

Original Article

Effects of *Sagunjang-Ga-Nokyong* on Neurologic Recovery in Rats after Spinal Cord Injury

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Objective : This study is investigate the effects of *Sagunjang-Ga-Nokyong*(SGJ-NY) treatment on regenerative responses of corticospinal tract(CST) axons in the injured spinal cord.

Methods : Using rats, we damaged their spinal cord, and then applied SGJ-NY extract to the lesion. Then we observed GAP-43 and NGF protein, astocyte, axonal regeneration responses and axonal elongation.

Result : Determination of GAP-43 and NGF protein levels were increased. And increased proliferation of astrocyte and enhanced processes in astrocytes were observed by SGJ-NY treatment. Higher number of astrocytes within the injury cavity in SGJ-NY treated group were showed, yet CSPG proteins were a weaker staining in the cavity in SGJ-NY. CST axons extended into the cavity and to the caudal area in SGJ-NY treated group were increased.

Conclusion : SGJ-NY treatment might increase neural activity in the injured spinal cord tissue, and improved axonal regeneration responses. In this process, activation of astrocytes may play a role in promoting enhanced axonal elongation. the current study show that SGJ-NY exerts positive activity on inducing nerve regeneration responses by elevating neural tissue migration activities.

Key Words : *Sagunjang-Ga-Nokyong*, spinal cord injury, neurologic recovery

Introduction

Axons in the central nervous system including the spinal cord are not able to regenerate when a certain levels of physical damage is given, and result in permanent functional defects in affected target organs¹⁾. To understand molecular mechanism underlying responses of the injured tissue in relation to axonal regeneration or degeneration processes, there have been numerous studies which were mostly devoted to identify molecular factors and tissue changes^{2,3)}.

One important finding to explain the failure of axonal regeneration in the central nervous system was the identification of myelin inhibitory factors

from oligodendrocytes and their signal transduction into the injured axons. Besides these factors however, many other factors including molecular components in the astrocytes and microglial cells are known to be directly or indirectly involved in axonal responses. For instance, chondroitin sulphate proteoglycan (CSPG), which is produced and secreted from astrocytes, is known to a major factor of glial scar that forms around the injury cavity(IC) and acts a barrier of axonal regrowth^{4,5)}.

While the identification and regulation of endogenous molecular factors might be critical for studies on axonal regeneration of injured spinal cord nerves, herbal drugs have been implicated as alternative, potential therapeutic agents for the cure

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of injured nerves⁶⁾. Several herbal drugs such as buyanghuanwutang and ginsenoside Rb1 have been shown to be useful for inducing facilitated axonal regrowth of injured peripheral nerve^{7,8)}.

Sagunjatang(*SijunZi-tang* ; *SGJ*) supplemented with *Nokyong*(*Cervi Pantorichum Cornu* ; *NY*) is one of the recipes which is currently used for clinical practice in the oriental medicine. According to recent studies in the oriental medicine, *SGJ* decoction is effective for the treatments of spinal cord function⁹⁾, blood coagulation¹⁰⁾, immune system disorders¹¹⁾, growth disorders¹²⁾. *NY* is a drug to nourish the 'Yangxu', and also supplement the 'Yin' in the kidneys which is known to be essential for functional activation of the nervous system¹³⁾. Yet, the examination of *SGJ* and/or *NY* in relation to neural responses at cellular and molecular levels after spinal cord injury has not been examined.

Thus in the present study, a possible growth promoting effect of *Sagunjatang-Ga-Nokyong*-treated animal group given spinal cord injury(*SGJ-NY*) on injured spinal cord nerve was investigated in an experimental rat model given spinal cord injury. And the current study show that *SGJ-NY* exerts positive activity on inducing nerve regeneration responses by elevating neural tissue migration activities.

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°C), 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 *NY* (*Cervi pantotrichum cornu*) 4 g and *SGJ* (*SijunZi-tang*) composed of *Insam* (*Ginseng radix*) 4 g, *Baikchul* (*Atractylodis macrocephalae*

rhizoma) 4 g, *Baikbokryung* (*Poria*) 4 g and *Gamcho* (*Glycyrrhizae radix*) 4 g were obtained from Daejeon University Oriental Medicine Hospital (Daejeon, Korea). Dried drugs were resuspended in 2 liters of water, heat and extracted with 2 liters of water for 3 h, and filtered three times. Concentrated solutions were frozen at -70°C for 4 h, and freeze-dried for 24 h. The yield for *SGJ-NY* was 7 g for 50 g of the initial raw materials respectively. Purified drug was kept at 4°C, and dissolved in water. The stock solution was stored at -20°C 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 the dura of the spinal cord at T9 - T10. A contusion injury was induced using a home-made *NYU* devise by dropping a 10 g weight from 25 mm onto the exposed dura of the spinal cord¹⁴⁾. 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) Drug administration

For drug treatment into the spinal cord, drug (10 $\mu\ell$ in PBS) or an equal volume of PBS was injected into the contused spinal cord by using micropipet. In case of spinal cord injury experiment, drugs (100 $\mu\ell$) were then i.p. supplemented every other days for a two week period.

3) Immunohistochemistry

For immunohistochemistry experiment, dissected tissues were frozen immediately at -75°C and embedded into the OCT medium. The spinal cord sections (20 μm) were cut using a cryostat and mounted on positively charged slides. Sections were used for immunofluorescence staining, hemotoxylin and eosin (H & E) staining, or Hoechst staining. Individual

experimental procedures are described below.

(1) Immunofluorescence staining

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, permeabilized 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 primary antibody, and then incubated with secondary antibody such as fluorescein-goat anti-mouse (Molecular probes, USA) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes, USA) 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 with corresponding specific secondary antibody reaction. For some experimental purpose, Hoechst staining reaction for nuclear visualization was performed after the first washing step after secondary antibody reaction (see below for the experimental details of Hoechst staining). Control sections treated with secondary antibody alone usually did not have any visible images. In cases when the nonspecific signals were high, all the data from those experiments were not further analyzed. Sections were observed with a Nikon fluorescence microscope and the images were captured by using Nikon camera (Nikon, Japan). The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5). The antibodies used for immunofluorescence staining were anti-GFAP antibody (Santa Cruz Biotech., USA), anti-CC1 antibody (Santa Cruz Biotech., USA), anti-CS56 antibody (Santa Cruz Biotech., USA).

(2) Hoechst staining

Hoechst 33258 dye (Sigma, USA) was used to visualize individual cells by staining nucleus. Tissue sections were treated with 25 $\mu\text{g}/\text{ml}$ of Hoechst in

0.1% triton X-100 in phosphate-buffered saline solution (PBST) for 10 min. Cell nuclei were observed blue color under the fluorescence microscope.

4) Western blot analysis

Nerve tissues were washed with ice-cold PBS, and sonicated under 50 - 200 μl 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 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 3 μM benzamidine, 0.5 mM DTT, 1 mM PMSF). Protein (10 μg) 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°C 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-GAP-43 antibody (Santa Cruz Biotech., USA), anti-NGF antibody (Santa Cruz Biotech., USA).

5) Anterograde tracing

DiI as anterograde tracer was treated into the sensorimotor cortical area in the rats 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 μl 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^{15,16}. Sections of brain and spinal cord sections at the low thoracic level were prepared and observed under the fluorescence microscope. To identify labeled cells, animals were anesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde in PBS. Sagittal brain sections were prepared and observed under the fluorescence microscope.

6) Astrocyte culture

35 mm culture dishes or 12 mm glass coverslips were precoated with polyornithine overnight at 37°C. The dorsal part of spinal cord T9-T11 was minced and dissociated with 2500 U/ml XI collagenase (Sigma, USA) for 30 min at 37°C, 5% CO₂ incubator for astrocyte cell culture. Then, the sample was centrifuged for 1 min at 3000 rpm and the supernatant was removed. Cells were resuspended in 500 µl DMEM containing type SII trypsin (Sigma, USA) to 0.5 mg/ml for 15 min and treated with EDTA (1 mM), soybean trypsin inhibitor (100 µg/ml), and DNase I (80 µg/ml) during the last 5 min. Cells were washed twice by resuspension and centrifugation. After resuspending with DMEM containing 10% FBS and cells were plate on precoated coverslip in 24 well for immunostaining, and incubated for 3 days at 37°C, 5% CO₂ incubator. Cells were fixed for immunofluorescence staining.

Results

1. Effects of SGJ-NY treatment on the production of regeneration-associated proteins in the injured nerves.

Growth Associated Protein 43 kDa (GAP-43) protein was not detected in the normal spinal cord tissue and then its synthesis was induced in the nerve tissue 7 days after contusion injury(Fig. 1A). When SGJ-NY were administered into the injury site, levels of GAP-43 protein were further enhanced. Similar changes in Nerve Growth Factor(NGF) protein was observed in the injured spinal cord tissue(Fig. 1B); levels of NGF protein in intact spinal cord tissue were low, and then increased after contusion injury. Treatment of SGJ-NY further upregulated NGF protein in the injury area.

2. Effects of SGJ-NY treatment on non-neuronal cell growth

To examine whether SGJ-NY affects on the growth of non-neuronal cells in the injured spinal cord tissues, cells were analyzed by *in vitro* primary culture and *in vivo* tissues. In cultured primary cells prepared from dissociated spinal cord tissues at the injury area, pretreatment of SGJ-NY showed increased number of GFAP-positive cells indicating increased responsiveness of astrocytes in culture(Fig. 2). It was also observed that CCI protein positive cells was detected in SGJ-NY treated group, but not in saline-treated group, indicating facilitated growth of



Fig. 1. Western blot analysis of GAP-43 and NGF protein in the injured spinal cord.

Seven days after contusion injury given saline or SGJ-NY, a dorsal half of the injured spinal cord tissue was excised and used for cell lysate preparation. Intact control group (CTL) was prepared as a control. (A) GAP-43 protein levels were induced after contusion (Saline-treated animal group given spinal cord injury; Sal) and further elevated by SGJ-NY treatment. (B) Increases in NGF protein levels were observed in the spinal cord tissues given SGJ-NY treatment.

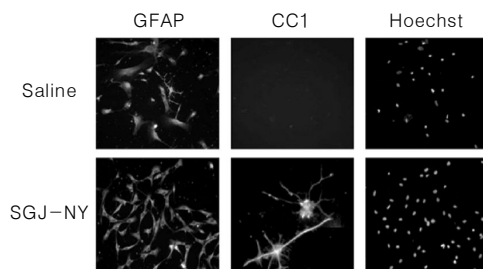


Fig. 2. Immunofluorescence staining of cultured cells prepared from the spinal cord tissue.

When the contusion injury was given, saline or SGJ-NY was injected into the injury site, and the tissue was prepared 7 days later. Dissociated cells were plated and cultured for two days, fixed, and used for immunofluorescence staining with anti-GFAP antibody for astrocyte and anti-CC1 antibody for oligodendrocyte identification respectively. Total individual cells in the plate were identified by Hoechst nuclear staining.

CC1 protein-positive oligodendrocyte by SGJ-NY treatment. Hoechst nuclear staining for culture cells showed increases in total cell number in the SGJ-NY treated group compared to saline treated group.

3. Histological comparison on the effects of SGJ-NY treatment on spinal cord tissues,

Effects of contusion injury on the spinal cord were investigated using the spinal cord sections. H & E staining in the areas of contusion showed clearly the injury cavity with increases in its size after 4 weeks injury when compared with that after 2 weeks injury (Fig. 3). It was also observed that hemotoxylin staining intensity (in blue) was increased in the injury area of the spinal cord tissues 2

weeks after injury. Then in the tissues 4 weeks later, hemotoxylin staining intensity was decreased although still higher than normal tissues. It was also observed in the tissue prepared 4 weeks after injury that non-specifically aggregated cell debris were seen around the injury area.

Possible changes in response pattern of astrocytes in the injured spinal cord area were examined by immunofluorescence staining with anti-GFAP antibody. Longitudinal spinal cord sections at thoracic level 9-10 prepared from the intact animal revealed the presence of moderate levels of GFAP-positive astrocytes in the white matter area which was the target of contusion injury and where the corticospinal tract (CST) was included. Contusion injury for 2 weeks

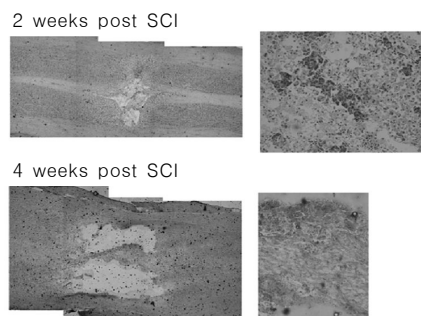


Fig. 3. Histological staining of injured spinal cord tissue.

Two or four weeks after spinal cord injury (SCI), horizontal sections of the spinal cord were prepared and used for histological staining with H & E.

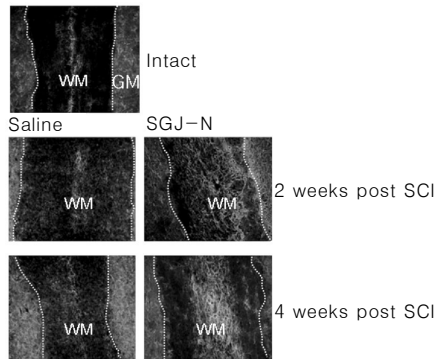


Fig. 4. Immunofluorescence staining of astrocytes in the spinal cord tissues.

Two or four weeks after contusion injury, spinal cord sections 5 mm rostral to the injury site were prepared and used for immunofluorescence staining with anti-GFAP-antibody to visualize astrocytes (shown in red). Spinal cord sections from an intact animal group were also prepared and compared at the similar levels of the spinal cord. Overall, the number of GFAP-positive astrocytes was much lower in the white matter (WM, marked by dotted lines) than the gray matter (GM).

resulted in slight increases in GFAP-positive cells in the white matter area (Fig. 4). Comparison between saline and SGJ-NY treated group did not show much more intense GFAP-staining of astrocytes in corresponding white matter area. In the sections prepared 4 weeks after injury, staining intensity and the number of astrocytes were higher in SGJ-NY treated group compared to saline treated group. In gray matter area, similar levels of GFAP staining of astrocytes were evenly observed in all the groups

examined.

A pattern of GFAP-immunostaining was investigated in the injury area of the spinal cord. As shown in Fig. 5, intense astrocyte staining in the epicenter of the cavity was observed. Some protuberance of tissue into the cavity (trabecula) was well observed in 4 week injury group (marked square). SGJ-NY treatment generated relatively unclear boundary of the cavity at 2 weeks after Spinal Cord Injury. At 4 weeks after SCI, a development of the trabecula

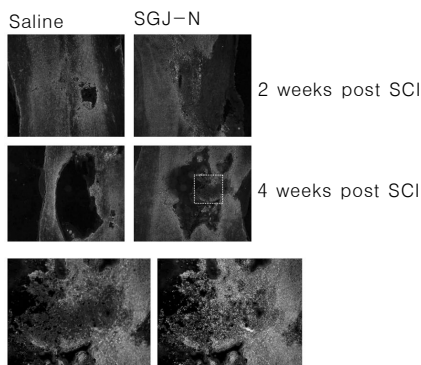


Fig. 5. Immunofluorescence staining with anti-GFAP antibody of injured spinal cord tissues.

At two or four weeks after contusion injury, longitudinal sections including the injury area were prepared and used for immunofluorescence staining. GFAP-positive astrocytes were seen in red. (Bottom picture) A section prepared 4 weeks after injury along with SGJ-NY administration was also treated with Hoechst dye for nuclear staining. An area with dotted square was enlarged and merged image with Hoechst staining (in blue) was presented.

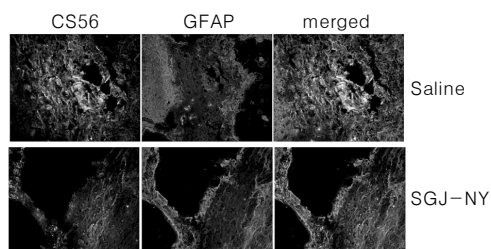


Fig. 6. Immunofluorescence staining of contused spinal cord sections with anti-CS56 and anti-GFAP antibodies. Four weeks after contusion injury, the sections were used for Immunofluorescence staining. Signals for CS56 protein (in green) and GFAP (in red) indicate the presence of CSPG protein and astrocytes in the tissue respectively. Merged images further demonstrate colocalization of two proteins.

structure was observed in SGJ-NY treated group. Hoechst nuclear staining of trabecula structure revealed strong signals in some regions where no astrocyte positive staining was present, indicating that a part of cavity zone was filled with cells other than astrocytes.

To determine whether SGJ-NY affected levels of chondroitin sulphate proteoglycan (CSPG) in the injury area, the spinal cord sections were used for immunofluorescence staining with anti-CS56 antibody that recognizes CSPG. In saline-treated group, the area of cavity showed strong intensity of CSPG staining along with moderate levels of GFAP sign-

aling whereas levels of GFAP staining was much weaker in SGJ-NY treated group (Fig. 6). A large portion CS56 positive area also showed GFAP-positive indicating the colocalization of two proteins in the tissue. CS56 stained area was restricted within the cavity rendering the epicenter confined in a limited area.

4. Effects of SGJ-NY on regrowth of corticospinal tract

To examine the potential facilitating effects of SGJ-NY on axonal regeneration after injury, regrowth of corticospinal tract (CST) axons after contusion

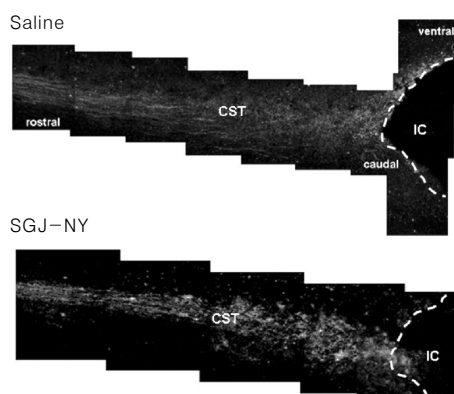


Fig. 7. Anterograde tracing of regenerating CST axons in the injured area.

At the time when the contusion injury was given, Dil was injected into the sensorimotor cortical area. Saline or SGJ-NY was administrated into the injury area at the time of injury and supplemented thereafter (see Material and Methods for details). Two weeks later, sagittal spinal cord sections were prepared and Dil-labeled CST axons were visualized under the fluorescence microscope. IC ; injury cavity

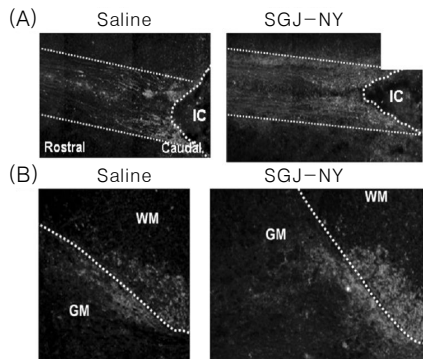


Fig. 8. Anterograde tracing of regenerating CST axons and observation of collateral sprouting of axons around the injured area.

At the time when the contusion injury was given, Dil was injected into the sensorimotor cortical area. Saline or SGJ-NY was administrated into the injury area at the time of injury and supplemented thereafter (see Material and Methods for details). Two weeks later, horizontal spinal cord sections were prepared and Dil-labeled CST axons were visualized under the fluorescence microscope. (A) Horizontal spinal cord sections at the injury site and adjacent rostral region. The region marked by the lines indicates CST and the injury cavity, marked 'C' with dotted boundary were shown in the Figure. (B) The CST axons in the transverse sections in the rostral area close to the injury cavity (within 3 mm distance from the injury site) were visualized under the fluorescence microscope. WM ; white matter, GM ; gray matter

injury was traced by fluorescence dye DiI. As shown in Fig. 7, in saline treated group, strong inhibition of CST axons' elongation was observed in an area of cavity formed by contusion injury. In SGJ-NY treated group, axon elongation was also prohibited, but some penetration and arborization into the cavity zone was observed.

To further examine possible differences between saline and SGJ-NY treated groups in CST axon's arborization, horizontal and transverse sections around the cavity was investigated. As shown in Fig. 8A,

more intense axonal arborization was observed in horizontal sections of SGJ-NY treated group compared with saline treated group. These collateral sprouting of axons were seen penetrating into the gray matter area. Also, the transverse sectional observation of spinal cord sections around the injury region showed more intense staining of CST axons in the gray matter area and collateral arborization in SGJ-NY treated group compared to saline treated group.

To determine whether the treatment of SGJ-NY

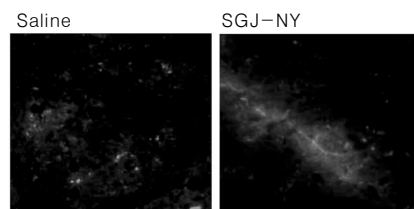
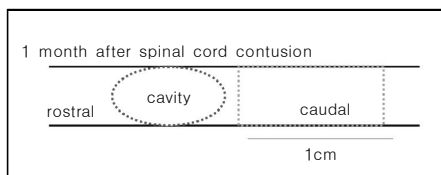


Fig. 9. Anterograde tracing of regenerating CST axons in the region caudal to the injured area.

At the time when the contusion injury was given, Dil was injected into the sensorimotor cortical area. Saline or SGJ-NY was administrated into the injury area at the time of injury and supplemented thereafter (see Material and Methods for details). Four weeks later, sagittal spinal cord sections were prepared and Dil-labeled CST axons in an area ca dal to the injury area were visualized under the fluorescence microscope.

had any effects of axons extension caudal to the injury area, DiI-labeled axons were examined in the sagittal spinal cord sections down to the injury area. As shown in Fig. 9, DiI labeled axons was seen at low levels in saline treated group and the treatment of SGJ-NY enhanced DiI-labeled CST axons in the caudal region.

Discussion

In general, injured nerves in the central nervous system do not regenerate, and consequently, the damage in the spinal cord results in serious functional deficits because there are several important axonal tracts conducting sensory information from the body and also motor activities transmitting from the brain¹⁷. Moreover, spinal cord is a thin and elongated structure and thus might be easily damaged despite the protective vertebrate structure. Indeed, there are increasing number of patients with spinal cord injury worldwide in part because of car and motor cycle accidents and severe physical activities¹⁸. Thus, it would be important to develop for the cure of spinal cord damage.

There have been extensive efforts to understand mechanism underlying CNS nerve injury and to develop therapeutic strategies for functional recovery of injured spinal cord. Using experimental animals such as rat and mouse, it was found that there are important molecular factors known to inhibit axonal outgrowth after injury in the CNS. Similarly, CSPG, produced from astrocyte, was shown to be secreted into the extracellular space and forms a macro0 molecular barrier structure which is called glial scar^{4,5}. In pathological point of view, glial scar is a defensive structure in that it can minimize inflammatory responses caused by spinal cord injury². Yet, it prevents injured axons from elongation beyond injury area. Several lines of studies, which has been studies in rats, show that the blockade of these inhibitory molecular factors by using drugs, gene inactivation techniques, antibody therapies and others was at least helpful for inducing the regrowth of

injured spinal cord axons¹⁹. Second, axon itself in the CNS, unlike peripheral nerve axons, may have intrinsic properties of limited regeneration property. For instances, peripheral axons after injury induce gene expression such as GAP-43 involved in axonal regeneration; however, CNS axons are not as efficient as PNS axons in responding to positive signals for axonal regeneration^{20,21}. Besides the factors as mentioned above, researchers believes that other unknown factors are involved in regenerating neural responses after spinal cord injury.

Due to the complexities of responsiveness of CNS neurons after damage, possibly reasonable approaches for the achievement of axonal regeneration would be combinatorial application of diverse therapies. Although several experimental approaches suggest possible improvement in recovery responses, it seems that there are many facts to be solved before further consideration into clinical application. Considering all these complexities, it would be important to consider application of oriental medicinal treatment. SGJ has been used as the prescription ever since Song dynasty in China, and used for the treatments for symptoms for cardiovascular¹⁰, immune¹¹ and skeletal muscle systems⁹. SGJ is composed of four herbal components; the Insam augments the digestive function and nourishes the vital essence, Baikchul and Baikbongryung regulates the spleen and adjust the moisture, and Gamcho supplements the vital essence. SGJ is known to be effective for diverse pathophysiological symptoms including the neurological disorders.

NY is also effective for nourishing the Yangxu, and supports the kidney Yin²². Thus, the combined used of SGJ and NY was expected to improve major physiological functions in the body and can be used for the treatments of diverse pathophysiological symptoms²³. It is particularly noted that NY, when applied via acupuncture therapy together with *Yanghyuljanggeunkeonbotang* (*Yangxuezhuangjin-jianbutang*), can improve physical activity and rehabilitation activity after spinal cord injury in the rats²⁴. NY, together with SGJ, may be more effective

for generating nerve regenerative activity.

In order to observe possible changes in neural responses in the injured spinal cord tissue, the present study was designed to examine biochemical and cell biological changes as well as histological responses by SGJ and NY treatment after spinal cord injury. Herbal drug, SGJ supplemented with NY (SGJ-NY) has been selected and examined whether they have any positive effects on neural responses of the injured spinal cord tissues.

The rat spinal cord was used as an experimental model. After contusion injury at thoracic 9-10 levels, accompanying biochemical, cell biological and histological changes were investigated to determine the effects of SGJ-NY treatment. As the first step, changes in levels of proteins known to be directly or indirectly involved in neural responses after nerve tissue injury were examined. GAP-43, NGF were chosen as target proteins. It has been well known that GAP-43 and NGF are important for axonal elongation in neurons during development and after peripheral axonal injury. GAP-43, an axonal growth-associated protein with an apparent molecular weight 43, was extensively studied and known to be strongly increased in the nerve tissues after injury²⁵. Previous studies have further shown that GAP-43 induction was regulated at gene expression levels at both transcriptional and posttranscriptional stages²⁶. Also, posttranslational modification featuring phosphorylation by protein kinase C and CaM kinase is also important for functional activation of the protein at subcellular level²⁷⁻²⁹. Yet, its effects in the central nervous system appears less clear than in the peripheral nervous system because CNS axons after injury cannot regenerate; yet GAP-43 increases were reported in the CNS axons undergoing sprouting after injury^{30,31}. NGF, nerve growth factor, is also known to be involved in neurite outgrowth and thus its upregulation in damaged neurons has been implicated to axonal regrowth activities in both PNS and CNS. In the present study, both GAP-43 and NGF proteins were increased in injured spinal cord tissues and further

upregulated by SGJ-NY treatment. These results suggest that SGJ-NY may have similar activity as the injury stimulation conferred to the injured nerves.

Then, the potential role of astrocytes in neural activation in the injured spinal cord was examined by cell biological and histological approaches. In cultured cells prepared from the spinal cord tissues, astrocytes were identified as the major cell type and the processes (; expanded cell body and outward peripheral protrusions) were more clearly seen in the cells treated with SGJ-NY. In cultured cells, oligodendrocytes were rarely observed, but the chances of finding these cells were increased by SGJ-NY treatment. Thus, it seems that SGJ-NY increased cell activity when evaluated under cultured condition.

To determine the effects of SGJ-NY on axonal growth activity, CST axons were investigated by using anterograde tracing technique. CST axons are one of the longest ones which extends from the sensorimotor cortex all the way to the ventral horn of the spinal cord at every levels. CST is permanently responsible for body muscle movement via transmitting synaptic activity to lower motor neurons in the spinal cord, and then directly innervates skeletal muscle in all body parts except the brain³². CST has been used for the investigation of axonal responsiveness after spinal cord injury since it can be relatively easier to histologically analyze and examine physiological consequences. That is, the CST is largely localized in the dorsomedial white matter area, and therefore they can be manipulated for contusion injury. Physiological activity-behavioral effects- can be assessed by well-developed methods such as BBB and motor score test and gridwalk test^{33,34}. However, most importantly, individual axons of the CST can be traced well by anterograde tracing because the single axons are extended throughout all the levels of the spinal cord.

In this study, CST axons were well traced DiI injection into the brain for 5 days or longer, and they were well visualized under the fluorescence microscope. In saline-treated injury group, most of

the CST axons stopped elongation before the injury cavity. Yet, in the horizontal sections, collateral close to the rostral area from the injury site were observed. This arborization or axonal sprouting processes were increased in SGJ-NY treated group. Yet in both groups, any clear extension of CST axons within in the injury cavity area were rarely observed although local DiI staining within in the cavity were seen more in SGJ-NY group than saline group. Final interesting finding was the observation of CST axons in the spinal cord area caudal to the injury site. DiI-labeled CST axons at the caudal area were seen more clearly in SGJ-NY group than saline group. These data raise the possibility that enhanced arborization which were developed well in SGJ-NY group, might extend their detour pathway around the epicenter of the injury site, and extend to the caudal portion of the spinal cord. This interpretation is consistent with previous reports that application of positive factors for regeneration after spinal cord injury induced axonal sprouting as an activity of increased neural plasticity, and sometimes, contributed to functional recovery.

Taken together, SGJ-NY treatment appeared to induce neural activity at several different levels - astrocyte activation, enhanced axonal elongation.

Considering that SGJ-NY is composed of five different herbal drugs, which of each contains numerous chemical ingredients it would not be surprising to observe divers possible activity of SGJ-NY on injured spinal cord tissue. Future study would be critical to characterize specific chemical components that are to confer growth-promoting activity of injured axons.

Conclusions

The present study was performed to investigate the effects of SGJ-NY treatment on regenerative responses of CST axons in the injured spinal cord. It was found that SGJ-NY treatment was involved in regulating the neural responses and showed

positive effects on axonal elongation. The major findings are summarized as below.

1. Determination of GAP-43 and NGF protein levels by Western blot analysis showed increases by SGJ-NY treatment.
2. Increased proliferation of astrocyte were observed by SGJ-NY treatment in cultured cells. Enhanced processes in astrocytes were observed by SGJ-NY treatment.
3. GFAP immunofluorescence staining of injured spinal cord tissues showed higher number of astrocytes within the injury cavity in SGJ-NY treated group than saline control group. Yet, CSPG protein staining as a marker for glial scar showed a weaker staining in the cavity in SGJ-NY group than saline group.
4. Anterograde tracing for the detection of CST axons showed increased CST axons extended into the cavity and to the caudal area in SGJ-NY treated group than saline control.

These data suggest that SGJ-NY treatment might increase neural activity in the injured spinal cord tissue, and improved axonal regeneration responses. In this process, activation of astrocytes may play a role in promoting enhanced axonal elongation. Further studies on identifying specific component in SGJ-NY associated with enhanced neural activity in the injured spinal cord may be helpful to develop therapeutic strategies for the treatment of spinal cord injury.

References

1. Takekazu Kubo, Atsushi Yamaguchi, Nobuyoshi Iwata, Toshihide Yamashita. The therapeutic effects of Rho-ROCK inhibitors on CNS. disorders Ther Clin Risk Manag. 2008;4(3):605-15.
2. Bareyre FM, Schwab ME. Inflammation, degeneration and regeneration in the injured spinal cord : insights from DNA microarrays. Trends Neurosci. 2003 Oct;26(10):555-63.

3. Schwab ME, Bartholdi D. Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev.* 1996 Apr;76(2):319-370.
4. Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci.* 2006 Aug;7(8):617-627.
5. Silver J, Miller JH. Regeneration beyond the glial scar, *Nat Rev Neurosci.* 2004 Skene, J.H.P. and Willard, M. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous system. *J. Cell Biol.* 1981a; 89:96-103.
6. Cheung ZH, So KF, Lu Q, Yip HK, Wu W, Shan JJ. Enhanced survival and regeneration of axotomized retinal ganglion cells by a mixture of herbal extracts. *J Neurotrauma.* 2002;19:369-378.
7. Chen YS, Wu CH, Yao CH, Chen CT. Ginsenoside Rb1 enhances peripheral nerve regeneration across wide gaps insilicone rubber chambers. *Int J Artif Organs.* 2002;25(11):1103-1108.
8. Cheng YS, Cheng WC, Yao CH, Hsieh CL, Lin JG, Lai TY, Lin CC, *Am J Chin Med.* Effects of buyang huanwu decoction on peripheral nerve regeneration using silicone rubber chambers. 2001;29(3-4):423-432.
9. Lee SG. The effect of Sagunjatang on spinal motoneurons damaged by oxidant-induced neurotoxicity. *Wonkwang Oriental Medicine College.* 1998.
10. Ha JY. Effect of Samultang and Sagunjatang on the Intravascular Caogulation Induced by Endotoxin in rats. *Journal of KyungHee Oriental Medicine College.* 1998;11:113-122.
11. Kim SH. The Effect Of Sagoonjatang, Samooltang and Palmooltang on Depressed Immune Response in Mice Induced by Prednisolone. *KOREAN JOURNAL OF ORIENTAL MEDICAL PATHOLOGY.* 1987.
12. Bae JK. Influence of Sakunza-Tang and Samul-Tang Extract Administration on Growth in Rats. *Journal of KyungHee Oriental Medicine College.* 1978.
13. Jung YP. The Antioxidant Effects of Cervi Parvum Cornu on GC-1 spg Cells. *Graduate school of Kyung Hee University.* 2006.
14. Gruner JA. A monitored contusion model of spinal cord injury in the rat. *J Neurotrauma.* 1992;9(2):123-126.
15. Paxinos, G and Watson, C. *The rat brain in stereotaxic coordinates*, 4th ed. Academic Press, San Diego. 1998.
16. Behar O, Mizuno K, Neumann S, Woolf CJ. Putting the spinal cord together again. *Neuron.* 2000 May;26(2):291-293.
17. Bradbury EJ, McMahon SB. Spinal cord repair strategies: why do they work?. *Nat Rev Neurosci.* 2006 Aug;7(8):644-653.
18. Schwab ME. Repairing the injured spinal cord, *Science.* 2002 Feb 8;295(5557):1029-1031.
19. Kim JE, Li S, GrandPre T, Qiu D, Strittmatter SM. Axon regeneration in young adult mice lacking Nogo-A/B. *Neuron.* 2003 Apr 24;38(2):187-199.
20. Smith DS, Skene JH. A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. *J Neurosci.* 1997;17(2):646-658.
21. Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JH. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat Neurosci.* 2001;4(1):38-43.
22. Shin MK. *Clinical Herbology.* Seoul:Younghlimsa. 1988:183-186.
23. Lee SI. *Herbal clinical application.* Seoul:Sungbosa, 1986:331-333.
24. Park IG. Effects of Yanghyuljanggeuncheonbotang (Yangxuezhuangjin-jianbutang) and Cervi Cornu Parvum pharmaco-acupuncture on the motor function recovery and nerve regeneration in rats induced spinal cord injury. *Graduate School of*

- Dongshin University. 2007.
25. Skene, J.H.P. and Willard, M. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous system. *J. Cell Biol.* 1981b;89:96-103.
 26. Basi, G.S., Jacobson, R.D., Virag, I., Schilling, J. and Skene, J.H.P. Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. *Cell.* 1987;49:785-791.
 27. Alexander KA, Wakim BT, Doyle GS, Walsh KA, Storm DR. Identification and characterization of the calmodulin-binding domain of neuromodulin, a neurospecific calmodulin-binding protein. *J Biol Chem.* 1988;263(16):7544-7549.
 28. Apel, E.D., Byford, M.F., Au, D., Walsh, K.A. and Storm, D.R. Identification of the protein kinase C phosphorylation site in neuromodulin. *Biochemistry.* 1990;29:2330-2335.
 29. Skene JH. Axonal growth-associated proteins. *Annu Rev Neurosci.* 1989;12:127-156.
 30. Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, BrennerHR, Caroni P. Overexpression of the neural growth-associated protein GAP-43 induces nervesprouting in the adult nervous system of transgenic mice. *Cell.* 1995;83(2):269-278.
 31. Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell.* 1995;10;80(3):445-452.
 32. Bareyre FM, Kerschensteiner M, Misgeld T, Sanes JR. Transgenic labeling of the corticospinal tract for monitoring axonal responses to spinal cord injury. *Nat Med.* 2005 Dec;11(12):1355-1360.
 33. Basso DM, Beattie MS, Bresnahan JC. Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. *Exp Neurol.* 1996 Jun; 139(2):244-256.
 34. Dijkstra S, Duis S, Pans IM, Lankhorst AJ, Hamers FP, Veldman H, Bar PR, Gispen WH, Joosten EA, Geisert EE Jr. Intraspinally administration of an antibody against CD81 enhances functional recovery and tissue sparing after experimental spinal cord injury. *Exp Neurol.* 2006 Nov;202(1):57-66.