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Protective effect of GCSB-5, an herbal preparation, against peripheral nerve injury in rats

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ABSTRACT

Aim of the study: GCSB-5 (traditional name: Chungpa-Juhn), an herbal medicine composed of 6 crude herbs (*Saposhnikovia divaricata* Schiskin, *Achyranthis bidentata* Blume, *Acanthopanax sessiliflorum* Seem, *Cibotium baromets* J. Smith, *Glycine max* Meriill, and *Eucommia ulmoides* Oliver), has been widely used in Asia for treatment of neuropathic and inflammatory diseases. This study investigated the protective effect of GCSB-5 against peripheral nerve injury *in vitro* and *in vivo*.

Materials and methods: After left sciatic nerve transection, rats received oral administration of GCSB-5 (30, 100, 300, and 600 mg/kg), or saline (vehicle), respectively, once daily for 8 weeks. Motor functional recovery and axonal nerve regeneration were evaluated by measurement of sciatic functional index (SFI), sensory regeneration distance, and gastrocnemius muscle mass ratio. The myelinated axon number was counted by morphometric analysis. In the *in vitro* study, the effects of GCSB-5 on H_2O_2-induced oxidative damage in SH-SY5Y cells were investigated by measurement of cell viability, production of reactive oxygen species (ROS), lipid peroxidation, release of lactate dehydrogenase (LDH), and cellular glutathione contents. Neurite outgrowth was also determined.

Results: After 8 weeks of nerve transection, SFI, regeneration distance, and gastrocnemius muscle mass ratio and myelinated axon number showed a significant decrease and these decreases were attenuated by GCSB-5. GCSB-5 significantly inhibited H_2O_2-induced cell death and oxidative stress, as evidenced by decreases in production of ROS and lipid peroxidation and release of LDH, and by increase in total GSH content.

Conclusions: The neuroprotective effect afforded by GCSB-5 is due in part to reduced oxidative stress.

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1. Introduction

Peripheral nerve injuries are commonly encountered in clinical practice due to accidental trauma, acute compression, or surgery, and can cause temporary or life-long neuronal dysfunctions that can subsequently lead to economic or social disability. Peripheral nerve injury sets in motion a dramatic change in the intracellular environment, including molecular composition in lesioned neurons, resulting in overproduction of reactive oxygen species (ROS) (Stoll and Muller, 1999). Release of ROS at the site of injury plays a role in modulation of the inflammatory response and is involved in necrotic and apoptotic cell death after nerve injury. Antioxidants counteract the detrimental effects of ROS and protect against oxidative injury, which is considered to be an essential role in the mechanisms of neuroprotection and functional recovery (Maher, 2006). Melatonin, a neurosecretory product of the pineal gland, which functions as an antioxidant and free radical scavenger, showed a beneficial effect on nerve recovery after sciatic nerve dissection (Atik et al., 2011).

Traditionally, nerve transection injuries have been remedied by a number of surgical methods, including nerve transfer, nerve grafts, and end-to-side neurorrhaphy (Matsumoto et al., 2000). Despite early diagnosis and improved modern surgical techniques, recovery of function can never reach the pre-injury level. Administration of nerve growth factor (NGF) and brain-derived neurotrophic factor has attracted the attention of researchers as a possible therapy for treatment of peripheral nerve injuries in order to accelerate axonal regeneration and target muscle reinnervation. However, systemically delivered recombinant neurotrophic factor has shown unexpected side effects in clinical trials (Wei et al., 2009). Therefore, it is important to find agents that can promote functional recovery without toxicity problems. Lu et al. (2010) have

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suggested Astragalus membranaceus extract as a potential nerve growth-promoting factor, being salutary in aiding growth of axons in the peripheral nerve. Recently, Ginsenoside Rg1, which showed ROS scavenging activity, was reported to promote nerve regeneration after nerve injuries (Ma et al., 2010).

GCSB-5 has been used clinically for treatment of arthritis, disc, and ankylosing spondylitis (Chung et al., 2010). It consists of a mixture of six crude drugs (root of *Saposhnikovia divaricata* Schiskin, root of *Achyranthis bidentata* Blume, bark of *Acanthopanax sessiliflorum* Seem, rhizome of *Cibotium baromets* J. Smith, seed of *Glycine max* Meriill, and bark of *Eucommia ulmoides* Oliver); each crude drug has been widely used for treatment of inflammatory diseases in Asia. Root of *Achyranthis bidentata* Blume can scavenge free radicals, such as $ONOO^-$, HOCl, and OH^- (Ida et al., 1998). Moreover, extracts of root of *Achyranthis bidentata* Blume accelerate peripheral nerve regeneration via enhancement of nerve growth, preventing neuron apoptosis, and inducing neuronal differentiation of PC12 cells (Ding et al., 2008). Bark of *Eucommia ulmoides* Oliver also shows ROS scavenging activity (Yen and Hsieh, 2000). Anti-inflammatory properties of bark of *Acanthopanax sessiliflorum* Seem (Kim et al., 1995) and bark of *Eucommia ulmoides* Oliver (Hong et al., 1998) have been reported. Anti-inflammatory activity of GCSB-5 in both *in vitro* and *in vivo* acute and chronic animal models has recently been reported (Chung et al., 2010).

Therefore, this study investigated the effect of GCSB-5 on peripheral nerve injury and molecular mechanisms of protection, using a rat *in vivo* sciatic nerve transection and repair model and human SH-SY5Y neuroblastoma cell line *in vitro*.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), and Pen/Strep (100 U/mL penicillin, and 100 μg/mL streptomycin, respectively) were obtained from Gibco BRL (Grand Island, NY, USA). Dulbecco's phosphate buffer saline (PBS) was purchased from PAA Laboratories GmbH (Pasching, Austria). 2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Nerve growth factor (NGF) was purchased from Promega (Madison, WI, USA). Unless stated otherwise, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation and composition of GCSB-5

Six crude herbs [root of *Saposhnikovia divaricata* Schiskin (Umbelliferae) 4.44 g, root of *Achyranthis bidentata* Blume (Amaranthaceae) 4.44 g, bark of *Acanthopanax sessiliflorum* Seem (Araliaceae) 4.44 g, rhizome of *Cibotium baromets* J. Smith (Dicksoniaceae) 2.78 g, seed of *Glycine max* Meriill (Fabaceae) 2.78 g, and bark of *Eucommia ulmoides* Oliver (Eucommiaceae) 1.39 g] was boiled in tap water (1 L) for 3 h and then the extract was free-dried and then subjected to ultrafiltration and components with molecular weight over 10,000 were excluded to obtain the extract of GCSB-5 (3.47 g, 17.1%). The filtrate was lyophilized as a powder and kept at 4 °C until use. The crude drugs were purchased from an herbal market, Seoul, Korea, and authenticated by Dr. S.H. Lee, Jaseng Hospital of Oriental Medicine, Seoul, Korea. The voucher specimens of the plants used in this study were deposited in the herbarium of Jaseng Hospital of Orential Medicine. The validation of GSCB-5 was performed by high-performance liquid chromatography analysis of each ingredient extract using six indicator biological components: cimifugin for root of *Saposhnikovia divaricata* Schiskin, 20-hydroxyecdysone (0.311–0.312 mg/g) for root of *Achyranthis bidentata* Blume, acanthoside D (0.577–0.578 mg/g) for bark of *Acanthopanax sessiliflorum* Seem, onitin-4-O-β-D-glucopyranoside for rhizome of *Cibotium baromets* J. Smith, genistin (0.0426–0.0427 mg/g) for seed of *Glycine max* Meriill and geniposide (0.431–0.432 mg/g) for bark of *Eucommia ulmoides* Oliver (Lee and Cha, 2008; Cha and Lee, 2009).

GCSB-5 was further standardized for quality control according to regulations imposed by the Korea Food and Drug Administration.

2.3. Animal treatments and surgical procedure

Male Sprague-Dawley rats, weighing 200–220 g, were supplied by Daehan Biolink Co., Ltd., Eumseong, Korea. All animal procedures were approved by the Sungkyunkwan University Animal Care Committee and were performed in accordance with the guidelines of the National Institutes of Health. In preparation for surgery, the animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The right sciatic nerve was exposed through a gluteal muscle-splitting incision. The nerve was sharply transected 10 mm distal to the sciatic notch using microsurgery scissors and immediately repaired with four epineurial 10/0 nylon sutures under an operating microscope (Kanaya et al., 1996). The wound was closed in layers using silk sutures and the animals were returned to their cages and allowed to recover from the anesthesia.

2.4. Administration of GCSB-5

GCSB-5 was dissolved in normal saline (vehicle) and administered orally once a day. Dose selection was based on a previous study (Chung et al., 2010). The animals were assigned randomly to the following groups (10 animals per group); (a) vehicle-treated sham (b) GCSB5-treated sham (c) vehicle-treated sciatic nerve transection (control), (d–g) GCSB-5 30, 100, 300, and 600 mg/kg-treated sciatic nerve transection. No differences were observed in any of the parameters between the vehicle- and GCSB-5-treated rats in the sham group; therefore, the results of groups (a) and (b) were pooled, and were referred to as sham.

2.5. Functional assessment

The sciatic functional index (SFI) was determined before surgery and 1, 2, 4, 6, and 8 weeks after surgery according to the method of Bain et al. (1989) for transected nerve injury. For calculation of SFI, paw prints were obtained by moistening the hind paws with water colors while rats walked along a white paper covered corridor (10 × 80 cm). Black paw prints were left on the paper. Paw print length (PL), total toe spread (TS), and intermediary toe spread (IT) on both the normal (N) and experimental (E) sides were measured and used for calculation of the SFI:

$$\mathrm{SFI} = -38.3([\mathrm{EPL} - \mathrm{NPL}]/\mathrm{NPL}) + 109.5([\mathrm{ETS} - \mathrm{NTS}]/\mathrm{NTS}) + 13.3([\mathrm{EIT} - \mathrm{NIT}]/\mathrm{NIT}) - 8.8$$

In this formula, an SFI of 0 indicates normal nerve function, and an SFI of −100 indicates total impairment.

2.6. Gastrocnemius muscle weight

At 8 weeks after surgery, the animals were euthanized with a lethal dose of ether. Skins were removed from the hindlimb, and scissors were inserted under the Achilles's tendon; the blade was used to separate the gastrocnemius muscle. Both the operated and un-operated sides of the gastrocnemius muscles of each animal

were weighed immediately while still wet at the time of euthanasia. Values were expressed as a ratio by dividing the wet weight of the gastrocnemius muscle of the operated side by that of the un-operated side.

2.7. Regeneration distance

Regeneration distance of the sensory nerve fibers was evaluated using the pinch-reflex test (Dahlin and Kanje, 1992). At 7 days after surgery, animals of each group were anesthetized and the sciatic nerve was re-exposed. The sciatic nerve was then pinched with microsurgical forceps in a distal-to-proximal direction at 0.5 mm intervals. When the tips of the fastest growing sensory axons were pinched, they elicited a reflex response, which could be observed as movement of the leg and contractions of the muscles on the back. The distance between the repair site and the place where the rat first responded was taken as the regeneration distance.

2.8. Neuromorphometry

After completion of functional assessments at 8 weeks after surgery, a 1 cm segment of sciatic nerve, including the segment distal and proximal to the coaptation site was excised and fixed in a solution of phosphate-buffered 2.5% glutaraldehyde overnight. Following fixation, the nerve tissue was post-fixed in 0.5% osmium tetroxide, and then dehydrated with gradient alcohol, and embedded in Spurr's resin. The tissue was then cut to a thickness of 2 μm using a microtome with a dry glass knife, and stained with toluidine blue for a light microscope (Olympus CKX 41, Olympus Optical Co., Tokyo, Japan). Myelinated axons were quantified according to the unbiased counting rule (Kaplan et al., 2005), and conducted by examiners who were blind to the experimental design.

2.9. Cell culture and treatments

The SH-SY5Y human neuroblastoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA). SH-SY5Y cells were cultured in DMEM medium supplemented with 10% FBS, 20 mM glutamine, and 1% Pen/Strep and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Experiments were carried out 24 h after the cells were seeded.

2.10. Neurite outgrowth

SH-SY5Y cells were plated in 6-well plates at a density of 6×10^4 cells/well and treated with 0.4 μM aphidicolin, a specific and reversible inhibitor of DNA polymerase α and δ, which ceases proliferation and extends long neurites (LoPresti et al., 1992). At 5 days, cells were washed and treated with NGF 10 ng/mL in the presence or absence of GCSB-5 or melatonin. At 96 h after incubation, the total number of cells and the number of cells provided with neurites (length at least double that of cell diameter) were counted on blind. After the incubation period, cultures were observed under phase-contrast, using an inverted microscope (200× magnification, Olympus BX51/Olympus DP71, Olympus, Japan) and then photographed by using a digital camera. The percentage of neurite-bearing cells was determined after at least 100 cells were counted. Triplicate wells were run in experiments and the data were averaged for each treatment group. The entire experiment was repeated twice.

2.11. Cell viability

Cells were pre-incubated with various concentrations of GCSB-5 (1×10^{-4}, 1×10^{-3}, 1×10^{-2} and 1×10^{-1} mg/mL), a vehicle, phosphate buffered saline (PBS), or melatonin (500 μM) for 2 h. After incubation, 500 μM of H_2O_2 was added to each well and the plates were further incubated for 24 h before determination of cell viability. Cell viability of SH-SY5Y cells was assessed using a modified MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide] assay (Mosmann, 1983). MTT is a yellow-colored tetrazolium salt that is reduced to a purple formazan. After incubation, MTT solution (dissolved in PBS) was added to each well with a final concentration of 5 mg/mL, and the incubation was continued for another 4 h at 37 °C. Finally, an equal volume of dimethyl sulfoxide was added to each well to solubilize any deposited formazan. The optical density of each well was measured at 560 nm using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA).

2.12. Lactate dehydrogenase (LDH) activity

Activity of LDH in the culture media was analyzed by spectrophotometry procedures using the ChemiLab LDH assay kit (IVDLab Co., Ltd., Suwon, Korea).

2.13. ROS production

SH-SY5Y cells were cultured to 70–80% confluence in 96-well plates, and treated with various concentrations of GCSB-5, a vehicle, or melatonin (500 μM) for 2 h; the plates were then further incubated with 25 μM H_2DCFDA, which becomes a fluorescent product, 2,7-dichlorofluorescein, in the presence of a wide variety of ROS, for 30 min at 37 °C. After removal of excess H_2DCFDA, the cells were washed twice with DMEM and were incubated with H_2O_2 (500 μM) for an additional 30 min. Cellular fluorescence was measured in a fluorescence microplate reader (SpecraMax M5, Molecular Devices, Sunnyvale, CA, USA) at excitation wavelength 485 nm and emission wavelength 530 nm.

2.14. Malondialdehyde (MDA) level

Lipid peroxidation was evaluated by measuring MDA concentrations according to the method of Buege and Aust (1978). MDA concentrations were calculated by the absorbance of thiobarbituric acid reactive substances at 532 nm.

2.15. Total glutathione (GSH) contents

Cells were incubated with H_2O_2 in the presence or absence of GCSB-5 (1×10^{-4}, 1×10^{-3}, 1×10^{-2} and 1×10^{-1} mg/mL) or melatonin (500 μM) for 30 min. After incubation, cells were washed twice with ice-cold PBS, collected by tripsin-EDTA, and resuspended in 1% picric acid. The homogenate was centrifuged at $10{,}000 \times g$ for 10 min, and an equal volume of 100 mM potassium phosphate buffer (pH 7.4) was added to the supernatant. Total GSH level was determined spectrophotometrically at a wavelength of 412 nm using the method reported by Tietze (1969) with yeast glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) and NADPH.

2.16. Statistical analysis

Results are reported as a mean ± S.E.M. The overall significance of the results was examined using two-way ANOVA. Differences between the groups were considered significant at $p < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons.

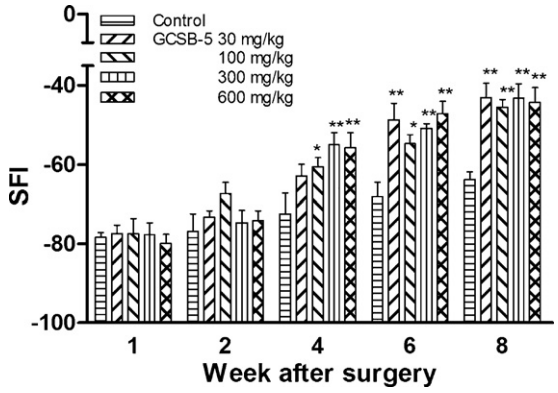

Fig. 1. Effects of GCSB-5 on the sciatic function index (SFI) 1, 2, 4, 6 and 8 weeks after surgery. Rats were administered orally with GCSB-5 30, 100, 300 and 600 mg/kg or saline once a day after surgery. The values are represented as means ± S.E.M for 10 rats per group. Significantly different (*$p < 0.05$, **$p < 0.01$) from control.

3. Results

3.1. Motor functional recovery

SFIs for the sham group were near zero at all time points (data not shown), which showed normal sciatic nerve function. As shown in Fig. 1, at 1 week after surgery, the SFI showed a dramatic decrease to approximately −78.4 ± 1.2, indicating complete loss of function. SFI values in the control group increased gradually over an 8-week period of observation, which indicates spontaneous motor functional recovery. No significant effects in the SFI were observed in the GCSB-5 group until 2 weeks after surgery. However, after 4, 6, and 8 weeks of surgery, the GCSB-5 group showed significantly greater improvement of SFI than the control group.

3.2. Gastrocnemius muscle mass ratio

As shown in Fig. 2A, the gastrocnemius muscle ratio between the operated and un-operated sides in the sham group at 8 weeks after surgery was 89.3 ± 8.7%. However, in the control group, the gastrocnemius muscle ratio showed a marked decrease to 44.4 ± 4.3%. This decrease was attenuated by treatment with GCSB-5 300 and 600 mg/kg (57.8 ± 6.0% and 56.8 ± 4.2%, respectively).

3.3. Sciatic nerve regeneration distance

In the control group, the sciatic nerve regeneration distance was 6.84 ± 0.03 mm at 7 days after surgery. When compared with the control group, the regeneration distance in the 100, 300, and 600 mg/kg GCSB-5 groups showed a marked increase (Fig. 2B).

3.4. Neuromorphometry

At 8 weeks after surgery, the total myelinated axon number per transverse section in the sham group was 5120.0 ± 143.8. However, in the control group, myelinated axon numbers showed a significant decrease to 2730.0 ± 219.2. This decrease was attenuated by GCSB-5 at the doses of 300 and 600 mg/kg (Fig. 3).

3.5. Neurotrophic activity

SH-SY5Y cells do not extend processes in the absence of exogenous NGF, with optimal efficacy being produced by NGF 10 ng/mL (data not shown). The number of neurite bearing cells in the control group was 9.7 ± 1.8%. However, at 96 h after NGF treatment, the number of neurite bearing cells in the control group increased

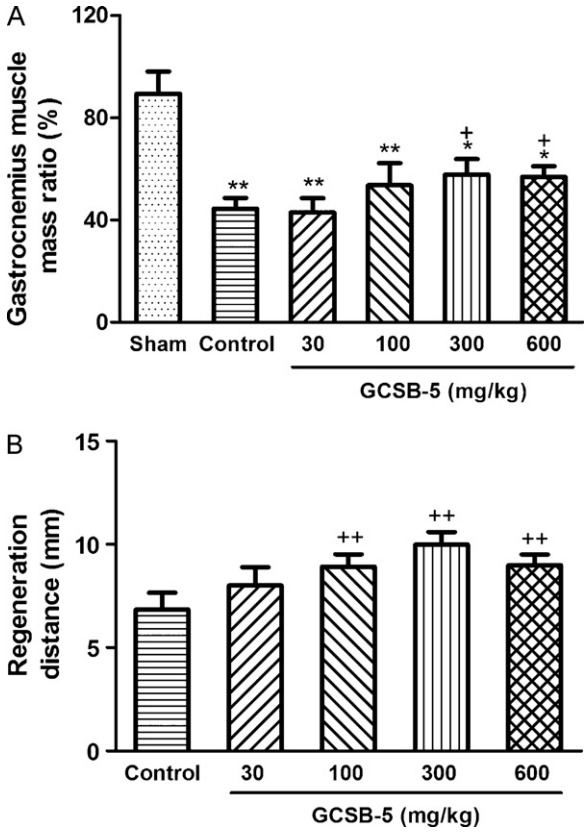

Fig. 2. Effects of GCSB-5 on the gastrocnemius muscle ratio (A) and sciatic nerve regeneration distance (B) 8 weeks and 7 days after surgery, respectively. Rats were administered orally with GCSB-5 30, 100, 300 and 600 mg/kg or saline once a day after surgery. The values are represented as means ± S.E.M for 10 rats per group. Significantly different (*$p < 0.05$, **$p < 0.01$) from sham. Significantly different ($^{+}p < 0.05$, $^{++}p < 0.01$) from control.

to 24.2 ± 2.6%. This increase was potentiated by treatment with GCSB-5 (1×10^{-3} and 1×10^{-1} mg/mL (38.6 ± 2.6% and 36.7 ± 3.3%, respectively)), and melatonin 500 μM (37.2 ± 2.1%) (Fig. 4).

3.6. Cell viability

When SH-SY5Y cells were treated with H_2O_2 for 24 h, cell viability decreased to 42.7% of control. This decrease was inhibited by pretreatment with GCSB-5 in a concentration-dependent manner, and melatonin 500 μM (Fig. 5A).

3.7. LDH release

As shown in Fig. 5B, 24 h incubation of cells with H_2O_2 significantly increased LDH release by approximately 1.5-fold compared with the control group. This increase was inhibited by treatment with GCSB-5 (1×10^{-3}, 1×10^{-2}, and 1×10^{-1} mg/mL), and melatonin 500 μM (Fig. 5B).

3.8. ROS production

Addition of H_2O_2 to cells resulted in a significant increase in ROS production by 1.6-fold compared with the control group. This increase was inhibited by treatment with GCSB-5 (1×10^{-3}, 1×10^{-2}, and 1×10^{-1} mg/mL), and melatonin 500 μM (Fig. 5 C).

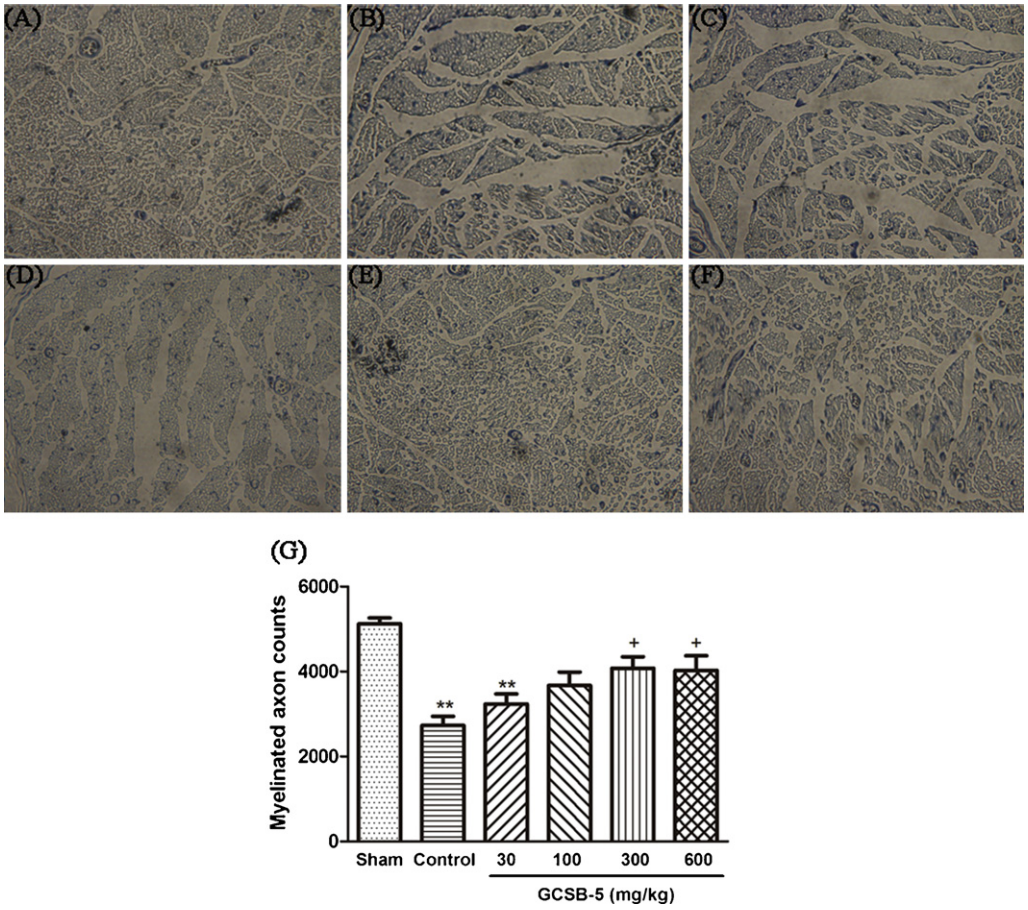

Fig. 3. Effects of GCSB-5 on the myelinated axon counts 8 weeks after surgery. (A-F), histological micrographs of nerve tissue stained with toluidine blue (magnification = 200×). (A) Sham group, (B) control group, (C–F) GCSB-30, 100, 300 and 600 mg/kg-treated group, respectively, (G) total myelinated axon counts. Rats were administered orally with GCSB-5 30, 100, 300 and 600 mg/kg or saline once a day after surgery. The values are represented as means ± S.E.M for 10 rats per group. Significantly different ($^{**}p < 0.01$) from sham. Significantly different ($^{+}p < 0.05$) from control.

3.9. MDA level

Addition of H_2O_2 to cells resulted in a significant increase in MDA level by 1.8-fold compared with the control group. This increase was inhibited by treatment with GCSB-5 (1×10^{-2} and 1×10^{-1} mg/mL), and 500 μM of melatonin (Fig. 5D).

3.10. Cellular total GSH contents

As shown in Fig. 5E, exposure to H_2O_2 resulted in a significant decrease of total GSH contents by 51.0% of that of the control group. This decrease was inhibited by treatment with GCSB-5 (1×10^{-3}, 1×10^{-2}, and 1×10^{-1} mg/mL), and melatonin 500 μM (Fig. 5E).

4. Discussion

Peripheral nerve injury is one of the most important and frequent injuries in industrial societies. Although modern repair strategies have reached a point of maximal benefit, the outcomes are often disappointing and any further advance in peripheral nerve regeneration will need to incorporate more than just mechanical manipulation. The search for effective drugs to promote nerve regeneration has become a therapeutic need. Recently, many studies have shown that application of traditional Chinese medicines is effective in treatment of peripheral nerve injury as well as nerve regeneration. Various herbal medicines, including Panax ginseng, Phoenix hanceana var. formosana extract, and Ginko biloba have shown potential for use as nerve injury repair agents through their antioxidant and anti-inflammatory properties (Lin et al., 2009). In Korea, GCSB-5 has also been used clinically for treatment of nervous diseases. However, pharmacological studies and underlying mechanisms of actions of the preparation have been poorly elucidated.

We first examined the effect of GCSB-5 on sciatic nerve transection injury *in vivo*. The rat sciatic nerve model is a widely used model for simultaneous evaluation of motor and sensory nerve function. The walking track analysis clearly demonstrated a direct relationship between individual hind limb muscle function and print measurement. Analysis of rat walking tracts by SFI has proven to be a reliable, repeatable, economical, and quantitative method of evaluating function following sciatic nerve injury and repair (Dinh et al., 2009). The SFI is considered an assessment of overall nerve function in the rat because walking requires complex motor-unit reinnervation coordinated by cortically integrated sensory feedback (Bain et al., 1989). Our data showed that at 2 weeks after surgery, function was still poor in both the control and GCSB-5 groups. At 4 weeks, an increasing ability to walk became evident. Better and faster functional recovery was shown in the GCSB-5 group, indicating that GCSB-5 has the effect of promoting functional recovery after transection of sciatic nerve fibers.

Denervation of a target muscle occurs as a consequence of peripheral nerve injury, accompanied by a series of histological and biochemical alterations, leading to final muscle atrophy. If the muscle is reinnervated, muscle function will be restored and atrophy will stop. Pathological changes occurring after peripheral nerve injury have been shown to result in acute, subacute, or chronic

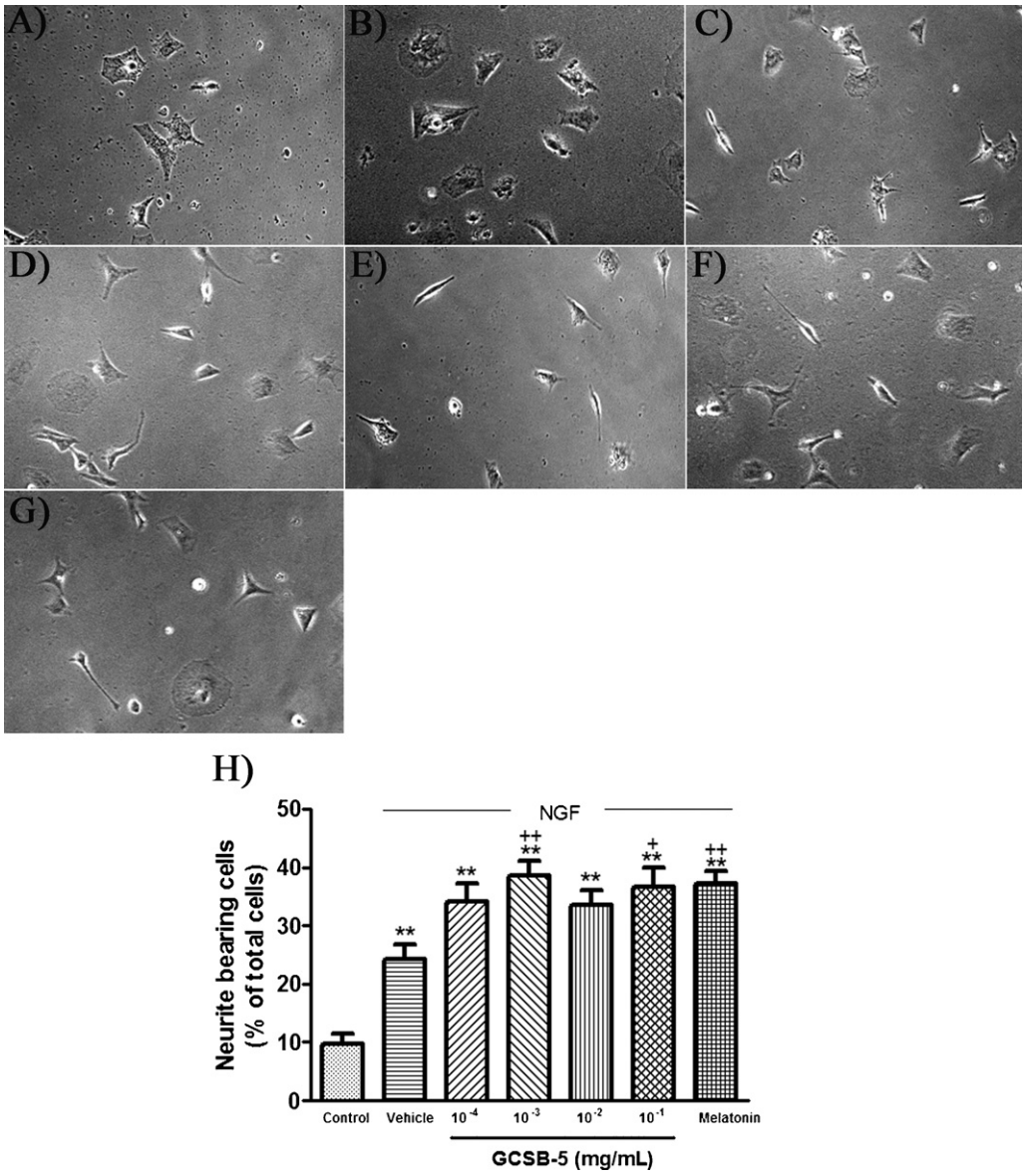

Fig. 4. Effects of GCSB-5 on the NGF-induced neurite outgrowth of SH-SY5Y cells (H). The percentage of neurite-bearing cells was determined after at least 100 cells were counted. The values are represented as means ± S.E.M. Triplicate wells were run in experiments and the data were averaged for each treatment group. Significantly different ($^{**}p < 0.01$) from control. Significantly different ($^{+}p < 0.05$, $^{++}p < 0.01$) from vehicle. Representative phase-contrast micrographs of untreated SH-SY5Y cells (control, A), treated with NGF alone (vehicle, B), treated with NGF and either GCSB-5 (1×10^{-4}, 1×10^{-3}, 1×10^{-2}, and 1×10^{-1}, C–F), or melatonin (500 μM, G).

denervation, which is in turn responsible for target muscle atrophy. The sciatic nerve crush, with the continuity of the nerve being preserved, allows for axonal reinnervation and restoration of the nerve-muscle interation (Yuan et al., 2010). The gastrocnemius muscle is supplied by the posterior tibial branch of the sciatic nerve. However, compared with the control group, treatment with GCSB-5 resulted in accelerated improvement of gastrocnemius muscle atrophy induced by sciatic denervation. These data correlate with our finding on SFIs and suggest that it may be due to appropriated reinnervation.

Injury to peripheral nerves results in partial or total loss of motor, sensory, and autonomic functions in involved segments of the body. Following traumatic or mechanically induced axonal degeneration in the peripheral nervous system, it is well established that axonal regeneration ensues, resulting in functional recovery (Gold et al., 1997). Thus, accelerated nerve regeneration is crucial in order to obtain satisfactory motor functional recovery. The regeneration distance of the leading axons was evaluated by the pinch test. This sensitive test reliably demonstrates the location of the most rapidly growing sensory axons and has frequently been used in studies evaluating the rate of nerve regeneration (Udina et al., 2002). In our study, GCSB-5 significantly increased nerve regeneration distance in transected sciatic nerves. Histological observations of sciatic nerve also strongly support the beneficial effect of GCSB-5 in axonal regeneration because the reduction in innervations of myelinated axons contributes to impaired functional performance (Horch and Lisney, 1981). Taken together, these data indicate that treatment with GCSB-5 promotes functional recovery by accelerating axonal regeneration after sciatic nerve transection in the rat.

Neuroprotective and nerve regeneration mechanisms of GCSB-5 were revealed using the human neuroblastoma SH-SY5Y cell line, which is a well-characterized model for neuronal differentiation, and is used extensively as a cellular model for the study of neurite outgrowth (Miglio et al., 2009). These cells differentiate into a more

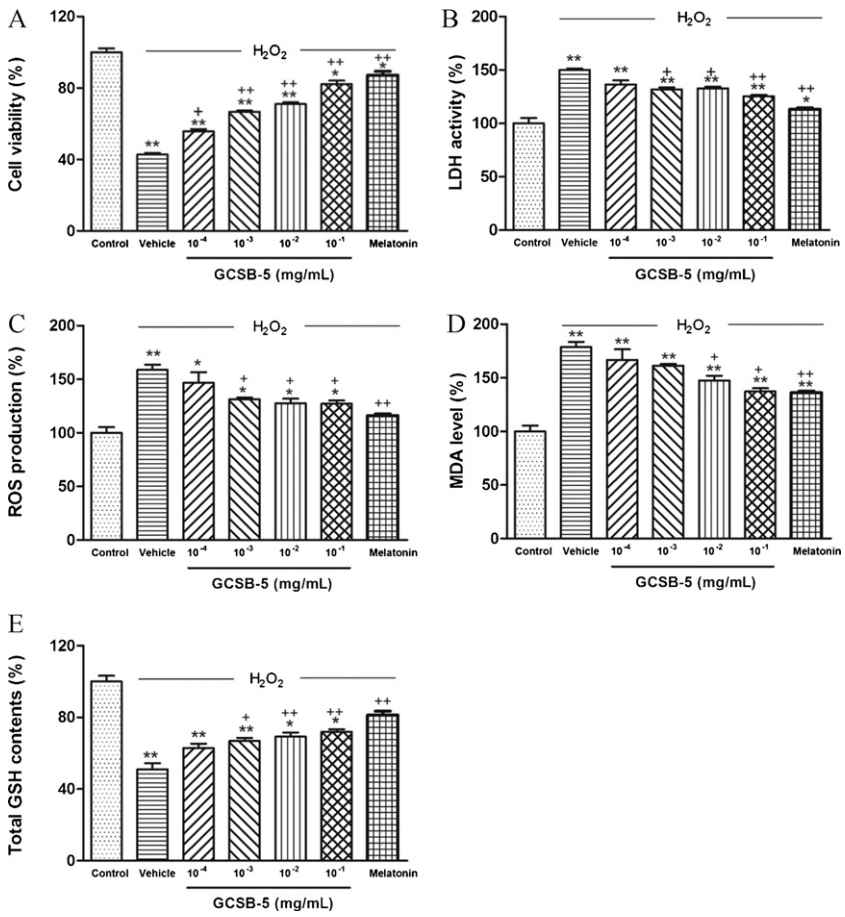

Fig. 5. Effects of GCSB-5 on the cell viability (A), LDH release (B), ROS production (C), MDA level (D), and total GSH contents (E) in H_2O_2-stimulated SH-SY5Y cells. The values are represented as means ± S.E.M. Triplicate wells were run in experiments and the data were averaged for each treatment group. Significantly different ($^{*}p < 0.05$, $^{**}p < 0.01$) from control. Significantly different ($^{+}p < 0.05$, $^{++}p < 0.01$) from vehicle.

neuronal phenotype when treated with differentiating factors, such as retinoic acid or brain-derived neurotrophic factor by an increase in neurite outgrowth (Monaghan et al., 2008). Neurite outgrowth is defined as the sprout of cell processes which will later become axons and dendrites, and is a key event of neuronal differentiation and nerve regeneration, and forms the basis of proper connectivity within a neuronal network (da Silva and Dotti, 2002). In our study, incubation with GCSB-5 elicited significant increases in the number of neurite-bearing cells compared with the NGF alone-treated group, indicating that GCSB-5 has nerve growth-promoting capability. These findings also correlate with *in vivo* data of GCSB-5 on nerve regeneration in the view of promotion of axonal regeneration.

It is generally accepted that peripheral nerve injury causes oxidative stress in axotomized motorneurons. Oxidative injury following peripheral nerve injury results from excessive ROS production, which leads to biochemical and structural. Production of ROS in lesioned neurons is a well known major cause of peripheral nerve injury-relevant neuronal damage (Martin et al., 2003). The healing process after nerve injury is reduced by mainly free oxygen radicals rather than inflammation and edema (Bagdatoglu et al., 2002). (Khodr and Khalil, 2001) reported that ROS could contribute to delayed recovery of injured nerves in old rats in areas innervated by the injured nerve. According to this evidence, neuroprotective therapies were aimed at interference with the oxidative stress process and special attention has been placed on the study of the neuroprotective actions of the antioxidant. Among various antioxidants, melatonin, a neurosecretory product of the pineal gland, limits neuronal loss after sciatic nerve transection in neonatal rats (Rogerio et al., 2002). Administration of melatonin seems to have a beneficial effect on nerve recovery after sciatic nerve dissection, and protects the sciatic nerve from I/R injury, which may be attributed to its antioxidant property (Atik et al., 2011). High concentrations of H_2O_2 induced rapid cell death, mainly necrotic cell death, with no evidence of apoptosis (Lee et al., 2008). Our study showed that human neuroblastoma SH-SY5Y cells treated with 500 μM H_2O_2, which is a relatively high concentration, caused a significant decrease in cell survival and elevation of oxidative stress characterized by the increase of LDH release, ROS production, and lipid peroxidation. However, GCSB-5 inhibited these cellular events. Cellular GSH plays a key role as a first defense antioxidant to counteract the deleterious effect of ROS. Depletion in GSH resulted in increased oxidative stress and ensuing neuronal cell death. In our study, incubation with GCSB-5 elicited significant increases in total GSH content. Taken together, our study demonstrated that GCSB-5 possesses potent ROS scavenging properties in an *in vitro* system, attenuating an increase of ROS production and a decrease of total GSH contents. These findings indicate that the antioxidant potential of GCSB-5 may contribute to its neuroprotection against H_2O_2 induced oxidative stress.

Overall, GCSB-5 appears to promote nerve regeneration and to accelerate motor functional recovery by reducing oxidative stress. This study provides evidence that GCSB-5 has potential for use as a nerve injury repair agent.

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