Neuroprotective Effect of *Buddleja officinalis* Extract on Transient Middle Cerebral Artery Occlusion in Rats

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The flower buds of *Buddleja officinalis* Maxim (Loganiaceae) are used to treat headache and inflammatory diseases in traditional Korean medicine. In the present study, the neuroprotective effects of the methanolic extract of *B. officinalis* (BOME) and of its hexane fraction (BOHF) were investigated in a middle cerebral artery occlusion (MCAo, 120 min occlusion, 24 h reperfusion) Sprague-Dawley rat model. BOME or BOHF (100 mg/kg, p.o.) was twice administered 30 min before the onset of MCAo and 2 h after reperfusion. BOME and BOHF treated groups showed infarct volumes reduced by 33.9% and 68.2%, respectively, at 2 h occlusion. In BOHF treated animals, cyclooxygenase-2 and iNOS inductions were inhibited in ischemic hemispheres at both the mRNA and protein levels. Furthermore, *in vitro* studies showed that BOME and BOHF both inhibited nitric oxide production in BV-2 mouse microglial cells. These results suggest that the anti-inflammatory and the microglial activation inhibitory effects of *B. officinalis* extract may contribute to its neuroprotective effects in brain ischemia.

Key words *Buddleja officinalis*; middle cerebral artery occlusion; cyclooxygenase-2; inducible nitric oxide synthase; anti-inflammatory; microglial

Stroke is one of the most frequent causes of death along with cancer and cardiac diseases, and the administration of neuroprotective agents within 6 h of the onset of stroke are known to reduce neuronal cell death caused by stroke-induced ischemia. However, no effective neuroprotective agent is available to treat cerebral ischemia.

Inflammation plays a critical role in ischemic brain injury, and anti-inflammatory measures reduce injury and enhance stroke recovery.1 Moreover, the inflammatory reactions involved are increased by reperfusion after focal brain ischemia and are due to a substantial influx of neutrophils and leukocytes into infarcted regions driven by specific adhesion molecules and cytokines. These species aggravate tissue damage by releasing oxygen radicals3 and cytotoxic products,3 and among these nitric oxides (NO) and the prostaglandins are two types of pleiotropic mediators produced at inflammatory sites by constitutive enzymes and by cyclooxygenase (COX-1), COX-2 and inducible nitric oxide synthase (iNOS).4 COX-2 and iNOS are known to be expressed in cerebral ischemia and contribute to the ischemic damage induced by postischemic inflammation. Microglia, resident brain inflammatory cells, also become activated in cerebral ischemia and release several inflammatory mediators, such as NO, tumor necrosis factor-α, interleukin-1β, and glutamate.5–7

The flower buds of *Buddleja officinalis* Maxim (Loganiaceae) are used to treat stroke, headache, and neurological disorders in traditional Korean medicine.8 B. officinalis contains terpenoids, flavonoids, phenylethanoids, and saponins,9 and has been reported to protect PC12 cells from apoptosis and the oxidative stress induced by the 1-methyl-4-phenylpyridinium ion (MPP+),10 as well as to inhibit eicosanoid generation by leukocytes.11

In the present study, the neuroprotective effects of the methanol extract of *B. officinalis* (BOME) and of its fractions were studied in ischemic brain injury using a middle cerebral artery occlusion (MCAo) rat model. We also investigated whether BOME inhibits the inductions of COX-2, OX-42 and iNOS in vivo and LPS-stimulated NO production in mouse BV2 microglial cells.

MATERIALS AND METHODS

Preparation of Plant Extracts The flower buds of *B. officinalis* were purchased at the Kyungdong Oriental drug store (Seoul, Korea). Dried buds (800 g) were extracted by sonication using an 85% methanol aqueous mix. The 85% methanol filtrate was evaporated *in vacuo* to give the methanol extract (BOME; 99.26 g, 12.41%). The extract was then fractioned sequentially into water (BOWF; 22.4 g, 2.8%), butanol (BOBF; 1.32 g, 0.16%), ethyl acetate (BOEF; 12.68 g, 1.56%), and hexane (BOHF; 14.35 g, 1.36%). Samples were dried *in vacuo* and stored at −20 °C until required for neuroprotective assessment.

Samples Treatment Samples were dispersed in an aqueous solution of Tween 20 (5% w/v) and administered orally at a dosage of 100 mg/kg at 30 min before the onset of MCAo and 2 h after the surgery. Rats in the control group received vehicle in the same volume and with the same time schedule as extract-treated animals.

Reagents and Cell Culture All chemicals used were of analytical grade and were purchased from Sigma (St. Louis, MO, U.S.A.). BV-2 mouse microglial cells, which were originally developed by Dr. V. Bocchini (University of Perugia, Perugia, Italy), were donated by Dr. E. Choi at Korea University (Seoul, Korea). Cells were grown in DMEM containing 10% FBS, 2 mM glutamine, and penicillin-streptomycin (100 units/ml) (Gibco-BRL, Gaithersburg, MD, U.S.A.) at 37 °C.
in 5% CO₂–95% O₂ in a humidified incubator.

**Transient Focal Brain Ischemia** All surgical procedures were conducted according to the animal welfare guidelines issued by the Korean National Institute of Health and the Korean Academy of Medical Sciences. Rats were housed under controlled conditions (20±2 °C; lighting 7:00—19:00), with food and water available *ad libitum*. Focal ischemia-reperfusion was produced using the method described by Zea-Longa *et al.* Briefly, rats were anesthetized with isoflurane (initiated with 5% and maintained at 2% isoflurane) in N₂O/O₂ (70:30). Rectal temperature was controlled at 37±0.5 °C throughout the experiment using a heating lamp and a homeothermic blanket system (Harvard Apparatus, Holliston, MA, U.S.A.). The right carotid bifurcation was exposed through a midline neck incision, and a filament with a rounded tip and a distal cylinder of silicon rubber (0.30 mm in diameter), was introduced into the external carotid artery. A suture was then inserted at least 19—20 mm from the carotid bifurcation, and was withdrawn 2 h later to allow reperfusion.

**Measurement of Infarct Size** Twenty four hours following the onset of ischemia, animals were anesthetized by 5% isoflurane inhalation and sacrificed by decapitation. Brains were removed quickly and chilled in ice-cold saline for 5 min, and 6×2 mm coronal sections were obtained using a tissue slicer, beginning 1 mm posterior to the anterior pole. The slices obtained were then immersed in a saline solution containing 2%, 2.3, 5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, U.S.A.) for 30 min at 37 °C. Brain slices were photographed using a digital camera and quantified using an image analyzing system (Optimas 6.5 Media Cybernetics, Silver Springs, MD, U.S.A.). The infarct area in each slice was calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral edema and was presented as the percentage of the infarct to the area of the contralateral hemisphere.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)** PCR products were directly synthesized from 2 μg of total RNA isolated from affected hemispheres using the SuperScript one step RT-PCR system with Platinum Taq (Intron, Seoul, Korea) and gene specific primers, by following the instructions provided by the manufacturer. Reaction mixtures (Intron, Seoul, Korea) consisting of 2 μg of total RNA, and 5 pmol primers were incubated at 45 °C, and then denatured at 94 °C for 5 min, and subjected to 32 amplification cycles (30 s at 94 °C, 30 s at 45 °C, 1 min at 70 °C); this was followed by a final elongation for 10 min at 70 °C. PCR products were analyzed by agarose gel electrophoresis (1.5%) followed by a final elongation for 10 min at 70 °C. PCR products were analyzed by agarose gel electrophoresis (1.5%), stained with ethidium bromide, and scanned with GelDoc (Bio-Rad, Richmond, CA, U.S.A.). The primers used in this study were as follows: COX-2 forward primer, 5′-CCATGTCAAAAACCGTGTTGAATGG-3′; COX-2 reverse primer, 5′-ATGGGAGTGGCGGACTATGCA-3′; GAPDH forward primer, 5′-GGTTATGAGGGTGTAACCCAGG-3′; and GAPDH reverse primer, 5′-CGCTGACTTCCCCTTCCACGCT-3′. The expected sizes of RT-PCR products were 374 bp for COX-2 and 234 bp for GAPDH. COX-2 mRNA levels were normalized versus GAPDH mRNA in the same samples. In preliminary studies, we established that the RT-PCR products obtained were of the correct size and corresponded to COX-2 mRNA by excising bands from gels.

**Immunohistochemistry** Twenty four hours after ischemic reperfusion, animals were anesthetized and brains were perfusion fixed with 4% paraformaldehyde after a transcardial wash-out with heparinized 5% sodium nitrite saline. Fixed brains were sectioned (40 μm) on a sliding microtome, and incubated with anti-COX-2 antibody (diluted 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) or with a mouse polyclonal antibody against OX-42 (against CD 11b antigen, diluted 1:100; Serotec, Oxford) overnight at room temperature. After incubation, sections were treated with anti-goat or anti-mouse antibody (diluted 1:200; Vector Laboratories, Burlingame, CA, U.S.A.) for 60 min and then with an avidin–biotin-peroxidase complex kit (diluted 1:50; Elite ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) at room temperature for 60 min. Avidin–biotin complex was visualized using 0.05% 3,3-diaminobenzidine (DAB; Sigma) and 0.02% H₂O₂.

**Measurement of NO** NO production was measured as nitrite (the stable end product of NO) level in culture supernatant. Nitrite concentrations were determined in culture supernatants using a spectrophotometric method based on the Griess reaction. Samples were mixed with equal volumes of Griess reagent and incubated at room temperature for 10 min. Nitrite concentrations were determined by measuring absorbances at 540 nm and comparing results with those of sodium nitrite standards prepared using the same medium.

**Statistical Analysis** All data are present as means± S.E.M. The effects of different treatments were compared using the Student’s *t*-test in GraphPad Prism 4 (GraphPad Software Inc., U.S.A.). *p* values less than 0.05 were considered to be statistically significant.

**RESULTS**

**Effect of *B. officinalis* Extracts on Infarct Volume in MCAO Rats** The neuroprotective effects of BOME were evaluated by measuring infarction volumes at 24 h after reperfusion. BOME and its fractions (water, butanol, ethyl acetate, hexane) were used to treat groups of rats. Figure 1A shows typical photographs of brain slices, and that the infarct areas in BOME or BOHF treated rats assessed 24 h post-ischemia were less than that in sham or non-treated controls. Figure 1B summarizes the effects of BOME (100 mg/kg, *p.o.*) and BOHF (100 mg/kg, *p.o.*) administered 30 min before and 2 h after surgery on cerebral infarction in this rat focal brain ischemia model.

Of the four fractions examined, the BOHF group showed a significant reduction in infarct volume after ischemic insult (Fig. 1A) on MCAo, whereas BOWF, BOBF or BOEF treated animals showed no reduction in infarct volume (data not shown). The control group (*n*=9) showed a 34.47±2.71% infarct volume, the BOME group (*n*=6) 23.77±1.49%, and the BOHF group (*n*=7) 12.96±3.59%. Animal body temperatures of animals were monitored for 6 h after cerebral reperfusion commenced, and no significant differences were observed between the BOME, BOHF, and control groups (data not shown); thus the observed neuroprotective effects of BOME and BOHF were not attributable to hypothermic effects.
**COX-2 and OX-42 Proteins**  COX-2 immunoreactivity increased in the ipsilateral neocortex after 2 h of ischemia. In these experiments, we observed COX-2 expression in BOME and BOHF-treated rats was reduced compared with that in vehicle-treated control rats. The sham group showed weak staining, but the vehicle-treated control group showed deep neocortex staining. The BOHF-treated group showed a marked reduction in staining intensity and surrounding areas were not stained. COX-2 expression was restricted to the boundary of the infarct area and the medial portion of affected hemispheres (Figs. 2A, B, C).

The histochemical marker of microglia, OX-42, in the vehicle-treated control group was upregulated 24 h after ischemia in the ischemic core and penumbra versus the sham group. Moreover, OX-42 immunoreactive cells had an amoeboid shape. BOHF reduced OX-42 immunoreactivity and the amoeboid-like cell shape (Figs. 2D, E, F).

**Effect of *B. officinalis* Extracts on NO Production in LPS-Stimulated BV-2 Cells**  The inhibition of proinflammatory cytokines is believed to protect neurons from cell death, and here we investigated this possibility by evaluating NO production in BV-2 cells. NO detection assays showed that the amount of nitrite increased from $9.05 \pm 0.65$ to $48.12 \pm 1.67 \mu M$ after exposure to LPS (100 ng/ml) for 24 h. Moreover, NO synthesis was reduced from $29.4 \pm 2.12$ to $12.4 \pm 1.52 \mu M$ by treating with *B. officinalis* extract at 100 mg/ml. The present results show that LPS enhanced NO synthesis in BV-2 cells and that *B. officinalis* (BOHF) suppressed LPS-induced NO synthesis (Fig. 3).

**Effects of BOME or BOHF on COX-2 and iNOS Gene Expression**  To further investigate the anti-inflammatory mechanism of *B. officinalis* extracts in the rat ischemic brain, we used RT-PCR analysis to quantify the expression levels of the COX-2 and iNOS genes, which encode anti-inflammatory proteins. Since our TTC results showed that large brain regions were damaged by 2 h of ischemia followed by 24 h of reperfusion, affected hemispheres were used for RT-PCR. Figure 4 shows the effects of BOME and BOHF on COX-2 and iNOS gene expressions in the ischemic rat brain. Both COX-2 and iNOS gene expression levels were significantly ($p<0.05$) reduced in the BOME and BOHF groups versus the vehicle-treated control and sham groups. However, the expression of the housekeeping gene, GAPDH, was unaltered.

These results indicate that the administration of BOHF at-
In the present study, rats administered BOME at 100 mg/kg (p.o.) at 0 h and 2 h post-occlusion showed a 32.8% reduction in infarction volume versus the vehicle-treated control rats. In subsequent studies, BOME was separated into four fractions, i.e., hexane, ethyl acetate, butanol and water fractions by sequential fractionation. BOHF rated into four fractions, 32.8% reduction in infarction volume versus the vehicle-treated control rats. In subsequent studies, BOME was separated into four fractions, i.e., hexane, ethyl acetate, butanol and water fractions by sequential fractionation. BOHF showed a 68.2% reduction in infarct volume compared with water fractions by sequential fractionation. BOHF treatments significantly inhibited LPS-induced NO production in the BV-2 cells (a mouse microglial cell line). Data are presented as the means±S.E.M. of rats in each group. Asterisks indicate statistically significant differences (p<0.05 vs. the vehicle-treated control group).

In rat models, neuronal damage occurs in vulnerable regions of the CNS. However, unlike neurons, glial cells survive transient ischemia, and subsequently give rise to prominent gliosis in regions of neuronal death. Microglia constitute approximately 5—20% of the total glial cell population, and unlike other glial cells, they are uniformly dispersed throughout the brain. Cerebral ischemia is known to induce microglial activation in regions of neuronal death. In the present study, OX-42, the histochemical marker of microglia, was found to be particularly induced in the late ischemic period after MCAO.

The NO released from activated microglia has a major toxic effect and plays deleterious roles in brain inflammation and neuronal cell death, and excessive NO produced during pathologic conditions may contribute to neuronal cell death. Therefore, NO production by iNOS provides a means of assessing the effects of drugs on brain inflammation. In the present study, iNOS mRNA was found to be upregulated in ischemic hemispheres, whereas BOHF (100 mg/kg, p.o.) administration inhibited iNOS induction in the ischemic rat brain. In addition, experiments with BV-2 mouse microglial cells showed that both BOME and BOHF reduced NO production, indicating that they inhibit proinflammatory microglial activation.

Following cerebral ischemia and reperfusion, COX-2 induction is intimately associated with postischemic inflammation and NO production. In the present study, COX-2 was found to be upregulated in the ischemic brain, which is consistent with the findings of a previous study, and the administration of BOHF inhibited this induction. This result suggests that the neuroprotective effect of BOHF may be due to the inhibition of COX-2 induction in cerebral ischemia.

In conclusion, we found that BOME and BOHF exhibited significant neuroprotective effects in a rat model of transient focal ischemia, and that its neuroprotective effect appears to involve the inhibitions of the inductions of COX-2, iNOS, and OX-42 in the ischemic brain. Our findings suggest that the flower buds of *B. officinalis* have a neuroprotective effect in the ischemic brain, which is due to the inhibition of inflammation and of microglial activation.

Further studies are needed to clarify the mechanism of the neuroprotective effect of the extract.

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**REFERENCES**


