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RESEARCH ARTICLE

Effect of electroacupuncture on the mRNA and protein expression of Rho-A and Rho-associated kinase II in spinal cord injury rats

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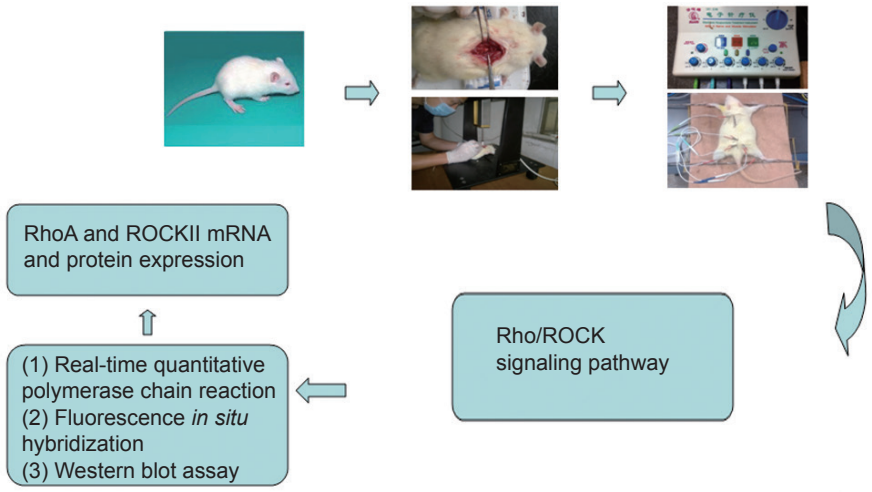
Graphical Abstract

Electroacupuncture inhitbits the mRNA and protein expression of RhoA and Rho-associated kinase II (ROCKII) in spinal cord injury rats



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Abstract

Electroacupuncture is beneficial for the recovery of spinal cord injury, but the underlying mechanism is unclear. The Rho/Rho-associated kinase (ROCK) signaling pathway regulates the actin cytoskeleton by controlling the adhesive and migratory behaviors of cells that could inhibit neurite regrowth after neural injury and consequently hinder the recovery from spinal cord injury. Therefore, we hypothesized electroacupuncture could affect the Rho/ROCK signaling pathway to promote the recovery of spinal cord injury. In our experiments, the spinal cord injury in adult Sprague-Dawley rats was caused by an impact device. Those rats were subjected to electroacupuncture at *Yaoyangguan* (GV3), *Dazhui* (GV14), *Zusanli* (ST36) and *Ciliao* (BL32) and/or monosialoganglioside treatment. Behavioral scores revealed that the hindlimb motor functions improved with those treatments. Real-time quantitative polymerase chain reaction, fluorescence *in situ* hybridization and western blot assay showed that electroacupuncture suppressed the mRNA and protein expression of Rho-A and Rho-associated kinase II (ROCKII) of injured spinal cord. Although monosialoganglioside promoted the recovery of hindlimb motor function, monosialoganglioside did not affect the expression of Rho-A and ROCKII. However, electroacupuncture combined with monosialoganglioside did not further improve the motor function or suppress the expression of Rho-A and ROCKII. Our data suggested that the electroacupuncture could specifically inhibit the activation of the Rho/ROCK signaling pathway thus partially contributing to the repair of injured spinal cord. Monosialoganglioside could promote the motor function but did not suppress expression of RhoA and ROCKII. There was no synergistic effect of electroacupuncture combined with monosialoganglioside.

Key Words: *nerve regeneration; spinal cord injury; electroacupuncture; Rho/Rho-associated kinase signaling pathway; monosialoganglioside; motor function; cytoskeleton; real-time quantitative polymerase chain reaction; western blot assay; hybridization in situ; neural regeneration*

Introduction

Spinal cord injury (SCI) is a serious neurological injury that often results in profound disability (Yoshimura et al., 2006; Gao et al., 2014, 2015). SCI results in high mortality and there is no effective treatment at present (Wu et al., 2014; Lin and Zhao, 2015). The regeneration of neurons in the adult central nervous system (CNS) is limited and their axons are unable to regenerate after severe injury (Domeniconi et al., 2005; Zhou and Snider, 2005; Chiba et al., 2010). The activities of the cytoskeleton influence the growth cone that is crucial for the growth of neural axons (Ito et al., 2004). The growth cone is susceptible to the surrounding environment (Monnier et al., 2003; James et al., 2008) and various signal pathways affect the cytoskeleton of the growth cone to regulate neuron axonal growth (Carmen et al., 2004; Lingor et al., 2008). The Rho/ROCK signaling pathway is a vital part in promoting the growth of neural axons and in the regulation of the cytoskeleton (Wettschureck et al., 2002; Doran et al., 2004; Liu et al., 2015). The two essential components of Rho/ROCK signaling pathway are Rho-A and Rho-associated kinase II (ROCKII) (Wettschureck et al., 2002; Hou et al., 2015; Jia et al., 2016). Rho GTPases are important regulators of the actin cytoskeleton and thereby control the adhesive and migratory behaviors of cells (EtienneManneville and Hall, 2002; Govek et al., 2005). Within these subfamilies of Rho GTPases, Cdc42, Rac and Rho-A have been shown to participate in regulating the growth of neural axons; Cdc42 and Rac regulate the actin to promote axon growth and stability (Nobes and Hall, 1995). Rho-A activates the downstream signaling molecule, ROCKII, and then triggers a series of reactions that cause the growth cone to collapse and retraction that result in limited regeneration of neural axons (Dickson, 2001). Although there is low mRNA expression of Rho-A in the normal spinal cord, Rho-A expression is significantly enhanced after SCI (Wu and Xu, 2016), which indicated that Rho/ROCK signaling pathway plays an essential role in the pathogenesis of SCI. How to promote the regeneration of axons is a key aim in treating SCI (Ng and Luo, 2004; Sun et al., 2008).

Electroacupuncture (EA) is widely used to treat SCI, and has been shown to be beneficial for the recovery of SCI (Paola and Arnold, 2003; Min et al., 2013). However, the underlying mechanism of EA in the treatment of SCI remains unclear (Zhang et al., 2012). In the present study, we investigated the effect of EA on the repair of SCI, and whether EA could inhibit the Rho/ROCK signaling pathway after SCI.

Materials and Methods

Animals

Eighty healthy, clean, male, Sprague-Dawley rats, aged 8 weeks, weighing 200 ± 20 g, were supplied by the Slack-Jingda Laboratory Animals Co., Ltd. of Hunan Province, China (certificate No. SCXK (Xiang) 2011-0003). The rats were fed with standard fodder and allowed free access to water and chow.

Following a 3-day adaptation, all rats were randomly divided into five groups: sham surgery (sham, n = 16), model control (SCI, n = 16), EA treatment (SCI + EA, n = 16), intramuscular monosialoganglioside (MI) injection treatment (SCI + MI, n = 16), EA combined with intramuscular monosialoganglioside injection treatment (SCI + EA + MI, n = 16). Eight rats in each group were randomly sacrificed at 7 and 14 days after SCI. At each time point, four rats from each group were used for real-time quantitative polymerase chain reaction (RT-qPCR) and *in situ* hybridization; the remaining four rats were used for western blot assay. All rats were evaluated for hindlimb motor function by Basso, Beattie, and Bresnahan (BBB) scores at 1 and 7 days, while the remaining rats were used for evaluation of hindlimb motor function at 14 days.

All procedures were conducted in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine, China.

Model establishment and treatment

Models of SCI were established in accordance with published methods (Shi et al., 2010). The 64 rats were anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneally) and an incision made. The T_{10} vertebral body of each rat was located by counting the ribs. The T_{10} vertebral body was removed by rongeur forceps to expose the spinal cord completely. Injury was induced by striking the spinal cord with an electric cortical contusion impactor. Strike parameters consisted of a strike tip with a diameter of 3 mm, velocity of 5 m/s, retention time of 0.5 seconds and compression of 1.5 mm. All rat models received intramuscular penicillin injection, twice a day, followed by suture of the incision. All rat models received assisted urination, three times daily, until the rat obtained the ability of automatic micturition. Rats in the SCI model group did not receive any treatment. EA and/or MI treatment were started 1 day after SCI.

In the SCI + EA group, the rats were restrained on a board. Stainless steel 0.18-mm-diameter needles (Hwato Disposable Acupuncture Needle; Jiangsu Medical Supplies Factory, Jiangsu Province, China) were inserted to a depth of approximately 5 mm at the acupoints: *Yaoyangguan* (GV3, posterior midline and in the depression below the spinous process of the fourth lumbar vertebra), *Dazhui* (GV14, posterior midline and in the depression below the spinous process of the seventh cervical vertebra) and *Zusanli* (ST36, bilaterally on hindlimbs below the fibular head 5 mm), *Ciliao* (BL32, corresponding to the second sacral posterior) on both sides (Gao et al., 2013). Then the needles were connected to a Hwato SDZ-IIEA apparatus (Suzhou, Jiangsu Province, China). The electric current frequency was 2 Hz, and the output voltage was 2 V. EA treatment was performed for 20 minutes once per day, lasting for 14 days.

Rats in the SCI + MI group received MI (Qilu Pharmaceutical Co., Ltd., Jinan, Shandong Province, China) *via* intramuscular injection (the 20 mg lyophilized powders were dissolved in 2 mL physiological saline solution; each rat received 0.54 mL/kg), once per day, lasting for 14 days.

Rats in the SCI + EA + MI group received both EA and MI

Table 1 Primer sequences for real-time quantitative polymerase chain reaction

Gene	Sequences (5′–3′)	Product size (bp)
Rho-A	Forward: GAT GGA GCT TGT GGT AAG Reverse: TCA GTG TCT GGG TAG GAG	150
ROCKII	Forward: ATC TCA TTT GTG CCT TCC Reverse: CTG GTG CTA CAG TGT CTC G	143
GAPDH	Forward: GCA AGT TCA ACG GCA CAG Reverse: GCC AGT AGA CTC CAC GAC AT	146

ROCKII: Rho-associated kinase II; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Primer sequences for hybridization *in situ*

Gene	Sequences (5′–3′)
Rho-A	AGG TAG AGT TGG CTT TAT GGG ACA CAG CTG GAC AGG AAG ATT ATG ACC GTC TGA GGC CTC TCT CCT ACC CAG ACA CTG ATG
ROCKII	TCT TTA TCA CTT CCC AAC CAA CTG TGA GGC CTG TAT GAA GCC ACT GTG GCA CAT GTT TAA ACC TCC TCC TGC TCT AGA GTG CCG TAG ATG CC

ROCKII: Rho-associated kinase II.

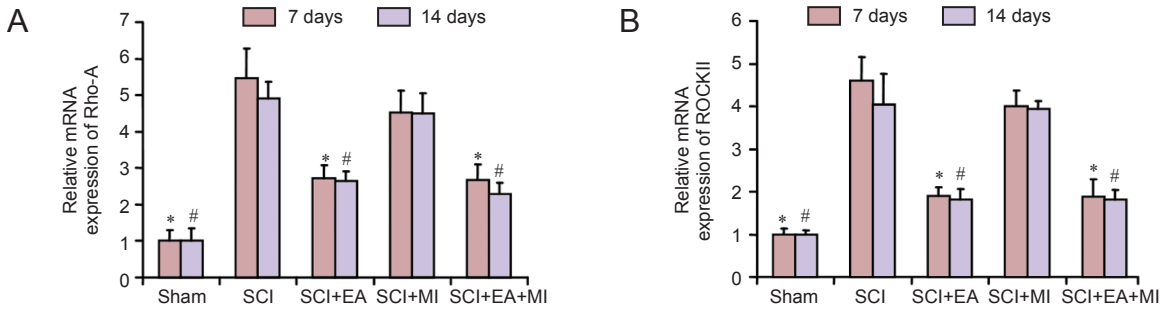

Figure 2 Effect of electroacupuncture on changes in mRNA expression of Rho-A and ROCKII of injured spinal cords.
The mRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ analytical method. *P < 0.05, *vs.* SCI group at 7 days; #P < 0.05, *vs.* SCI group at 14 days. Data are expressed as the mean ± SD (n = 4; one-way analysis of variance and Student-Newman-Keuls *post hoc* test). SCI: Spinal cord injury; EA: electroacupuncture; MI: monosialoganglioside; ROCKII: Rho-associated kinase II.

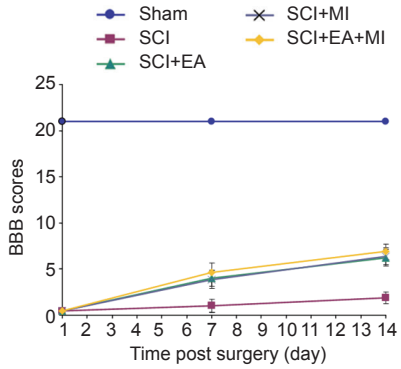

Figure 1 Effect of electroacupuncture on the motor function of spinal cord injury rats.
BBB score was significantly lower in the SCI group than in those treatment groups (SCI + EA, SCI + MI, SCI + EA + MI groups) (P < 0.05). Data are expressed as the mean ± SD (n = 16 of each group at 7 days; n = 8 of each group at 14 days; one-way analysis of variance and Student-Newman-Keuls *post hoc* test). BBB: Basso, Beattie, and Bresnahan; SCI: spinal cord injury; EA: electroacupuncture; MI: monosialoganglioside.

treatments, lasting for 14 days.

In the sham group, 16 rats received anesthesia, incision, removal of T_{10} vertebral body and suture, but no strike injury.

Motor function assessment and tissue preparation

The hindlimb motor function of rats was assessed at 1, 7 and 14 days after SCI using the open field locomotor test developed by Basso, Beattie, and Bresnahan (Basso et al., 1995). Double independent BBB scores were recorded, and the average values are presented. A random selection of eight rats in each group were sacrificed at 7 and 14 days after SCI, and the impaired spinal cords were harvested for RT-qPCR, *in situ* hybridization and western blot assay. The tissues were frozen in liquid nitrogen and stored at –80°C.

RT-qPCR

RT-qPCR was performed using SYBR Green system. Total RNA was isolated from the spinal cords of rats in each group at 7 and 14 days using TRIzol solution (Invitrogen, Carlsbad, CA, USA). The mRNA expression levels of Rho-A and ROCKII were measured using a RT-qPCR system with SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was amplified by PCR using primers for each target gene. RT-qPCR conditions are as follows: 94°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 45 seconds and 72°C for 30 seconds. Fluorescence signal was detected at 60°C and samples were finally extended at 72°C for 7 minutes. The amplification efficiency was compared between the target and reference control GAPDH (glyceraldehyde 3-phosphate dehydrogenase) using the delta-delta Ct (ΔΔCt) method (Min et al., 2016). Primers employed are listed in **Table 1**.

mRNA expression of Rho-A and ROCKII, as determined by fluorescence *in situ* hybridization

The sections were dewaxed by xylene three times, 5 minutes

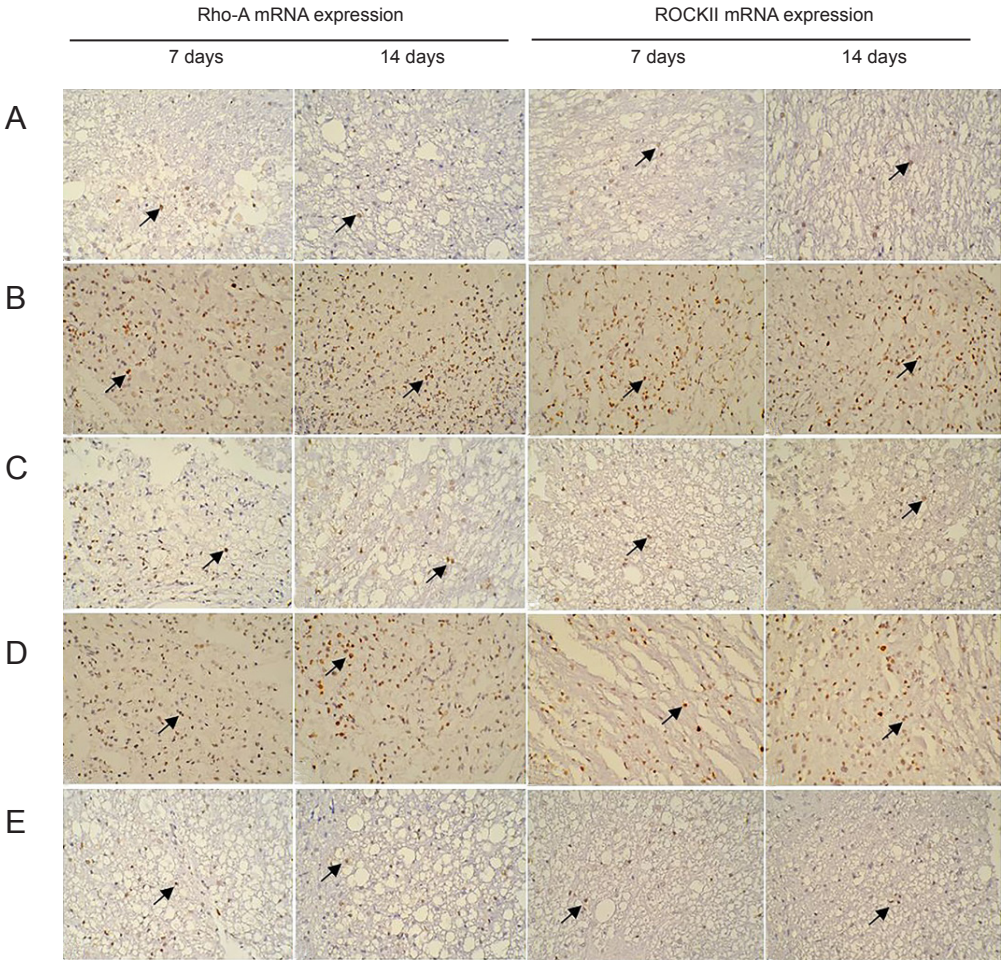

Figure 3 mRNA expression of Rho-A and ROCKII in injured spinal cords at 7 and 14 days (*in situ* hybridization, × 200).
In situ hybridization positive cells contain brown spot or particles as indicated by the arrows. (A) Weak expression of Rho-A and ROCKII in spinal cords of sham-operated rats at 7 and 14 days; (B) injury induces increased Rho-A and ROCKII mRNA expression, and there was no significant difference between 7 and 14 days. Treatment of EA or EA + MI could decrease mRNA expression of Rho-A and ROCKII (C, E). MI alone could not alter the mRNA expression of Rho-A and ROCKII (D). (A) Sham group; (B) SCI group; (C) SCI + EA group; (D) SCI + MI group; (E) SCI + EA + MI group. SCI: Spinal cord injury; EA: electroacupuncture; MI: monosialoganglioside; ROCK-II: Rho-associated kinase II.

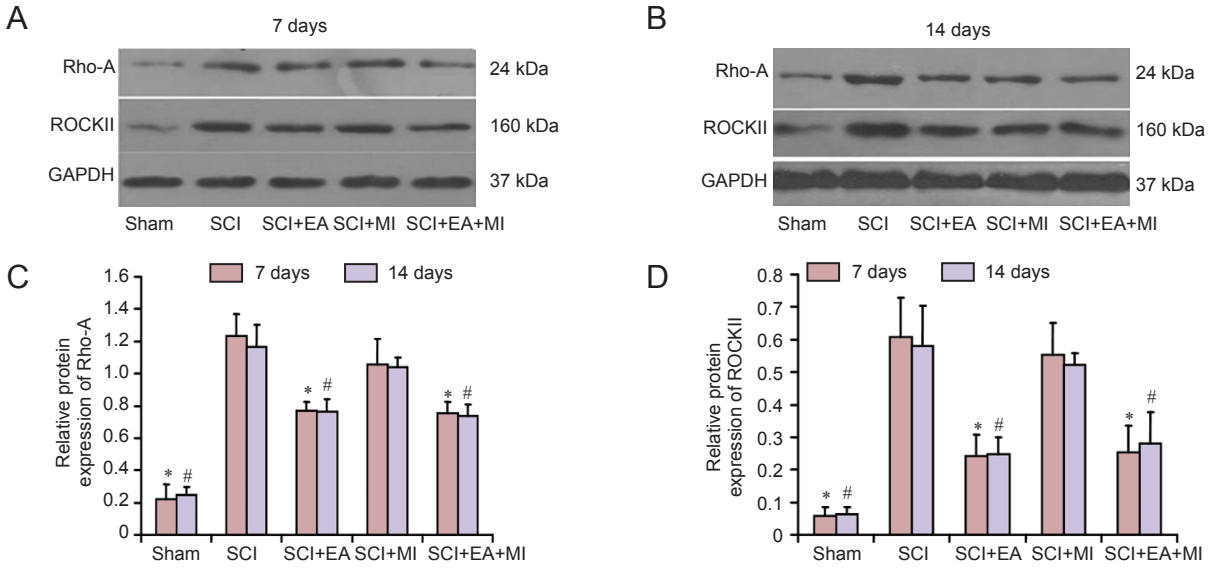

Figure 4 Effect of Electroacupuncture on changes in protein expression of Rho-A and ROCKII of injured spinal cords.
(A, B) Western blotting bands for Rho-A and ROCKII in different groups at 7 and 14 days. (C, D) Quantification of protein expression of Rho-A and ROCKII. Y-axis indicates the relative grayscale value of protein bands (the ratio of target grayscale values to loading control grayscale values). *$P < 0.05$, *vs.* SCI group at 7 days; #$P < 0.05$, *vs.* SCI group at 14 days. Data are expressed as the mean ± SD ($n = 4$; one-way analysis of variance and Student-Newman-Keuls *post hoc* test). SCI: Spinal cord injury; EA: electroacupuncture; MI: monosialoganglioside; ROCKII: Rho-associated kinase II.

per time. Xylene was removed in gradient alcohol of 100%, 96% and 70%. The sections were dried in the air, washed for 2–5 minutes with phosphate buffered saline (PBS) to remove protease, and then dehydrated in gradient alcohol. Each section was incubated in 50 μL hybridization buffer containing oligonucleotide probe 10 μm at 95°C for 5 minutes and 37–40°C for 12 hours, washed three times with 5×, 1× and 0.2× saline sodium citrate respectively, and treated with blocking buffer at 37°C for 15 minutes. The blocking buffer was blotted off with paper. Each section was then incubated in 30 μL biotinylated anti-digoxin (1:50) at 37°C for 1 hour, washed four times with 0.5 M PBS, incubated in streptavidin-biotin-peroxidase complex at 37°C for 30 minutes, and rinsed four times in 0.5 M PBS. Subsequently, the sections were developed in 3,3′-diaminobenzidine, counterstained with hematoxylin, dehydrated in alcohol, permeabilized in xylene, mounted with quench-proof mounting agent, and photographed with a fluorescence microscope. Negative controls were incubated in 0.01 M PBS without primary antibody. Primers employed are displayed in **Table 2**.

Western blot assay

All spinal cord tissues obtained at 7 and 14 days in each group were homogenized in a lysis buffer (JRDUN Biotechnology, Shanghai, China). Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies overnight at 4°C. Monoclonal antibodies included rat monoclonal anti-Rho-A (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat monoclonal anti-ROCKII (1:1,000; Santa Cruz Biotechnology) and rat monoclonal anti-GAPDH (1:2,000; Santa Cruz Biotechnology). The membranes were washed 3 times for 5 minutes with Tris-buffered saline Tween, incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) (1:1,000; Beyotime Biotechnology, Shanghai, China) and goat anti-mouse IgG-HRP PS1 (C-20) (1:1,000; Beyotime Biotechnology) antibodies at 37°C for 1 hour. The immunoreactive bands were visualized using an enhanced chemiluminescence reagent (Beyotime Biotechnology). The grayscale values of bands were quantified using Image J software (Fujifilm, Tokyo, Japan). The relative expression of protein was calculated based on the grayscale value ratio of target to loading control.

Statistical analysis

All data, presented as the mean ± SD, were analyzed by a one-way analysis of variance, followed by a *post hoc* Student-Newman-Keuls test using the SPSS 13.0 software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Effects of treatment on behavior in SCI rats

Behavioral scores (BBB scores) in each group are shown in **Figure 1**. BBB scores of SCI rats were lower than that of the normal rats ($P < 0.05$). After treatment with EA or MI, the hindlimb motor function of rats improved in each treatment group. BBB score was significantly lower in the SCI group ($P < 0.05$) than in any of the treatment groups (SCI + EA, SCI + MI, and SCI + EA + MI). However, there was no significant difference in BBB score among those three treatment groups ($P > 0.05$).

EA suppressed mRNA and protein expression of Rho-A and ROCKII in injured spinal cords of rats

Relative mRNA expression of Rho-A and ROCKII in injured spinal cords was determined by RT-qPCR, *in situ* hybridization; and protein expression of Rho-A and ROCKII by western blot assay. As shown in **Figures 2**, **3**, the mRNA expression levels of Rho-A and ROCKII were significantly increased in the SCI, SCI + EA, SCI + MI, and SCI + EA + MI groups compared with the sham group at 7 days ($P < 0.05$) and 14 days ($P < 0.05$). EA treatment significantly down-regulated the mRNA expression of Rho-A and ROCKII compared with the SCI group ($P < 0.05$). The mRNA expression of Rho-A and ROCKII between SCI and SCI + MI groups was not significantly different ($P > 0.05$).The expression of mRNA for Rho-A and ROCKII showed no significant difference between 7 days and 14 days for each group ($P > 0.05$). Western blot assay results exhibited the same trend as did in the RT-qPCR (**Figure 4**). The above results showed that the Rho-A and ROCKII was activated rapidly after SCI and inhibited after EA treatment.

Discussion

In this study, we investigated the mechanism underlying the effects of EA on SCI at the molecular level. Our results demonstrated that EA treatment improved hindlimb motor function of SCI rats. In our experiments, the mRNA and protein expression of Rho-A and ROCKII were enhanced after SCI compared with sham group. This confirms other studies showing that although there was low mRNA expression of Rho-A in the normal spinal cord, Rho-A expression was significantly enhanced after SCI (Conrad et al., 2005; Erschamer et al., 2005). This indicates that the Rho/ROCK signaling pathway plays an essential role in the pathogenesis of SCI. We found that EA treatment suppressed Rho-A and ROCKII mRNA and protein expression in the injured spinal cord of rats. These results suggest that a possible mechanism of inducing the recovery from SCI by EA treatment could be by down-regulating the expression of Rho-A and ROCKII. We also found that hindlimb motor function improved following inhibition of Rho-A and ROCKII in SCI models.

MI is frequently used in the treatment of SCI, and MI has a significant effect on promoting neural repair (Walker et al., 1993). Gangliosides are sialic acid derivatives of endogenous glycolipids and are present predominantly in the cell membrane in the central nervous system (Wang et al., 2015). MI is already used as a therapeutic option for treatment of central nervous system injuries because of its anti-neurotoxic, anti-inflammatory and neuroprotective effects. Moreover,

MI promotes the development, growth, differentiation and maturation of neurons (Barros et al., 2016). Our results indicated that although ganglioside promoted the recovery of hindlimb motor function of SCI rats, it did not affect the expression of Rho-A and ROCKII. SCI results in a complex pathophysiological process. Therefore different treatments may aid recovery from SCI in different ways. Although EA and MI each improved the hindlimb motor function, there was no additional effect with the combined treatment. Regeneration of the central nervous system is difficult, and the recovery from SCI takes a much longer time in humans. In our experiment, it was not possible to observe the recovery after SCI rats for long enough to observe a better efficacy or further recovery of motor function.

Our results suggest that the Rho/ROCK signal pathway is a target for EA in the treatment of SCI. The synergistic effects of EA and other therapies could be explored in subsequent experiments.

Precisely where EA acts in inhibiting the Rho/ROCK pathway requires further research. Dent and Gertler (2003) indicated that the growth of axons is closely associated with cytoskeleton. The neurons in the adult central nervous system fail to regenerate their axons after an injury, which is a major difficulty in the repair of SCI. Axonal regeneration was inhibited in the glial scar after cerebral or spinal cord injury (Schwab et al., 1993). The amount of growth inhibitory molecules (growth-IMs), such as myelin-associated glycoproteins (McKerracher et al., 1994), Nogo (Chen et al., 2000) and oligodendrocytic myelin glycoprotein (Wang et al., 2002), is crucial in the promotion of axonal regeneration for treating SCI. Growth-IMs have been identified as activators of the intracellular Rho/ROCK signaling pathway that induces reorganization of the actin cytoskeleton causing growth cone collapse and consequently inhibits axonal regeneration (Domeniconi et al., 2005). Many extracellular orientation information molecules, such as lysophosphatidic acid, thrombin receptor activator protein and activated prostaglandin E receptor, also affect Rho/ROCK signaling pathway leading to the collapse of the growth cone (Katoh et al., 1998).

After Rho-A has been activated through the receptor on the cell membrane by growth-IMs, the Rho-A activates the downstream signal molecule ROCKII. The ROCKII phosphorylates myosin light chain phosphatase so it is unable to dephosphorylate myosin. The elevated level of phosphorylated myosin increases its contractility, enhancing microfilament retraction in the cytoskeleton of the axonal growth cone. Finally, it leads to the collapse of axonal growth cone and inhibits regrowth of the axon (Wettschureck et al., 2002).

In conclusion, after SCI, the Rho/ROCK signaling pathway is activated that contributes to the inhibition of regeneration of axons. EA could inhibit activation of Rho/ROCK signaling pathway after SCI by suppressing expression of Rho-A and ROCKII, thereby promoting axon regrowth and inducing recovery from SCI. These findings provide evidence to support the widespread clinical utilization of EA. These research findings augment our understanding of the neuromodulatory effects of EA and also provide us with an enhanced insight into the biology of SCI and repair.

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Author contributions: *YJM conceived and designed the study and revised the paper. LLQD wrote the paper, provided data and revised the paper. WPX, HZ, ZYM, and JP participated in the experiment. WPX provided the guidance of model establishment. LHC and XWH provided some advices for the study. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*

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