Improved Regenerative Responses of Injured Spinal Cord Nerve Fibers by the Treatment of Sukjihwang(*Rehmanniae radix preparat*)

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In oriental medicine, Sukjihwang (SJH, *Rehmanniae radix preparat*) has been used as one of the key ingredients for the prescription of several herbal decoctions and applied clinically for the treatment of several diseases including nervous system and cardiovascular disease. Here, possible growth-promoting effects of SJH on injured spinal cord axons were investigated in the rats. SJH administration increased levels of active form of ERK1/2 protein and Cdc2 proteins in the injured spinal cord tissue. Anterograde DiI-tracing of corticospinal tract axons showed that SJH-treatment enhanced axonal arborization in the injury area and extensive axonal extension into the caudal area. In SJH-treated group, glial scar formed after spinal cord injury was confined in a smaller area compared to the control group, and the trabecula structure was well observed within the injury cavity. Furthermore, increased proliferation and migration of astrocytes in the injury cavity were observed by SJH treatment. Thus, these present data provide a biological evidence on potential importance of SJH therapy for the treatment of injured spinal cord.

Key words : Sukjihwang (SJH, *Rehmanniae radix preparat*), injured spinal cord, ERK1/2 protein

Introduction

Axons in the central nervous system (CNS) fail to regenerate after injury. Numerous studies have focused on elucidating the mechanisms underlying regeneration failure of injured CNS axons and recently begun to identify molecular factors in the injured spinal cord[1]. For instances, Nogo and myelin-associated glycoprotein (MAG) which are produced from oligodendrocyte in the injured spinal cord are known to inhibit axonal growth. Vimentin, chondroitin sulphate proteoglycan (CSPG), and EphA4, the cognate neuronal receptor for ephrin B3, are all produced from reactive astrocytes and known to inhibit axonal regeneration[2-5].

Based on identification of molecular factors preventing axonal regrowth, studies on artificial induction of axonal regeneration have been devoted to block artificially their activities. Mice lacking Nogo or Nogo receptor protein expression were generated and examination of axonal regrowth showed some improvement after spinal cord injury[6-8]. Intrathecal delivery of ChABC, a digestive enzyme for CSPG, resulted in significant improvement of electrophysiological and behavioral recovery of damaged corticospinal tract as well as axonal elongation after spinal cord injury[9]. While these and other studies strongly suggest that axonal regeneration after spinal cord injury is feasible, a consistent target-specific functional recovery has not been demonstrated. It was reported in many cases that only a small portion of axons were seen to be regenerated and thus, innervation of target tissue in terms of functional recovery was rarely achieved. Moreover, results among different researchers are not consistent, which is in part due to variation of experimental treatments for in vivo experiments.

It has been reported that the treatment of herbal drugs can improve the nerve function. For instance, herbal drugs such as Hominis placenta and ginseng on injured peripheral nerves have been shown to promote axonal regeneration[10,11], and neuroprotective effects of some herbal drugs ginkgo leaf extract, dansheng, and ginseng components in the injured spinal cord tissues were reported[12-14]. However, potential effects of axonal regrowth after spinal cord injury remain to be explored.

Sukjihwang (SJH; Rehmanniae radix preparat) is prepared by processing the roots of Saengjihwang (*Rehmanniae radix*). In oriental medicine, SJH is known to act on organs such as heart, liver, and kidneys, and recognized as being effective for supplementing blood circulation, strengthening heart function, and micturition. SJH is known to improve bone marrow

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function and thus helps erythropoetic activity. SJH is also applied for the treatment of symptoms of malnutritional weakness, larynx dryness, and poor pulsation[15]. It is believed that the physiological function of SJH is augmented by combined use of other herbal drugs to make Samultang and Yukmijihwangtang, for instance.

According to oriental medicinal theory, the blood-supplementing function of SJH is associated with stimulating nervous system function. Thus in the present study, potential function of SJH on injured nerves was investigated using injured spinal cord model in the rats. Rats were given spinal cord injury by contusion and affected axonal tracts were examined by biochemical and histological methods. SJH administration appeared to facilitate axonal elongation in the injured spinal cord tissue.

Materials and Methods

1. Materials

1) Experimental animals

Sprague-Dawley rats (8 weeks old) were used in this experiment. They were placed in an animal room with regulated temperature (22℃), 60% of humidity, and 12-h light and 12-h dark cycle. They were allowed to eat commercial rat chow (Samyang Co., Korea) and drink water ad libitum.

2) Drugs

Dried Sukjihwang (SJ; Rehmanniae radix preparat) was obtained from Daejeon University Oriental Medicine Hospital (Daejeon, Korea). Dried drug was resuspended in 2 liters of water, heat extracted with 2 liters of water for 3 h, and filtered three times. The filtered fluid was distilled using the rotary vacuum evaporator, and freeze-dried. The yield for SJH was 8 g for 50 g of the initial raw material. The product was kept at 4℃ and dissolved in water. The stock solution was stored at -20℃ and used for experiment by diluting with physiological saline solution before use.

2. Methods

1) Spinal cord injury procedure

Rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). Using aseptic technique, a laminectomy was performed to expose spinal cord at T10 - T11. A contusion injury was induced using a home-made NYU devise by dropping a 10 g weight from 25 mm onto the exposed dura mater of the spinal cord[16]. The wound was closed in anatomical layers and rats were placed until further treatment in standard plastic cages and given food and water ad libitum.

2) Immunohistochemistry

For immunohistochemistry experiment, dissected tissues were frozen immediately at -75℃ and embedded into the OCT medium. The spinal cord sections (20 ㎛) were cut using a cryostat and mounted on positively charged slides. For immunofluorescence staining, sections on a slide were fixed with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS) at room temperature for 40 min, permeablized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 hr at room temperature. Sections were incubated with anti-glial fibrillary acidic protein (GFAP) antibody or anti-CSPG antibody (Santa Cruz Biotech, USA), then incubated with fluorescein-goat anti-mouse (Molecular probes, USA) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes) in 2.5% horse serum and 2.5% bovine serum albumin for 1 hr at room temperature and cover-slipped with gelatin mount medium. Primary antibody reaction was performed with single or double antibodies depending on the experimental purposes, and followed by corresponding specific secondary antibody reaction. Sections were observed with a Nikon fluorescence microscope and the images were captured by using Nikon camera. The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5). For some experimental purpose, Hoechst staining reaction for nuclear visualization was performed after the first washing step after secondary antibody reaction. Tissue sections were treated with 25 ㎍/ml of Hoechst 33258 dye in 0.1% triton X-100 in phosphate-buffered saline solution (PBST) for 10 min. Cell nuclei were observed blue under the fluorescence microscope.

3) Western blot analysis

Nerve tissues were washed with ice-cold PBS, and sonicated under 50 - 200 ㎕ of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.14, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% triton X-100, 10% glycerol, 5 ㎍/ml leupeptin, 5 ㎍/ml aprotinin, 3 ㎕M benzamidine, 0.5 mM DTT, 1 mM PMSF). Protein (10 ㎍) was used resolved in 12% SDS polyacrylamide gel and transferred to Immobilon polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). Blots were blocked with 5% nonfat dry milk in PBST (17 mM KH_2PO_4, 50 mM Na_2HPO_4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween-20) for 1 hr at room temperature and then incubated overnight at 4℃ in 0.1% triton X-100 in PBS plus 5% nonfat dry milk containing antibodies. Protein bands were detected using the Amersham ECL kit (Amersham Pharmacia Biotech, USA), with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Transduction Laboratories,

USA). Relative intensities of the protein bands were analyzed by autoradiography. The antibodies used in the present study were anti-Cdc2 antibody, anti-Erk1/2 antibody, anti-phospho Erk1/2 antibody, and anti-actin antibody (clone no. C4, ICN Biomedicals, USA).

4) Anterograde tracing

DiI as anterograde tracer was treated into the sensorimotor cortical area in the rat brain at the time when the spinal cord injury was given. The rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic instrument (Harvard Instrument, USA). Rat's head skin was incised, the skull was drilled, and the glass capillary filled with DiI was lowered through the drilled holes. Labeling of corticospinal tract (CST) was performed by injecting DiI (5 ㎕ of 3% in DMSO) into the sensorimotor cortex (anterior-posterior (AP) 2.0 mm, medial-lateral (ML) 2.3 mm, depth 1.5 mm from the dura surface) on the left side of the brain by using picoinjector (Harvard Instrument, USA.), according to the rat brain atlas[17]. Brain and spinal cord sections at the low thoracic level were prepared and observed under the fluorescence microscope.

5) Drug administration

For drug treatment into the spinal cord, drug (10 ㎕ in PBS) was injected into the contused spinal cord by using micropipet. In case of spinal cord injury experiment, drugs (10 ㎕) were intraperitoneally supplemented every other days for two week period.

Results

1. SJH-mediated Regulation of Erk1/2 and Cdc2 proteins in the contused spinal cord tissues.

To investigate the effect of SJH treatment on Erk1/2 and Cdc2 protein levels, spinal cord tissue within and around the injury site were prepared 1 and 2 weeks after injury. Fig. 1A shows Western blot analysis of phospho-Erk1/2 and total Erk1/2 protein levels in the injured spinal cord tissue. At 1 week time point after contusion injury, phospho-Erk1/2 protein levels were increased in the injury site and adjacent rostral area in both saline- and SJH-treated animal groups compared with non-injury control group (Fig. 1A). Comparison between saline and SJH group showed similar levels of phospho-Erk1/2 protein levels. Similar comparison was made in the injured spinal cord tissues at 2 weeks after injury. SJH-treated, but not saline-treated, animals showed stronger phospho-Erk1/2 protein levels in rostral and injured regions compared with saline-treated and non-injured animal groups (Fig. 1A). In both 7 and 14-day treated groups, total Erk1/2 protein levels were consistent and did not show any difference among various treatments.

To investigate Cdc2 protein regulation in the injured spinal cord region, the tissues were prepared 7 and 14 days after contusion injury. As shown in Fig 1B, a robust induction of Cdc2 was observed in the injured and rostral regions of the spinal cord in saline treated group at 7 days post injury. In SJH group, a slight increase was observed in the injury site, but decreased Cdc2 protein levels were observed in the rostral region. At 14 days after injury, Cdc2 protein was mostly downregulated in both saline and SJH-treated groups.

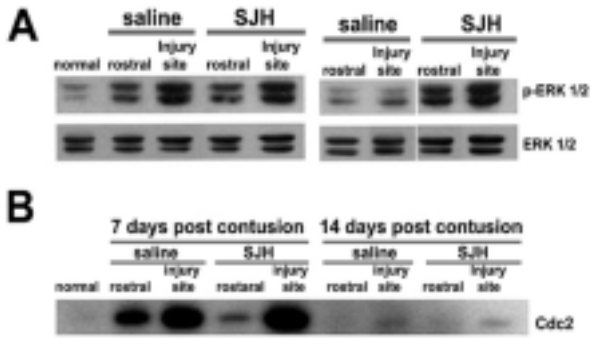

Fig. 1. Western blot analysis of Erk1/2 and Cdc2 protein levels in the injured spinal cord. After contusion injury, dorsal half of the injured spinal cord tissue was excised and used for cell lysate preparation. Spinal cord tissue 1 cm rostral to the injury site was prepared as well. (A) Phospho-Erk1/2 (p-Erk1/2) and Erk1/2 protein levels in the injured spinal cord area 7 and 14 days after injury. Dual Erk1/2 protein bands were detected at 42 and 44 kDa. (B) Western blot analysis of Cdc2 protein in the injured spinal cord. Protein lysates in the injured spinal cord were prepared 7 and 14 days after contusion injury and used for Western analysis with Cdc2 antibody.

2. Effects of SJH treatment on astrocyte activation in the injured spinal cord

To examine the reactivity of astrocytes in the spinal cord after injury, proliferation and possible morphological changes of astrocytes were investigated. Two or four weeks after spinal cord injury, cross sections at the injury area of the spinal cord were prepared and used for immunofluorescence staining with anti-GFAP antibody as a marker of astrocytes. In sections prepared 2 weeks after injury, the number of cells positive to GFAP in an area of dorsomedial CST was higher in both saline- and SJH-treated groups compared to non-injury control (Fig 2A). Similar difference was seen when total cells were labeled by nuclear Hoechst staining. In the tissues prepared 4 weeks after contusion injury, similar differences were observed in the cell numbers positive to GFAP-positive astrocytes and total cells (Fig 2B). The number of astrocytes and total cells in saline-treated group was higher than non-injury control. Comparison between saline- and SJH-teated group showed stronger intensity of GFAP-staining in SJH group than saline group.

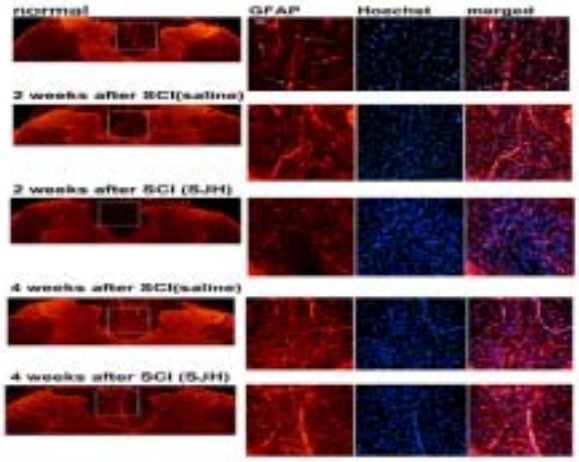

Fig. 2. GFAP staining of astrocyte cells in the injured spinal cord area. Horizontal spinal cord sections were prepared 2 weeks or 4 weeks after spinal cord injury (SCI) by contusion and immunostained with anti-GFAP antibody to identify astrocytes and counterstained with Hoechst dye for nuclei. The number of astrocytes and nuclei in SJH-treated group was higher than saline group or intact control . Rectangular areas in cross sections were seen in enlarged views after GFAP and Hoechst staining.

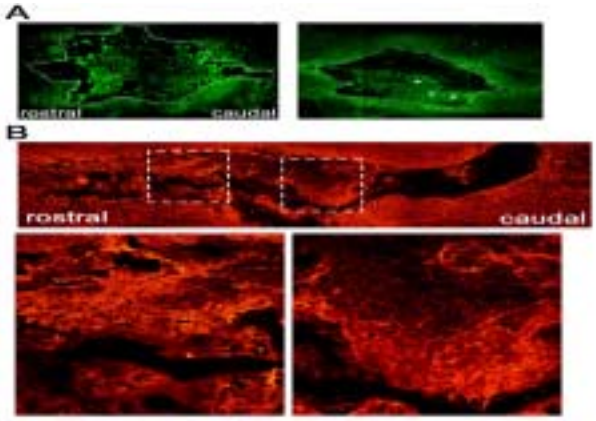

Fig. 3. Immunofluorescence staining of cavity area in the spinal cord. (A) CSPG immunostaining defined glial scarring in saline- and SJH-treated spinal cord 2 or 4 weeks after contusion injury. CSPG immunopositivity within the cavity was also visualized trabecula structure (marked as asterisks). (B) GFAP-staining of astrocytes in the contused cavitation area. Spinal cord sections were prepared 4 weeks after injury. Stronger GFAP staining was observed within the cavity in SJH-treated tissue than saline-treated group.

It has been previously shown that contused spinal cord tissue develops glial scar lasting several weeks to longer than a month. To examine the effect of SJH on injury development in terms of glial scarring, tissue distribution of chondroitinsulphate proteoglycan (CSPG), a major component of glial scar in the injured spinal cord, was determined by immunofluorescence staining. As shown in Fig 3A, the trabecula structure was well observed in the cavity in the contused area in SJH-treated group at 4 weeks after injury. It was further observed that the size of injury cavity, as a boundary of glial scarring surrounding the cavity, was confined in a smaller area in SJH-treated group than saline-treated group. GFAP staining was further investigated in the epicenter cavity. Staining intensity of GFAP-positive cells were much higher in SJH-treated group than saline group. Astrocyte staining was particularly noted in trabecula within the epicenter in SJH-treated group(Fig. 3B).

3. Effects of SJH treatment on CST axonal elongation after spinal cord injury

To identify CST in lower thoracic level, DiI fluorescence dye was microinjected into the sensorimotor cortical area in rat brain and axonal tracts were observed 5 days later. Fig 4A shows anterograde tracing of DiI-labeled CST axons in the spinal cord sections. Heavy DiI staining was seen in an area dorsomedial to the gray matter in the cross section.

To determine differences in axonal re-growth after contusion injury, DiI-labeled axons were observed in the cross section of the spinal cord rostrally adjacent to the epicenter area 4 weeks after injury. In saline-treated group, moderate level of axonal branches were observed in gray matter area (Fig 4B). Axonal arborization and elongation were largely increased by SJH treatment. Having confirmed that the arborization of CST axons in the vicinity of the epicenter, we investigated caudal extension of regrowing CST axons (Fig 4C).

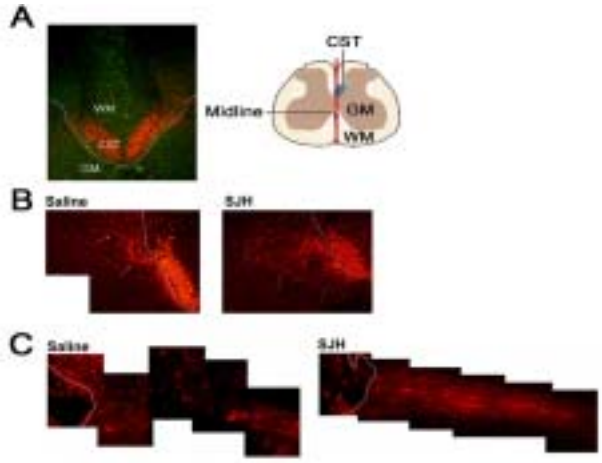

Fig. 4. Anterograde tracing of CST axons after SCI in the rats. (A) Identification of CST axons in the spinal cord by anterograde tracing. Five days after injection of DiI fluorescence dye into the motor cortex, spinal cord sections prepared at lower thoracic level was used for identification of DiI-labeled CST (in red) under the fluorescence microscope. DiI-labeled CST axons in cross sectional view of spinal cord at T11 were seen in the dorsomedial white matter (WM) (left). A diagram of spinal cord showing the location of CST (right). (B) DiI-labeled CST axons in the rostral boundary of the epicenter of the spinal cord were compared between saline- and SJH-treated animals. Transverse spinal cord sections were prepared 4 weeks after injury. CST axonal sprouting (marked as arrows) was seen in larger area penetrating into gray matter (GM) in SJH-treated group compared to saline-treated animal. (C) CST axons in the caudal region close to the injury site. Horizontal sections were prepared 4 weeks after injury and analyzed for axonal tracts in the caudal region. Longer and clearer DiI-labelled axons were seen in SJH group than in saline control.

CST axons were examined caudal area adjacent to the epicenter in saline and SJH-treated groups at 4 weeks after injury. It was observed that the CST axons were more clearly extended into the caudal part to the epicenter in SJH treat

group compared to saline treated group. Together these data suggest that SJH treatment may have growth promoting activity of CST axons after spinal cord injury.

Discussion

The present study was designed to investigate possible function of SJH in CST axonal responses after spinal cord injury. CST is one of the longest axonal tracts in the CNS and physiologically important for transmitting movement command from the brain to the whole body parts except the brain. We chose CST axons for the present study because a majority of CST fibers in rodents are positioned to the dorsomedial white matter in the spinal cord, which is thus advantageous for applying external manipulations such as trauma and drug administration[18,19]. Furthermore, CST injury can easily be monitored by several behavioral tests.

In oriental medicine, SJH has been used as one of the major components for making up many herbal decoctions. The extract is known to contain several monosaccharide and disaccharide including glucose, galactose, and sucrose, catalpol, vitamin A, beta-sitosterol and others[20]. SJH is known to supplement the function of erythropoiesis, liver, and kidneys, and the production of sperms and bone marrow. A range of its clinical application includes strengthening of the stamina, diabetes therapy, maintenance of regular menstrual cycle, and treatment of vestibular function such as dizziness and tinnitus. Although specific descriptions in relation to neurological disorders are lacking, physiological activities in the nervous system are governed in many aspects by kidneys and bone marrow, according to oriental medicinal theory, and thus, SJH could mediate neuronal functions.

In the present study, SJH was administered in vivo into the injury site of spinal cord at low thoracic level. Biochemical and histological analyses on the effects of SJH on injured nerve showed potentially positive function for axon elongation after spinal cord injury. SJH treatment on the injured nerve altered induction levels of phospho-Erk1/2 protein in the injured spinal cord area. Erk1/2 has diverse function including cell proliferation, differentiation, and survival[21,22]. Although more precise analysis would be required, the present data indicate upregulation of Erk1/2 at the axon terminal, which might interact with proteins such as actin in the growth cone and promote axonal migrating activity.

SJH treatment also enhanced Cdc2 protein levels in the injured spinal cord. Cdc2 is a protype of cell cycle proteins and is important for the transition of G2 to M phase in the cell cycle[23]. It has been recently shown that Cdc2 activity was upregulated in injured sciatic nerves and involved in axonal regeneration in rats[24]. As shown in Fig 2, stained nuclei and GFAP-positive astrocytes were increased by spinal cord injury and further upregulated by SJH treatment. A role of astrocyte in the injured CNS tissue is controversial; after spinal cord injury, the number of astrocytes is increased and involved in forming glial scar. Glial scar can limit inflammation responses and minimize pathologically harmful effects after spinal cord injury, but at the same time, inhibit axonal elongation across the injury area cavity. Adverse effect of CSPG-glial scar on axonal regeneration was experimentally demonstrated by inducing enhanced axonal regeneration of CST axons after spinal cord injury by administration of chondroinase ABC to lyse glial scar[9]. In contrast, a recent study demonstrated that Stat3-mediated astrocyte activity, possibly migrating activity, plays a role for axonal elongation after spinal cord injury, suggesting a beneficial function of astrocyte for regeneration[25]. According to our data, the number of astrocytes was increased by SJH treatment, suggesting positive correlation in nerve activities between astrocyte increase and SJH treatment.

In order to determine whether SJH treatment altered axonal elongation after spinal cord injury, CST axons were examined by anterogradely tracing technique. In the injured area, elongation of most of the CST axons was hampered in the cavity which was well observed 2-4 weeks after injury. However, careful observation revealed axonal sprouting possibly inducing detour path into the gray matter or toward the ventral zone in the white matter. This observation is consistent with previous reports showing that spinal cord injury with different intensity can generate axonal sprouting. Yet, this plasticity of axonal regrowth could be a different mechanism from axonal regeneration because axonal sprouting can begin from spared neurons[26-28]. According to our data, CST axons at the caudal area from the injury site showed increased arborization in SJH-treated group compared to saline group, suggesting that SJH treatment may contribute to induce axonal regrowth after spinal cord injury.

The present study further indicates that SJH treatment regulated the progression of inflammatory responses in the contused areas. The size of cavity bound by CSPG-stained scar was much reduced in SJH-treated group compared to saline group. Moreover, trabecula structure, which may potentially function as a bridge connecting between proximal and distal portion of the CST, was observed in the cavity area in SJH-treated tissues. In conclusion, our findings thus suggest that SJH may exert positive effects for possible removal of CSPG-mediated glial scarring in the injured spinal cord and further play a role in axonal regrowth after spinal cord injury

by regulating non-neuronal cell activities.

Conclusion

In oriental medicine, SJH has been used as one of the key components for the prescription of several herbal decoctions and applied for the treatment of several diseases including nervous system and cardiovascular disease. Here in the present study, possible growth promoting effects of SJH on injured spinal cord axons were investigated in rats. SJH administration appears to be effective for facilitating axonal regrowth beyond the epicenter of the injury area. The major findings are summarized as follows.

Phospho-Erk1/2 protein levels in the injury site of the spinal cord were elevated in saline and SJH-treated group compared to non-injury control in the spinal cord 7 days after injury. At 14 days after injury, elevated levels of phospho-Erk1/2 remained higher in SJH-treated group than saline control. Phospho-Edk1/2 protein levels in the motor cortex were higher in SJH- and saline-treated groups than non-injury control. Cdc2 protein was strongly induced in the injured spinal cord with saline or SJH-administered group at 7 days post injury, and then at 14 days, Cdc2 was sharply down-regulated to basal level. Cdk2 kinase was increased in the injured spinal cord with saline injection, and further increased by SJH-treatment. Cdk5 levels were not influenced by either injury or SJH-administration. In the motor cortex, levels of Erk1/2, Cdc2, Cdk2, and Cdk5 were not altered by SJH-treatment in the injured spinal cord. SJH-treatment enhanced axonal arborization and elongation of CST axons into and throughout the epicenter cavity of injured spinal cord compared to saline control. Extensive axonal sprouting and caudal extension were observed in SJH-teated group. In SJH-treated group, astrocytes in vivo and in culture showed increased proliferation compared to saline control and extensive cytoplasmic structures in cultured individual cells. In SJH-treated group, glial scarring when observed 1 month after injury was confined in a smaller area than saline-treated group. Behavioral scores on gridwalk 1 month after SCI showed significant improvement In SJH-treated group compared with saline control. Motor function and BBB tests did not show any signifiant differences between saline and SJH-treated groups.

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