoplasm and nucleus, where it catalyzes serine/threonine phosphorylation at specific sites of its protein substrates. This step further upregulates expression of the apoptosis-inhibitive protein Bcl-2 and downregulates and degrades activated caspase-3. Many previous studies have indicated that Akt-mediated signaling pathways play crucial roles in SCI, as suppression of Akt activity markedly potentiates neuronal damage [2]. On the contrary, the upregulation of Akt activity can significantly improve the SCI prognosis [7]. Our study revealed that LM22A-4 significantly upregulated Akt activity, which is a possible mechanism of its neuroprotection. In addition, TrkB excitation can upregulate ERK activity. Consequently, the ERI signaling pathway is also activated during SCI and plays neuroprotective roles in spinal cord trauma. Tae et al. [8] has reported that the administration of estrogenic drugs to enhance ERK activity can generate neuroprotection, which is supported by our finding that LM22A-4 significantly augmented ERK activity. This function might be another neuroprotective mechanism of LM22A-4.

In summary, the TrkB-specific agonist LM22A-4 may play a prominent neuroprotective role in SCI. Because LM22A-4 can effectively penetrate the blood-brain barrier, it can be administered via the abdominal or venous route, which tremendously simplifies the administration procedure. Furthermore, LM22A-4 has a relatively long half-life and can therefore exert its effects over a long period, which would reduce the frequency of administration. Furthermore, we did not observe any apparent side effects of LM22A-4. Given these advantages, we speculate that LM22A-4 may be a novel drug for clinically treating SCI, but this possibility requires clinical trials for further verification.

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### Disclosure of conflict of interest

None.

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