The effect of lithium chloride on the motor function of spinal cord injury–controlled rat and the relevant mechanism

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Abstract

The objective of this study is to discuss the effect and mechanism of lithium chloride on the rehabilitation of locomotion post spinal cord injury (SCI) by observing the effect of lithium chloride on the expression of the brain-derived neurotrophic factor (BDNF)/tropomyosin receptor kinase B (TrkB) pathway. In total, 36 Sprague-Dawley (SD) rats were randomly divided into the sham operation group (n = 12), model group (n = 12), and lithium chloride group (n = 12). The sham operation group underwent laminectomy, while for the model group and the lithium chloride group with the NYU spinal cord impactor the SCI model was established. Basso, Beattie, and Bresnahan (BBB) score was used to evaluate locomotion after administration for 1, 3, 5, and 7 days, and the tissues were gathered for Nissl staining, transmission electron microscopy, immunofluorescence, and Western blot. With a statistical difference (P < 0.05) on the 3rd day and significant difference (P < 0.01) on the 5th day post administration, a higher BBB score was observed in the lithium chloride group indicating that lithium chloride improved the locomotion function after SCI. A better structure and morphology of neuron were observed by Nissl staining in the lithium chloride group. Lithium chloride promoted BDNF secretion from neurons in the spinal cord anterior horn with a significant difference compared to the model group (P < 0.01). Compared with the model group, lithium chloride significantly promoted the expression of BDNF protein and phosphorylated TrkB protein (P < 0.05), but no difference in the expression of TrkB was detected. Lithium chloride can alleviate the locomotion function after SCI with a mechanism that it can promote BDNF secretion from neurons in the spinal cord anterior horn and phosphorylation of TrkB to upregulate the BDNF/TrkB pathway supporting survival of neurons and regeneration and remyelination of axons.

Keywords

brain-derived neurotrophic factor (BDNF), lithium chloride, remyelination

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Introduction

Spinal cord injury (SCI) refers to spinal fracture or dislocation and can be divided into two groups: primary SCI and secondary SCI, in which very complicated pathophysiological reactions are involved, including a series of cascade responses, like the spinal cord edema, ischemia, ion imbalance, electrolyte disturbance, excitatory amino acid poisoning, inflammations, cellular necrosis, cellular apoptosis, and disturbance of the central nervous system. SCI can cause various complications, including motor function loss, numbness, pain, bladder and bowel dysfunction, sensory disturbances, and respiratory complications. The impact of SCI on social and economic aspects is significant, and it is a major public health concern. Several treatments have been developed to improve the quality of life of SCI patients, including rehabilitation therapy, surgical intervention, and pharmacological therapy. However, the efficacy of these treatments is limited, and the development of new treatment strategies is still required.

Lithium chloride, a drug that is widely used in psychiatry, has been found to have neuroprotective effects in SCI models. Lithium chloride can reduce the production of pro-inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1beta (IL-1β), and it can also reduce the expression of tissue necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) receptors. Lithium chloride can also promote the expression of brain-derived neurotrophic factor (BDNF), which is an important growth factor that promotes the survival, differentiation, and regeneration of neurons.

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and apoptosis finally evoking neural deficiency in the spinal cord and disruption of axonal connections. Generally, since SCI can lead to paraplegia in patients, or even death, it has been considered quite devastating. Nevertheless, there remains no ideal method for the treatment of SCI, which brings heavy burdens to patients, their families, and the society. Brain-derived neurotrophic factor (BDNF), as a member of the neurotrophic factor family, is considered as the richest neurotrophic factor in the body and widely spread inside the central nervous system. BDNF, through binding with the tyrosine receptor kinase B (TrkB) with a high affinity, can cause phosphorylation of TrkB, thus activating other intracellular signaling pathways to exert the functions. Previous studies have shown that, after SCI, the combination of BDNF and TrkB (BDNF/TrkB) can promote neuronal survival, axonal growth, and synaptic plasticity, thereby facilitating axonal repair after SCI and recovery of limb functions. Thus, BDNF/TrkB signaling expression has become a hotspot in research on the regeneration and repair of axons after SCI.

BDNF generally serves as a potential target for anti-depression drugs. Zhu et al. have shown that the expression of BDNF is downregulated in depression patients, and anti-depression effect can be observed after the BDNF is injected into the hippocampus in animals with depression. Lithium chloride, as an important anti-depression drug, has been utilized in clinical practice for a long time. In addition, it is also applied in the treatment of central nervous system diseases, such as Alzheimer’s disease, Huntington’s disease, and stroke.

Materials and methods

Experimental animals

A total of 36 healthy, adult, female SPF Sprague-Dawley (SD) rats with an average weight of 220 ± 20 g were purchased from Slac Laboratory Animal Co., Ltd (China; License No. SCXK (Shanghai) 2014-0006) and raised in Laboratory Animal Center of Shandong University. Procedures and methods of experiment were in accordance with the requirements of Animal Ethics Committee. According to the random digit table, 36 SD rats were divided into three groups: the sham group (n = 12), the control group (n = 12), and the lithium group (n = 12). Rats in the sham group only received laminectomy without damages to the spinal cord, while for those in other two groups the NYU impactor was utilized to damage the spinal cord to establish the SCI models.

Major reagents and apparatus

Lithium chloride (Abcam, USA); primary antibodies, namely, rabbit anti-rat BDNF antibody (Abcam), rabbit anti-TrkB antibody (Abcam), rabbit anti-rat p-TrkB antibody (Abcam), and rabbit anti-rat β-actin antibody (Abcam); secondary antibodies, namely, goat anti-rabbit Alexa Fluor 688 antibody (Thermo Fisher, USA) and goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fisher); DAPI (4′,6-diamidino-2-phenylindole) staining solution (Boster, China); Nissl staining solution (Solarbio, China); Triton X-100 (Sigma, USA); NYU impactor (WM Keck Center for Collaborative Neuroscience, USA), fluorescence microscope (DMI 4000B/DFC425C; Leica, Germany); and Image Lab and Image-Pro image analysis systems (Bio-Rad, USA) were used in this study.

Establishment of the SCI control model

According to the literature, we established the SCI models using the NYU impactor in the following procedures: rats first received the intraperitoneal injection of 7% chloral hydrate (5 mL/kg) for anesthesia; after regular disinfection, a longitudinal incision of 2–3 cm was made via sequentially excising the skin, subcutaneous fascia layer, muscle, and muscle tendon from T9 to T11; the bilateral vertebral plates of T10 were removed using a small bone rongeur to fully expose the T10 segment of the spinal cord; rats were then placed onto a fixed station of the NYU impactor, and the exposed T10 spinal cord segment was confused by an impactor from the height of 12.5 mm; retraction or flutter of hind legs and the spastic swing of tails in rats suggested that the models were successfully established; finally, the incision was rinsed using normal saline followed by suturing of the incision layer by layer. After the operation, each rat model was placed into a single cage for rearing, in which heat preservation measures were taken, and the bladder of the rat was massaged artificially twice per day for defecation until the capability of independent
defecation was recovered. After the operation, penicillin (40,000 U/day) was given to each rat for prophylaxis of postoperative infection.

**The intervention method**

On the first day after the operation, rats in the lithium group received intraperitoneal injection of lithium chloride at a dose of 3 mg/kg once a day, while for rats in the control and sham groups, gavage using the normal saline of the same volume was performed once a day.

**Ethological analysis**

Assessment for limb performance was carried out for individual rat every day before and after the operation using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale to evaluate the hindlimb performance in the following procedures: rats were placed in an open field to observe the limb activities of rat, including all joint motions and foot placement of hindlimb, coordination between the forelimb and hindlimb, trunk stability, and position of rat tail; and each observation lasted for 4 min. Scores were given according to the performance of the rat: 21 points—normal; 0 point—completely paralyzed. BBB assessment was carried out by two evaluators who were familiar with the BBB assessment rules but not the staff in this study. The average of scores given by two evaluators was taken as the final score of the rat.

**Sample preparation**

At 7 days after postoperative intervention, six rats in each group were utilized for collecting samples for the heart perfusion experiment. The injured spinal cord at a length of about 2 cm was excised with the injury as the center and then placed in 4% paraformaldehyde at 4°C for 24 h, followed by sequential dehydration, and, respectively, in 20% and 30% sucrose solution for 24 h. Paraffin section of 5 μm thickness was prepared via the conventional method. For the remaining six rats in each group, excision was performed with the injury site of the spinal cord as the center to obtain the spinal cord segment at a length of about 2 cm, which was then placed in an Eppendorf tube and rapidly transferred into liquid nitrogen for preservation at −80°C.

**Detection methods**

**Nissl staining**

After being dehydrated in the sucrose solution, the spinal cord was taken out for repair, dehydration, transparency, waxing, embedding, and paraffin sectioning, followed by incubation at 37°C overnight. On the next day, dewaxing and hydration were performed, and the sections were placed into a box containing the staining solution. The staining box was then placed into an incubator for staining at 60°C for 50 min, and the sections were rapidly rinsed three times using distilled water. Then the sections were placed in the differentiating fluid for 1 or 2 min and dried and sealed using neutral balsam after the background of vision under the microscope was well adjusted. The sections were then observed under the microscope and the pictures were recorded using Leica DM4000B.

**Immunofluorescence staining**

Paraffin sections, after being dewaxed, hydrated, rinsed in 0.01 mol/L phosphate buffered saline (PBS), and repaired under high pressure using citrate buffer, were cooled to room temperature and then washed using PBS again. Then, the samples were placed in 0.3% Triton X-100 solution at room temperature for rupture of membranes for 10 min and washed with PBS; blocking serum was added into the samples followed by incubation at 4°C overnight. On the next day, the samples were again placed in an oven for incubation at 37°C for 1 h. After the liquid on the surface of tissues was removed, rabbit anti-BDNF primary antibody (1:200) was added into the samples for incubation at 4°C overnight. On the next day, the samples were again placed in an oven for incubation at 37°C for 30 min and washed using PBS. In a dark place, Alexa Fluor 688 (1:200) was added for incubation in an oven at 37°C for 1 h and then the samples were washed again using PBS. DAPI staining solution was added for staining for 3 min at room temperature. Finally, after the section was rinsed using PBS and the liquid spread on the surface of the section was removed, the section was blocked using the mounting medium and placed under the microscope for observation. Images were collected using Leica DMI 4000B/DFC425C and the analysis was carried out using the Image-Pro analytic system.
**Western blot**

To 100 mg of spinal cord tissues, 1 mL cell lysate and 10 μL phenylmethanesulfonyl fluoride (PMSF) were added. Tissue samples, after being sufficiently ground, were centrifuged at 12,000 r/min for 5 min with the sediment being discarded. In the supernatant, protein concentration was detected via the Coomassie brilliant blue method. Spinal cord tissues were heated at 100°C for 5 min for complete degeneration of protein, and 50 μg sample protein was taken from each tissue for loading sample to perform 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis). The membrane was then transferred onto the polyvinylidene fluoride (PVDF) membrane and blocked using 5% skimmed milk for 2 h. Thereafter, primary antibodies of BDNF (1:1000), TrkB (1:1500), p-TrkB (1:1000), and β-actin (1:1000) were, respectively, added onto the membrane for incubation at 4°C overnight. After the membrane was washed using TBST (tris-buffered saline with Tween 20) three times, fluorescence-labeled secondary antibody (1:2000) was added for incubation at room temperature for 1 h. In a dark place, the substrate was prepared and covered with the PVDF membrane, and after 1-min reaction with the membrane, the color development was performed. The Image-Lab software was employed for the calculation of optical density of protein stripes, and data analysis and processing. With β-actin as an internal reference, we calculated the ratios of BDNF and TrkB to it and the ratio of p-TrkB to TrkB.

**Statistical analysis**

Statistical analysis was performed using SPSS 19.0 software, and data were presented as mean ± standard deviation. Since scores of the BBB locomotor rating scale did not accord with the normal distribution, we adopted the non-parametric test for differences among the groups, while, for the remaining data in accordance with the normal distribution, we adopted the one-way analysis of variance (ANOVA). Tukey’s honestly significant difference (HSD) test is used in conjunction with an ANOVA to find means that are significantly different from each other. P < 0.05 was considered statistically significant.

**Ethics committee approval**

This study was approved by the institutional ethical review board of Taizhou Municipal Hospital, Taizhou, Zhejiang, China. All the experiments were conducted as per National Institutes of Health (NIH) principles of laboratory animals (Reference No. 1034/ERB/TMH/2017).

**Results**

**Scores of the BBB locomotor rating scale**

Compared with the sham group, the scores of the BBB locomotor rating scale in the lithium chloride group and the control group were significantly decreased; on the first day after the operation, we found no statistically significant difference in the comparison of the scores of the BBB locomotor rating scale between the two groups (P > 0.05); with extension in the time of treatment, gradual increases were identified in the scores of the BBB locomotor rating scale in the lithium chloride group and the control group, and ever since the 3rd day after the operation, the score of the BBB locomotor rating scale in the lithium chloride group was superior to that in the control group with a statistically significant difference (P < 0.05); on the 5th day after the operation, a significant elevation was observed in the score of the BBB locomotor rating scale in the lithium chloride group, which was significantly (P < 0.01) different from that of the control group as shown in Figure 1.

**Nissl staining**

In the sham group, gray matter of the spinal cord was in the shape of butterfly, the structure was integral, and the boundary between the gray and white matters was visible; in the cytoplasm of neurons, we could identify the plaque or macular Nissl substance.
and, inside the large nuclear, the nucleolus was visible, but there was no gliocyte proliferation or glial scar; in the control group, the difference was statistically significant ($P < 0.01$). We found that the structure of the spinal cord was severely destructed, and neurons were in vesicular shape due to shrinkage, necrosis, dissolution, and liquidation; in the lithium chloride group, cellular swelling was relatively slight, most neurons maintained better shape, and vesicular changes were scarcely seen, but in the recovery of injured neurons we found that the nuclei of some neurons were swollen without the nucleolus as shown in Figure 2.

**Detection of BDNF immunofluorescence staining**

BDNFs with a positive response were indicated in red and with an oval or spindle shape, and mainly distributed in the cytoplasm of cells in the anterior horn of the spinal cord of the rat. After 7 days of intervention, fewer BDNFs with a positive response were identified in the sham group, and the expression of BDNF was also decreased; compared with the sham group, the quantities of BDNFs with a positive response were higher in the control group and the lithium chloride group, and the expression of BDNF was also increased significantly with a statistically significant difference ($P < 0.01$); in comparison with the control group, we found that the quantity of BDNFs with a positive response in the lithium chloride group was dramatically increased, and the difference was statistically significant ($P < 0.01$) as shown in Figure 3 and Table 1.

**Detection of the protein expressions of BDNF, TrkB, and p-TrkB via Western blot**

According to the results of Western blot detection, we found that, after 7 days of intervention, the protein expression of BDNF in the sham group was downregulated; in comparison with the sham group, the protein expression of BDNF in the control group was increased ($P < 0.05$), and a significant increase was identified in the protein expression of BDNF in the lithium group ($P < 0.01$), and the differences were statistically significant; compared with the control group, we found that the protein

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**Figure 2.** Nissl staining of neuron morphology.

**Figure 3.** Immunofluorescence for BDNF-positive cells (200×).
expression of BDNF was much higher in the lithium chloride group with a statistically significant difference \( (P < 0.05) \). After 7 days of intervention, the differences in protein expressions among the three groups disappeared \( (P > 0.05) \); however, the protein expression of p-TrkB in the sham group was relatively low, which was significantly different from those in the control and lithium chloride groups \( (P < 0.01) \); in comparison with the control group, a dramatic increase was found in the protein expression of p-TrkB in the lithium chloride group with a statistically significant difference \( (P < 0.05) \) as shown in Table 2.

### Discussion

A series of pathological reactions after SCI can generate a large quantity of harmful substances in the injury region, including the inflammatory factors, inhibitory proteins of axonal regeneration, and glial scars.\(^9\) The results of this study showed that lithium chloride can promote the recovery of locomotor functions of SCI-controlled rats; ever since the 3rd day after intervention, lithium chloride can improve the locomotor function of limbs. The observations of Nissl staining sections under the microscope revealed that lithium chloride has a protective effect on the neurons and axonal myelin sheath, which is conducive to the survival and regeneration of neurons and regeneration and remyelination of axons. Immunofluorescence results indicated that lithium chloride can augment the quantity of BDNF-positive cells after SCI, and those cells are mainly distributed in the anterior horn of gray matter in the spinal cord, suggesting that lithium chloride can facilitate the secretion of BDNF in the motor neurons in the anterior horn of gray matter in the spinal cord, thus improving the microenvironment in the injury region and promoting the survival and regeneration of motor neurons in the anterior horn of the spinal cord. In addition, Western blot detection showed that lithium chloride can increase the protein expressions of BDNF and p-TrkB after SCI, indicating that it can upregulate the signal expression of BDNF/TrkB. As a key member of the neurotrophic factor family, BDNF can exert its functions via binding with its specific receptor, TrkB, which is very important to the survival, proliferation, and synaptic plasticity of neurons\(^10\) and can promote the axonal regeneration and repair, and recovery of the locomotor function of limbs after the injuries of the central nervous system. Based on the regulatory effect of the BDNF/TrkB signaling pathway on the nervous system, the upregulated signal expression of BDNF/TrkB can relieve the apoptosis and necrosis of neurons, axonotmesis, and axonal demyelination caused by SCI, thus enhancing the efficacy on the treatment of SCI. Therefore, the BDNF/TrkB signaling pathway has become a new target in the treatment of SCI. After SCI, a rapid increase would first be detected in the expression of BDNF in the injury region, and a peak level can be attained on the 7th day after SCI. Not only can BDNF facilitate the regeneration of neurons in the injury region, but it can also protect the distal neurons of the injury region, thereby promoting axonal growth, regeneration, and remodeling.\(^11,12\) Lithium chloride might boost the secretion of BDNF in motor neurons of the anterior horn in the spinal cord after SCI to activate the BDNF/TrkB signaling pathway, and the relevant downstream signaling pathways, thereby promoting the survival and regeneration of neurons as well as axonal regeneration and remyelination to advance recoveries in locomotor functions of hindlimbs after SCI. Nevertheless, further studies are still required to investigate the roles of lithium chloride in the activation of relevant signaling pathways through BDNF/TrkB signals and the relevant mechanisms.

### Table 1. The number of BDNF-positive cells \((x \pm s)\).

| Group   | N  | Positive cell number |
|---------|----|----------------------|
| Sham    | 6  | 12.13 ± 5.33         |
| Control | 6  | 23.00 ± 5.40*        |
| Lithium | 6  | 35.50 ± 8.21*#       |

BDNF: brain-derived neurotrophic factor. Compared with sham, *\(P < 0.01\); compared with control, #\(P < 0.01\).

### Table 2. The expression of related proteins.

| Group | BDNF       | TrkB       | p-TrkB     |
|-------|------------|------------|------------|
| Sham  | 0.624 ± 0.057 | 0.456 ± 0.089 | 0.468 ± 0.093 |
| Control | 0.708 ± 0.075* | 0.475 ± 0.107 | 0.603 ± 0.059* |
| Lithium | 0.795 ± 0.100* | 0.481 ± 0.112 | 0.676 ± 0.071*# |

BDNF: brain-derived neurotrophic factor; TrkB: tropomyosin receptor kinase B. Compared with sham, *\(P < 0.05\), #\(P < 0.01\); compared with control, Δ\(P < 0.05\).
Declaration of conflicting interests
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