● **RESEARCH ARTICLE**

Exercise promotes motor functional recovery in rats with corticospinal tract injury: anti-apoptosis mechanism

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Abstract

Studies have shown that exercise interventions can improve functional recovery after spinal cord injury, but the mechanism of action remains unclear. To investigate the mechanism, we established a unilateral corticospinal tract injury model in rats by pyramidotomy, and used a single pellet reaching task and horizontal ladder walking task as exercise interventions postoperatively. Functional recovery of forelimbs and forepaws in the rat models was noticeably enhanced after the exercises. Furthermore, TUNEL staining revealed significantly fewer apoptotic cells in the spinal cord of exercised rats, and western blot analysis showed that spinal cord expression of the apoptosis-related protein caspase-3 was significantly lower, and the expression of Bcl-2 was significantly higher, while the expression of Bax was not signifiantly changed after exercise, compared with the non-exercised group. Expression of these proteins decreased with time after injury, towards the levels observed in sham-operated rats, however at 4 weeks postoperatively, caspase-3 expression remained significantly greater than in sham-operated rats. The present findings indicate that a reduction in apoptosis is one of the mechanisms underlying the improvement of functional recovery by exercise interventions after corticospinal tract injury.

Key Words: nerve regeneration; spinal cord injury; corticospinal tract; exercise; functional recovery; apoptosis; Bcl-2; Bax; caspase-3; NSFC grants; neural regeneration

Introduction

The corticospinal tract is a descending motor pathway in the anterior horn of the spinal cord, which controls voluntary movement of the body by direct and indirect synaptic transmission. Injury to the corticospinal tract is the leading cause of functional loss of voluntary movement (Carmel and Martin, 2014; Darian-Smith et al., 2014; Yeo et al., 2014) and can be devastating to patients and a burden to society (Zompa et al., 1997; Kent and Dorstyn, 2014; Singh et al., 2014). As such, repair of corticospinal tract injury remains a significant unsolved problem in the field of neuroscience.

Functional rehabilitation training is considered an important method by which to promote the functional recovery of limbs and improve survival rate and quality of life for patients (Dornbos and Ding, 2012; Hung et al., 2013; Tahamtan et al., 2013; Chang et al., 2014; Egan et al., 2014; Gokbel et al., 2014; Wong et al., 2014). Several studies have focused on the effect of exercise interventions on neuroplasticity after central nervous system damage; for example, strengthening exercises can promote functional recovery after spinal cord injury (Engesser-Cesar et al., 2005) or stroke (Komitova et al., 2005) and can restore motor function after brain injury more effec-

tively when combined with stimulation and sensory exercises (Maegele et al., 2005). Others have shown that functional recovery after central nervous system injury requires specific exercise interventions such as grasp-strengthening training other than usual horizontal ladder walking, which could realize the recovery of fetch function (Girgis et al., 2007). Girgis and colleagues also found that exercise had an important influence on the regulation and guidance of axonal growth and extension after spinal cord injury (Girgis et al., 2007; Maier et al., 2008). Furthermore, in animal models, exercise effectively restored motor function by promoting axonal sprouting from proximal lesions in the corticospinal tract and supporting the repair of brain regions receiving input from the cerebral cortex. However, the mechanisms underlying these effects of exercise remain unclear. Proposed mechanisms include the elevation of nerve growth factor expression, and the reconstruction of motor function-related neural networks in the spinal cord (Griesbach et al., 2004).

Previous studies demonstrated that spinal cord injury causes death of cortical neurons in the rat brain in a time-dependent manner, suggesting that such damage may induce apoptosis in cortical motor neurons (Lee et al., 2004;

Nishimura and Isa, 2012). Neuronal survival is the foundation of structural and functional recovery after spinal cord injury (Ward et al., 2014), and one explanation for the failure of motor function to recover fully after damage to the spinal cord is that neurons in corresponding nerve bundles may be altered as a result of the injury (Zhang and Bai, 2013).

The aim of the present study was to determine whether exercise improves functional recovery from spinal cord injury by influencing the neuronal targets of the denervated zone. We explored the mechanism underlying the restorative effect of exercise on corticospinal tract injury, and performed functional behavioral assessment after corticospinal tract injury in rats.

Materials and Methods

Experimental animals
Seventy-two adult male clean-grade Sprague-Dawley rats, weighing 220–250 kg, were provided by the Experimental Animal Center of Jilin University, China (license No. SCXK (Ji) 2008-0005). All animals were housed in individual cages at 20°C and with a relative humidity of 40%, under a 12-hour light/dark cycle, and allowed free access to food and water. All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), and the protocol was approved by the Institutional Animal Care Committee from the China-Japan Union Hospital of China. Rats were randomly divided into three groups: sham ($n = 8$), model ($n = 32$) and trained ($n = 32$).

Establishment of unilateral corticospinal tract injury models
Three weeks before spinal cord surgery, animals in the model and trained groups were deeply anesthetized with 10% chloral hydrate (0.3 mg/kg) and injected with a 10% solution of biotinylated dextran amine (BDA; Vector, North Hollywood, CA, USA), as described previously (Maier et al., 2008). Each animal received eight intracortical injections of 0.4 μL BDA tracer (total injection volume, 3.2 μL). The syringe remained in place for 3 minutes after completion of each injection. On the day of spinal cord surgery, all animals were anesthetized with 10% chloral hydrate (0.3 mg/kg) and fixed in the supine position on the laboratory bench. An anterior incision was made in the skin of the neck, anterior muscles were separated and the trachea and esophagus were pulled to the right to expose the occipital bone. The base of the occipital bone was removed using a burr drill to expose the vertebral body. A small hook was inserted into the vertebral body to a depth of 0.1 cm, then the left side of the pyramidal tract was cut (McKenna et al., 2000). In sham-operated rats, the vertebral body was exposed without damage to the pyramidal tract. Hemostatic sutures were used to close the incision and an adjustable heating pad was placed under the rat. The rats were housed at 22°C in individual cages with good ventilation.

Exercise intervention
Single pellet reaching task
Rats were placed in a test box made according to the design of Girgis et al. (2007). Preoperative training began 2 weeks before surgery, with ten sessions per day, conducted 6 days per week. Each session lasted 10 seconds, followed by 10 seconds of rest. The training was considered successful when all rats could retrieve and eat the food pellet. Preoperative training continued until the success rate reached 65% or more, calculated as the number of successful grasps/total number of grasping attempts × 100%. Postoperative training began 3 days after surgery, with one session every other day. The animal's movements were observed and evaluated according to the methods of Metz and Whishaw (2000), and behavior was scored as follows: 0, no movement; 0.5, abnormal movement; 1, normal movement. The final score for each rat was a mean of three measurements (**Figure 1A**).

Horizontal ladder walking task
A horizontal ladder was made as described by Girgis et al. (2007). Rats performed the task 2 weeks before surgery and from 3 days after surgery, with three training sessions per day, every other day. Gait was classified as normal when all four digits of the forepaw gripped the surface of the metal rod. The following behavior was classed as incorrect: (1) One or more digits not grasping the bars; (2) wrist or forelimb used instead of paw to provide physical support; (3) inability to grasp, leading to the forelimb falling between bars (Maier et al., 2008). Success rate in the task was calculated as follows: success rate (%) = number of normal steps/total number of steps × 100%. Each rat was scored three times and the average of the three scores was recorded (**Figure 1B–E**).

DAB staining
At 1, 2, 3 and 4 weeks postoperatively, rats in the model and trained groups were injected intraperitoneally with 10% chloral hydrate (0.3 mg/kg), and C_5–T_1 spinal cord tissue was harvested. There were four rats in each group at each time point. Tissue was harvested from all rats in the sham group at 4 weeks postoperatively, using the same procedure. The tissue was fixed in 4% paraformaldehyde overnight at 4°C and then transferred to 0.1 M phosphate buffer containing 30% sucrose for 5 days. Frozen sections, 40 μm thick, were cut and rinsed with TBS-TX solution (50 mM Tris, 0.9% NaCl, 0.5% Triton X-100, pH 8.0) three times for 10 minutes each time, then sections were incubated in avidin-biotin-peroxidase complex (1:100 in TBS-TX; Vector) overnight at 4°C. After another three 10-minute washes with TBS-TX, sections were incubated with 50 mM Tris-HCl, pH 8.0, 0.004% H_2O_2, 0.4% nickel ammonium sulfate solution and 0.015% DAB solution (Vector) for 5 minutes, and the reaction was terminated with 50 mM Tris-HCl solution. The sections were dried, mounted and observed under a light microscope (BH-2; Olympus, Tokyo, Japan).

TUNEL staining
The remaining rats in the model and trained groups were injected intraperitoneally with 10% chloral hydrate (0.3 mg/kg) at 1, 2, 3, and 4 weeks postoperatively, and $C_{3–6}$ spinal cord tissue was harvested. There were four rats in each group at each time point. Spinal cord was harvested 1 week after surgery for all rats in the sham group. Tissue was fixed in

4% paraformaldehyde, embedded in paraffin, and cut into 5 μm thick sections for staining using a TUNEL kit (Roche, Madison, WI, USA), according to the manufacturer's instructions. A DNase I degraded sample was used as a positive control, and PBS was added instead of the primary antibody for a negative control. Slides were viewed at high magnification (400×) under a light microscope (BH-2; Olympus). Five fields of vision were selected at random to calculate the number of positive cells per unit area (1 mm^2).

Western blot analysis

The undamaged C_6–T_1 spinal cord tissues harvested for TUNEL staining at the same time points were used for western blot analysis. Tissue was washed with freezing saline and frozen in liquid nitrogen. Samples were extracted and protein content was determined using the Bradford method (Yamauchi et al., 2007). The samples were boiled with 2.5% sodium dodecyl sulfate and 5% β-mercaptoethanol, and electrophoresed in 10% polyacrylamide for 90 minutes at 20 mA. The proteins were transferred onto a polyvinylidene fluoride membrane (LC2002; Invitrogen Life Technologies, Carlsbad, CA, USA) using transmembrane buffer (3 g Tris, 14.4 g glycine, 200 mL methanol, adding ddH$_2$O to 1,000 mL) and 10% methanol. The membrane was incubated with rabbit anti-Bcl-2, anti-Bax, anti-caspase-3, and anti-β-actin polyclonal antibodies (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature, and washed with PBST. It was then incubated with goat anti-rabbit IgG (1:1,000; Pierce, Rockford, IL, USA) for 90 minutes at room temperature. After the second antibody was discarded, the membrane was rinsed three times with PBST, and DAB color-substrate solution (5 mg DAB powder (Vector), 10 mL distilled water, and 8–10 μL H$_2$O$_2$) was added until the target band was visible. The membrane was scanned and the optical density of the stained protein bands was measured using a gel imaging analysis system (Shanghai Day Technology Co., Ltd., Shanghai, China), and the optical density ratio of target protein to β-actin was calculated.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Measurement data were expressed as the mean ± SD, and differences between groups were analyzed using one-way analysis of variance and Tukey-Kramer *post-hoc* test. $P < 0.05$ indicated statistical significance.

Results

Validation of the unilateral corticospinal tract injury model

After BDA tracer injection and DAB staining, corticospinal tract neurons were clearly labeled on both sides of the spinal cord in rats in the sham group. In the model and trained groups, integral corticospinal tract neurons were observed rostral and contralateral to the injury site. However, the ipsilateral side of the tract was visibly lesioned at the site of the injury. Furthermore, caudal to the injury site, no corticospinal tract neurons were labeled by the BDA tracer on the ipsilateral side. These results demonstrate the success of our experimental animal models of unilateral corticospinal tract injury (**Figure 2**).

Exercise intervention improved motor function in rats with corticospinal tract injury

Scores in the single pellet reaching task and success rate in the horizontal ladder test were significantly lower in rats in the model and trained groups than in the sham group ($P < 0.05$). Compared with the model group, rats in the trained group had significantly higher scores and horizontal ladder success rates ($P < 0.05$; **Figure 3**).

Exercise interventions reduced apoptosis in spinal cord tissue of rats with corticospinal tract injury

TUNEL-stained apoptotic cells were rarely visible in the sham group. One week after surgery, there were significantly more TUNEL-positive cells in the model rats than in the sham-operated rats ($P < 0.01$). The number of apoptotic cells decreased over time, but remained higher than that in the sham group until at least 4 weeks after injury ($P < 0.05$). From 1 week postoperatively, in trained rats there were significantly fewer TUNEL-positive cells than in model rats ($P < 0.05$). The number again decreased over time, but remained lower than that in the model group until at least 4 weeks postoperatively ($P < 0.05$; **Figure 4**).

Effect of exercise interventions on Bcl-2, Bax and caspase-3 expression in spinal cord tissue of rats with corticospinal tract injury

Western blot analysis showed that Bcl-2, Bax and caspase-3 were poorly expressed in the spinal cord tissue of rats in the sham group. In the model and trained groups, expression of all three proteins was significantly greater than in the sham group from 1 week after surgery ($P < 0.01$); caspase-3 expression remained greater than the sham group, while expression of the other two proteins was not different from the sham group at 4 weeks ($P < 0.05$). Bcl-2 expression in the trained group was greater than that in the model group, but diminished with time ($P < 0.01$ at 1 and 2 weeks; $P < 0.05$ at 3 weeks) and was not significantly different from the model group by 4 weeks ($P > 0.05$). Bax expression in trained rats was not significantly different from that in the model group at any time point ($P > 0.05$) and caspase-3 expression in the spinal cord tissue of rats in the trained group was significantly lower than that in the model group 1 week after surgery ($P < 0.01$) and decreased over time (**Figure 5**).

Discussion

Several studies have found that the neurons and axons remaining after spinal cord injury exhibit functional plasticity (Jones and Schallert, 1994; Raineteau and Schwab, 2001; Bareyre et al., 2004; Celnik and Cohen, 2004; Ballermann and Fouad, 2006; Darian-Smith, 2009). They play a role in the reconstruction of neural circuits and compensatory limb function *via* alterations in plasticity, a critical process in the repair of spinal cord injury. This plasticity can be promoted by outside intervention. The key to rebuilding neural circuits is that target neurons remain active until newborn axons

connect with them (Seki et al., 2002; Kwon et al., 2005). In the present study, we investigated whether exercise protects target neurons when innervation is lost through injury.

The molecular mechanisms underlying the induction of apoptosis and regulation of cell death have become a hot topic in recent research (Beesoo et al., 2014; Childs et al., 2014; Greenberg et al., 2014; Saeed and Jun, 2014; Temajo and Howard, 2014). Bax and Bcl-2 are members of the Bcl-2 gene family (Seki et al., 2003). Bcl-2 family proteins can inhibit or promote apoptosis and interact with proteins involved in determining cell survival or death (Willis et al., 2003). Bcl-2 and Bax are mutually antagonistic, and together play an important role in the regulation of apoptosis (Belka and Budach, 2002; Wang et al., 2003; Ma et al., 2005). Bcl-2 is the main anti-apoptosis gene associated with the inhibition of a variety of apoptotic processes. The functional role of Bcl-2 and Bax together depends on their expression relative to one another (Hou et al., 2003). High expression of Bcl-2 in the central nervous system helps to regulate apoptosis induced by injury and plays a key role in protecting local damaged neurons (Michaelidis et al., 1996; Yang et al., 1999). After spinal cord injury, Bcl-2 protects neurons from death caused by free radical damage (Kane et al., 1993), hypoxia (Zhong et al., 1993b), glutamate toxicity (Zhong et al., 1993a; Kane et al., 1995; Moriishi et al., 1999) and lack of growth factors (Allsopp et al., 1993; Goldblum and Rice, 1995). Bcl-2 transgenic rats exhibit less neuronal loss after spinal cord injury than their wild-type counterparts, and a high expression of Bcl-2 plays a protective role against pathological processes in neurons after spinal cord injury (Moriishi et al., 1999). We therefore examined the effects of exercise on Bcl-2 and Bax protein expression after unilateral corticospinal tract injury, to determine whether protection of target neuron cell activity underlies the therapeutic effects of exercise interventions after spinal cord injury.

The present study showed that the number of apoptotic cells in the spinal cord of rats in the trained and model groups was greater than that in the sham group 1 week after injury, indicating that the injury activated neuronal apoptotic pathways. At the same time, expression of Bcl-2 and Bax proteins in the trained and model groups was considerably higher than in the sham group after injury for the first 3 weeks post-surgery. This indicates that the apoptotic pathway induced by corticospinal tract injury may involve a change in expression of Bcl-2 and Bax proteins in the rat spinal cord. The expression of Bcl-2 protein in the trained group was higher than that in the model group 1–3 weeks after injury, whereas there was no significant difference in Bax expression between the trained and model groups at any time point after injury. These results indicate that exercise intervention has an anti-apoptotic effect by increasing the Bcl-2/Bax ratio, thus preserving more active neurons in the spinal cord gray matter, which are then able to connect with newborn axons to reconstruct neural circuits.

We also analyzed the changing expression of cleaved caspase-3 as a downstream effector protein in apoptosis, to confirm our hypothesis. Caspase family members are the main proteases in the process of apoptosis (Hengartner, 2000; Friedlander, 2003). Fourteen members of the caspase family have been identified to date, 11 of which exist in the human body (Yuan et al., 1993). Caspase-3 is a common downstream effector in various apoptotic pathways and its main substrate is poly(ADP-ribose) polymerase, which is associated with DNA repair and genetic integrity monitoring (Guo et al., 2013; Liu et al., 2013). Caspase-3 is the main terminal shear enzyme in apoptosis, and it is also the principal activator of the caspase cascade reaction (Wei et al., 2014). In the present study, expression levels of cleaved caspase-3 protein in the trained group were lower than those in the model group 1–3 weeks after injury, and TUNEL staining revealed that the number of apoptotic cells in the trained group was significantly lower than that in the model group at the same time points. Together with the results of Bcl-2 and Bax expression, these data indicate that one mechanism by which exercise intervention promotes functional recovery after corticospinal tract injury involves the upregulation of the anti-apoptotic protein Bcl-2, which reduces the number of apoptotic neurons, thus ensuring more neural circuits can be reconstructed. When new axons grow into the denervated area, the increased Bcl-2/Bax ratio in the spinal cord may weaken the role of pro-apoptotic Bax and reduce the expression of the downstream effector, cleaved caspase-3 protein.

In summary, we have shown that exercise intervention has a neuroprotective role after spinal cord injury, and promotes functional recovery by reducing apoptosis. However, various apoptotic pathways are activated after spinal cord injury, and involve a large number of proteins, so our results elucidate only one of several possible mechanisms by which exercise affects target neurons. Other apoptotic pathways remain to be studied in the future. Furthermore, our results provide evidence supporting the implementation of exercise programs to aid functional recovery after spinal cord injury.

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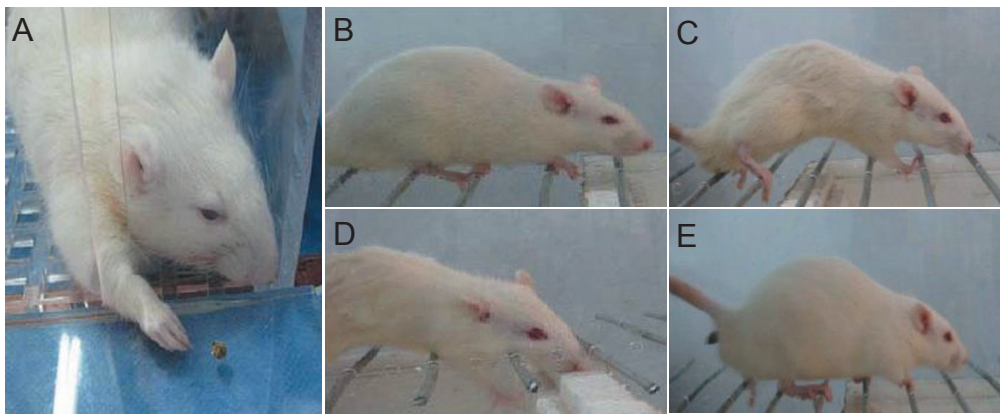
Figure 1 Exercise interventions in rats.
(A) Single pellet reaching task. (B–E) Horizontal ladder walking: (B) forearm support, failure to place the palm of the paw directly onto the rung; (C) misplaced digits, one or more digits incorrectly placed on the rung; (D) slip, paw slipped off the rung or placed between rungs; (E) normal, accurate grasp of each rung with all four digits placed over the front of the metal bar.
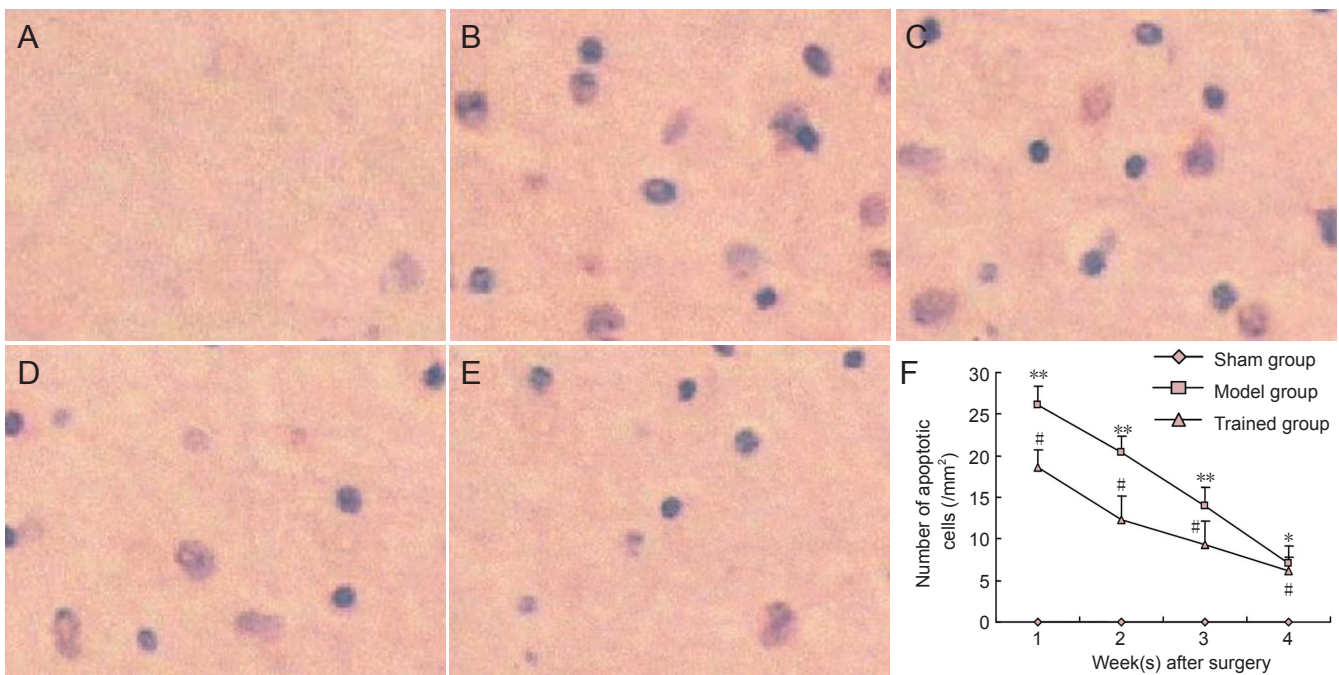

Figure 4 Effect of exercise interventions on the number of apoptotic cells in spinal cord tissue of rats with corticospinal tract injury.
(A–E) Apoptotic cells in the spinal cord of rats in the sham group (A) and in the trained group, 1 (B), 2 (C), 3 (D), and 4 (E) weeks after surgery (TUNEL staining, × 400). (F) Number of apoptotic cells in spinal cord. Data are expressed as the mean ± SD (n = 4 rats per group at each time point). Groups were compared using one-way analysis of variance and Tukey-Kramer *post-hoc* test. ∗$P < 0.05$, ∗∗$P < 0.01$, *vs.* sham group; #$P < 0.05$, *vs.* model group.
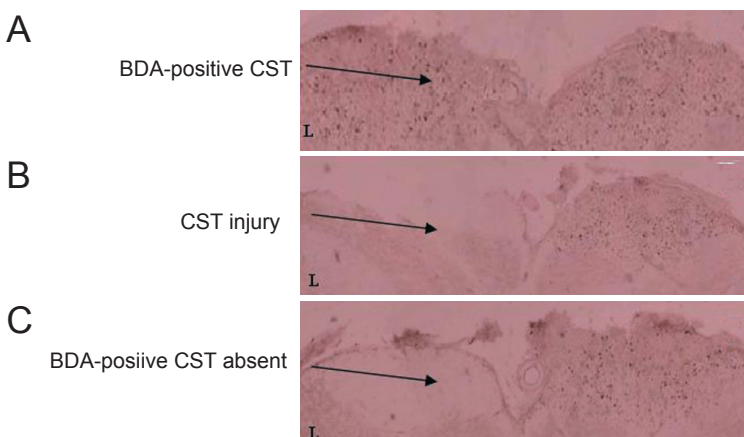

Figure 2 Unilateral corticospinal tract (CST) injury model in rats (DAB staining, × 100).
(A) Rostral part of injury site; bilateral BDA injections into the forelimb motor cortex labeled left and right CST tracts. (B) Injury level; complete interruption of left (ipsilateral) CST. (C) Caudal part of injury site; interruption of BDA transport to this region. L: Left side; BDA: biotinylated dextran amine.
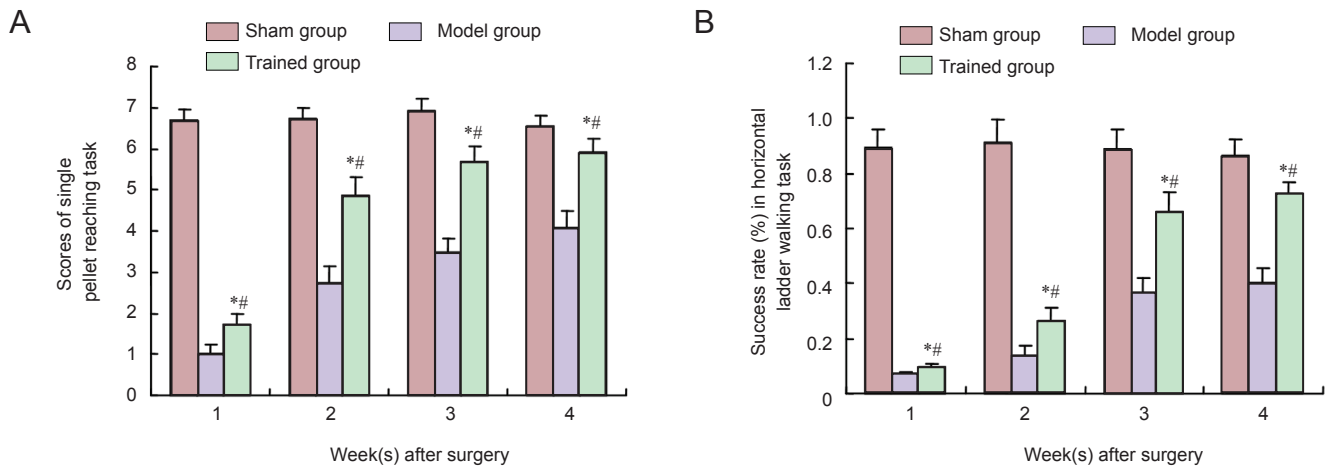
Figure 3 Effect of exercise interventions on motor function score of rats with corticospinal tract injury.
(A) Single pellet reaching task; higher score indicates greater motor coordination. (B) Horizontal ladder task; higher success rate indicates better motor function. Data were expressed as the mean ± SD (n = 8 rats per group at each time point). Groups were compared using one-way analysis of variance and Tukey-Kramer *post-hoc* test. ∗P < 0.05, *vs.* sham group; #P < 0.05, *vs.* model group.
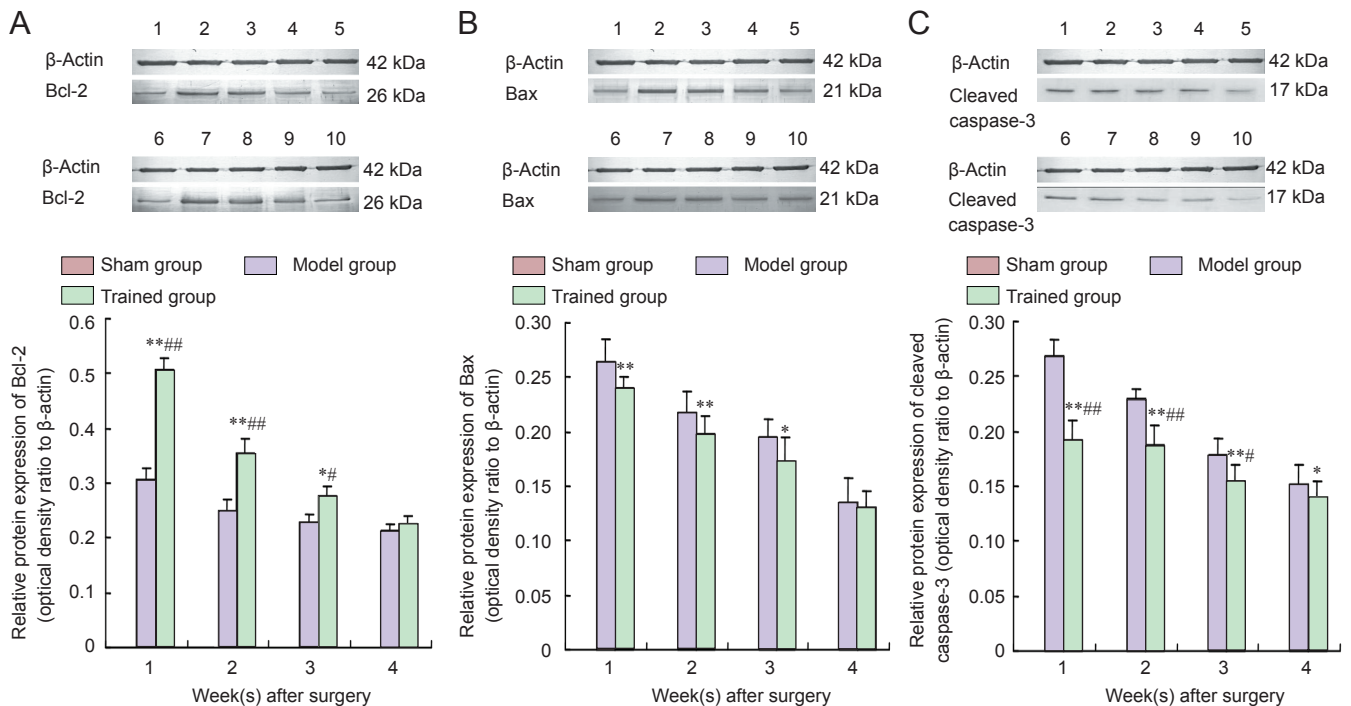

Figure 5 Effect of exercise interventions on Bcl-2 (A), Bax (B) and caspase-3 (C) expression in spinal cord tissue of rats with corticospinal tract injury (western blot assay).
Data are expressed as the mean ± SD (n = 4 rats per group at each time point). Groups were compared using one-way analysis of variance and Tukey-Kramer *post-hoc* test. #P < 0.05, ##P < 0.01, *vs.* model group; *P < 0.05, **P < 0.01, *vs.* sham group. Western blots: 1, 6: sham group; 2, 3, 4, 5: model group at 1, 2, 3, 4 weeks, respectively; 7, 8, 9, 10: trained group at 1, 2, 3, 4 weeks, respectively.

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