Stachys sieboldii (Labiatae, Chorogi) Protects against Learning and Memory Dysfunction Associated with Ischemic Brain Injury

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Summary Stachys sieboldii (Labiatae; Chinese artichoke, a tuber), “chorogi” in Japanese, has been extensively used in folk medicine, and has a number of pharmacological properties, including antioxidative activity. However, few studies have examined the neuroprotective effects of S. sieboldii tuber extract (chorogi extract), and it remains unknown whether the extract can alleviate learning and memory dysfunction associated with vascular dementia or Alzheimer’s disease. Therefore, in this study, we investigated the neuroprotective effects of chorogi extract, and examined its protection against learning and memory dysfunction using Ginkgo biloba leaf extract (ginkgo extract) as a positive control. Mice were subjected to bilateral carotid artery occlusion (BCAO) for 30 min. Oral administration of chorogi extract or ginkgo extract significantly reduced post-ischemic glucose intolerance on day 1 and neuronal damage including memory impairment on day 3 after BCAO, compared with the vehicle-treated group. Neither herbal medicine affected locomotor activity. Furthermore, neither significantly alleviated scopolamine-induced learning and memory impairment. In primary neurons, neuronal survival rate was significantly reduced by hydrogen peroxide treatment. This hydrogen peroxide-induced neurotoxicity was significantly suppressed by chorogi extract and ginkgo extract. Taken together, our findings suggest that chorogi extract as well as ginkgo extract can protect against learning and memory dysfunction associated with ischemic brain injury through an antioxidative mechanism.

Key Words Stachys sieboldii, Ginkgo biloba, dementia, cerebral ischemia, chorogi

Stachys sieboldii (Labiatae; Chinese artichoke) has been extensively used in folk medicine in China (1). The tuber of S. sieboldii (“chorogi” in Japanese) is used as a food item in Japan. S. sieboldii has been found to be effective against the common cold and heart disease, provides pain relief, and has substantial nutritional value (1). The methanolic extract of the tuber of S. sieboldii, containing phenylethanoid glycosides, including acteoside and stachyoside C, was shown to significantly inhibit lethality induced by potassium cyanide in mice (1). This extract was reported to inhibit hyaluronidase activity, exert anti-inflammatory effects, and protect against kidney disease (2, 3). However, the therapeutic potential of S. sieboldii tuber extract (chorogi extract) in most diseases remains unknown.

Self-medication is defined by the World Health Organization as the practice whereby individuals treat their ailments and conditions with medicines that are approved and available without prescription. The awareness among the general public that professional care for minor ailments is often unnecessary has contributed to the increasing prevalence of self-medication (4). Dietary supplements are widely used for the prevention of lifestyle-related diseases, such as diabetes and high blood pressure (5). Therefore, it is important to investigate the pharmacological effects of nutrients and herbal supplements on such diseases. Diabetes, high blood pressure and obesity are major risk factors for cerebral stroke (6).

In turn, cerebral stroke is a major risk factor for dementia. Progressive impairment in memory and cognition is a key clinical feature of dementia, and is reflective of degeneration within the central nervous system. Vascular dementia and Alzheimer’s disease (AD) are two of the most common forms of dementia (7). Cerebral stroke and AD have similar pathogenetic mechanisms that underlie neuronal loss and dysfunction, including elevated levels of oxidative stress and a heightened inflammatory response (8, 9). Thus, we hypothesized that chorogi extract may protect against cerebral stroke and AD-associated dementia.

Ginkgo biloba leaf extract (ginkgo extract) has been widely used in the treatment and prevention of neurodegenerative dementias associated with ageing, AD, peripheral vascular diseases and neurosensory problems (10, 11). Ginkgo extract has been standardized to contain flavonoid glycosides (including quercetin, kaempferol andisorhamnetin), terpenoids (including the ginkgolides A, B, C and J, and bilobalide) and organic acids (12), and has robust antioxidant and
anti-apoptotic properties (13). The antioxidative effects of ginkgo extract appear to be mediated by its ability to directly scavenge reactive oxygen species (ROS) (14), chelate prooxidant transition metal ions (15), promote the expression of antioxidant proteins such as superoxide dismutase, and increase levels of antioxidant metabolites such as glutathione (15, 16). In addition, ginkgo extract protects against apoptosis through a number of different mechanisms (13). These varied properties of ginkgo extract may underlie its neuroprotective effects (17).

In this study, we investigate the neuroprotective effects of chorogi extract, and we examine whether it can protect against learning and memory dysfunction, using ginkgo extract as a positive control.

**MATERIALS AND METHODS**

*Animals.* Male ddY mice (5 wk old) were obtained from SLC (Shizuoka, Japan). Animals were housed at a temperature of 23–24 °C with a 12-h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. In addition, all experiments were approved by the ethics committee for animals at Kobe Gakuin University (approval number A13-25).

*Chorogi extract and ginkgo extract administration.* Dried *S. sieboldii* (tuber, 1.5 kg) was extracted with H₂O (1.5 L) for 1 h at 60 °C. Then, the water extract was dehydrated, sterilized and passed through a sieve. The final yield was 640 g of dry powder, containing 0.313% acteoside, a major constituent of *S. sieboldii* (Lot. 241106). The ginkgo extract was purchased from Maruzen Pharmaceuticals Co., Ltd. (Onomichi City, Hiroshima, Japan).

Both extracts were dissolved in water (resulting in a 20 g/kg suspension of *S. sieboldii*). Chorogi extract (1, 5, 10 or 20 g/kg) or ginkgo extract (100, 200 or 400 mg/kg) was orally administered once a day for 5 d. The vehicle group received water (0.1 mL/10 g) orally.

*Assessment of locomotor activity.* Spontaneous locomotor activity was quantified for 30 min using the open field test on day 1 after final administration of chorogi extract or ginkgo extract, as previously described (18). The open field box (30 cm × 30 cm × 30 cm) consisted of a floor divided into 16 squares (7.5 cm × 7.5 cm/square) illuminated by white light. Each mouse was gently placed in the exact center of the box and activity was scored as the number of line crossings (i.e., when a mouse removed all four paws from one square and entered another). The open field session was recorded with a camera and the data were analyzed after the session. The open field arena was cleaned with ethanol solution and dried after testing each mouse.

*Measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine levels.* Serum AST, ALT and creatinine levels were measured using commercially available kits (AST and ALT: Transaminase CII-Test; creatinine: LabAssay Creatinine [both from Wako, Tokyo, Japan]) on day 1 after final administration of chorogi extract or ginkgo extract according to the manufacturer’s protocol.

*Preparation of global cerebral ischemia model using bilateral carotid artery occlusion (BCAO).* A model of transient global cerebral ischemia was established using BCAO, as previously described (19). In brief, mice were anesthetized with pentobarbital (60 mg/kg) and the bilateral common carotid arteries were occluded for 30 min with standard surgical aneurysm clips (Mizuhu Ikakogyo Co., Ltd., Tokyo, Japan). Mice were kept under a heating lamp to maintain core body temperature at 37.0 ± 0.5 °C. Sham-operated mice were subjected to the same procedure without BCAO. Surgery was performed on day 1 after final administration of chorogi extract or ginkgo extract.

*Learning and memory tests.* A one-trial step-through-type passive avoidance learning test was used as previously described (20). The apparatus (Ohara Co., Ltd., Tokyo, Japan) consisted of illuminated and dark compartments (each 4 × 13 × 10 cm) adjoining each other through a small gate (3 cm in diameter) with a grid floor consisting of 2.5-mm stainless steel rods set 7 mm apart. In the training trial (on day 2 after BCAO or 30 min after administration with scopolamine), mice were placed in the illuminated compartment facing away from the dark compartment. When mice entered the dark compartment, an electric shock (50 V, 3 s duration) was delivered. Then, the mice were shut in the dark compartment for 5 s and carried back to the home cage. In the test trial (24 h after the training trial), the mice were again placed in the illuminated compartment, and the time taken for the mice to enter the dark compartment (maximum 600 s) was recorded.

*Analysis of pyknotic cell death.* Pyknotic cell death was evaluated by hematoxylin-eosin (HE) staining as previously reported (21). Mice were decapitated, and brains were dissected immediately on day 3 after BCAO. The brain slices were incubated in 4% paraformaldehyde (pH 7.4) dissolved in ice-cold phosphate buffer over night at 4 °C. Paraffin-ﬁxed brain tissue was sectioned at 6 μm thickness using a sliding microtome and deparafﬁnized with xylene and ethanol, then stained with HE (Carrazzi’s Hematoxylin Solution, Sakura Finetek Japan Co., Ltd., Tokyo, Japan) for histological study. After HE staining, we counted the number of shrunken neurons with pyknotic nuclei to assess neuropathy.

*Measurement of blood glucose levels.* Mice were fasted for 15 h before the test day (on day 1 after BCAO), and 1.5-μL samples of blood were obtained from the tail vein to measure blood glucose levels using the Glucose Pilot (Avenir Biotech, Carlsbad, CA), as previously described (20). The change in blood glucose level was calculated using the following formula: change in fasting blood glucose (FBG) = FBG on day 1 after BCAO − FBG before BCAO.

*Scopolamine administration.* Scopolamine (0.5 or 1 mg/kg, Sigma-Aldrich, St. Louis, MO) was dissolved in saline and given intraperitoneally 30 min before the
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Primary cortical neuronal cultures and analysis of survival rate. Cortical neurons were prepared from fetuses obtained from 18-d pregnant ddY mice, as previously described (22). Briefly, dissociated cortical neurons were plated in 96-well plates, for survival experiments at a density of 1.2 × 10^5 cells/cm². Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (with l-glutamine, sodium pyruvate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Nacalai Tesque, Kyoto, Japan). The medium was supplemented with 5% heat-inactivated bovine serum (Biowest, Nuaillé, France), 5% heat-inactivated horse serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Invitrogen) and 0.1 mg/mL streptomycin (Invitrogen) and maintained in a humidified incubator with 5% CO₂ at 37°C. Plates were coated with poly-d-ornithine (100 μg/mL; Sigma-Aldrich). Cytosine β-d-arabinofuranoside (0.1 μM; Sigma-Aldrich) was added to cultures on the second day after seeding to inhibit the proliferation of non-neuronal cells. In this culture preparation, >90% of cells are neurons (22). Cortical neurons were cultured for 5 d before drug treatment. On day 5, 100 μM hydrogen peroxide (H₂O₂) and chorogi extract (0.025, 0.25, 2.5 mg/mL) or ginkgo extract (1, 10, 100 μg/mL), in a volume of 100 μL/well, was added to the primary neurons. Cell survival rates were estimated using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Nacalai Tesque) reduction assay (Dojindo, Tokyo, Japan) 24 h after drug treatment.

Statistical analysis. Locomotor activity, serum AST levels, serum ALT levels, serum creatinine levels, FBG and cell survival rate were compared using one-way

![Fig. 1. Effects of chorogi extract and ginkgo extract on locomotor activity. Mice were orally administered chorogi extract, ginkgo extract or vehicle alone (water) once a day for 5 d. A: Locomotor activity on day 1 after final administration of chorogi extract or ginkgo extract. B: Total count for A. Results are presented as the mean ± SE; n = 4.](image1)

![Fig. 2. Effects of chorogi extract and ginkgo extract on liver and kidney function. Mice were orally administered chorogi extract, ginkgo extract or vehicle alone (water) once a day for 5 d. Serum samples were prepared on day 1 after final administration of chorogi extract or ginkgo extract. A: Aspartate aminotransferase (AST). B: Alanine aminotransferase (ALT). C: Creatinine. Results are presented as the mean ± SE; n = 10.](image2)
analysis of variance followed by Scheffe’s test. Results are presented as means±standard error of the mean (SE). Results of the one-trial step-through passive avoidance test were compared using the Steel-Dwass test with post hoc nonparametric multiple comparison tests. Data are presented as medians (25–75%). Differences were regarded as statistically significant when the p value was less than 0.05.

RESULTS

Effects of chorogi extract and ginkgo extract on locomotor activity

The various doses of chorogi extract and ginkgo extract used in the present study did not affect locomotor activity on day 1 after final administration (Fig. 1).

Effects of chorogi extract and ginkgo extract on liver and kidney function

The various doses of chorogi extract and ginkgo extract used in the present study did not affect serum AST, ALT or creatinine levels on day 1 after final administration (Fig. 2).

Effects of chorogi extract and ginkgo extract on cerebral ischemia-induced memory impairment

There was a significant decrease in the response latency in the passive avoidance test on day 3 after BCAO. This decrease in response latency was significantly and dose-dependently suppressed by chorogi extract (Fig. 3A) and by the ginkgo extract (Fig. 3B), compared with the water-treated control group. In the BCAO group, pyknotic cell death (arrows) was observed

Fig. 3. Effects of chorogi extract and ginkgo extract on memory impairment and neuronal damage on day 3 after global cerebral ischemic stress. Mice were orally administered chorogi extract, ginkgo extract or vehicle alone (water) once a day for 5 d. On day 1 after final administration, mice were subjected to BCAO. A: Effect of chorogi extract on memory impairment on day 3 after BCAO (n=6–11). B: Effect of ginkgo extract on memory impairment on day 3 after BCAO (n=6–8). A and B: The boxes contain the values between the 25th and 75th percentile, the line across the boxes represents the median, and the vertical bars extend between the highest and lowest values. *p<0.05, #p<0.05; Steel-Dwass test of post hoc nonparametric multiple comparison tests. C–G: HE-stained sections from the hippocampal CA3 region showing shrunken neurons with pyknotic nuclei (black arrow) from one representative animal in each group. C: Water-treated sham group. D: Water-treated BCAO group. E: 10 g/kg chorogi extract-treated BCAO group. F: 20 g/kg chorogi extract-treated BCAO group. G: 400 mg/kg ginkgo extract-treated BCAO group. H: Quantitative analysis of shrunken neurons with pyknotic nuclei. *p<0.05, #p<0.05; Scheffe’s test. Results are presented as the mean±SE; n=4. Scale bar= 50 μm.
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in the hippocampal CA3 region on day 3 after BCAO, but not in the sham group (Fig. 3C and D). This cell death was decreased by chorogi extract and by ginkgo extract (Fig. 3E, F and G). Quantitative analysis showed that the number of cells undergoing pyknotic cell death was significantly increased in the BCAO group compared with the sham group, and this increase was significantly suppressed by chorogi extract and ginkgo extract (Fig. 3H).

Effects of chorogi extract and ginkgo extract on the transient increase in FBG after cerebral ischemic stress

FBG levels were significantly increased on day 1 after BCAO compared with those of the sham group (Fig. 4). Chorogi extract and ginkgo extract significantly suppressed this elevation in FBG levels on day 1 after BCAO compared with the water-treated BCAO group (Fig. 4). Effects of chorogi extract and ginkgo extract on scopolamine-induced memory impairment

A significant decrease in the response latency in the passive avoidance test was observed after administration of scopolamine (0.5 mg/kg; Fig. 5A; 1 mg/kg; Fig. 5B). Chorogi extract or ginkgo extract did not significantly affect this decrease in response latency (Fig. 5).

Effects of chorogi extract and ginkgo extract on H2O2-induced cell death in primary cultured neurons

Cell survival rate was significantly decreased by 100 μM H2O2 compared with the control group. Treatment with chorogi extract (Fig. 6A) or ginkgo extract (Fig. 6B) significantly and dose-dependently suppressed the reduction in survival rate induced by 100 μM H2O2.

DISCUSSION

Recently, there has been a dramatic increase in the use of dietary supplements and natural medicines for the prevention of lifestyle-related diseases such as cerebral ischemia. Therefore, it is important to gather pharmacological data on the effects of these supplements and natural medicines on the diseases that they target. It has been reported that many herbal medicines (e.g., ginkgolides from the leaves of Ginkgo biloba, blueberry and naoxinqing extract from Diospyros kaki leaves) protect against cerebral ischemic neuronal damage (23–25). The neuroprotective effects are thought to be mediated by the antioxidative properties of these various natural medicines. In this study, we investigated...
the effects of chorogi extract on cerebral ischemia and scopolamine-induced memory impairment, and we used ginkgo extract as a positive control. We found that chorogi extract, similar to ginkgo extract, attenuates learning and memory dysfunction.

Neurodegeneration after ischemic stroke results from a complex series of pathological events. These events include glutamate excitotoxicity and inflammation, which may lead to calcium overload, depolarization in the infarcted region, oxidative stress, neovascular changes and neuronal cell death (apoptosis/necrosis) (26). Glutamate is the major excitatory neurotransmitter in the mammalian brain and a key mediator of intracellular communication, plasticity, growth and differentiation (27). It is well known that memory impairments, including learning and memory dysfunction, result from cerebral ischemia (19, 20). In the present study, we found that chorogi extract alleviates cerebral ischemia-induced learning and memory dysfunction, but not scopolamine-induced impairment, similar to ginkgo extract. ROS are considered to play a pivotal pathogenetic role in ischemia-reperfusion-induced neuronal injury (28–30). Many herbal medicines capable of suppressing ROS production have been found to alleviate cerebral ischemic neuronal damage (20). In addition, it has been reported that ischemic neuronal damage can be suppressed by vitamins and polyphenols possessing antioxidative properties (31–33). Oxidative stress induces the activation of effectors that trigger inflammatory pathways, which induce further cell damage.

It is known that chorogi extract possesses antioxidative activities. Our present results clearly demonstrate that chorogi extract and ginkgo extract significantly suppress H$_2$O$_2$-induced cell death in primary cultured cortical neurons. In a previous report, the methanolic chorogi extract, containing phenylethanoid glycosides, including acteoside and stachyoside C, was found to significantly inhibit lethality induced by KCN in mice (1). Using high-performance liquid chromatography, various sugars, including stachyose, sucrose and raffinose, were identified in chorogi extract (34). Acteoside and stachyoside C have a significant effect in the KCN-induced anoxia model (1). Raffinoside family oligosaccharides are present at high levels under normal and stress conditions, and act as antioxidants to protect plant cells from oxidative damage and maintain redox homeostasis (35). Furthermore, ginkgo extract, which contains ginkgolides, has been reported to suppress cerebral ischemic damage and has very strong antioxidative effects (11). Thus, the neuroprotection provided by chorogi extract and ginkgo extract against learning and memory dysfunction may be mediated by their ability to reduce oxidative stress.

We have recently focused on the regulation of blood glucose levels as a novel therapeutic strategy for cerebral ischemia. We previously reported that cerebral ischemic neuronal damage can be triggered by glucose intolerance following ischemic stress (20). The suppression of post-ischemic glucose intolerance significantly ameliorates neuronal damage (20). Chorogi extract and ginkgo extract may alleviate post-ischemic glucose intolerance and protect against cerebral ischemia-induced learning and memory dysfunction. The bioactive compounds in these extracts may scavenge oxidants such as free radicals after ischemic stress directly. Chorogi extract and ginkgo extract may also have an indirect antioxidative effect by increasing expression levels of antioxidants such as superoxide dismutase and glutathione-peroxidase. The extracts may also provide neuroprotection by increasing the expression of neurotrophic factors such as brain-derived neurotrophic factor and nerve growth factor. It is possible that the amelioration of post-ischemic glucose intolerance may be secondary to the neuroprotective action of chorogi extract and ginkgo extract. Consequently, further study is required to clarify whether these extracts directly or indirectly alleviate glucose intolerance.

We found that chorogi extract and ginkgo extract did not affect scopolamine-induced learning and memory dysfunction. Scopolamine is commonly used to induce cholinergic deficits and generate AD-like animal models (36, 37). Dysfunction of the cholinergic system in the CNS is implicated in the development of cognitive impairment in AD (38). The administration of several cholinotoxins, including scopolamine, was reported to induce memory impairment in rodents and humans (39, 40). Consequently, mice treated with these cholinotoxins are also used widely as AD models. Acteoside,
present in chorogi extract, was shown to protect against amyloid-β-induced cellular injury (41). Although chorogi extract and ginkgo extract suppressed the development of cerebral ischemia-induced memory dysfunction, they did not suppress scopolamine-induced memory dysfunction. This suggests that these extracts may not impact acetylcholine levels in the brain. However, the effects of chorogi extract and ginkgo extract in AD remain unclear, and additional studies using AD models, such as amyloid-β-induced AD, are required to clarify the therapeutic properties of these natural medicines.

Conclusion
We found that chorogi extract, similar to ginkgo extract, protects against learning and memory dysfunction associated with ischemic brain injury. This neuroprotection is likely mediated by the antioxidative properties of these extracts. Taken together, our findings suggest that these traditional medicines may have substantial therapeutic potential for ischemic brain injury.

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