Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2009 | 110 | 1276–1287 doi: 10.1111/j.1471-4159.2009.06214.x

Neuroprotective effect of *Scutellaria baicalensis* on spinal cord injury in rats

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Abstract

Inflammation has been known to play an important role in the pathogenesis after spinal cord injury (SCI). Microglia are activated after injury and produce a variety of proinflammatory factors such as tumor necrosis factor-α, interleukin-1β, cyclooxygenase-2, and reactive oxygen species leading to apoptosis of neurons and oligodendrocytes. In this study, we examined the neuroprotective effects of total ethanol extract of *Scutellaria baicalensis* (EESB), after SCI. Using primary microglial cultures, EESB treatment significantly inhibited lipopolysaccharide-induced expression of such inflammatory mediators as tumor necrosis factor-α, IL-1β, IL-6, cyclooxygenase-2, and inducible nitric oxide synthase. Furthermore, reactive oxygen species and nitric oxide production were significantly attenuated by EESB treatment. For *in vivo* study, rats that had received a moderate spinal cord contusion injury at T9 received EESB orally at a dose of 100 mg/kg. EESB inhibited expression of proinflammatory factors and protein carbonylation and nitration after SCI. EESB also inhibited microglial activation at 4 h after injury. Furthermore, EESB significantly inhibited apoptotic cell death of neurons and oligodendrocytes and improved functional recovery after SCI. Lesion cavity and myelin loss were also reduced following EESB treatment. Thus, our data suggest that EESB significantly improve functional recovery by inhibiting inflammation and oxidative stress after injury.

Keywords: apoptotic cell death, inflammation, neuroprotection, oxidative stress, *Scutellaria baicalensis*.
J. Neurochem. (2009) **110**, 1276–1287.

Spinal cord injury (SCI) induces apoptotic cell death of neurons and oligodendrocytes resulting in demyelination and axon degeneration, thereby leading to spinal cord dysfunction (Liu *et al.* 1997; Springer *et al.* 1999; Casha *et al.* 2001). Inflammation and oxidative stress are major factors exacerbating the pathogenesis of SCI by inducing apoptosis of neurons and oligodendrocytes (Bareyre and Schwab 2003; Bao and Liu 2004). Microglia play a pivotal role in inducing an inflammatory response and are believed to contribute to the neurodegenerative process by releasing both proinflammatory cytokines (Block and Hong 2005) and reactive oxygen species (ROS) (Min *et al.* 2003, 2004; Qin *et al.* 2004). Therefore, it is reasonable to assume that microglial inactivation may represent a viable therapeutic strategy for treating acute SCI.

Flavonoids are a group of low molecular weight polyphenolic compounds of plant origin. They have a variety of biological activities such as anti-inflammatory, antioxidant, antiviral, and anti-tumor effects (Middleton *et al.* 2000). Among the many known herbal remedies in Oriental medicine, *Scutellaria baicalensis* is one of the most widely used against bacterial infections and inflammatory diseases. Baicalein, baicalin, and wogonin are three major flavonoids isolated from the roots of *S. baicalensis*. The neuroprotective effects of these components have been demonstrated *in vitro*

Received December 16, 2008; revised manuscript received April 9, 2009; accepted June 3, 2009.

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Abbreviations used: BBB, Basso–Beattie–Bresnahan; COX-2, cyclooxygenase-2; DCF-DA, dichlorodihydrofluorescein diacetate; DMSO, dimethylsulfoxide; EESB, ethanol extract of *Scutellaria baicalensis*; GM, gray matter; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; ROS, reactive oxygen species; SCI, spinal cord injury; TNF-α, tumor necrosis factor-α; TUNEL, transferase-mediated dUTP biotinylated nick end labeling; WM, white matter.

and *in vivo* (Kim *et al.* 2001a; Lee *et al.* 2005; Suk 2005). For example, wogonin suppresses activation of microglia *in vitro* as well as *in vivo*, provides neuroprotection against ischemic brain damage, and suppresses lipopolysaccharide (LPS)-induced cell death in C6 glial cells by inhibiting nitric oxide (NO) production (Kim *et al.* 2001a; Lee *et al.* 2003a). Baicalein attenuates inflammation-mediated degeneration of dopaminergic neurons by inhibiting microglial activation (Li *et al.* 2005) and protects cortical neurons against apoptosis from glutamate or glucose deprivation (Lee *et al.* 2003b). In addition, wogonin protects cortical neurons from excitotoxic and oxidative damage *in vitro* (Cho and Lee 2004) and SH-SY5Y cells from hydrogen peroxide-induced oxidative stress (Gao *et al.* 2001).

The methanol extract isolated from the roots of *S. baicalensis* has been shown to protect CA1 hippocampal neurons from cerebral ischemia (Kim *et al.* 2001b). The extract, especially, at low concentrations, exerts significant neuroprotective effects *in vivo* (Kim *et al.* 2001b). As *S. baicalensis* has been used in Oriental medicine to treat inflammatory diseases, we hypothesized that *S. baicalensis* may have neuroprotective effects as demonstrated by inhibiting microglial activation after SCI. In this study, we tested our hypothesis using a contusion spinal injury model in rats.

Materials and methods

Preparation of ethanol extract from *Scutellaria baicalensis*

Ethanol extract of *S. baicalensis* (EESB) was extracted from dried roots of *S. baicalensis* with 70% ethanol as described previously (Kim *et al.* 2001b). The ethanol filtrate was evaporated *in vacuo*, and powdered EESB was stored at −20°C until use. By HPLC analysis, EESB contains baicalein (1.8%), baicalin (12.6%), and wogonin (0.8%).

Primary microglia cultures and drug treatment

Primary microglia were isolated from the cerebral cortices of postnatal day 1 rats (Sprague–Dawley rats; Samtako, Osan, Korea) as described previously (Lee *et al.* 2004). Microglia were seeded into six-well plates (1.2×10^6 cells/well) and pre-treated with EESB for 30 min followed by stimulation with lipopolysaccharide (LPS, 1 μg/mL, *Escherichia coli* 0111:B4; Sigma, St Louis, MO, USA). EESB was dissolved in dimethylsulfoxide (DMSO) and then diluted in phosphate-buffered saline (the final concentration of DMSO was 0.1%). For control, diluted DMSO (0.1%) without EESB was used.

Assay for reactive oxygen species and nitric oxide production

The production of ROS was measured fluorometrically using dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probe, Eugene, OR, USA) as described previously (Kim *et al.* 2007). NO production was measured as described previously (Lee *et al.* 2004). Briefly, 100 μL of culture medium was allowed to react with 100 μL of Griess reagent (Sigma). The optical density was read at 540 nm in a microplate reader (Molecular devices, Sunnyvale, CA, USA) after 15 min. Cellular nitrite production was quantitated by subtracting the level of nitrite present in the media (in the absence of cells) from the total nitrite level. Nitrite concentrations were calculated from a standard curve derived from the reaction of sodium nitrite in fresh media.

Spinal cord injury

Adult Sprague–Dawley rats (male; 230–250 g; Samtako) were subjected to contusion injury (10 g × 25 mm) as described previously (Yune *et al.* 2007). For the sham-operated controls, animals underwent a T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Polices for Rodent Survival Surgery provided by the Animal Care Committee of the Kyung Hee University.

Ethanol extract of *Scutellaria baicalensis* administration

Powdered EESB were suspended in sterile deionized water and administrated orally at a dose of (30, 100, or 300 mg/kg) beginning at 2 h after SCI and then once a day for 2 weeks. Control groups received equivolumetric administration of sterile deionized water at the corresponding time points. It has been shown that 100 mg/kg of EESB is an optimal dose for neuroprotection after ischemic insult (a personal communication with Dr Hochoel Kim at Kyung Hee University College of Oriental Medicine, Seoul, Korea).

Tissue preparation

After SCI, spinal cord sections were prepared as described previously (Yune *et al.* 2007). Frozen tissue longitudinal and cross-sections were then cut at 10 or 20 μm on a cryostat (CM1850; Leica, Wetzlar, Germany).

RNA isolation and RT-PCR

RNA isolation using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis were performed as previously described (Lee *et al.* 2003c). The primers used for β-actin, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were synthesized by the Genotech Corp (Daejeon, Korea). The sequences of the primers were: 5′-CTCCATGACTCTCAGCACAGAG-3′ (sense) and 5′-GCACCGAAGATATCCTCATGAT-3′ (antisense) for iNOS; 5′-CCATGTCAAAACCGTGGTGAATG-3′ (sense) and 5′-ATGGG AGTTGGGCAGTCATCAG-3′ (antisense) for COX-2; 5′-CCCA GACCCTCACACTCAGAT-3′ (sense) and 5′-TTGTCCCTTGAA GAGAACCTG-3′ (antisense) TNF-α; 5′-GCAGCTACCTATGTC TTGCCCGTG-3′ (sense) and 5′-GTCGTTGCTTGTCTCTCC TT GTA-3′ for IL-1β; 5′-AAGTTTCTCTCCGCAAGATACTTCCAGCC A-3′ (sense) and 5′-AGGCAAATTTCCTGGTTATA TCCAGTTT-3′ (antisense) for IL-6; and 5′-CTTCTGCATCCTGTCAGCGATGC-3′ (sense) and 5′-AGAAGAGCTATGAGCTGCCTGACG-3′ (antisense) for β-actin, which was used as an internal control. Experiments were repeated three times and the values obtained for the relative intensity were subjected to statistical analysis. The gels shown in figures are representative of results from three separate experiments.

Western blot

Whole-cell extracts from primary microglial cells and spinal cords (1 cm) were prepared for western blotting as described previously

(Yune *et al.* 2007). The primary antibodies used were anti-β-tubulin (1 : 10 000; Sigma), anti-iNOS (1 : 10 000; Transduction Laboratory, Lexington, KY, USA), and anti-IL-1β (1 : 400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot analysis of protein carbonylation was performed according to the protocol for the OxyBlot™ Protein Oxidation Detection Kit (Millipore, Billerica, MA, USA). Tubulin was used as an internal control. Relative intensity of each band on western blots and of carbonylated proteins were measured and analyzed by AlphaImager software (Alpha Innotech Corporation, San Leandro, CA, USA). Background in films was subtracted from the optical density measurements.

TUNEL and immunohistochemical staining

Either 1 or 5 days after injury, serial spinal cord sections (10-μm thickness) were collected and every 50 μm section was processed for transferase-mediated dUTP biotinylated nick end labeling (TUNEL) (Oncor, Gaithersburg, MD, USA) and then for immunocytochemistry using specific cell type markers: anti-neuronal neuclei (NeuN) (1 : 1000; Millipore) for neurons and anti-CC1 (1 : 100; Oncogene, Darmstadt, Germany) for oligodendrocytes. Only double labeled cells were considered and counted as TUNEL-positive neurons (1 day) in the gray matter (GM) or oligodendrocytes (5 days) in the white matter (WM). The areas counted included 2 mm to the lesion epicenter in the GM (from centromedial to ventral region) and 5 mm to the lesion epicenter in the WM rostrally and caudally. For double labeling, FITC or cyanine 3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used. Some sections were processed for immunohistochemistry using antibody against nitrotyrosine (1 : 1000; Millipore) or OX-42 (1 : 100; Millipore). Nitrotyrosine-positive neurons were counted as previous described (Yune *et al.* 2008). Also, nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI) according to the protocol of the manufacturer (Molecular Probe). In all controls, reaction to the substrate was absent if the primary antibody was omitted or if the primary antibody was replaced by a non-immune, control antibody.

Quantitation of the proportion of resting and activated microglia

Percentage of field analysis was used to provide a quantitative estimate (proportional area) of changes in the activation state of microglia. Resting and activated microglia were classified based on previous report (Hains and Waxman 2006). Briefly, resting microglia displayed small compact somata bearing long, thin, ramified processes. Activated microglia exhibited marked cellular hypertrophy and retraction of processes such that the process length was less than the diameter of the soma compartment. Cells were sampled only if the nucleus was visible within the plane of section and if cell profiles exhibited distinctly delineated borders.

DNA laddering

At 1 day after injury, spinal cords were isolated and processed for DNA laddering as described previously (Yune *et al.* 2003).

Behavioral tests

To examine functional deficits after injury, behavioral analyses were performed by trained investigators who were blind as to the experimental conditions. For testing of hindlimb locomotor function, open-field locomotion was evaluated by using the 21-point Basso–Beattie–Bresnahan (BBB) locomotion scale as previously described (Basso *et al.* 1995). The ability to control and place the hindlimb precisely was tested on a horizontal grid as previously described (Merkler *et al.* 2001). Analysis was performed by counting the number of foot falls (mistakes) in foot placing. Footprint analysis was performed as previously described (de Medinaceli *et al.* 1982; Stirling *et al.* 2004). Both the animal's forepaws and hindpaws were dipped in red and blue dye (non-toxic) and then allowed to walk across a narrow box (1 m long and 7 cm wide).

Assessment of lesion volume

Lesion volume, using rats employed for behavioral analyses, was assessed as described previously (Yu and Geddes 2007; Yune *et al.* 2008). Serial longitudinal sections (10 μm) through the dorsoventral axis of the spinal cord were used to determine lesion volume. Every 50 μm, sections were stained with Cresyl violet acetate and were studied with light microscopy. With a low-power (1.25×) objective, the lesion area was determined by MetaMorph software (Molecular devices). Areas at each longitudinal level were determined, and the total lesion volume derived by means of numerical integration of sequential areas.

Luxol-fast blue staining

To assess the loss of myelin, serial transverse cryosections (16 μm thickness) were stained with Luxol-fast blue staining as previously described (Yune *et al.* 2007).

Statistical analysis

Data are presented as mean ± SD values. Quantitative data from behavioral tests were evaluated for statistical significance using two-way ANOVA with a *post hoc* Tukey test; data from other experimental results were evaluated for statistical significance using Student's paired *t*-test. In all analyses, a value of $p < 0.05$ was considered statistically significant.

Results

Ethanol extract from *Scutellaria baicalensis* inhibits the production of inflammatory factors and ROS in primary microglia

As flavonoids isolated from *S. baicalensis* have been shown to exert neuroprotective effects by inhibiting activation of microglia (Kim *et al.* 2001b; Lee *et al.* 2003a; Li *et al.* 2005; Suk 2005), we first examined whether EESB exhibit anti-inflammatory effects on primary microglia cultures. We used an *in vitro* model consisting of LPS-induced microglia (Watters *et al.* 2002; Kim *et al.* 2006) and examined the effect of EESB on the production of inflammatory cytokines and mediators. Primary microglia were treated with LPS (100 ng/mL) followed by treatment with EESB (1, 5, 10, and 20 μg/mL). As shown in Fig. 1, LPS induced both mRNA and protein expression of TNF-α, IL-1β, COX-2, and iNOS. EESB treatment (5 and 20 μg/mL) significantly inhibited TNF-α, IL-1β, COX-2, and iNOS expression (Fig. 1b and d). EESB (1, 5, 10, and 20 μg/mL) inhibited expression of proinflammatory factors in a dose-dependent manner but the

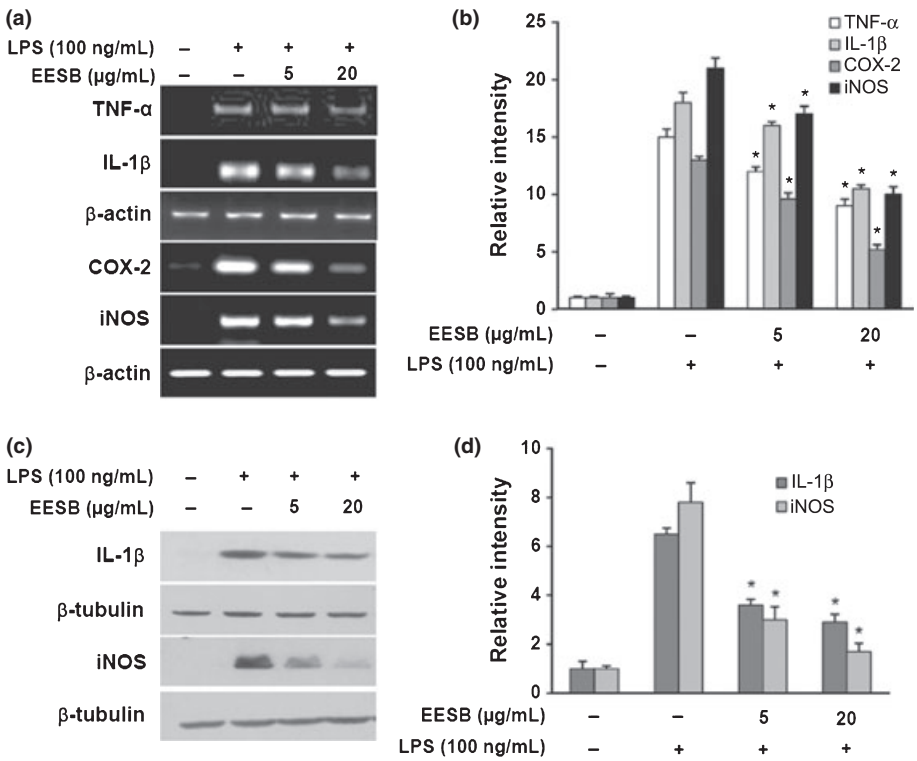

Fig. 1 EESB treatment inhibits expression of proinflammatory cytokines and mediators in primary microglia activated by LPS. RT-PCR (a) and western blot (c) analysis showed that expression of proinflammatory cytokines and mediators was increased after LPS treatment in primary microglia. Quantitative analyses of RT-PCR (b) and western (d) gels show that both protein and mRNA expression of TNF-α, IL-1β, iNOS, and COX-2 were significantly inhibited by EESB treatment when compared with that of vehicle control. Data represent mean ± SD from three separate experiments; $^*p < 0.05$ versus LPS-treated control.

inhibitory effect of EESB at 1 μg/mL was not statistically significant (data not shown). Next, we examined whether EESB exhibit antioxidant effect on LPS-induced primary microglia by examining the production of ROS using DCF-DA. As shown in Fig. 2a and b, LPS treatment increased ROS production 12 h after activation with LPS; EESB treatment (10 μg/mL) significantly attenuated this increase in ROS. The intensity of DCF-DA was decreased by EESB treatment (1, 5, 10, and 20 μg/mL) in a dose-dependent manner, but EESB treatment at 1 μg/mL showed no significant decrease in the DCF-DA intensity (data not shown). Similarly, NO production was increased by LPS-activation and LPS-induced NO production was significantly inhibited by EESB in a dose-dependent manner (Fig. 2c).

EESB inhibits protein carbonylation and nitration and expression of proinflammatory factors after SCI

Reactive oxygen species induce damage to such macromolecules as proteins, lipids, and DNA resulting in functional impairments of these molecules (Valko *et al.* 2007). In particular, proteins are vulnerable to be carbonylated and nitrosylated on tyrosine residues by ROS (Berlett and Stadtman 1997; Dalle-Donne *et al.* 2006). We examined the extent of protein carbonylation and nitration in injured spinal cord as an index of protein oxidation. As shown in Fig. 3a, carbonylated proteins were barely detectable in the sham control. By contrast, carbonylated proteins – varying in molecular weight from 28 to 250 kDa – were observed in the spinal cord 1 day after injury; EESB (100 mg/kg) post-treatment significantly decreased protein carbonylation when compared with that in the vehicle control (Figs. 3a and b). Also, immunohistochemistry using a specific antibody for nitrotyrosine revealed that nitrotyrosine-positive neurons were observed in the ventral horn of the injured spinal cord at 1 day after injury (Figs. 3c). However, nitrotyrosine-positive neurons were not observed in the sham-operated spinal cord (data not shown). Quantitative analysis showed that EESB treatment significantly decreased the number of nitrotyrosine-positive neurons in ventral horn (Fig. 3d).

Microglia are activated following SCI and produce proinflammatory cytokines and mediators which lead to neuronal and oligodendrocyte cell death (Lee *et al.* 2003c; Festoff *et al.* 2006). As EESB inhibit LPS-induced microglial activation *in vitro* (see Fig. 1), we hypothesized that EESB might also inhibit microglial activation after SCI. To

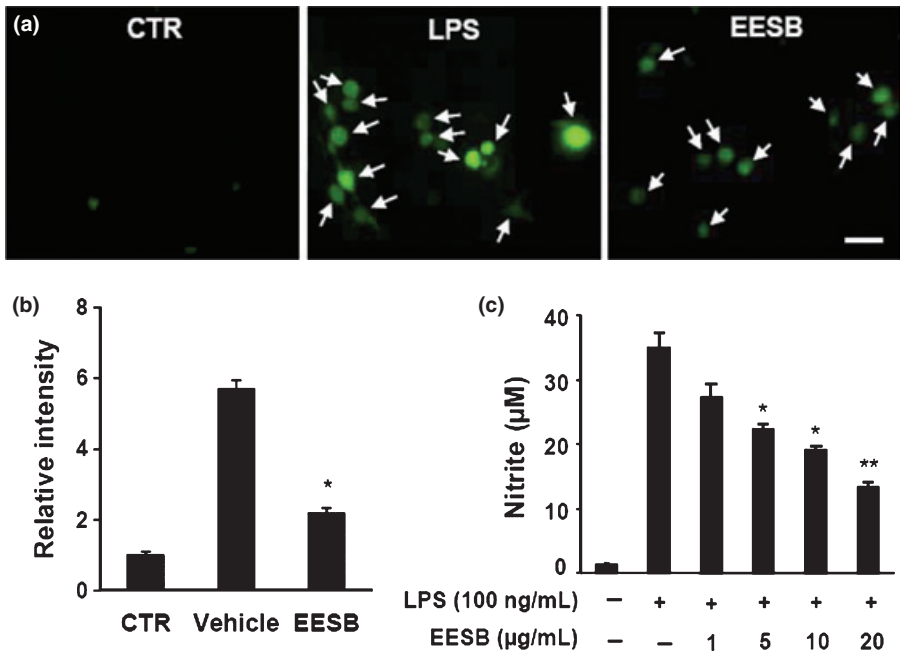

Fig. 2 EESB treatment inhibits production of ROS and NO in primary microglia activated by LPS. (a) DCF fluorescence in microglia was increased by LPS treatment and decreased by EESB treatment. Arrows indicate DCF-positive microglia. Scale bar, 10 μm. (b) Quantitative analysis of DCF fluorescence shows that EESB (10 μg/mL) significantly decreased ROS production when compared with that of LPS control. (c) NO production was increased by LPS treatment and significantly inhibited by EESB in a dose-dependent manner. Data represent mean ± SD from three separate experiments; $^{*}p < 0.05$ and $^{**}p < 0.001$ versus LPS-treated control.

evaluate whether EESB inhibits microglial activation after SCI, expression of proinflammatory cytokines and mediators at 4 and 8 h after injury was examined. Expression of TNF-α, IL-1β, IL-6, COX-2, and iNOS mRNA after SCI was markedly increased as reported (Lee *et al.* 2003c; Yune *et al.* 2003) (Fig. 4a). Furthermore, EESB treatment significantly inhibited mRNA expression of TNF-α, IL-1β, COX-2, and iNOS at 4 h and IL-6 at 8 h after injury when compared with those of vehicle control (Fig. 4b). Immunostaining using OX-42 antibody, a microglia marker, revealed that after SCI, a number of activated microglia exhibiting marked cellular hypertrophy and retraction of cytoplasmic processes were observed in both the GM and the WM as reported (Fig. 4c) (Hains and Waxman 2006). EESB treatment significantly reduced the proportion of activated microglia when compared with vehicle-treated control (Fig. 4d).

EESB inhibits apoptotic cell death after SCI

Trauma to the spinal cord results in extensive apoptotic cell death (Liu *et al.* 1997; Lee *et al.* 2003c). As the methanol extract of *S. baicalensis* has been shown to protect hippocampal neurons after cerebral ischemia (Kim *et al.* 2001b), we examined whether EESB alleviated apoptotic cell death after SCI. As expected, following SCI, TUNEL-positive neurons, and oligodendrocytes were observed in the GM (1 day) and in the WM (5 days), respectively (Fig. 5a) as reported (Yune *et al.* 2003, 2004, 2007; Stirling *et al.* 2004). EESB (100 mg/kg) post-treatment significantly reduced the number of TUNEL-positive neurons and oligodendrocytes when compared with the vehicle control (Fig. 5b). DNA gel electrophoresis also revealed a marked reduction of DNA laddering following EESB treatment 1 day after injury (Fig. 5c).

EESB improves functional recovery after SCI

Rats exposed to the 25 mm weight-drop insult were treated with EESB (30, 100, and 300 mg/kg) beginning 2 h after injury and then once daily for 14 consecutive days. Functional recovery was then evaluated for 35 days after the injury using the BBB rating scale, the grid walk test and footprint analysis. The hindlimbs were paralyzed immediately after injury, but the rats recovered extensive movement of the hindlimbs within 7–14 days after injury (Fig. 6a). The BBB scores were significantly higher in rats treated with 100 or 300 mg/kg of EESB by 14–35 days after SCI than in vehicle-treated rats (Fig. 6a). However, the BBB scores of EESB treatment (30 mg/kg) were not significantly higher that those of vehicle-treated group. In addition, there was no significant difference between 100 and 300 mg/kg of EESB-treated groups ($p > 0.05$). Other behavioral tests were plotted against both the EESB (100 mg/kg) and the vehicle-treated

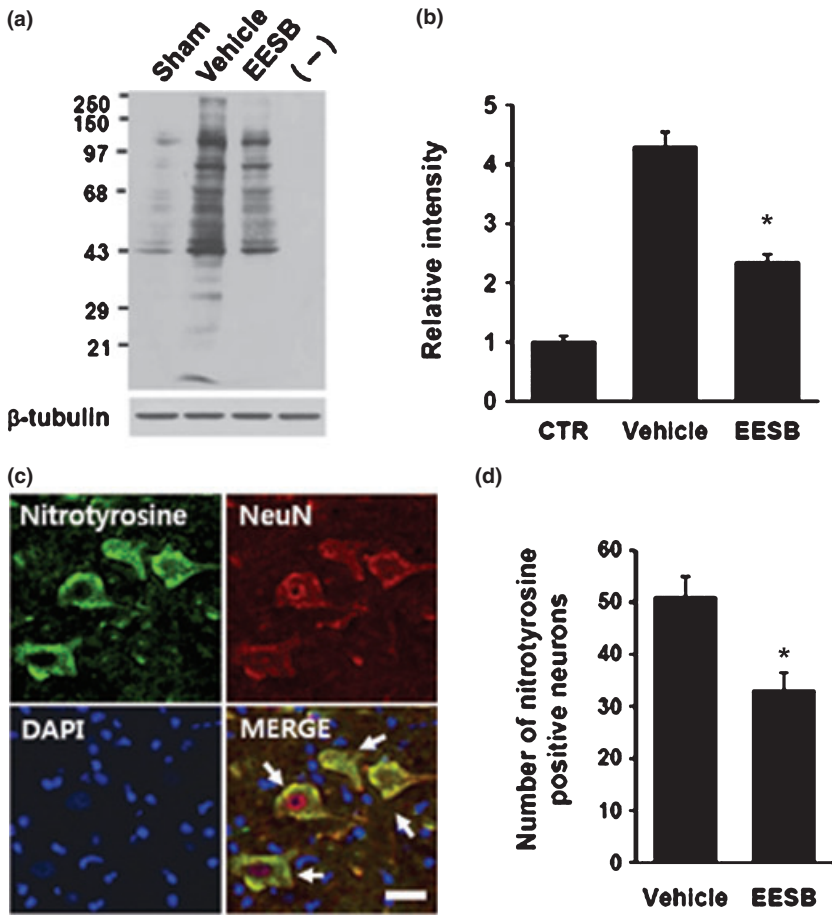

Fig. 3 EESB reduces protein carbonylation and nitration after SCI. (a) Total lysates from spinal cord samples at 1 day after injury were processed as described in the Materials and methods sections (n = 3/group). Western blot analysis shows that carbonylated proteins in the molecular weight range of 28–250 kDa were increased 1 day after injury. The lane (–) is a negative control that shows that carbonylated proteins were not detected on the membranes without 2, 4-dinitropheneylhydrazine derivatization at 1 day after injury. (b) Quantitative analysis of western gel shows that EESB (100 mg/kg) significantly reduced the level of protein carbonylation when compared with vehicle control. Data are presented as mean ± SD from three separate experiments; *$p < 0.05$. (c) Immunohistochemical analysis of protein nitration in ventral motor neuron at 1 day after SCI (n = 3/group). A representative photograph of nitrotyrosine (green)- and neuronal nuclei (NeuN) (red)-positive neurons (arrows) in the injured spinal cord was taken from a location 2 mm rostral to the lesion site. Bar, 30 μm. (d) Serial transverse sections (20-μm thickness) were collected every 500 μm from 2.5 mm rostral to 2.5 mm caudal to the lesion epicenter (total 11 sections) and the number of nitrotyrosine-positive neurons (over 30 μm in diameter) was counted. Data are presented as mean ± SD from three separate experiments; *$p < 0.05$. Quantitative analysis shows that EESB (100 mg/kg) treatment significantly decreases the number of nitrotyrosine-positive neurons when compared with that of vehicle control.

group. The ability to control and place the hindlimbs precisely was tested on a horizontal grid 35 days after injury. As shown in Fig. 6b, the number of mistakes (footfalls on the grid walk) in the EESB-treated group (100 mg/kg) was significantly lower than the number observed in the vehicle-treated group. Finally, footprint analyses obtained from sham-treated, vehicle-treated, and EESB-treated rats 35 days after SCI reveal that EESB-treated rats showed more consistent forelimb–hindlimb coordination and less toe drag which compares more favorably to what is observed in the normal animal (Fig. 6c). By contrast, the footprints obtained from vehicle-treated animals showed inconsistent coordination and extensive drags as revealed by ink streaks extending from both hindlimbs.

Histological analyses also revealed that the lesion volume of EESB-treated groups at 38 days after injury was significantly smaller than that of the vehicle-treated control (Figs. 6d and e). Next, the extent of myelin loss after injury was assessed by Luxol-fast blue staining. As shown in Fig. 6f, extensive myelin loss near the lesion area was evident in the vehicle-treated group at 38 days after injury when compared with the sham control (Fig. 6f, Vehicle)

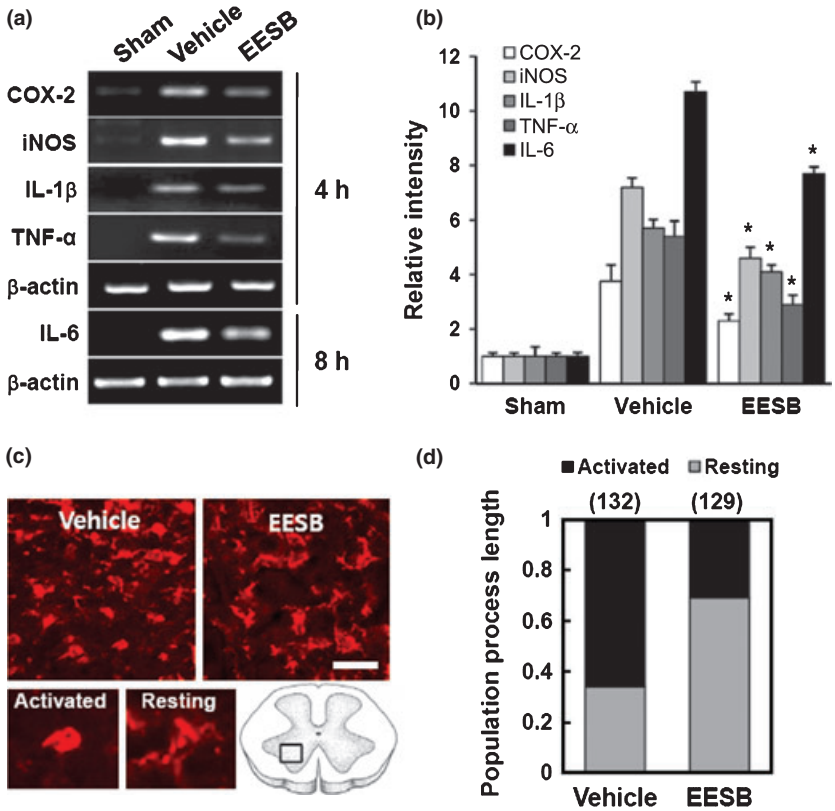

Fig. 4 EESB inhibits expression of inflammatory factors and reduces microglial activation after SCI. (a) Total RNA isolation from spinal cord samples at 4 and 8 h after injury were processed as described in the Materials and methods sections (n = 3/group). RT-PCR analysis shows that expression of TNF-α, IL-1β, iNOS, and COX-2 at 4 h and IL-6 at 8 h was reduced by EESB (100 mg/kg) treatment when compared with that of vehicle control. (b) Quantitative analysis of RT-PCR shows that expression of proinflammatory factors was significantly reduced by EESB. Data represent mean ± SD from three separate experiments; *$p < 0.001$. (c) Immunolabeling for OX-42-positive microglia in the ventral horn (right bottom panel, box) (n = 5/group). Sections were taken 1 mm caudal to the lesion epicenter. Microglia exhibited activated phenotype with characteristics: marked cellular hypertrophy and retraction of processes at 4 h after injury (Vehicle). (d) Quantification of the proportion of resting and activated microglia. After SCI, there is a large shift from resting to activated microglia, which is significantly reversed by EESB. Scale bar, 30 μm. Data are presented as mean ± SD from five separate experiments.

whereas EESB treatment apparently attenuated myelin loss (Fig. 6f, EESB).

Discussion

As *S. baicalensis* has been shown to protect hippocampal neurons from cerebral ischemia (Kim *et al.* 2001b), we examined the neuroprotective effects of the EESB after SCI. Our results showed that EESB provided significant neuroprotection against moderate spinal contusion injury in rats. Also, we demonstrated that EESB inhibited the production of inflammatory cytokines/mediators and ROS in both primary microglial cultures and in injured spinal cord. Furthermore, EESB significantly inhibited apoptotic cell death of both neurons and oligodendrocytes, thereby improving functional recovery after SCI. EESB treatment also reduced lesion volume and extent of myelin loss at 38 days after injury. Following SCI, the involvement of proinflammatory cytokines and ROS in the cell death of neurons and oligodendrocytes has been well documented (Bao and Liu 2002; Lee *et al.* 2003c; Yune *et al.* 2003). Thus, our study indicates that after SCI, the inhibition of proinflammatory factors and of ROS production may account in part for the neuroprotective mechanism of EESB.

In pharmacological view, the therapeutic dose of a drug is often determined by analyzing the dose–response relationship. The dose–response curve is a plot of drug effect versus drug concentration and typically sigmoidal. Our data showed that both 100 and 300 mg/kg of EESB significantly improved hindlimb motor function and 30 mg/kg of EESB was not effective. In addition, there was no significant difference between 100 and 300 mg/kg of EESB-treated groups. In an animal model of global cerebral ischemia, 100 mg/kg of EESB is an optimal dose for neuroprotection after ischemic insult, and a range between 100 and 500 mg/kg of EESB is the plateau state

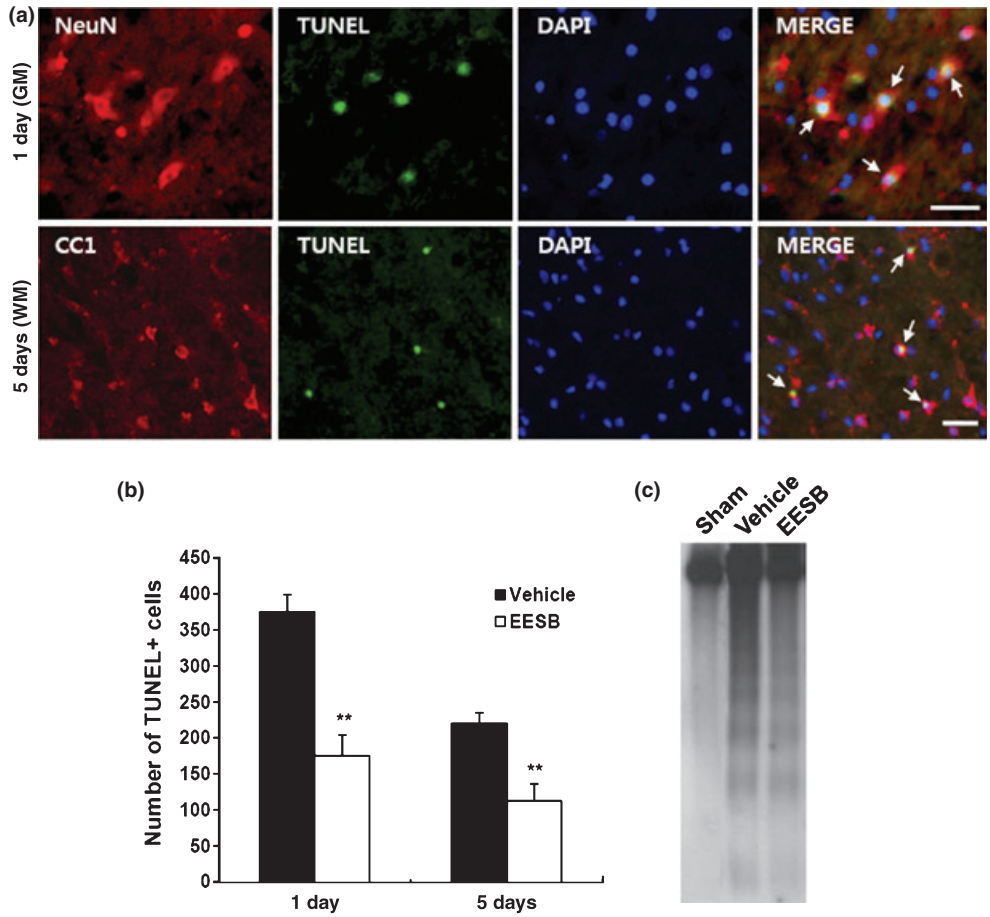

Fig. 5 EESB reduces apoptotic cell death of neurons and oligodendrocytes after SCI. Spinal cord samples at 1 or 5 days after SCI were processed for TUNEL staining as described in the Materials and methods sections (n = 5/group). (a) TUNEL labeling of neurons in the ventral horn area at 1 day after injury (upper panel). Representative images were from the sections selected 1 mm rostral to the lesion epicenter. TUNEL-positive oligodendrocytes in the WM at 5 days after SCI (bottom panel). Representative images were from the sections selected 5 mm rostral to the lesion epicenter. Arrows indicate TUNEL-positive neurons or oligodendrocytes. Bars, 30 μm. (b) Quantitative analysis of TUNEL-positive neurons at 1 day or oligodendrocytes at 5 days after injury. Note that the number of TUNEL-positive cells were significantly reduced in the EESB (100 mg/kg)-treated group when compared with vehicle control. Data represent mean ± SD obtained from five separate experiments; $^{**}p < 0.001$. (c) The extent of DNA laddering was increased after injury when compared with sham control; however, the intensity of DNA laddering was less in the EESB (100 mg/kg)-treated group when compared with that in the vehicle control.

in a dose–response curve (a personal communication with Dr Hocheol Kim at Kyung Hee University College of Oriental Medicine, Department of Herbal Pharmacology, Seoul, Korea). Based on this observation, we believe that both 100 and 300 mg/kg of EESB might be the plateau state in dose–response effect of EESB in our *in vivo* system so that there was no significant difference between two doses.

Three major flavonoids of *S. baicalensis*: baicalein, baicalin, and wogonin have been shown to exert neuroprotective effects both *in vitro* and *in vivo* (Gao *et al.* 2001; Kim *et al.* 2001b; Lee *et al.* 2003b, 2005; Li *et al.* 2005). In this study, we did not examine and compare the neuroprotective effects of an individual flavonoid within the EESB fraction on the functional recovery after SCI. However, our previous *in vivo* study (Kim *et al.* 2001b) and preliminary results *in vitro* (data not shown) suggest that the entire EESB, when compared with any particular flavonoid present within the EESB fraction, appears to be more efficacious on functional recovery after SCI.

It has been shown that total extract isolated from the roots of *S. baicalensis* protects CA1 hippocampal neurons from cerebral ischemia (Kim *et al.* 2001b). Wogonin, one of the major components of *S. baicalensis*, also shows the neuroprotective effect in brain injury models, transient global ischemia, and excitotoxic injury by kainite (Lee *et al.* 2003a). Furthermore, a recent report shows that baicalin, one of the major components of *S. baicalensis*, passes

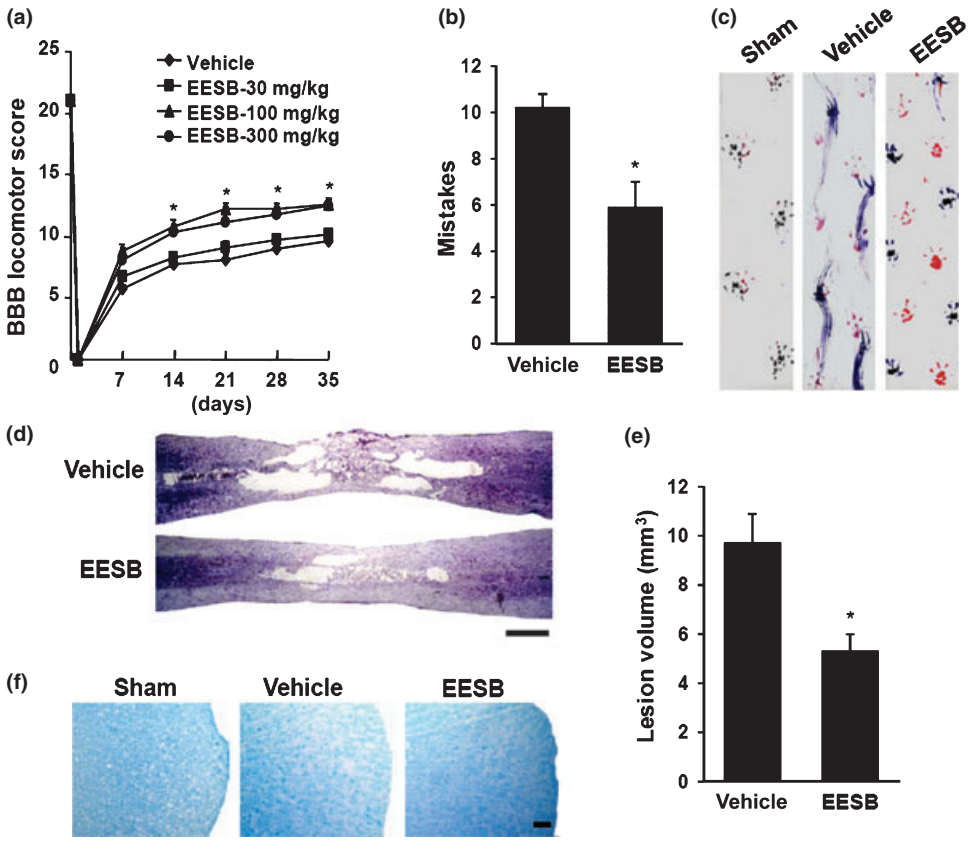

Fig. 6 EESB improves functional recovery after SCI. After SCI, EESB was administered orally 2 h after injury, and recovery was assessed by BBB, grid walk test, and footprint analysis (n = 10/group). (a) The BBB scores were significantly increased in rats treated with EESB (100 and 300 mg/kg) at 14–35 days when compared with vehicle-treated rats. Data represent mean ± SD; *$p < 0.05$ versus vehicle control. (b) Grid walk test of the vehicle- and EESB-treated groups at 35 days after injury. The number of footfalls (mistakes) was significantly decreased in the EESB (100 mg/kg)-treated group when compared with that of vehicle control group; *$p < 0.05$. (c) Representative footprints obtained from each group at 35 days after SCI show a more consistent 'plantar stepping,' conducive to weight support, and to fewer toe drags in the EESB (100 mg/kg)-treated group when compared with the gait pattern observed in vehicle-treated animals. (d) Representative spinal cord tissues (1.2 mm from dorsal surface) showing cavitation in the lesion site at 38 days after injury. Scale bar, 1 mm. (e) Quantitative analysis of lesion volumes at 38 days after injury. Data are presented as mean ± SD from five separate experiments; *$p < 0.05$. (f) Luxol-fast blue staining shows that myelin loss in lateral funiculus was extensive in the vehicle control when compared with that in sham control after injury. EESB treatment decreased the extent of myelin loss when compared with that in vehicle control. Scale bar, 30 μm.

through the blood–brain barrier and is detectable in brain dialysate (Huang *et al.* 2008). Based on these observations, we suggest that EESB can cross the blood–brain barrier and exerts its neuroprotective effect after SCI.

Inflammation has been implicated in a number of neurodegenerative conditions such as Alzheimer's disease (Hull *et al.* 2002), Parkinson's disease (McGeer *et al.* 2001), and brain ischemia (Liao *et al.* 2001). Following SCI, acute inflammatory responses involve the recruitment of neutrophils and macrophages to the site of injury and activate resident microglia (Taoka and Okajima 1998). Also, injury to the spinal cord triggers a rapid and robust up-regulation of such proinflammatory cytokines as IL-1β, TNF-α, and IL-6 (Wang *et al.* 1996; Hayashi *et al.* 2000). Moreover, anti-inflammatory drugs, such as minocycline, reduce apoptotic cell death by inhibiting microglial activation after SCI (Lee *et al.* 2003c; Stirling *et al.* 2004; Festoff *et al.* 2006; Yune *et al.* 2007). In this study, we observed that EESB significantly inhibited microglial activation after injury (see Fig. 4), and EESB treatment decreased production of proinflammatory cytokines and mediators after SCI. Thus, the results indicate that the neuroprotective effects of EESB may be attributable in part to inhibition of microglial activation. In addition, as infiltrating neutrophils involve inflammatory response and oxidative damage after SCI (Taoka and Okajima 1998), we examined the effect of EESB on neutrophil infiltration by myeloperoxidase activity assay. However, EESB treatment had no significant effect on

myeloperoxidate activity, suggesting that EESB may not affect neutrophil infiltration after injury (data not shown). Furthermore, as baicalin can distribute into the CSF very quickly and reaches its peak concentration within 30 min after its intravenous injection (Huang *et al.* 2008), we suggest that the effect of EESB on cytokine expression (4 h post-injury) may be attributable to its rapid delivery and inhibition of microglial activation.

Reactive oxygen species play a critical role in the apoptotic process in a variety of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and SCI (Swerdlow *et al.* 1996, 1997; Bao and Liu 2002, 2004). After CNS injury, apoptosis is triggered by the accumulation of such free radicals as ROS, which includes superoxide anion, hydroxyl radical, and reactive nitrogen species such as NO and peroxynitrite (Lewen *et al.* 2000). The oxidation of proteins is generally accepted to be a major pathogenic mechanism of oxidative stress, and this phenomenon has been well studied in aging and in cerebral ischemia (Sadek *et al.* 2003; Crack and Taylor 2005). The carbonylation of proteins is a result of irreversible oxidative damage and is considered as an indicator of severe oxidative damage and disease-derived protein dysfunction (Berlett and Stadtman 1997; Dalle-Donne *et al.* 2006). A number of neurodegenerative diseases have been associated with the accumulation of proteolysis-resistant aggregates of carbonylated proteins in tissues (Choi *et al.* 2004; Jiang *et al.* 2004). After SCI, the levels of NO have also been found to increase and to form peroxynitrite, a strong oxidant when it reacts with superoxide anion ($O_2 \cdot^-$) (Bao and Liu 2002; Yune *et al.* 2003). Peroxynitrite is also known to mediate several destructive chemical reactions including protein carbonylation (Berlett and Stadtman 1997; Szabo 2003; Dalle-Donne *et al.* 2006). Our data demonstrated that protein carbonylation and nitration were dramatically increased after SCI whereas EESB treatment significantly attenuated the extent of protein carbonylation and nitration. Thus, these data indicate that the neuroprotective effect of EESB may also be partly ascribed to antioxidant activity.

We first demonstrated that EESB exerts neuroprotective effects after SCI. Also, our study indicates that neuroprotective effects of EESB appear to be attributable to both its anti-inflammatory and antioxidative activities after injury. However, it should be noted that components of EESB other than flavonoids and unknown to us may have similar neuroprotective effects after injury. Our study thus suggests that EESB may be a candidate for use as an orally administered therapeutic agent for acute human SCI. Although herbal remedies are usually perceived as natural and thus devoid of side effects, they may contain such toxic materials as heavy metals (Bayly *et al.* 1995; Abbot 1996). Also, indications for using a given herbal remedy are often ill-defined and dosage is arbitrary because the concentrations of the active components are unknown. However, this type of uncertainty need no longer be an issue, for the active ingredients of *S. baicalensis* have been identified as particular flavonoid compounds. It may be the case that the main flavonoids from *S. baicalensis* could be utilized as pro-drugs for use in the development of other novel neuroprotective agents for the treatment of neurodegenerative diseases and acute CNS injuries.

Acknowledgements

The authors wish to thank Dr George Markelonis at the University of Maryland School of Medicine for his editorial assistance. This study was supported by grants from the Korea Science and Engineering Foundation (KOSEF) No. R01-2007-000-20617-0, R11-2008-036-02001-0 (SRC) 2009K001286 (Brain Research Center of the 21st Century Frontier Research Program), Seoul R&BD Program (No.10524), and Post BK21 Program.

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