The Study on Regenerative Effects of Ginseng on Injured Axonal and Non-Neuronal cell

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Objective: This study was carried out to understand effects of ginseng (hereinafter; GS, Panax Ginseng) extract on regeneration responses on injured sciatic nerves in rats.

Methods: Using white mouse, we damaged sciatic nerve & central nerve, and then applied GS to the lesion. Then we observed regeneration of axon and non-neuron.

Results:
1. NF-200 protein immunostaining for the visualization of axons showed more distal elongation of sciatic nerve axons in GS-treated group than saline-treated control 3 and 7 days after crush injury.
2. GAP-43 protein was increased in the injured sciatic nerve and further increased by GS treatment. Enhanced GAP-43 protein signals were also observed in DRG prepared from the rats given nerve injury and GS treatment.
3. GS treatment in vivo induced increased neurite outgrowth in preconditioned DRG sensory neurons. In vitro treatment of GS on sensory neurons from intact DRG also caused increased neurite outgrowth.
4. Phospho-Erk1/2 protein levels were higher in the injured nerve treated with GS than saline. Phospho-Erk1/2 protein signals were mostly found in the axons in the injured nerve.
5. NGF and Cdc2 protein levels showed slight increases in the injured nerves of GS-treated group compared to saline-treated group.
6. The number of Schwann cell population was significantly increased by GS treatment in the injured sciatic nerve. GS treatment with cultured Schwann cells increased proliferation and Cdc2 protein signals.
7. GS pretreatment into the injured spinal cord generated increased astrocyte proliferation and oligodendrocytes in culture. In vitro treatment of GS resulted in more differentiated pericytoplasmic processes compared with saline treatment.
8. More arborization around the injury cavity and the occurrence at the caudal region of CST axons were observed in GS-treated group than in saline-treated group.

Conclusion: GS extract may have the growth-promoting activity on regenerating axons in both peripheral and central nervous systems.

Key Words: ginseng, axon, non-neuron

Introduction

Axons in the peripheral nervous system can regenerate, elongate their original target and induce functional recovery\textsuperscript{1,2}. This regeneration capability appears to be contributed to combined activity of axonal property and environmental influence\textsuperscript{1,2}. After nerve injury, lesion signals are known to retrogradely transported into the cell body and induce target gene expression whose products then

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are involved in axonal regeneration. As extrinsic environmental factors, activation of Schwann cells is critical. In an injury area, proliferation of Schwann cells followed by myelination of axons are important for regeneration process. Proliferating Schwann cells function to remove degenerating axonal debris at the distal portion of the axons and also provide a guide for axonal extension toward the original target.

Peripheral nerve regeneration, however, is known to depend on several factors. Even though axons are successfully extended distally, correct targeting is not always successful, and thus the optimization for axonal regeneration is important. In oriental medicine, ginseng (hereinafter; GS, Panax Ginseng) is one of the most important herbal drugs used in oriental medicine, and known to improve circulation, increase blood supply, revitalize and aid recovery from weakness after illness, stimulate the body by promoting yang energy and reducing yin energy. Ginseng is now also indicated for use as tonic or a prophylactic and restorative agent for enhancement of mental and physical capacities, in case of weakness, exhaustion, tiredness, loss of concentration, impotence, cold limbs, during illness or convalescence. And many comprehensive study were made about ginseng’s antioxidant activity, anticancer effect, anti-fatigue, immunological intervention and homeostatic effect.

Recent days, researches about ginseng’s effect on the cell cycle regulation and cell nerve regeneration were conducted. But nerve regeneration related studies were rarely done. In relation to axonal regeneration, ginsenoside Rb1 was reported to be effective for peripheral axonal regeneration and increased ginsenoside Rb1 and Rg1 were effective for facilitated peripheral nerve regeneration, Schwann cell proliferation, and for the survival of injured spinal cord neurons. Yet, systemic analysis of nerve regeneration has not been demonstrated in these studies. Thus in the present study, potential effects of GS extract were investigated whether they have any effects on regeneration responses on injured sciatic nerves in rats. The present data show that GS extract induced several important biochemical and physiological changes in the nerves undergoing axonal regeneration and related tissues. These responses may be associated with promoting regeneration activities either directly or indirectly.

### Materials and Methods

1. Materials

   (1) Experimental animals
   Sprague-Dawley rats (male, 7 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 GS was obtained from Daejeon University Oriental Medicine Hospital (Cheongju, Korea). GS 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 (Büchi 461, Eyela, USA). Concentrated solutions were frozen at -70°C for 4 h, and freeze-dried for 24 h. The yield for GS was 7 g for 82 g of the initial raw materials respectively. The product 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) Surgery
   1) Sciatic nerve surgery
   Sprague-Dawley rats were housed individually in cages of a temperature-controlled room. Animals were anesthetized with a mixture of ketamine (80 g/kg) and xylazine (5 mg/kg). Sciatic nerve was exposed and crushed with a pair of forceps held tightly for
30 sec twice at 1 min intervals. Then, 5 μl GS (10 mg/ml) in saline solution or the equal volume of saline were microinjected into the crush sites. Animals were recovered from anesthesia and sacrificed 7 days later. Animals were deeply anesthetized with a mixture of ketamine and xylazine, and sciatic nerves were separately dissected, immediately frozen, and kept at -70°C until use. For purpose of immunohistochemistry experiments, the sciatic nerve was prepared and longitudinal sections were prepared.

2) 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 - T11. A contusion injury was induced using a home-made NYU device by dropping a 10 g weight from 25 mm onto the exposed dura of the spinal cord23. A NYU device is an experimental device that automates the weight-drop rod. The wound was closed in anatomical layers and rats were placed in standard plastic cages until further treatment and given food and water ad libitum.

(2) Cell culture
1) Primary DRG sensory neuron culture

General principles for nerve culture were referenced as published previously. Glass coverslips were precoated with a mixture of poly-L-ornithine (0.1 mg/ml, Sigma) and laminin (0.02 mg/ml, Collaborative Research, USA) in a 37°C, 5% CO2 incubator. L4 and L5 Dorsal Root Ganglia (DRG) were removed from adult male rats, and placed in ice-cold DMEM medium (Gibco, USA). The ganglia were treated with DMEM containing type XI collagenase (2,500 U/ml, Sigma) for 90 min at 37°C. Tissues were then washed with DMEM medium and centrifuged at 800 rpm for 1 min to remove the supernatant. Cells were then treated with DMEM containing type SII trypsin (0.5 mg/ml) for 10 min followed by DMEM containing trypsin inhibitor (100 μg/ml), EDTA (1 mM) and DNase I (80 mg/ml) for 5 min. After washing cells with culture medium (DMEM containing 5% heat-inactivated FBS (Gibco, USA), 5% horse serum, 2 mM glutamine and 1% penicillin-streptomycin), 800-1200 neurons were plated onto 12 mm round coverslips and cultured for 12 h in a 37°C, 5% CO2 incubator and changed with fresh culture medium. DRG neurons were treated with GS (50 μg/ml) or saline vehicle and cultured for 24-48 h. Cells grown on the coverslips were fixed with 4% paraformaldehyde 4% sucrose solution for 45 min at room temperature and used for immunofluorescence staining. The images of immunostained cells were captured on the digital camera, and neurite arborization and length were quantitatively assessed by using the i-Solution software (Image & Microscope Technology, USA).

2) 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 2,500 U/ml XI collagenase for 30 min at 37°C, 5% CO2 incubator for astrocyte cell culture24,25. Then, the sample was centrifuged for 1 min at 3,000 rpm and the supernatant was removed. Cells were resuspended in 500 μl DMEM containing type SII trypsin 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) for 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 harvested and fixed for immunofluorescence staining.

3. Histochemistry

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 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 PBS (phosphate-buffered saline) at room temperature for 40 min, permeabized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4 h at room temperature. Sections were incubated with GFAP (anti-glial fibrillary acidic protein) antibody (Santa Cruz Biotech), then incubated with fluorescein-goat anti-mouse (Molecular probes) or rhodamine-goat anti-rabbit secondary antibodies (Molecular probes) in 2.5% horse serum and 2.5% bovine serum albumin for 1 h at room temperature and cover slipped with gelatin mount medium. Primary antibody reaction was performed with single or multiple antibodies depending on the experimental purposes, and followed with corresponding specific secondary antibody reaction. For some experimental purposes, Hoechst staining reaction for nuclear visualization was performed after the first washing step then 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. The merged images were produced by using layer blending mode options of the Adobe Photoshop (version 5.5).

Primary antibodies used in the present study were the ones raised against to following proteins: NF-200, GAP-43, β tubulin, phospho-Erk1/2, NGF, S100β, CC1, GFAP, and Cdc2. Secondary antibodies were rhodamine-conjugated or fluorescein-conjugated IgG’s.

2) Hoechst staining

Hoechst 33258 dye was used to visualize individual cells by staining nucleus. Tissue sections were treated with 25 μg/ml of Hoechst in 0.1% triton X-100 in PBST (phosphate-buffered saline solution) for 10 min. Cell nuclei were observed blue 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 Na3VO4, 1% triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/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 PVDF (polyvinylidenedifluoride) membranes (Millipore, Bedford, USA). Blots were blocked with 5% nonfat dry milk in PBST (17 mM KH2PO4, 50 mM Na2HPO4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween-20) for 1 h 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, Piscataway, USA), with horseradish peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibodies (Transduction Laboratories, Lexington, USA). Relative intensities of the protein bands were analyzed by autoradiography. The antibodies used in the present study were anti-Cdc2 antibody, anti-GAP-43 antibody, anti-NGF antibody, anti-Erk1/2 antibody, anti-phospho Erk1/2 antibody, and anti-actin antibody (clone no. C4, ICN Biomedicals).

5. Anterograde and retrograde tracing

DiI as anterograde tracer was treated into the...
sensorimotor cortical area in 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 CST (cortico spinal tract) was performed by injecting DiI (5 μl of 3% in DMSO) into the sensorimotor cortex (anterior-posterior 2.0 mm, medial-lateral 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. Sections of brain and spinal cord sections at the low thoracic level were prepared and observed under the fluorescence microscope. Retrograde labeling of neuronal cell bodies in the sensorimotor cortex was performed by injecting DiI (5 μl of 3% in DMSO) into the spinal cord at the thoracic level 9 - 11. Labeling of cortical neuron cell bodies was allowed to diffuse for at least 3 days. To identify labeled cells, animal was anesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde in PBS. Sagittal brain sections were prepared and observed under the fluorescence microscope.

6. Drug administration

Individual herbal drugs (5 μl in PBS) was injected into the crush injury site of the sciatic nerve by using micropipet. For drug treatment into the spinal cord, drug (10 μl) was injected into the contused spinal cord by using micropipet. In case of spinal cord injury experiment, drugs (1 μl) were then i.p. supplemented every other days for 2 weeks period.

Results

1. Observation of GS-mediated axonal elongation by NF-200 immunofluorescence staining in the injured sciatic nerves

NF-200 protein immunostaining for the visualization of axons showed more distal elongation of sciatic nerve axons in GS-treated group than saline-treated control 3 and 7 days after crush injury (Fig. 1, 2).

2. Changes of GAP-43 synthesis in relation to axonal regeneration and GS treatment

GAP-43 protein was increased in the injured sciatic nerve and further increased by GS treatment. Enhanced GAP-43 protein signals were also observed in DRG prepared from the rats given nerve injury and GS treatment (Fig. 3, 4).
Fig. 3. Examination of GAP-43 protein synthesis in the sciatic nerve.

The sciatic nerve was prepared 7 days after injury. When the injury was given, saline or GS was treated into the nerve.

(A) Western blot analysis of GAP-43 protein in the nerve. Protein lysate (10 μg) prepared from the nerve tissue (1 mm length around the crush injury) was used for analysis and GAP-43 protein band (43 kDa) was detected by anti-GAP-43 antibody.

(B) Immunofluorescence staining of sciatic nerve tissues prepared 7 days after crush injury and GS treatment. Axons were visualized with NF-200 staining and the merged images (in yellow) showed colocalization of GAP-43 and NF-200 signals. The image labeled ‘merged-enlarged’ indicates the enlarged view for the rectangular area in lower right image.

3. Effects of GS treatment on neurite outgrowth of cultured DRG sensory neurons and GAP-43 protein levels

GS treatment in vivo induced enhanced neurite

Fig. 4. GAP-43 protein expression in the DRG tissues.

The DRG from lumbar 4 and 5 were prepared from the rats which had undergone 3 (A) and 7 days (B) after crush injury of the sciatic nerve. Saline or GS were treated at the time of crush injury. Intact animals were used as a control. The sections (20 μm) were used for double immunofluorescence staining with Tubulin selective antibody (TuJ1) and anti-GAP-43 antibody.

Fig. 5. Pattern of neurite outgrowth of cultured DRG sensory neurons.

DRG culture was prepared from the rat which had given sciatic nerve crush injury for 7 days. Saline or GS was treated after nerve injury. Dissociated cells were prepared and cultured for 1 (A) or 2 days (B) and used for immunofluorescence staining with NF-200 and Hoechst nuclear staining. The images for NF-200 immunostaining were seen in green in the Figure and the images for Hoechst nuclear staining was merged onto the NF-200 stained image (labeled NF-200/Hoechst in A).
outgrowth in preconditioned DRG sensory neurons. In vitro treatment of GS on sensory neurons from intact DRG also caused increased neurite outgrowth (Fig. 5-7).

4. Effects of GS treatment on Erk1/2 protein synthesis in the injured sciatic nerves

Phospho-Erk1/2 protein levels were higher in the injured nerve treated with GS than saline. Phospho-Erk1/2 protein signals were mostly found in the axons in the injured nerve (Fig. 8).

5. Effects of GS treatment on NGF, and Cdc2 protein synthesis in the injured sciatic nerves

NGF and Cdc2 protein levels showed slight increases in the injured nerves of GS-treated group compared to saline-treated group (Fig. 9, 10).

6. Effects of GS on non-neuronal cell activity in the nervous system

The number of Schwann cell population was significantly increased by GS treatment in the injured sciatic nerve. GS treatment with cultured Schwann
Fig. 9. NGF protein expression in the injured sciatic nerve and the effects of GS treatments.

The sciatic nerve was prepared from the intact animal or crush injury for 7 days plus saline or GS treatment.
(A) Western blot analysis of NGF protein in nerve lysate in three different groups.
(B) Immunofluorescence staining of NGF protein in the sciatic nerve tissues. The nerve tissues were prepared as the same way as above and the longitudinal sections were used for immunofluorescence staining. Overlapping images for NF-200 (in green) and NGF (in red) were analyzed by merging images (in yellow).

Fig. 10. Cdc2 protein expression in the injured sciatic nerve and the effects of GS treatments.

The sciatic nerve was prepared from the intact animal or crush injury for 7 days plus saline or GS treatment.
(A) Western blot analysis of Cdc2 protein in nerve lysate in three different groups.
(B) Immunofluorescence staining of Cdc2 protein in the sciatic nerve tissues. The nerve tissues were prepared as the same way as above and the transverse sections were used for immunofluorescence staining. Overlapping images for Cdc2 (in green) and S100β (in red) were analyzed in merged images (in yellow).

cells increased proliferation and Cdc2 protein signals (Fig. 11, 12).

7. Effects of GS treatment on neural tissues in the injured spinal cord

GS pretreatment into the injured spinal cord generated increased astrocyte proliferation and oligodendrocytes in culture. In vitro treatment of GS resulted in more differentiated pericytoplasmic processes compared with saline treatment(Fig. 13, 14).

8. Effects of GS treatment on regenerative responses of injured CST axons

More arborization around the injury cavity and the occurrence at the caudal region of CST axons were observed in GS-treated group than in saline-treated group(Fig. 15-17).
In oriental medicine, GS has been widely used by the clinical evidence that it can improve the vital energy. It is further known that GS can protect major organs (liver, heart, spleen, lung and kidneys), stabilize the mental status, and thus strengthen the body by increasing resistance against external insults.

In relation to nervous system function, several studies have demonstrated that GS or its active constituents can positively influence nerve cells and nerve tissue. GS, in particular, has been shown to have neuroprotective effects against various insults, including traumatic brain injury and spinal cord injury. These effects are likely due to its ability to promote the survival of neural cells, inhibit apoptosis, and improve functional outcomes.

**Discussion**

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component, ginsenoside is important for regulating synaptic transmission and also has a beneficial effect on protecting neurons or non-neuronal cells from degeneration\textsuperscript{16,17}. Rb1 and Rg1 were shown to be effective for protecting hippocampal neurons and spinal cord neurons, and also can enhance learning and memory and neurogenesis\textsuperscript{18}.

The importance of studies on peripheral axonal regeneration is further extended, considering that it can provide useful information for axonal regeneration in the central nervous system\textsuperscript{19,20}.

Blockade of these molecules by gene knockout approach or by application of inhibitory molecules such as antibodies showed a certain level of axonal regeneration after spinal cord injury\textsuperscript{21}. Inhibitory factors were also found from astrocytes which are activated after spinal cord injury\textsuperscript{22}. Glial scar protein called CSPG (chondroitin sulphate proteoglycan) was shown to inhibit axonal extension beyond the injury cavity\textsuperscript{22}. Schwann cells, a major non-neuronal cell type in the PNS are rapidly proliferated and migrated into the distal portion of the injured axons undergoing regeneration response\textsuperscript{23,24}. Schwann
cells can remove the tissue debris and provide the pathway for regrowing axons. Thus, it is very important to understand the similarities and differences in responses of injured axons between PNS and CNS.

Here in the present study, GS extract was examined whether it has any effects on neural responses in the sciatic nerves as a model for examining the peripheral nerve responses. Then, the study was extended to the regeneration responses of injured axons in the spinal cord tissues. All these investigations were performed in rats and the data demonstrate activation of neural activities in terms of neurite outgrowth and non-neuronal cell growth.

The first major finding from the present investigation was that GS treatment into the injured sciatic nerve tissue induced enhanced axonal elongation and GAP-43 protein levels in the injured sciatic nerve. Axonal GAP-43 (growth associated protein) was initially identified as a protein strongly induced in the nerves given injury. In peripheral nerves after injury, GAP-43 is increased in the axons undergoing regeneration. Immunohistochemical studies showed that GAP-43 protein is expressed in the cell body and selectively transported into the axons. GAP-43 protein is also known to be phosphorylated by protein kinase C or CaM kinase and phosphorylated form of GAP-43 was shown to be highly localized in the presynaptic terminal or growth cones.

It was found in the present study that GAP-43 protein levels were increased by GS treatment in the injured sciatic nerve. The time course and spatial distribution of GAP-43 protein levels along the regenerating were highly correlated with NF-200 axonal staining pattern in the nerve. It was also observed that GAP-43 protein signals were similarly upregulated in the DRG at lumbar 5 where sensory neurons for sciatic motor nerve axons were located. Importantly, GAP-43 levels in both regenerating nerve area and DRG sensory neurons were upregulated by in vivo administration of GS treatment in the injury site. Thus, these data suggest that GAP-43 upregulation by GS treatment is induced possibly at gene expression level. GS treatment may induce some signaling event which could be transmitted to the nucleus for target gene expression including GAP-43 gene.

In this study, in vivo administration of GS increased neurite outgrowth compared to saline-treated group, suggesting that GS increased regeneration-related neuronal responses. It was also found that DRG sensory neurons given nerve injury plus GS treatment showed higher levels of GAP-43 protein levels compared to injury control group. Thus, GAP-43 may be one of the targets which may respond to GS treatment in the injured sciatic nerve.

As other target molecules for examining regeneration responses after GS treatment, levels of Erk1/2 were investigated in the injured sciatic nerve tissue. Erk1/2, together with p38 and JNK (c-Jun N-terminal kinase) belongs to MAP (mitogen activated protein) kinase. MAP kinase family proteins have a common property which is activated by phosphorylation by upstream protein kinases and in turn phosphorylates and activates their substrate proteins such as CREB (cAMP responsiveness element binding) protein. In cultured PC12 cells, JNK and p38 kinase are important for neuronal apoptosis whereas Erk1/2 protein is more important for cell survival. Since these MAP kinase family proteins are activated by phosphorylation, their activity is determined by measuring levels of phospho-Erk1/2 protein levels. In regenerating peripheral neurons, several studies have indicated that Erk1/2 activation is important for regenerative responses as evidenced by increased levels of phospho-Erk1/2 after nerve injury. These data suggest that phospho-Erk1/2 in the injured nerve may be induced by GS treatment and involved in axonal elongation. As mentioned above, any specific target substrate protein of phospho-Erk1/2 is not known although CREB are known to be activated by phosphorylation. Further studies will be helpful to identify downstream signaling and physiological consequences.

As other potential target proteins that might be
associated with axonal regeneration, NGF and Cdc2 were examined. NGF is a prototypical neurotrophic factor known to promote neurite outgrowth in cultured cells and target-directed axonal elongation \textit{in vivo} during development\textsuperscript{3}. Also, NGF has been shown to be upregulated in the injured nerve and important for neurite outgrowth.

Cdc2 is the prototype as the cell cycle protein and is important for the cell cycle transition of G2 to M phase. Cdc2 is activated by the binding of cyclin B proteins\textsuperscript{32,33}. In the nervous system, activation of Cdc2 was reported to be involved in neuronal apoptosis\textsuperscript{34}. Furthermore, Cdc2 was shown to be expressed in Schwann cells in the regenerating nerves\textsuperscript{34}. The present data showed that Cdc2 was strongly induced in the peripheral nerve after injury and further increased by GS treatment. Immunofluorescence staining showed Cdc2 protein signals which were completely overlapped with S100\textsubscript{\beta} protein signal\textsuperscript{35}. Since S100\textsubscript{\beta} protein is known to be exclusively expressed in the Schwann cells, these data suggest GS-mediated Cdc2 expression in Schwann cells. Thus, NGF and Cdc2 activation by GS treatment may be involved in increased Schwann cells in the injured nerve.

Direct effects of GS on Schwann cell were investigated using \textit{in vitro} cultured Schwann cell system. It was first observed that injury preconditioning increased Schwann cell proliferation in culture. Then, GS treatment further increased cell proliferation along with increased Cdc2 protein signals in the cells. In this aspect, GS appears to act in a similar way as injury stimulation for Schwann cells in order to enhance their supportive role for axonal regeneration. Thus, increased Schwann cell population in the injured nerve and in the presence of GS may be critical for successful axonal regeneration.

Astrocytes are one of the major types of non-neuronal cells (\textit{aka} glial cells)\textsuperscript{21}. In this aspect, astrocytes may be inhibitory for axonal regrowth processes. Yet, recent studies in the mice given spinal cord injury showed that astrocytes restricts pathological inflammatory responses in the injury cavity and thus are supportive for regeneration processes\textsuperscript{36}. Thus, a possible dual role of astrocytes in the neural tissues after spinal cord injury may function in a different manner depending on the severeness of injury as well as the time point and tissue type of injuries.

The CST is one of the longest axon tracts beginning its tract at motor cortical area in the brain all the way to the lowest part of the spinal cord to relay motor command to lower motor neurons located at the ventral horn of the spinal cord\textsuperscript{37}.

Retrograde tracing experiments by injecting DiI at the caudal part from the injury site and determining labeled cortical neurons in the brain would be useful to resolve this issue. Analysis of DiI-labeled axons at caudal part from the injury cavity revealed more staining signals in GS-treated group than saline-treated group, suggesting more axonal regrowth and elongation to caudal part to the injury cavity by GS treatment.

Thus, anterograde tracing data suggest that GS may function as positive effects for possible axonal regeneration in the injured spinal cord. These results further suggest that GS-mediated enhanced processes of astrocytes might be associated with potential function of these cells supporting for axonal regrowth in the injured spinal cord. Finally together with positive growth promoting activity of peripheral axons, somehow, GS may activate the common neural pathway(s) to induce axonal regeneration in both PNS and CNS.

GS has been used as one of critical herbal drugs for the treatments of diverse symptoms in human. The current finding therefore may be useful to develop therapeutic agents for the treatments of injured nerves.

**Conclusions**

The present study was performed to examine the effects of GS extract on axonal regeneration in both
The responses of neuronal cells including axons and non-neuronal cells were investigated in the peripheral sciatic nerves and corticospinal tract axons in the spinal cord. The major findings are summarized as below.

1. NF-200 protein immunostaining for the visualization of axons showed more distal elongation of sciatic nerve axons in GS-treated group than saline-treated control 3 and 7 days after crush injury.

2. GAP-43 protein was increased in the injured sciatic nerve and further increased by GS treatment. Enhanced GAP-43 protein signals were also observed in DRG prepared from the rats given nerve injury and GS treatment.

3. GS treatment in vivo induced enhanced neurite outgrowth in preconditioned DRG sensory neurons. In vitro treatment of GS on sensory neurons from intact DRG also caused increased neurite outgrowth.

4. Phospho-Erk1/2 protein levels were higher in the injured nerve treated with GS than saline. Phospho-Erk1/2 protein signals were mostly found in the axons in the injured nerve.

5. NGF and Cdc2 protein levels showed slight increases in the injured nerves of GS-treated group compared to saline-treated group.

6. The number of Schwann cell population was significantly increased by GS treatment in the injured sciatic nerve. GS treatment with cultured Schwann cells increased proliferation and Cdc2 protein signals.

7. GS pretreatment into the injured spinal cord generated increased astrocyte proliferation and oligodendrocytes in culture. In vitro treatment of GS resulted in more differentiated pericytoplasmic processes compared with saline treatment.

8. More arborization around the injury cavity and the occurrence at the caudal region of CST axons were observed in GS-treated group than in saline-treated group.

These data suggest that GS extract may have the growth-promoting activity on regenerating axons in both peripheral and central nervous systems. Further studies at molecular levels to identify specific ginsenoside chemicals may be useful for the application to therapeutic strategies.

References


8. Hee-Jin Choi, Ho-Suk Han, Jung-Hye Park, Jun-Ho Son, Jong-Ho Bae, Tae-Su Seung, Cheong Choi. Antioxidantive, Phospholipase A2 Inhibiting, and Anticancer Effect of Polyphenol Rich


27. Alexander KA, Wakim BT, Doyle GS, Walsh KA, Storm DR. Identification and characterization of the calmodulin-binding domain of neur-