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Original Article

Therapeutic effects of PTN and its effects on neurological function, morphology of spinal cord cells, and bax pathways in rats with spinal cord injuries

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Abstract: Objective: The aim of the current study was to explore the therapeutic effects of pleiotrophin (PTN), along with its effects on neurological function, morphology of spinal cord cells, and Bax pathways, in rats with spinal cord injuries. Methods: Selected rats were divided into the sham surgery group (SS group), model group (MO group), and treatment group (TR group), with 5 rats in each group. Rats in the SS group were not treated after the spinal incision. Rats in the MO group had spinal cord injuries. Rats in the TR group were given PTN based on spinal cord injuries. PTN, neurological function, motor function, Bax positive expression, Bax messenger ribonucleic acid (mRNA), cytopathic degree, and differences in morphological changes in spinal cord cells of the three groups were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR), neurological severity scores (NSS), Basso-Beattie-Bresnahan (BBB) locomotor rating scale, immunohistochemistry, hematein eosin (HE) staining, and electron microscopy. The aim was to explore the therapeutic effects of PTN on rats with spinal cord injuries, examining its effects on neurological function, morphology of spinal cord cells, and Bax pathways. Results: Compared with SS and TR groups, PTN expression in the spinal cord cells of the MO group significantly decreased ($P<0.05$). PTN in the TR group was significantly lower than that in the SS group ($P<0.05$). NSS scores in the MO group were significantly higher than those in the TR group ($P<0.05$). The BBB locomotor rating scale was significantly lower than that in the TR group ($P<0.05$). Bax positive expression and mRNA expression levels were the highest in spinal cord tissues of the MO group, while they showed the lowest expression levels in the SS group. Bax positive expression and mRNA expression levels were significantly higher in the TR group than those in the SS group ($P<0.05$). Spinal cord cells of the rats in the MO group were obvious abnormal. Massive hemorrhaging, mild edema, and loose structure in the gray matter were observed, while the number of neuronal necrosis increased significantly. The spinal edema of rats in the TR group was lightened and the injury degree began to weaken with decreased neuronal necrosis. The spinal cord cell nuclei of rats in the SS group were large and round with intact nuclear membrane and normal structure. Moreover, there were no gaps between the cells. Mitochondria of spinal cord cells in the MO group were significantly decreased with a large amount of layered edema appearing. The number of organelles significantly decreased. There was mild edema in spinal cord cells of the TR group, while the nuclear membrane and nucleoli were more normal. The number of organelles decreased. Conclusion: PTN showed significant therapeutic effects on rats with spinal cord injuries. These effects may dramatically improve neurological function, reduce spinal cord injuries, and inhibit neuronal apoptosis.

Keywords: Pleiotrophin, spinal cord injury, neurological function, spinal cord cell morphology, therapeutic effects

Introduction

Spinal cord injuries (SCI) are a common disease in trauma [1]. In recent years, incidence rates of SCI have risen yearly, having a huge impact on patients and causing serious economic burden on society [2]. There are two types of spinal cord injuries in clinic, primary and secondary injury. Primary injuries are caused by trauma, including various external shock, tears, and compression of the spinal cord, resulting in hemorrhaging, electrolyte damage, and other

symptoms. Secondary injuries are caused by spinal cord ischemia and calcium ion overflow, resulting from various causes or diseases. This leads to ischemia, degeneration, and necrosis of the spinal gray white matter [3, 4]. Secondary injuries can prevent spinal cord injuries by prevention and intervention, but primary injuries are inevitable. Comparing the two types, the former has a longer duration of attack and wider damage. Moreover, the degree of tissue damage is more serious [5]. However, primary injuries can gradually develop into secondary injuries, leading to the combination of the two injuries in the same patient. This not only increases the difficulty of treatment, but also reduces the success rate of healing [6]. PTN belongs to the developmental gene family, playing an important role in the biological process of cells [7]. During neural development, PTN holds a dominant position in proliferation, migration, and axon growth of nerve cells. Expression of this multi-functional nutrient after spinal cord injuries should be modified to significantly decrease. This stimulates the application of PTN in neural regeneration. Therefore, in this study, qRT-PCR, NSS scores, BBB locomotor rating scale, immunohistochemistry, HE staining, and electron microscopy were used to detect PTN content, neurological function, motor function, Bax positive expression, Bax mRNA expression, cytopathic degree, and differences of morphological changes in the spinal cord cells of the three groups. The above indexes were used to explore the therapeutic effects of PTN on spinal cord injury rats, examining the effects on neurological function, Bax, and morphology of spinal cord cells.

Materials and methods

General information

A total of 15 male Sprague-Dawley (SD) rats, weighing 231-289 g, aged 3-4 weeks old, were purchased from Guangdong Medical College Experimental Animal Center.

Model preparation and grouping

Selected rats were randomly divided into 3 groups, with 5 rats in each group. The first group was the MO group, with spinal cord injury rat models. The second group was the SS group. No treatment was performed to the rats after spinal incision. The third group was the TR group. Rat models with spinal cord injuries in this group were treated with PTN. The present study was approved by the Animal Ethics Committee of The Second Affiliated Hospital of Nantong University.

After fasting for 12 hours, the rats were anesthetized. The dorsal hair was removed and disinfected with alcohol with face down. After disinfection, the vertebrae were found in the lower edge of the ribs. The skin in the middle of the back was opened and 9-11 spines and laminae were then exposed. The laminae were removed and spinal cord was exposed. Rats in the MO TR groups were fixed on the foundation bed of the percussion device, then the corresponding parameters were adjusted.

The spinal cord was hit by a 20 g heavy weight from a height of 5 cm (injury energy was 100 gcm). An alarm sound should prevail after the bat hit the spinal cord. After causing spinal cord injury, the wound was routinely irrigated and sutured. Rats were maintained in normal life, food, and drinking, and their abdomens were massaged to assist urination and defecation after rat modeling was completed [8]. At the same time, rats in the TR group were given subcutaneous injections of PTN (Tengyun Kechuang Information Consulting (Shenzhen) Co., Ltd.) at a concentration of 80 μg/mL once a day. PTN was given at a dose of 80 μg/kg for 4 weeks [9]. The other two groups were injected subcutaneously with 0.2 mL normal saline. During this period, all rats lived in an equivalent environment. Cages were kept clean and hygienic. After 4 weeks of treatment, nerve and motor scoring experiments were performed. After the experiment, rats in the three groups were decapitated. The injured spinal cord tissues were taken for subsequent detection.

Detection of neurological function

NSS scores were used to detect neurological function, with a full score of 18 points. Scoring criteria included: 0 points (no injury); 1-6 points (mild); 7-12 points (moderate); 13-18 points (severe); 18 points (complete neurological function loss).

Detection of motor function

BBB scores were used to evaluate the motor function of rats [10]. Criteria: 0-7 points: Joints

could move gradually and the intensity of activities gradually strengthened; 8-13 points: Load-bearing movement could be conducted, but there was no coordinated movement in the fore and hind limbs; 14-21 points: Fore and hind limbs were gradually coordinated while moving, which kept the body balanced.

Detection of PTN in spinal cord cells by western blot analysis

A total of 50 μg of spinal cord tissue at the injury center was harvested. The electrophoresis tank and other components were washed and dried. Moreover, 12% separation gel was prepared with 1 cm water layer. The sample suspension was added into the well with 50 μg in each well. Bromophenol blue (Nantong Runfeng Petrochemical Co., Ltd.) was poured into the separation gel with 90 V electrophoresis. Electrophoresis was stopped after the voltage rose to 120 V. After electrophoresis was terminated, the gel was taken out. A piece of polyvinylidene fluoride (PVDF) membrane (Shanghai Fangang Plastic Materials Co., Ltd.) was incubated in methanol (Shandong Tonghua Chemical Co., Ltd.) for 5-10 seconds. Next, the PVDF membrane was incubated in transfer buffer for 15 minutes. The PVDF membrane was transferred into a transfer membrane tank within 10 minutes. Buffer was added to conduct an ice bath in 70 V for 1.5 hours. When the transfer was completed, the PVDF membrane was taken out from the membrane tank and washed twice with tris buffered saline tween (TBST), at 10 minutes per time. The PVDF membrane was then incubated in blocking buffer and rocked gently in a horizontal shaker for 2 hours.

The PVDF membrane was then packed into a self-made hybridization bag with primary antibody (Abcam, UK) incubation at 4°C overnight. The primary antibody was diluted with hybridization solution (glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 1:1,000). After the PVDF membrane was taken out, it was washed with 1xTBST 3 times, at 10 minutes per time. The TBST was poured off, then the membrane was placed with the corresponding secondary antibody (KPL, USA) and incubated at room temperature for 1.5 hours, with gentle shaking. After incubation, the membrane was washed 3 times with TBST, at 10 minutes per time. Solutions A and B of the electrochemiluminescence (ECL) developing solution (Shanghai Xitang Biotechnology Co., Ltd.) were mixed as the working solution. It was added at a ratio of 1:1 and incubated for 1 minute. Automatic chemiluminescence imaging analysis was used for PVDF membrane detection.

Detection of Bax positive expression in spinal cord tissues by immunohistochemistry

Conventional dewaxing of paraffin sections: Tissue chip was immersed in dimethylbenzene (Jinan Aohui Chemical Co., Ltd.) for 10 minutes. The process was repeated three more times. It was immersed in ethanol at a concentration of 100%, 90%, and 80% for 10 minutes, separately. The tissue chip was immersed in 100 mL solution, which was compounded with 0.01 mol/L citric acid buffer, 3mL water, and 10 μL Triton X-100 (Haian Petrochemical Plant, Jiangsu Province) for 20 minutes. It was then rinsed 3 times with 0.01 mol/L phosphate buffer solution (PBS), at 3 minutes per time. Afterward, it was put into 0.01 mol/L citric acid buffer (pH 6.0-6.4) to perform heat repair in 95°C water bath for 15 minutes. It was then cooled down to room temperature. The tissue chip was rinsed 3 times with 0.01 mol/L PBS, at 3 minutes per time, then blocked. The primary antibody (Abcam, UK) was added and the chip was incubated at 37°C for 30-40 minutes. It was rinsed 3 times with 0.01 mol/L PBS, at 3 minutes per time. The biotinylation secondary antibody (KPL, USA) was added and the chip was incubated at 37°C for 40 minutes. It was rinsed 3 times with PBS. The streptavidin (Shanghai Shifeng Biotechnology Co., Ltd.) was labeled by peroxidase at 37°C for 40 minutes. Color development was conducted with diaminobenzidine (DAB) until the positive cells and negative tissues had a clear contrast. The reaction was then stopped by washing with double-distilled water. The tissue chip was counterstained, differentiated, and blued by hematoxylin (Chengdu Biyang Biotechnology Co., Ltd.). Next, it was dewatered and dried with gradient ethanol (50%-60%-70%-80%-90% absolute ethanol, 5 minutes per time). The tissue chip was made transparent with dimethylbenzene twice, at 5 minutes per time. Finally, it was sealed with neutral balsam (Beijing Culaibo Technology Co., Ltd.).

Table 1. Primer sequences

Protein	Gene	Primer sequences
Bax	F	5'-AGAGGGGCCGAGTGGGAT-3'
	R	5'-TCAAAGAGGCCACAATCCTC-3'
GAPDH	F	5'-TGAACGGGAAGCTCACTGG-3'
	R	5'-TCCACCACCCTGTTGCTGTA-3'

Note: GAPDH for glyceraldehyde-3-phosphate dehydrogenase.

Detection of Bax mRNA expression in spinal cord tissues by qRT-PCR

The injured spinal cord tissue of the rats was homogenized and chloroform (Beijing Tiandi Shouhe Technology Development Co., Ltd.) was added. The tissue suspension was shaken and centrifuged to make it milky. The tissue suspension was centrifuged at 4°C at a speed of 1,200 r/min. Next, isopropanol (Jinan Jinghao Chemical Co., Ltd.) was added and centrifuged. Afterward, 75% ethanol was added and centrifuged. The issue sample was finally dried and stored at -80°C. Total RNA was extracted and reversely transcribed into complementary deoxyribonucleic acid (cDNA). The study was performed according to kit instructions. The internal reference was GAPDH. Conditions: Predegenerate at 96°C for 35 seconds, denature at 96°C for 10 seconds, and refold at 70°C for 25 seconds. The number of cycles would be subject to 40 for at least 3 times. Bax expression was calculated by relative quantitative $2^{-\Delta\Delta CT}$, see **Table 1**.

Detection of pathological changes of spinal cord cells by HE staining

Injured spinal cords in the three groups were fixed for 15 minutes and embedded in paraffin. Then they were cut into 5 μm slices and sealed with goat serum (Shanghai Yuanye Biotechnology Co., Ltd.). The slices were counterstained with hematoxylin for 6-8 minutes. Finally, the morphology of the spinal cord cells was observed.

Observation of morphological changes of spinal cord cells by electron microscopy

Injured spinal cords in the three groups were cryopreserved. They were then fixed with 2.5% glutaraldehyde fixative (Jinan Chenglian Chemical Co., Ltd.) and rinsed. The process was repeated two more times. Tissues were cut into slices after dehydration, replacement, and embedding. Slices were counterstained with uranyl acetate (Beijing Zhongjing Keyi Technology Co., Ltd.). Pathological changes of the spinal cord cells were then observed under an electron microscope.

Statistical analysis

Data was analyzed by SPSS 22.0 statistical analysis software. Single factor analysis of variance was used for comparisons of motor function, neurological function, PTN content, Bax positive expression, Bax mRNA expression, cytopathic degree, and differences in morphological changes in the spinal cord cells among the three groups. Bonferroni's t-tests were carried out for pairwise comparisons. $P<0.05$ indicates statistical significance.

Results

Detection of PTN in spinal cord cells by western blot analysis

PTN in spinal cord cells was detected by Western blot analysis. The gray scale showed that PTN in the three groups was 1.21±0.56, 0.68±0.22, and 0.23±0.08, respectively. Compared with SS and TR groups, expression of PTN in the MO group significantly decreased. Compared with the SS group, the TR group had a significantly lower PTN than the SS group (both $P<0.05$). See **Figure 1**.

Comparison of NSS scores in the three groups

NSS scores in the three groups were 0.00± 0.00, 15.69±4.57, and 8.24±2.13, respectively. NSS scores were the lowest in the SS group and highest in the MO group. NSS scores in the MO group were significantly higher than those in the TR group ($P<0.05$). See **Figure 2**.

Detection of motor function by BBB scores

Results showed that BBB scores in the three groups were 21.00±0.00, 2.14±0.38, and 14.68±3.84, respectively. BBB scores were the highest in the SS group and lowest in the MO group. BBB scores in the MO group were significantly lower than those in the TR group ($P< 0.05$). See **Figure 3**.

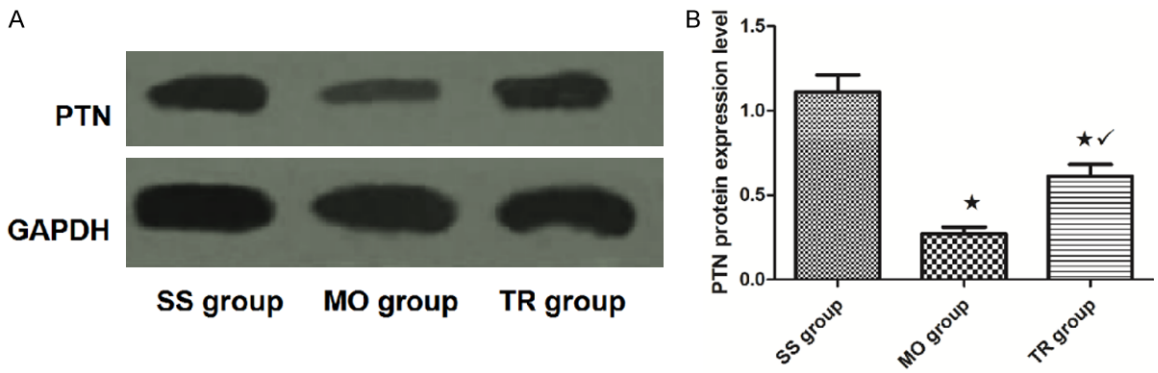

Figure 1. PTN in spinal cord cells of the three groups. Note: Compared with the SS group, ★P<0.05; Compared with the MO group, √P<0.05. A is for expression of PTN in spinal cord cells of the three groups; B is for the comparisons of PTN in the spinal cord cells of the three groups. PTN for pleiotrophin; GAPDH for glyceraldehyde-3-phosphate dehydrogenase; SS group for the sham surgery group; MO group for model group; TR group for the treatment group.

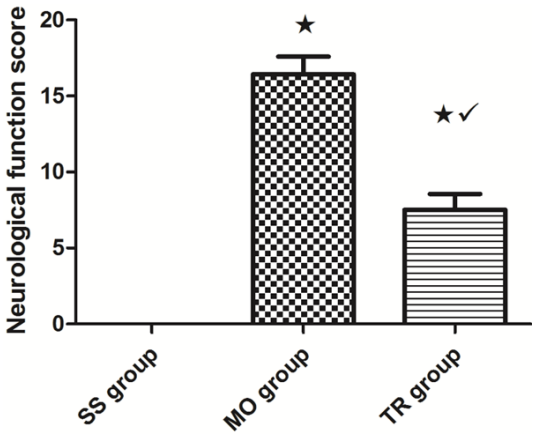

Figure 2. Comparison of NSS scores in the three groups. Note: Compared with the SS group, ★P<0.05; Compared with the MO group, √P<0.05. SS group for the sham surgery group; MO group for the model group; TR group for the treatment group.

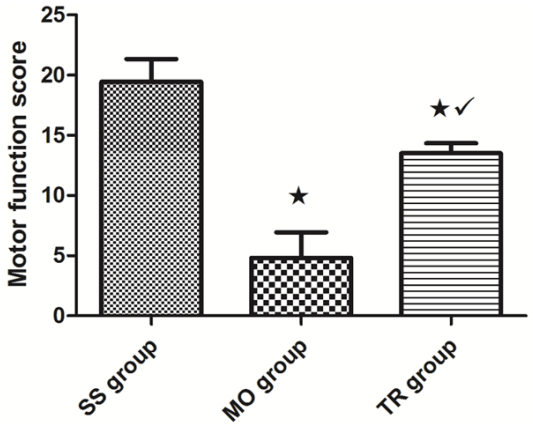

Figure 3. Comparisons of motor function in the three groups. Note: Compared with the SS group, ★P<0.05; Compared with the MO group, √P<0.05. SS group for the sham surgery group; MO group for the model group; TR group for the treatment group.

Detection of Bax positive expression in spinal cord tissues by immunohistochemistry

Results showed that Bax positive expression in the spinal cord tissues of the MO group was the highest and the staining was deeper. Expression was the lowest in the SS group and the staining was lighter. Bax positive expression levels in the MO group were significantly more than those in SS and TR groups. Bax positive expression in the TR group was significantly more than that in the SS group. See **Figure 4**.

Detection of Bax mRNA expression in spinal cord tissues by qRT-PCR

Results of qRT-PCR showed that Bax mRNA expression was the highest in spinal cord tissues of the MO group and lowest in the SS group. Bax mRNA content in the MO group was significantly higher than that in SS and TR groups and it was significantly higher in the TR group than that in the SS group (all P<0.05). See **Figure 5**.

Detection of pathological changes in spinal cord cells by H&E staining

Spinal cord cells in the SS group were normal without obvious changes. Proliferation of glial cells was noted. Spinal cord cells in the MO group had obvious abnormalities. Massive hemorrhaging, mild edema, and loose structure in the gray matter were observed. The number of neuronal necrosis increased significantly. Spinal edema of rats in the TR group

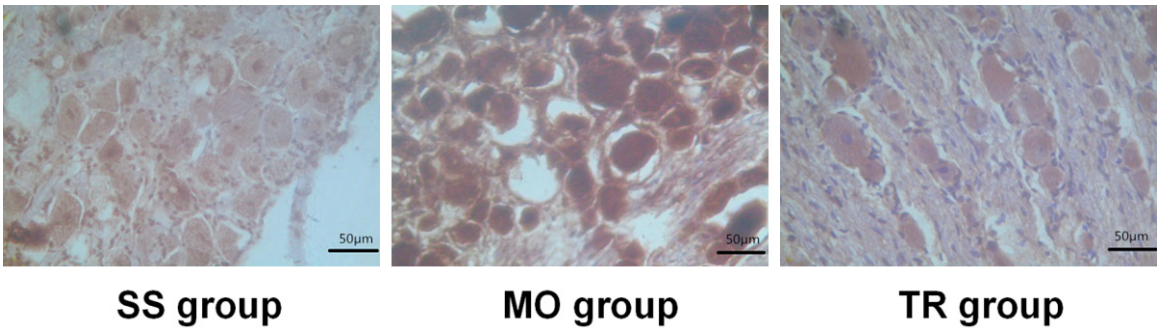

Figure 4. Bax positive expression in the spinal cord tissues of three groups (×400). Note: SS group for the sham surgery group; MO group for the model group; TR group for the treatment group.

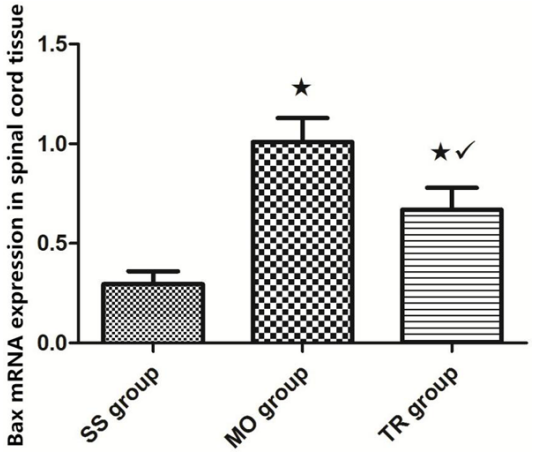

Figure 5. Bax mRNA expression in spinal cord tissues of the three groups. Note: Compared with the SS group, ★P<0.05; Compared with the MO group, √P<0.05. SS group for the sham surgery group; MO group for the model group; TR group for the treatment group; mRNA for messenger ribonucleic acid.

had lightened and the injury degree began to weaken with decreased neuronal necrosis. See **Figure 6**.

Observation of morphological changes in spinal cord cells by electron microscopy

Spinal cord cell nuclei of rats in the SS group were large and round with intact nuclear membrane and normal structure. There were no gaps between the cells. Mitochondria of the spinal cord cells in the MO group were significantly reduced with large amount of layered edema appearing. The number of organelles significantly decreased. There was mild edema in the spinal cord cells of the TR group, while the nuclear membrane and nucleoli were more normal. The number of organelles decreased. See **Figure 7**.

Discussion

Spinal cord injuries are serious complications of spinal injuries. There are many causes of SCI, including violent trauma, such as compression and tears or abnormal changes and diseases of body. Clinical manifestations of SCI include spinal cord concussion and shock. Spinal cord injuries vary with the location. For example, if the first and second spinal cords are injured, most of the injured patients die immediately. There is little hope for treatment [11]. SCI causes great harm to the body and carries a poor prognosis. Therefore, early treatment at the onset of disease plays an important role throughout the entire treatment. Patients can be treated with on-site rescue, emergency treatment, and other professional operations. In clinic, corticosteroids, gangliosides, and other drugs are often adopted to treat SCI [12].

PTN content in the MO group was significantly lower than that in the SS group and it was significantly higher in the TR group than that in the MO group. NSS scores were the lowest in the SS group and highest in the MO group. NSS scores and BBB scores in the MO group were significantly higher than those in the TR group. Bax positive expression and mRNA expression was the highest in the spinal cord tissues of the MO group, but the lowest in the SS group. Bax positive expression and mRNA expression in the TR group was significantly higher than that in the SS group. Previous studies have reported that, during neurodevelopment, PTN had the main position in the proliferation, migration, and axonal growth of nerve cells. Expression of this multi-nutrient after spinal cord injuries is increased significantly, stimulating the application of PTN in neural regeneration [13]. Studies

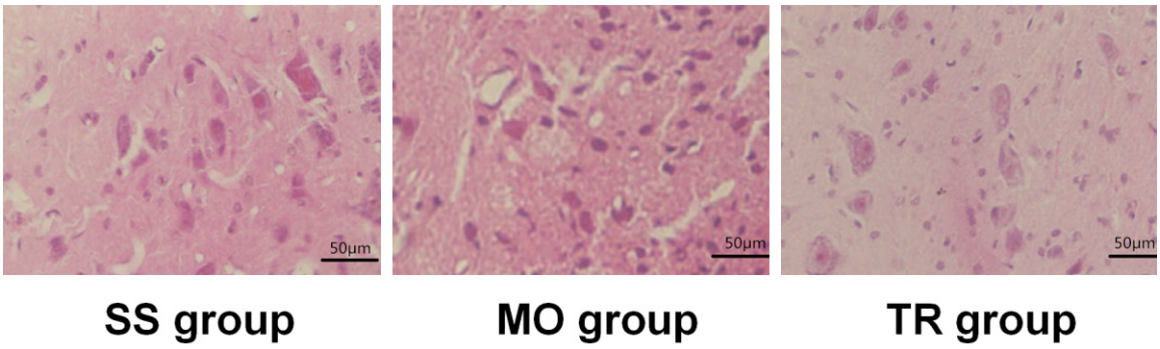

Figure 6. Pathological changes of spinal cord cells in the three groups (×400). Note: SS group for the sham surgery group; MO group for the model group; TR group for the treatment group.

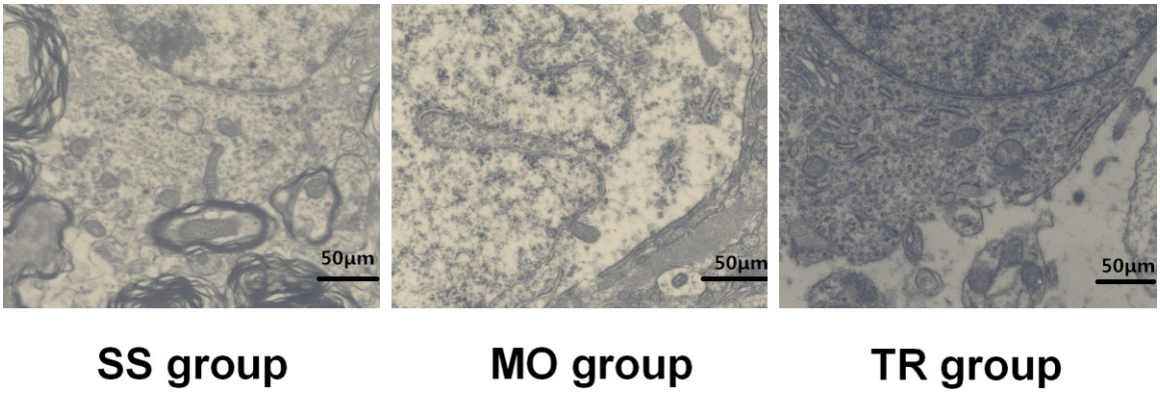

Figure 7. Morphological changes of spinal cord cells in the three groups (×400). Note: SS group for the sham surgery group; MO group for the model group; TR group for the treatment group.

on PTN have mainly focused on fundamental research at home and abroad. PTN has been shown to be related to a variety of neurofunctional problems in rats, which could affect pheochromocytoma cells, cortex, and neurite outgrowth of dopaminergic neurons. PTN may also mediate many cellular effects, including mitogenic origin, cell survival, oncogenic origin, inflammation, differentiation, and stem cell renewal [14, 15]. Results of animal experiments have shown that expression of PTN in rats with spinal cord injuries was significantly reduced. It was conjectured that PTN was also involved in the occurrence and development of neurological diseases, such as spinal cord injuries [16]. Studies have shown that PTN participates in the proliferation and apoptosis of a variety of cells in the body. PTN is highly expressed in healthy organisms and lowly expressed in diseased organisms, indicating that low expression can significantly promote apoptosis and reduce cells lifespan. Therefore, expression levels of PTN have an important effect on cell growth and apoptosis [17, 18]. It was found that when PTN acted on diseased rats, expression of PTN increased accordingly. With the extension of effect time, scores of motor function and neurological function had significantly risen, indicating that PTN provides certain therapeutic effects on rats with spinal cord injuries. Many studies have shown that PTN could inhibit Bax positive expression and mRNA expression in rats with spinal cord injuries, significantly improving morphological changes of spinal cord cells [19, 20]. The relationship between PTN and spinal cord injuries provides new research directions for the diagnosis and treatment of spinal cord injuries [21].

Present results are consistent with previous conclusions. There were also some limitations to the current study. Other neurological functions of rats were not tested due to limited time. In the future, more experimental methods should be added to investigate the effects of PTN on rats with spinal cord injuries, examining

the mechanisms of neurological function. The aim should be to provide a more favorable experimental basis for treatment of spinal cord injuries. In summary, PTN showed significant therapeutic effects on rats with spinal cord injuries. Thus, PTN may significantly improve neurological function, inhibit neuronal apoptosis, and reduce spinal cord injuries.

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Disclosure of conflict of interest

None.

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