Neuroprotective effect of the ethanol extract of *Artemisia capillaris* on transient forebrain ischemia in mice via nicotinic cholinergic receptor

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[ABSTRACT] *Artemisia capillaris* Thunberg is a medicinal plant used as a traditional medicine in many cultures. It is an effective remedy for liver problems including hepatitis. Recent pharmacological reports have indicated that *Artemisia* species can exert various neurological effects. Previously, we reported a memory-enhancing effect of *Artemisia* species. However, the mechanisms underlying the neuroprotective effect of *A. capillaris* (AC) are still unknown. In the present study, we investigated the effect of an ethanol extract of AC on ischemic brain injury in a mouse model of transient forebrain ischemia. The mice were treated with AC for seven days, beginning one day before induction of transient forebrain ischemia. Behavioral deficits were investigated using the Y-maze. Nissl and Fluoro-jade B staining were used to indicate the site of injury. To determine the underlying mechanisms for the drug, we measured acetylcholinesterase activity. AC (200 mg·kg⁻¹) treatment reduced transient forebrain ischemia-induced neuronal cell death in the hippocampal CA1 region. The AC-treated group also showed significant amelioration in the spontaneous alternation of the Y-maze test performance, compared to that in the untreated transient forebrain ischemia group. Moreover, AC treatment showed a concentration-dependent inhibitory effect on acetylcholinesterase activity *in vitro*. Finally, the effect of AC on forebrain ischemia was blocked by mecamylamine, a nonselective nicotinic acetylcholine receptor antagonist. Our results suggested that in a model of forebrain ischemia, AC protected against neuronal death through the activation of nicotinic acetylcholine receptors.

[KEY WORDS] *Artemisia capillaris*; Transient forebrain ischemia; Acetylcholinesterase; Nicotinic acetylcholine receptor

[Introduction] Ischemic brain damage is caused by a decreased blood supply to the brain, leading to a severe reduction in the supply of nutrition and oxygen [¹-²]. Within minutes, this may cause severe brain damage through necrotic neuronal cell death [³]. Therefore, a prompt restoration in blood supply is critical. Once the blood supply is restored, secondary damage occurs through the generation of reactive oxygen species, neuroinflammation, and excitotoxicity [¹, ⁴-⁶]. This secondary damage could be a target for neuroprotective drug development. Various antioxidants, anti-inflammatory agents, and glutamate receptor antagonists have been developed as therapeutic can-
didates against secondary damage. However, so far none of these compounds have progressed past clinical trials, owing to a lack of clinical effects and severe side effects [7]. Therefore, a new drug target is required to overcome these drawbacks.

The nicotinic cholinergic receptor is an acetylcholine receptor subtype expressed throughout the central (CNS) and peripheral nervous systems (PNS). In the CNS, this receptor mediates various neurological functions, including mood regulation, learning, and memory [8]. Moreover, this receptor is associated with various neuroprotective mechanisms including antioxidative activity [9], phosphoinositide 3-kinase (PI3K)/Akt signaling, extracellular signal–regulated kinases (ERK)/mitogen-activated protein kinase (MAPK) signaling, and Janus kinase-2 (JAK-2)/signal transducer and activator of transcription-3 (STAT-3) pathways [10-11]. Inhibition of the PI3K/AKT pathway in neuronal cells and the administration of nicotinic acetylcholine receptor (nAChR) blockers blocks the neuroprotective effect of acetylcholinesterase inhibitors [12]. Several studies suggest that activation of the nicotinic acetylcholine receptor in a model of ischemic stroke can exert a neuroprotective effect [13-15].

Artemisia capillaris Thunberg belongs to the Compositae family and grows in dry or semiarid habitats. A. capillaris is traditionally used for the treatment of liver diseases, including hepatitis, jaundice, and cholecystitis [16]. A. capillaris has been reported to be beneficial in various conditions such as lipa-poptosis, obesity, skin cancer, atopic dermatitis, and liver injury [17-21]. Moreover, studies have reported anti-inflammatory, antioxidant, and anti-cancer effects of A. capillaris [22-23]. Several components of A. capillaris, including scoparone (6, 7-dimethylesculetin) and capillarisin [24-25], have positive effects on brain diseases, lipid composition, and lipid metabolism [20-26]. However, the effect of A. capillaris on cerebral ischemia has not previously been studied.

In our previous study, we found that an ethanol extract derived from another Artemisia species, A. princeps, can enhance memory through the facilitation of acetylcholine signaling [27]. This suggests that the neuroprotective effect of A. capillaris may be due to its modulation of brain acetylcholine levels. In the present study, we tested the neuroprotective effect of an ethanol extract of A. capillaris on mouse model of transient forebrain ischemia. We found that A. capillaris inhibited acetylcholinesterase activity and protected against ischemic injury-induced neuronal cell death through the activation of cholinergic receptor.

Material and Methods

Preparation of AC extract

Aerial parts of A. capillaris Thunberg (Compositae) were purchased from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). The animals were housed four per cages and allowed access to water and food ad libitum. The cages were maintained at a constant temperature (23 ± 1 °C) and relative humidity (60 ± 10%) under a 12-h/12-h light/dark cycle (lights on from 07 : 30 to 19 : 30). The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University, Korea (approved No. KHP 2010-10-14). All efforts were made to minimize animal suffering.

Surgery

The mice were anesthetized with 2.0% isoflurane and 70% nitrous oxide in oxygen and subjected to transient forebrain ischemia, as previously described [28]. Transient forebrain ischemia was induced by bilateral common carotid artery occlusion (BCCAO) with aneurysm clips for 25 min, and circulation was restored by removing the clips. The mice that received the same surgical operation without carotid artery clipping served as sham-operated controls. During the surgical procedure, rectal temperature was maintained at 37 ± 0.5 °C with heating pad (Biomed S.L., Alicante, Spain). Regional cerebral blood flow (rCBF) was monitored using laser Doppler flowmetry (LDF; Perimed, PF5010, Jarfalla, Sweden). The mice which showed between 80% and 95% of rCBF alternation of ABC but not BAB). Maze arms were thoroughly cleaned with heating pad (Biomed S.L., Alicante, Spain). Regional cerebral blood flow (rCBF) was monitored using laser Doppler flowmetry (LDF; Perimed, PF5010, Jarfalla, Sweden). The mice which showed between 80% and 95% of rCBF alternation of ABC but not BAB). Maze arms were thoroughly cleaned

Y-maze test

The Y-maze is a three-arm horizontal maze (40-cm-long and 3-cm-wide with 12-cm-high walls) in which the arms are symmetrically disposed at 120° angles from each other. The maze floor and walls were constructed from dark opaque polyvinyl plastic. The mice were initially placed within one arm, and the sequence (i.e., ABC, CAB, etc.) and number of arm entries were recorded manually for each mouse over an 8-min period. An actual alternation was defined as entries into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). Maze arms were thoroughly cleaned between tasks to remove residual odors. One hour after the last administration of each drug or vehicle, the mice were gently placed in the maze. The percentage of alternations was defined according to the following equation: % Alternation =
[(Number of alternations)/(Total arm entries−2)] × 100. The number of arm entries served as an indicator of locomotor activity.

**Tissue preparation**

At pre-designated time points after reperfusion, the mice were anesthetized with an intramuscular injection of Zoletil 50° (10 mg kg⁻¹) and perfused transcardially with phosphate buffer (100 mmol·L⁻¹, PH 7.4) followed by ice-cold 4% paraformaldehyde and then decapitated. The brains were removed and post-fixed in phosphate buffer (50 mmol·L⁻¹, PH 7.4) containing 4% paraformaldehyde overnight, immersed in 30% sucrose solution (in 50 mmol·L⁻¹ PBS), and then stored at 4 °C until sectioning. Frozen sections were prepared in the coronal plane (30 μm) using a cryostat (Leica, Nussloch, Germany) and kept in storage solution at 4 °C. 45 sections were obtained from each mouse. 5 sections by 9-section intervals (270 μm) were used for each immunohistochemical analysis.

**Nissl staining**

To detect neuronal degeneration, the sections were stained with Fluoro-Jade B (FJB). In brief, the sections were immersed in a series of solutions, including 1% sodium hydroxide in 80% alcohol, 70% alcohol, 0.06% potassium permanganate, and 0.0004% FJB. After washing, the sections were placed on a slide warmer, and neuronal degeneration in the CA1 region was examined using confocal microscopy.

**Immunohistochemistry**

For the immunohistochemistry, free floating sections were rinsed in PBS at room temperature. Then they were incubated overnight with anti-caspase-3 antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. The sections were then incubated with FITC-conjugated secondary antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C until sectioning. Frozen sections were prepared in the coronal plane (30 μm) using a cryostat (Leica, Nussloch, Germany) and kept in storage solution at 4 °C. 45 sections were obtained from each mouse. 5 sections by 9-section intervals (270 μm) were used for each immunohistochemical analysis.

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**Acetylcholinesterase activity assay**

Acetylcholinesterase activity assays were carried out using an acetylthiocholine iodide substrate by a colorimetric method, as described previously [30]. Whole mouse brains were homogenized in a glass Teflon homogenizer (Eyela, Tokyo, Japan) containing 10 volumes of homogenization buffer (12.5 mmol·L⁻¹ sodium phosphate buffer, PH 7.0, 400 mmol·L⁻¹ NaCl) and then centrifuged at 1,000 g for 10 min at 4 °C. The supernatant was used as source of enzyme for the assay. AC was initially dissolved in distilled water and diluted to various concentrations in Buffer A (100 mmol·L⁻¹ sodium phosphate buffer, PH 8.0) immediately before use. An aliquot of diluted AC solution in Buffer A (1.5 mL) was then mixed with 2.6 mL of Buffer A, 20 μL of acetylthiocholine iodide solution (75 mmol·L⁻¹), and 100 μL of buffered Ellman’s reagent (10 mmol·L⁻¹ 5, 5'-dithio-bis [2-nitrobenzoic acid] and 15 mmol·L⁻¹ sodium bicarbonate) and reacted at room temperature for 30 min. Absorbance was measured at 410 nm immediately after adding the enzyme source (400 μL) to the reaction mixtures (OPTIZEN 2120UV; Mecasys Co., Ltd., Seoul, Korea). Readings were taken at 30-s intervals for 5 min. The concentration of AC required inhibiting acetylcholinesterase activity by 50% (IC₅₀) was calculated using an enzyme inhibition dose-response curve.

**Statistics**

The data are expressed as means ± SEM. The differences among experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test for multiple comparisons. In drug interaction test, repeated two-way ANOVA followed Bonferroni’s post hoc test was used. Statistical significance was set at P < 0.05.

**Results**

**Effect of AC on transient forebrain ischemia-induced cell death**

Initially, we examined the effect of AC against transient forebrain ischemia-induced neuronal cell death. The mice underwent 25 min of BCCAO and reperfusion. AC was orally administered for seven days, beginning one day prior to surgery. Seven days after the reperfusion surgery, hippocampal slices were prepared for Nissl staining and FJB staining. The BCCAO-treated group showed a significantly lower number of viable cells in the CA1 region than the sham group (F₅,₃₀ = 8.129, n = 6/group, P < 0.05, Figs. 1A and 1C). Donepezil (5 mg·kg⁻¹, p.o.) was used as a positive control, and prevented the BCCAO-induced reduction in viable cells (P < 0.05, Figs. 1A and 1B) and the increase in degenerative neurons (P < 0.05, Figs. 1A and 1C) in the CA1 region. To observe apoptotic neuronal cell death, we stained slices with an anti-caspase-3 antibody. AC treatment blocked the BCCAO-induced increase of caspase-3-positive cells in CA1 region (F₅,₃₀ = 21.8, n = 6/group, P < 0.05, Figs. 1A and 1D). Donepezil also prevented the BCCAO-induced increase of caspase-3-positive cells (P < 0.05, Figs. 1A and 1D) in the CA1 region.

Fig. 1  Effects of AC on transient fore brain ischemia-induced cell death. The mice were suffered 25 min duration of bilateral carotid artery occlusion (BCCAO) and reperfusion. AC was administered immediately after the reperfusion by orally and once a day for 7 days. Seven days after reperfusion, mice hippocampal slices were prepared to examine Nissl, Fluoro-Jade B (FJB), and caspase-3 staining. (A) Microphotographs of Nissl staining and FJB staining. Bar = 100 μm (n = 6). (B) Quantitative analysis of the percentage of viable cells in CA1 region from Nissl staining (n = 6). (C) Quantitative analysis of the number of FJB + cells in CA1 region (n = 6). (D) Quantitative analysis of the number of caspase-3 + cells in CA1 region (n = 6). The data represent means ± SEM. *P < 0.05 vs sham group, #P < 0.05 vs BCCAO only-treated group

Effects of AC on transient forebrain ischemia-induced spontaneous alternation deficit

Hippocampal damages can affect spontaneous alternation [31]. Previously, we found that the transient forebrain ischemia model showed a significant reduction in spontaneous alternation in Y-maze test [32–33]. Therefore, to test the effect of AC on BCCAO-induced reduction in spontaneous alternation, a Y-maze test was conducted seven days post reperfusion. The BCCAO-treated control group showed significantly reduced spontaneous alternation compared to the sham group (F_{5,54} = 4.685, n = 10/group, P < 0.05, Fig. 2A). AC treatment prevented this reduction in a dose-dependent manner (P < 0.05). Donepezil also prevented the reduction in spontaneous alternation (P < 0.05). Total arm entry, which represents locomotor activity, was not significantly different among groups (F_{5,54} = 0.1780, n = 10/group, P > 0.05, Fig. 2B).

Fig. 2  Effects of AC on transient forebrain ischemia-induced memory deficit. Seven days after reperfusion, Y-maze test was conducted. Spontaneous alternation behavior (A) and the number of total arm entries (B) during an 8-min session were measured. Data represent means ± SEM, n = 10. *P < 0.05 vs sham group, #P < 0.05 vs BCCAO only-treated group
Effect of AC on acetylcholinesterase

To investigate the mechanism underlying the neuroprotective effect of AC, we tested the effect of AC on acetylcholinesterase activity in vitro and ex vivo. AC inhibited acetylcholinesterase activity in a concentration-dependent manner in vitro ($IC_{50} = 157 \mu g \cdot mL^{-1}$, Fig. 3A). Moreover, in ex vivo samples, we observed that the oral administration of AC significantly blocked acetylcholinesterase activity in the hippocampus ($F_{3, 16} = 6.214, n = 5/\text{group}, P < 0.05$, Fig. 3B). Donepezil, a positive control, also significantly inhibited acetylcholinesterase activity ($P < 0.05$, Fig. 3B).

Effect of blockade of nAChR on the neuroprotective effect of AC

Acetylcholinesterase inhibitors exert their neuroprotective effects via the corresponding increase in nAChR activation [34]. Therefore, to further investigate the neuroprotective effect of AC, we antagonized nAChRs using the nonselective nAChR antagonist, mecamylamine (Mec, 10 mg·kg$^{-1}$, i.p.). Mec administration did not affect BCCAO-induced neuronal death, which was shown by Nissl ($F_{4, 20} = 17.13, n = 5/\text{group}, P < 0.05$, Figs. 4A and 4B) and FJB ($F_{4, 20} = 26.65, n = 5/\text{group}, P < 0.05$, Fig. 3B).
underlies the protective effect of nAChR against neuronal cell survival pathways. The phosphorylation of PI3K and Akt damages [37]. The may exert a neuroprotective effect in various disease models. 

Further, we tested the effect of nAChR antagonism on the memory enhancing effect of AC. Mec (10 mg·kg⁻¹) administration alone did not affect the BCCAO-induced reduction in spontaneous alternation (F₁,₄₅ = 5.229, P < 0.05, n = 10/group, Fig. 5A). However, when co-administered with AC, Mec significantly blocked the beneficial effect of AC on memory impairment induced by BCCAO (P < 0.05). Moreover, significant drug interactions in spontaneous alternation was observed after conducting two-way ANOVA for the following groups: BCCAO + vehicle; BCCAO + Mec; BCCAO + AC; and BCCAO + AC + Mec (Mec, F₁,₃₆ = 3.478, P < 0.05; AC, F₁,₃₆ = 5.009, P < 0.05; interaction, F₁,₃₆ = 4.209, P < 0.05). Total arm entry did not differ between groups (F₁,₄₅ = 0.1299, n = 10/group, P > 0.05, Fig. 5B).

Fig. 5  Effect of blockade of nAChR on the memory enhancing effect of AC. AC and/or mecamylamine (Mec) were treated immediately after reperfusion and once a day for 7 days. Seven days after reperfusion, Y-maze test was conducted. Spontaneous alternation behavior (A) and the number of total arm entries (B) during an 8-min session were measured. The data represent means ± SEM, n = 10. * P < 0.05 vs sham group, # P < 0.05 vs BCCAO only-treated group, $ P < 0.05 vs BCCAO + AC-treated group with the PI3K-Akt signaling cascade [38]. It plays a major role in learning and memory [39-41]. Activation of this nicotinic receptor subtype has been shown to exhibit neuroprotection against various in vitro toxic challenges, including brain injury [42-43], oxygen-glucose deprivation [44], oxidative stress [45], amyloid β toxicity [46], and 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) insult [47]. Moreover, α7 nAChR activation and the consequent PI3K-Akt activation, has a neuroprotective effect in models of Alzheimer’s disease [39, 48-49] and Parkinson’s disease [50]. 

Ischemic brain damage is mediated through excessive oxidative stress, inflammatory responses, and excitotoxicity [51]. Several studies suggest the activation of nicotinic receptors may produce a protective effect against ischemic brain damage [52-53]. Nicotinic receptor activation may protect the brain against ischemia through stimulation of the PI3K-Akt signaling cascade. Previous studies have reported that the active components of AC induce PI3K-Akt signaling in various cell lines [54-57], indicating that the neuroprotective effect of AC against ischemic brain damage may be mediated by nicotinic receptor-activation and the consequent activation of the PI3K-Akt pathway.

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References


