Journal of Ethnopharmacology 139 (2012) 6–11

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Neuroprotective effects of *Eleutherococcus senticosus* bark on transient global cerebral ischemia in rats

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ARTICLE INFO

Article history:
Received 3 January 2011
Received in revised form 11 May 2011
Accepted 19 May 2011
Available online 27 May 2011

Keywords:
Eleutherococcus senticosus
Rats
Global cerebral ischemia
Neuroprotection
Functional recovery
COX-2
Glia

ABSTRACT

Ethnopharmacological relevance: Eleutherococcus senticosus Maxim., classified into the family of Araliaceae, is used in a variety of diseases in traditional Korean medicine including ischemic heart disease.
Aim of the study: To determine the neuroprotective effects of *Eleutherococcus senticosus* on global cerebral ischemia.
Materials and methods: A four-vessel occlusion (4-VO) rat model was used to evaluate the potential protective effects against transient global cerebral ischemia ethanol extracts of *Eleutherococcus senticosus* was orally administered at doses of 3, 30, and 300 mg/kg twice at times of 0 and 90 min after reperfusion. The effects on memory deficit were investigated by using a Y-maze neurobehavioral test after brain ischemia, and the effects on hippocampal neuronal damage were measured 7 days after ischemia. The expressions of glial fibrillary acid protein (GFAP), CD11b antibody (OX-42), and cyclooxygenase-2 (COX-2) were investigated by immunohistochemistry.
Results: Oral administration of *Eleutherococcus seticosus* at 30, 100 and 300 mg/kg significantly reduced hippocampal CA1 neuronal death by 3.5%, 25.9% and 53.1%, respectively, compared with a vehicle-treated group. Oral administration of *Eleutherococcus senticosus* at 300 mg/kg inhibited 81.9% of the decrease in spontaneous alternation induced by 4-VOin the Y-maze test, and also attenuated ischemia-induced activation of COX-2, GFAP and OX-42 in the hippocampal CA1 region.
Conclusion: Eleutherococcus senticosus protects delayed neuronal death in the CA1 region of the hippocampus against global cerebral ischemia in rats with the recovery of spatial memory, which can be considered as the normal functioning of the hippocampus. Regarding the immunohistochemical study, the effect of *Eleutherococcus senticosus* may be attributable to its anti-inflammatory properties through the inhibition of COX-2 expression, microglia and astrocyte expression.

1. Introduction

Transient global cerebral ischemia, arising in humans, can be a consequence of cardiac arrest or severe systemic hypotension. It leads to major neuropsychological dysfunctions, including learning and memory disabilities (Peskine et al., 2004). Although cardiac arrest and cerebral ischemia remain as leading causes of adult disability in industrialised countries, little progress has been achieved in translation of promising experimental therapies into clinical practice (O'Collins et al., 2006; Rosamond et al., 2008). The main focus of drug development to protect ischemia-induced injury has been the investigation of neuroprotective sources capable of protecting salvageable neurons from ischemic cell death. Natural products, especially medicinal plants, could be an ideal source to develop safe and effective agents for neuroprotection of ischemia-induced injury (Kim, 2005).

Eleutherococcus senticosus Maxim., known as Siberian Ginseng, is a medicinal herb with a long history of use including ischemic heart disease due to its traditional Korean medical effects such as tonify *qi*, strengthen muscle and bone, and tranquilize (Yi et al., 2001; Wang et al., 2010). *Eleutherococcus senticosus* has also been reported to possess anti-stress, anti-tumor, hypoglycemic, and anti-arrhythmic effects (Hibasami et al., 2000; Kimura and Sumiyoshi, 2004; Park et al., 2006; Maslov and Guzarova, 2007). The major active components of *Eleutherococcus senticosus* are eleutherosides, chiisanoside, acanthosides, daucosterin, β-sitosterol, sesamin, and they are responsible for its diverse biological activities (Davydov and Krikorian, 2000). It has been reported that eleutheroside E, liriodendrin, isofraxidin, chiisanoside and β-sitosterol have been reported to have

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doi:10.1016/j.jep.2011.05.024

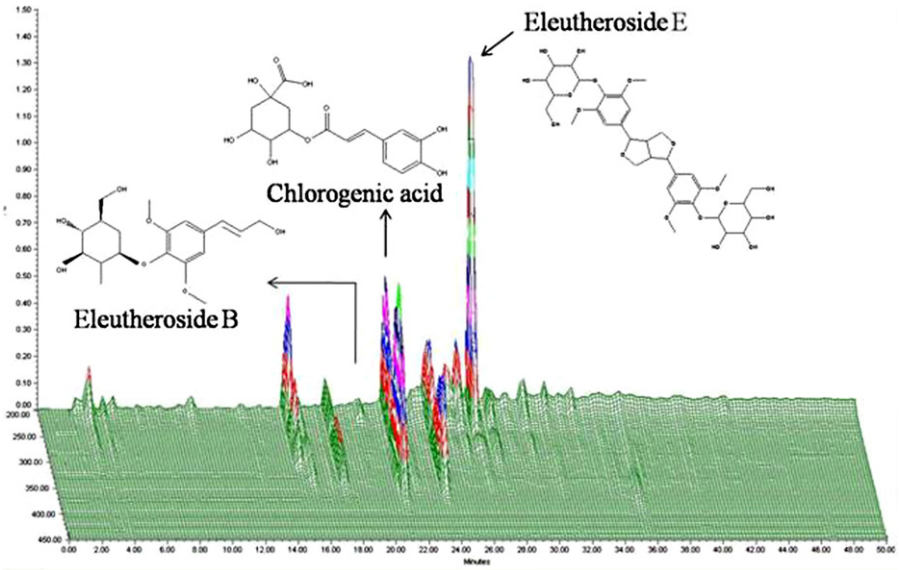

Fig. 1. 3-D HPLC chromatogram for standardization of *Eleutherococcus senticosus*. Detection was performed by using a photodiode array detector. *X*-axis is retention time; *Y*-axis is wavelength, and *Z*-axis is absorbance unit. Analytical conditions were as follows: column, C_{18} Φ4 250 mm; mobile phase, solvent A (1% H_3PO_4) and solvent B (CH_3CN); flow rate, 1 ml/min; program, 0–60 min 5–50% B; 60–61 min 50–70% B; 61–85 min 70% B.

anti-inflammatory effects (Tokiwa et al., 2006; Jung et al., 2003; Yamazaki et al., 2007). Especially, *Eleutherococcus senticosus* has been reported to protect against neuronal death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and amyloid beta (Fujikawa et al., 2005; Tohda et al., 2008).

Recently, our group found that *Eleutherococcus senticosus* reduces the infarct volume in transient focal cerebral ischemia (Bu et al., 2005). However focal ischemia is a far more complex insult than global ischemia with several unresolved issues such as extracellular edema, vascular damage while global ischemia involves a short very intense insult in which ATP is severely lowered and is quite uniform as a delayed neuronal death (Lipton, 1999). Global ischemia is characterized by a slow development of cell death during reperfusion, which shows great selectivity. There is no intimation of involvement of the vasculature, although there is a real possibility that microglia/macrophages play a role in the process (Swan et al., 1988).

The aim of the present study was to determine the neuroprotective effects of *Eleutherococcus senticosus* in global cerebral ischemia in rats. We used a four-vessel occlusion (4-VO) rat model to evaluate the potential protective effects against transient global cerebral ischemia (Katsuta et al., 2003). Y-maze test was used to investigate spatial memory impairment. We observed the expression of glial fibrillary acid protein (GFAP), CD11b antibody (OX-42), and COX-2 by immunohistochemistry to find out the inhibitory effects on microglia activation, astrocyte activation and COX-2 upregulation which are related in inflammation.

2. Materials and methods

2.1. Plant material

The dried stem bark of *Eleutherococcus senticosus* was purchased from *Yaksudang* Co. (Seoul, Korea). It was identified by Dr. Ho-Young Choi, Department of Herbal Pharmacology, College of Oriental Medicine, Kyung Hee University where the voucher specimen (#HP060) is deposited.

2.2. Sample preparation

Eleutherococcus senticosus (150 g) was extracted with 70% ethanol (3000 ml) for 3 h at 80 °C in a reflux apparatus. The extract was filtrated and concentrated under reduced pressure, then, lyophilized to yield a dark brown powder. The yield of extract was 9.26%. The sample was then stored at −20 °C for further use. The quantitative authentication of *Eleutherococcus senticosus* was performed by a high performance liquid chromatography (HPLC) analysis system equipped with a Waters 600 controller, a 717 autosampler and a 996 PDA detector. The chromatic separation was achieved at room temperature on Hypersil Gold C_{18} column (250 mm × 4 mm i.d., 5 μm particle size). Mobile phases A and B were 1% H_3PO_4 (v/v) and CH_3CN, respectively. Gradient elution was as follows: 0–60 min 5–50% B; 60–61 min 50–70% B; 61–85 min 70% B. The flow rate was 1.0 ml/min and the sample injection volume was 10.0 μl. The isolated compounds were monitored with a photodiode array detector (926; Waters, Milford, MA, USA). In HPLC analysis, 3 compounds were identified in *Eleutherococcus senticosus*: eleutheroside E, eleutheroside B and chlorogenic acid. Among them, the content of eleutheroside E was calculated for standardization. *Eleutherococcus senticosus* was standardized to contain 0.486 ± 0.046% eleutheroside E. A 3-D HPLC chromatogram and the structures of the constituent compounds are shown in Fig. 1.

2.3. Animals

Male Wistar rats (170–190 g) were obtained from Samtako Co. (Osan, Korea). Animals were allowed to have an access to water and food *ad libitum*, and maintained under a constant temperature (23 ± 1 °C), humidity (60 ± 10%) and a 12 h light/dark cycle (light on 07:00–19:00 h). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University.

2.4. Surgery

Transient global cerebral ischemia was induced by 4-VO, as described in previous (Pulsinelli and Brierley, 1979). Briefly, under 1.5–2.0% isoflurane anesthesia in a mixture of 70% N_2O/30%O_2, vertebral arteries were electrocauterized and common carotid arteries were exposed. On the following day, both carotid arteries were occluded with aneurysm clips to induce cerebral ischemia. After

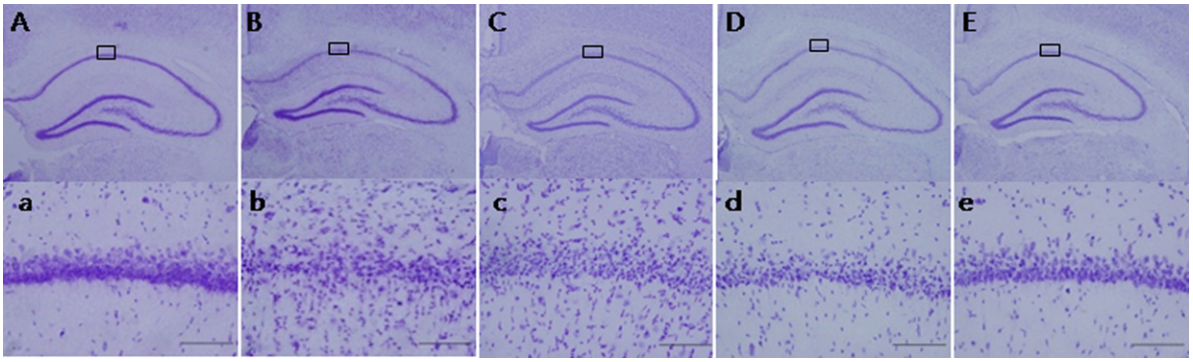

Fig. 2. Neuroprotective effects of *Eleutherococcus senticosus* against ischemic brain injury. Representative photomicrographs of Cresyl violet-stained hippocampal regions of sham-operated group (A, a) or vehicle-treated group (B, b) or *Eleutherococcus senticosus* [3 (C, c), 30 (D, d), and 300 (E, e) mg/kg]-treated group. Boxed regions in A, B, C, D and E (×40) are shown in a, b, c, d and e (×400), respectively. Scale bar is 100 μm.

10 min of occlusion, the aneurysm clips were removed for reperfusion. In order to minimize the variability among animals, the following criteria were strictly applied for the 10 min ischemic period and the 20 ± 5 min postischemic coma (loss of righting reflex and bilateral pupil dilation). Rectal temperature was maintained at 37 ± 0.5 °C until 6 h after ischemia.

Samples were dissolved in distilled water and administered orally twice at dosages of 3, 30 and 300 mg/kg at 0 and 90 min after reperfusion. The rats in the vehicle-treated group were administered distilled water. Sham-operated group was performed using the same surgical procedures, except that carotid arteries were not occluded.

2.5. Y-maze test

Four days after ischemia, a Y-maze was used as a test for short-term spatial memory. The maze was acrylic three-arm maze with equal angles between all arms. Each was 20 cm long, 5 cm wide and 10 cm high. Each rat was placed in the center of Y and allowed to explore freely during an 8 min session. The sequence and total number of arms entered were recorded. Arm entry was considered to be complete when the hind paws of the mouse were completely within the arm. The percentage of alternation was calculated as [successive triplet sets/(total number of arm entered − 2)] × 100 (Mori et al., 2001). The assessment of outcome was blinded.

2.6. Histology

Seven days after ischemia, the animals were anesthetized, and their brains were fixed with 4% paraformaldehyde (PFA) after transcardial wash-out with heparinized 0.5% sodium nitrite saline. The fixed brains were cut into 30 μm sections on a cryostat (CM3050 S; Leica, Heidelberg, Germany) and the sections, stained with cresyl violet (CV). Neuronal cell density was measured by counting viable cells in a total of 6 frames (1.0 mm × 1.0 mm) of the left and right CA1 regions of 3 coronal sections (approximately +3.3, 3.5, and 3.7 mm caudal to the bregma) for each animal. Neuronal cell density was equivalent to the average number of viable cells in 1 frame. The assessment of outcome was blinded.

2.7. Immunohistochemistry

Free-floating (40 μm) sections were reacted with a goat polyclonal antibody against COX-2 (diluted 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, USA) at two days after ischemia, a mouse polyclonal antibody against OX-42 (1:100, Serotec, Oxford, UK), or a rabbit polyclonal antibody against GFAP (1:500, Sigma, Saint Louis, USA) at seven days after ischemia overnight at room temperature. After incubation, the sections were reacted with anti-goat antibody, anti-mouse antibody or anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, USA) for 60 min, respectively and then reacted with an avidin–biotin–peroxidase complex kit (Elite ABC kit; 1:50, Vector Laboratories) at room temperature for 60 min. The avidin–biotin complex was visualized with 0.05% 3,3-diaminobenzidine (DAB; Sigma) and 0.02% H_2O_2.

2.8. Statistical analysis

All data were presented as the mean ± standard deviation (SD). The effects of different treatments were compared by one-way ANOVA followed by the Tukey's *post hoc* test using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects on neuronal cell density in the CA1 region

Typical images of hippocampal slices stained with CV from rats of Sham operated, and of 7 days after 4-VO for 10 min with distilled water and *Eleutherococcus senticosus* at dosages of 3, 30 and 300 mg/kg are shown in Fig. 2. The extent of the neuronal damage was quantified by counting the number of surviving pyramidal neurons in the CA1 region, which was expressed as a neuronal cell density (Fig. 3). A marked reduction in the cell densities in the CA1 region was observed in vehicle-treated group (88.0 ± 6.4 cells/mm^2) in comparison to the sham-operated group (415.6 ± 12.6 cells/mm^2, $p < 0.001$). Viable neurons had round and intact morphology, whereas dying neurons in the ischemic area showed a shrunken morphology (Fig. 2A vs. B). When rats were given *Eleutherococcus senticosus* orally at 0 and 90 min after reperfusion, there was a dose-dependent inhibition against the reduction of cell density due to ischemia that reached maximal levels (261.9 ± 26.0 cells/mm^2, 53%, $p < 0.001$) at 300 mg/kg. These results indicate that *Eleutherococcus senticosus* inhibited the neuronal loss in the CA1 region of hippocampus after 4-VO.

3.2. Effects on Y-maze test

To see whether the protective effects of *Eleutherococcus senticosus* on neuronal cell density in CA1 region associate with any functional recovery, we investigated hippocampal-dependent spatial learning and memory performance. Because spatial learning and memory can be assessed by tests of spontaneous alternation (Lalonde, 2002), we used a Y-maze to quantify spontaneous alternation at 4 days after ischemia. During an 8-min session of Y-maze

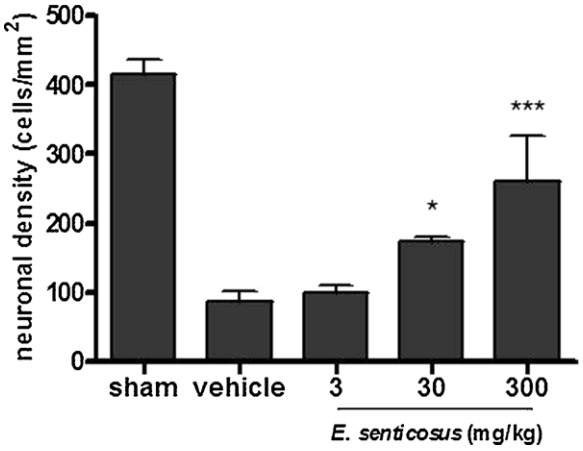

Fig. 3. Neuronal density of hippocampal CA1 region 7 days after ischemia. Either vehicle or *Eleutherococcus senticosus* (3, 30 and 300 mg/kg) was orally administered at 0 and 90 min after 10 min ischemia. Seven days later, neuronal cell density in CA1 region was measured by CV staining and cell counting. The values are mean ± SD [$F(4,16) = 45.09$, $^{*}p < 0.05$ or $^{***}p < 0.001$ vs. vehicle-treated group]. Sham, sham-operated group ($n = 5$); vehicle, vehicle-treated group ($n = 5$), *Eleutherococcus senticosus* (mg/kg), *Eleutherococcus senticosus* [3 ($n = 5$), 30 ($n = 6$) and 300 ($n = 8$) mg/kg]-treated group.

test, vehicle treated group (41.8 ± 2.4%) showed significant reduction compared to sham-operated group (64.5 ± 5.6%, $p < 0.0001$). However treatment with *Eleutherococcus senticosus* at 300 mg/kg significantly inhibited the decrease in spontaneous alternation induced by ischemia (60.4 ± 10.7%, $p < 0.001$). These results indicate that *Eleutherococcus senticosus* attenuated spatial memory loss (Fig. 4).

3.3. Effects on COX-2, GFAP and OX-42 expression

To define early changes in COX-2, immunohistochemical studies were performed at early reperfusion times in rats subjected to 4-VO for 10 min. We evaluated immunoreactivity for COX-2 which is involved in the mechanisms of neurotoxicity associated within flammation. Expression of COX-2 significantly increased in ischemic brain 48 h after 4-VO, which was markedly inhibited by *Eleutherococcus senticosus* (Fig. 5B vs. C). Seven days after ischemia, we performed immunohistochemical staining of GFAP and OX-42 in rat brain slices to investigate whether *Eleutherococcus senticosus* has an effect on the inhibition of CA1 astrocytes and

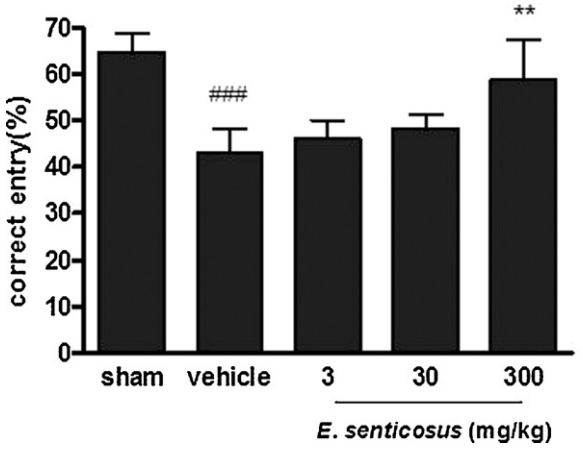

Fig. 4. Changes in spontaneous alternation in the Y-maze test after 4-VO-induced global ischemia. Each group comprises 6–8 animals. The values are mean ± SD. [$F(4,18) = 11.11$, $^{###}p < 0.001$ vs. sham-operated group, $^{**}p < 0.01$ vs. vehicle-treated group].

microglia activation. Immunohistochemical staining with GFAP showed only few GFAP-positive astrocytes in the sham-operated groups (Fig. 5G). However, ischemia induced an increase in GFAP-positive astrocytes, with hyperplasia and hypertrophy flanking the pyramidal neurons around hippocampal CA1 (Fig. 5H). Ischemic that were treated with *Eleutherococcus senticosus* showed a marked decrease in reactive astrocytes compared with the ischemic group (Fig. 5H vs. I). With OX-42 as a marker, no microglial cells were found in the sham-operated group (Fig. 5D) and ischemia caused recruitment of microglial cells, which were especially clustered in the CA1 area with dying neurons (Fig. 5E). However, *Eleutherococcus senticosus* markedly reduced this activated microglia (Fig. 5F). In short, *Eleutherococcus senticosus* attenuated ischemia-induced COX-2 upregulation, and ischemia-induced astrocyte and microglial activation in hippocampal CA1 region.

4. Discussion

Our findings demonstrated that *Eleutherococcus senticosus* reduced neuronal cell death in the CA1 region of hippocampus and spatial memory loss on Y-maze at 7 days after 4-VO in rats. *Eleutherococcus senticosus* attenuated ischemia-induced COX-2 upregulation at 2 days after 4-VO and overactivation of GFAP and OX-42 at 7 days after 4-VO in hippocampal CA1 region.

Global ischemia leads to selective cell death of hippocampal CA1 pyramidal neurons while other neurons are much less vulnerable in humans and rodents. Since hippocampal CA1 neuronal death usually peaks at7 days after an initial ischemic insult, this phenomenon is referred to as delayed neuronal death (Lipton, 1999). Delayed neuronal death is related with high-energy metabolic failure, ATP depletion, ion imbalance, inflammation and other biochemical changes including an increase of free radicals, mitochondrial dysfunction, lactic acidosis, and inhibition of proteosynthesis as a consequence of endoplasmic reticular stress (DeGracia et al., 2002). The 4-VO rat model which causes delayed neuronal death in the hippocampal CA1 region has been developed to evaluate neuroprotective effects against transient global cerebral ischemia by reducing CA1 cell death (Kirino, 1982; Pulsinelli et al., 1982). In the present study, marked reduction in cell densities in the CA1 region was observed in vehicle-treated group in comparison to the sham-operated group at 7 days after 4-VO. Oral administration of *Eleutherococcus senticosus* at dosages of 3, 30 and 300 mg/kg at 0 and 90 min after reperfusion, showed dose dependent inhibition against reduction of cell density due to ischemia that reached 53.1% at 300 mg/kg. This result suggests that *Eleutherococcus senticosus* has neuroprotective effects against delayed neuronal death induced by global cerebral ischemia.

Global cerebral ischemia not only leads to neuronal damage in the hippocampus, but also results in a deficit in spatial learning and memory (Wang et al., 2008). It has been known that 4-VO impairs rat learning and memory (Mori et al., 2001; Gulinello et al., 2006). We also observed 4-VOcaused reduction in spontaneous alternation in the Y-maze test. Y-maze reveals impairment in spatial learning and memory which requires normal functioning of hippocampus by tests of spontaneous alternation (Wall et al., 2004). Oral administration of *Eleutherococcus senticosus* at the dosage of 300 mg/kg inhibited 81.9% of the decrease in spontaneous alternation induced by 4-VO. This outcome was consistent with morphological result, and the dosages that protected against ischemic neuronal death were analogous to those associated with spatial memory. Our result indicates that protective effects of *Eleutherococcus senticosus* in CA1 region is associated with a restoration of the ischemia-induced impairment of spatial learning and memory performance, suggesting that *Eleutherococcus senticosus* provides functional restoration after ischemia.

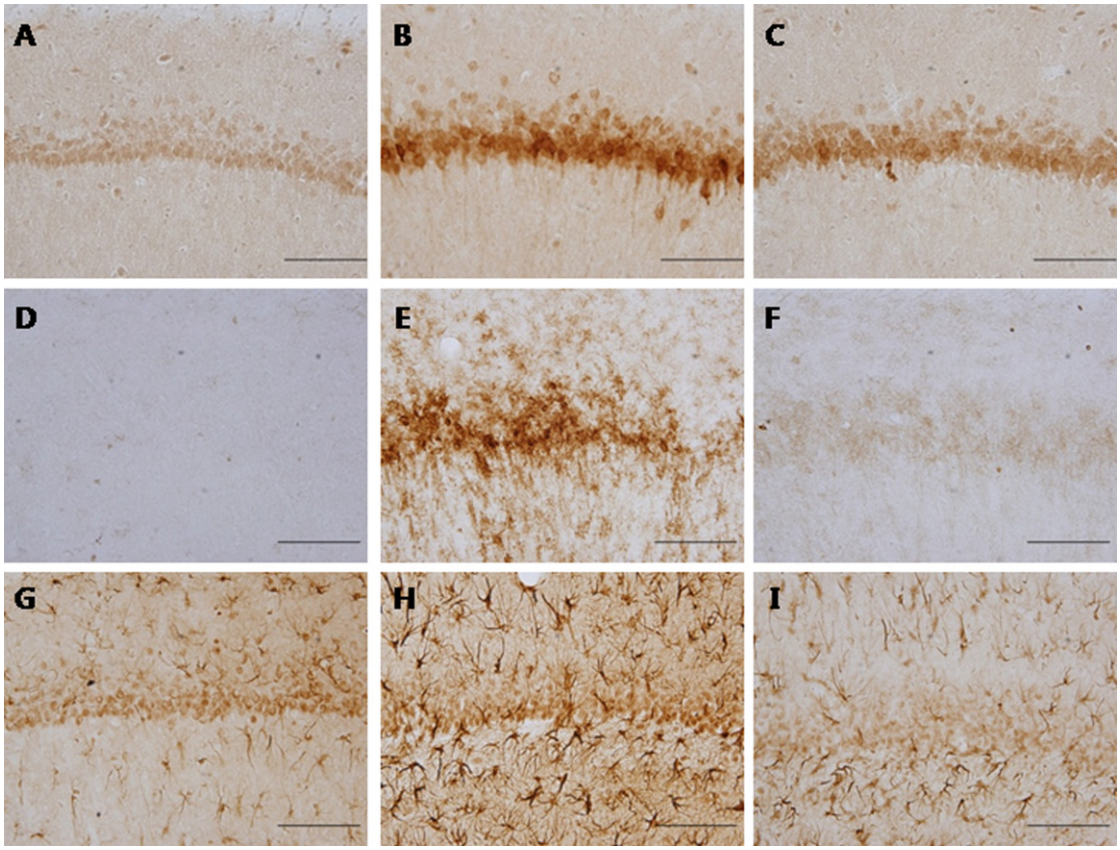

Fig. 5. Inhibitory effect of *Eleutherococcus senticosus* on COX-2 expression (A, B, C) in neocortex after 48 h, OX-42 expression (D, E, F) in ipsilateral hemisphere, and GFAP expression (G, H, I) in astrocyte after 7 days following 10 min of ischemia. Sham-operated group (A, D, G), vehicle-treated group (B, E, H), or *Eleutherococcus senticosus* 300 mg/kg treated group (C, F, I). Scale bar = 100 μm.

Global cerebral ischemia also triggers an inflammatory reaction that progresses for days after the onset (del et al., 2000; Iadecola and Alexander, 2001). COX-2 is rate-limiting enzyme involved in arachidonic acid metabolism, thereby generating prostaglandins and thromboxanes, molecules that play important roles in inflammatory reaction (Vane et al., 1998) and reported to upregulate in CA1 hippocampal cells until 3days after ischemia (Candelario-Jalil and Fiebich, 2008; Cheng et al., 2010). Treatments aimed at inhibiting COX-2 are viable strategies for targeting the late stages of ischemic injury (Araki et al., 2001; Candelario-Jalil, 2008). In this study, *Eleutherococcus senticosus* inhibited postischemicCOX-2 upregulation at 48 h after ischemia in hippocampal CA1 region. It is consistent with previous study that *Eleutherococcus senticosus* reduces infarct volume in transient focal cerebral ischemia with inhibition of COX-2 upregulation (Bu et al., 2005). Our results suggest that neuroprotective effects of *Eleutherococcus senticosus* after global ischemia might be attributable to interrupting inflammatory reaction by the inhibition of COX-2 expression.

Global cerebral ischemia is accompanied by reactive astrogliosis and activation of microglial cells in the hippocampal area (Jorgensen et al., 1993; Herrmann et al., 2000; Sulkowski et al., 2002). Reactive gliosis can produce excess amounts of cytokines as well as inflammatory products that exacerbate ischemic damage (Walker and Rosenberg, 2009; Wang et al., 2009). Several studies have suggested that inhibiting glial activation attenuates ischemic injury (Suk, 2004; Fox et al., 2005; Wang et al., 2005). In this study, are markable increase in GFAP (marker for activated astrocytes) and OX-42 (marker for activated microglia) immunoreactivity were observed in vehicle-treated group in comparison to sham-operated group while marked reduction were observed in *Eleutherococcus senticosus* treated group after 4-VO. There were increase in COX-2 expression at 2 days after 4-VO and microglia and astrocytes activations at 7 days after 4-VO, which suggests that these inflammatory responses were caused by 4-VO and that they in turn cause neuronal cell death in CA1 region. *Eleutherococcus senticosus* reduced the expressions of COX-2, microglia and astrocytes, which is consistent with past studies demonstrating protection against ischemic brain damage by targeting inflammatory response also show reductions in glial activation (Yrjanheikki et al., 1998, 1999; Pei and Cheung, 2004; Weng and Kriz, 2007).

In summary, *Eleutherococcus senticosus* protects delayed neuronal death in the CA1 region of hippocampus against global cerebral ischemia in rats with recovery of spatial memory, which can be considered as normal functioning of hippocampus. Regarding the immunohistochemical study, the effect of *Eleutherococcus senticosus* may be attributable to its anti-inflammatory properties by the inhibition of COX-2 expression, microglia and astrocyte expression. Based on these findings, it is tempting to speculate that *Eleutherococcus senticosus* may be a therapeutic candidate for the neuroprotection of tissue and the recovery of function in the neuroprotection in patients with global ischemia, such as a cardiac arrest, ischemic stroke or various neuroinflammatory disorders.

Acknowledgements

This work was supported by 2nd Stage of Brain Korea 21 Project (Ministry of Education, Korea) and a grant from the Kyung Hee University in 2008 (KHU-20080601).

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