Differential Effects of Buckwheat and Kudingcha Extract on Neuronal Damage in Cultured Hippocampal Neurons and Spatial Memory Impairment Induced by Scopolamine in an Eight-Arm Radial Maze

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We have reported the neuroprotection provided by an extract of buckwheat (BWE, Fagopyrum esculentum Moench) on neuronal damage in the repeated cerebral ischemia model and demonstrated that BWE inhibited the excess glutamate release induced by repeated cerebral ischemia, suggesting that BWE had a free radical-scavenging action in addition to anti-glutamate action. In the present study, we studied the neuroprotective effects of Kudingcha extract (KDE, Ligustrum purpurascens Y. C.) on scavenging by the 2,2-diphenyl-1-picylhydrazyl (DPPH) radical in primary cultured hippocampal neurons, and on spatial memory impairment induced by scopolamine in an eight-arm radial maze in comparison with BWE. The effects of KDE (0.01–1 mg/ml) were more potent than those of BWE (0.01–1 mg/ml) in scavenging the DPPH radical (1 mM). KDE (100 μg/ml) prevented the cell damage induced by glutamate (300 μM) or kainate (1 mM), which was more potent than BWE (100 μg/ml), but BWE suppressed the cellular damage induced by β-amyloid (25-35) (20 μM) more potently than KDE (100 μg/ml). BWE (600 mg/kg), but not KDE, significantly suppressed the increase in errors induced by scopolamine (0.5 mg/kg i.p.) in the eight-arm radial maze. The results suggest that BWE may protect against cholinergic dysfunction and that KDE protects more effectively against glutaminergic dysfunction.

Key words —— buckwheat extract, Kudingcha extract, 2,2-diphenyl-1-picylhydrazyl radical, primary cultured hippocampal neuron, scopolamine

INTRODUCTION

Buckwheat (Fagopyrum esculentum Moench) noodles are a traditional food in Japan and contains catechin, epicatechin, quercetin, rutin and an oligomer of catechin and epicatechin. Catechin, epicatechin, quercetin and rutin have flavonoid structures, and their antioxidant action and neuroprotection have been widely studied.1–11 Buckwheat extract (BWE) has been also reported to have antioxidant activity in vivo and in vitro.12,13) Flavonoids have been shown to reduce elevated blood pressure, stimulate endothelium-dependent vasodilation, and inhibit vascular smooth muscle cell proliferation. These beneficial effects of flavonoids reduce the risk of coronary artery disease.14) Studies have confirmed that catechin, epicatechin, rutin, and quercetin are beneficial in the treatment of stroke, and we demonstrated that BWE ameliorates spatial memory impairment induced by cerebral ischemia in the eight-arm radial maze in rats by inhibiting excess glutamate release,15) suggesting that BWE has free radical-scavenging in addition to anti-glutamate activity.
Kudingcha (*Ligustrum purpurascens* Y. G.), a popular beverage consumed in south China, contains acteoside, isoacteoside, and ligupurpuroside (A–D), with phenylethanoid glycoside structures (Fig. 1). Phenylethanoid glycosides are a class of water-soluble polyphenolic compounds. It has been reported that the antioxidant effects of Kudingcha are equal to those of green tea. Acteoside exhibits effects against the hepatotoxicity induced by carbon tetrachloride and anti-glomerular basement membrane (GBM) nephritis in rats, and anti-nephrine action via the suppression of the accumulation of leukocytes in the glomeruli. However, there have been no reports on the effects of Kudingcha on neuronal damage.

In the present study, we compared the effects of BWE on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the neurotoxicity induced by glutamate, kainate, and β-amyloid in primary hippocampal cell cultures with Kudingcha extract (KDE) and investi-
Materials AND METHODS

Materials —— The BWE (Fagopyrum esculentum Moench) mixture was obtained from Amino Up Chemical Co., Ltd. (Sapporo, Japan). The preparation consisted of 25% polyphenols, including oligomers of catechin and epicatechin, catechin, epicatechin, quercetin, rutin and hyperoside. Extraction and structural confirmation were performed following the method of Yokozawa et al. The remaining 75% of the BPE mixture contained carbohydrates (50%), proteins (2.8%), lipid (2.6%), dietary fiber (11.4%) and ash (8.2%). KDE contained about 28% polyphenols, including acteoside, and ligupurpurside (A–D), and others. Polyphenols contained in BWE and KDE were measured using the Folin-Ciocalteu reagent reported by Mukoda et al. and calculated using catechin as a standard. BWE or KDE was dissolved in distilled water to make a solution of 100 mg/ml and filtered. The filtrate was further diluted to 1 mg/ml with culture medium. After the cells had been incubated for 6–8 days, they were exposed to 180 µl of the culture medium with the addition of BWE (1 mg/ml) 20 µl for 24 hr. Glutamate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), kainate (Sigma Chemical Co., St. Louis, MO, U.S.A.), were dissolved in neurobasal medium containing phenol red as an indicator for the change in pH. β-Amyloid25–35 (AnaSpec, Inc., San Jose, CA, U.S.A.) was dissolved in the distilled water to make a 10-mM solution and further diluted with neurobasal medium. All agents were added to the neurobasal medium for 24 hr in the experiments as described in the MATERIALS AND METHODS. DPPH was purchased from Sigma Chemical Co.

Animals —— Pregnant female Wistar rats and male Wistar rats (7 weeks old) were purchased from Kyu Do Co., Ltd. (Saga, Japan). The female rats were housed individually in cages and the male rats were housed 4 or 5 to a cage in a room with controlled temperature (23 ± 2°C) and relative humidity (60 ± 10%), and maintained on a 12-hr light-dark cycle. All other procedures regarding animal care and use were carried out according to regulations dictated by the Experimental Animal Care and Use Committee at the Facilities of Fukuoka University.

DPPH Radical-Scavenging Effects —— MeOH solutions of DPPH (0.01–3 mM) were prepared and the absorbance was measured spectrophotometrically at 520 nm (U-2000A, Hitachi, Tokyo, Japan) after storage at room temperature for 30 min in air. Based on the absorbance curve, the maximum absorbance of DPPH was at a concentration of 1 mM. Then MeOH polyphenol solutions at various concentrations (0.005–5 mg/ml) were added to a solution of DPPH (1 mM) in MeOH (1 ml), and the reaction mixture (total volume 5 ml) was shaken vigorously. After storage at room temperature for 30 min in air, the remaining DPPH was determined at 520 nm. The radical-scavenging activity of each polyphenol was expressed as the ratio of lowered DPPH activity (%), relative to the absorption (100%) of DPPH solution.

Primary Culture of Hippocampal Neurons —— Primary cultures of hippocampal neurons were prepared from the hippocampus of 17-day-old embryos of Wistar rats as described previously, with some modifications. Briefly, whole embryos were placed in a Petri dish containing basic medium (neurobasal medium; Gibco, Gaithersbury, MD, U.S.A.) containing 6%(v/v) horse serum (Gibco), glutamine 2 mM (Sigma Chemical Co.), glucose 6 mg/ml (Sigma Chemical Co.), and antibiotics (Penicillin/Streptomycin, Gibco). Pieces of the hippocampus were collected, transferred to a 15-ml plastic centrifuge tube, and triturated 10 times with a wide-bore fire-polished Pasteur pipette. Trituration was then repeated 10 times with a narrow-bore fire-polished Pasteur pipette. After centrifuging at 720 rpm for 7 min, the deposits were triturated 10 times with the Pasteur pipette. Viable cells were stained with trypan-blue dye and counted with a hemocytometer after plating on 4-well chamber slides. The cell suspension was diluted from the calculated average to a density of 6 × 10⁴ cells/cm², and plated in chambers (96 well) (Nalge Nunc International, Rochester, NY, U.S.A.), which were coated with poly-D-lysine (10 µg/ml, Sigma Chemical Co., lot 17H5521) overnight with laminin solution (Becton Dickinson, San Jose, CA, U.S.A.) for 2 hr. Cell cultures were incubated for 24 hr in 12% horse serum/neurobasal medium [12% horse serum (Gibco, lot 46K6762), neurobasal medium (Gibco, lot 1006455)] at 37°C in a 5% CO₂ humidified atmosphere. The culture medium was then replaced with B-27 supplement/neurobasal medium [B-27 supplement (Gibco, lot 1016205)]. All experiments were performed in serum-free medium after 6–8 days of culture. After growth to
confluence, the cells were exposed to test agents for 24 hr before the lactate dehydrogenase (LDH) assay.

**LDH Assay** —— Cellular injury was quantitated by measuring the activity of LDH released into the extracellular environment. A sample of the culture medium (50 µl) from each well was placed on a new microplate and reacted with a color former containing nitroblue-tetrazolium (0.74 mg/ml), diaphorase, and NDA dissolved in DL-lithiumlactate (LDH-Cytotoxic Test, Wako Pure Chemical Industries, Ltd.) for 45 min, and 1 mol/l hydrochloric acid (100 µl) was then added to stop the reaction. Tween20 (0.2%) was used as the control. The release of LDH into the medium was estimated based on the production of blue diformazane, which was measured by the absorbance at a wavelength of 560 nm with a microplate reader (Model 550, Bio-Rad, Tokyo, Japan).

**Eight-Arm Radial Maze Task** —— Male Wistar rats (240–250 g) were placed on a restricted food intake regimen (10–12 g/day, CE-2; Clea Japan, Tokyo, Japan). Behavioral testing was conducted in the apparatus as reported previously15) in an eight-arm radial maze (Neuroscience Co., Tokyo, Japan). The maze consisted of a central platform 24 cm in diameter, with eight arms extending radially. Rats were allowed to visit each arm to eat all eight pellets in the food cups located near the end of each arm. Each test animal was trained once per day to memorize the apparatus. The performance of the test animals in each trial was assessed using three parameters: the number of correct choices in the initial eight chosen arms; number of errors, defined as choosing arms that had already been visited; and time elapsed before the rat all eight pellets. When the test animals made seven or eight correct choices and less than one error in three successive sessions, i.e., had memorized the maze, they were used for the next experiment. BWE and KDE were administered orally to rats that had acquired spatial memory, and vehicle (water) was administered to the control group. After 30 min, scopolamine 0.5 mg/kg i.p. was administrated and the rats were confined in the eight-arm radial maze.

**Statistical Analyses** —— All data for cell analysis are expressed as mean ± S.E. Dunnett’s test was used to analyze the cell damage rate in cultured hippocampal neurons, and the Mann-Whitney U-test was used to analyze the data in the behavioral test.

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**RESULTS**

**Effects of BWE and KDE on the DPPH Radical**

DPPH is a stable free radical containing an odd electron in its structure and is usually used for detecting radical-scavenging activity. The absorbance of DPPH radicals showed dose–dependent curve at concentrations of 0.05–1 mM (data not shown). The DPPH radical (1 mM)-scavenging effects of KDE (0.01–1 mg/ml) were more potent than those of BWE (0.01–1 mg/ml) (Fig. 2).

**Protective Effects of BWE and KDE against Glutamate-Induced Cell Damage**

Glutamate (100–300 µM) dose dependently induced cell damage in a primary culture of hippocampal neurons (100 µM, 20.5 ± 1.9%; 300 µM, 23.0 ± 1.4%). BWE or KDE 1–100 µg/ml alone did not induce the cell damage, respectively (data not shown). When BWE 1, 10 and 100 µg/ml was examined for the protective effects against the cell damage induced by glutamate 100 µM (data not shown), 100 µg/ml BWE significantly suppressed the damage (13.3 ± 1.9%, p < 0.05). Therefore we used the concentrations of BWE and KDE 100 µg/ml in the present study. BWE and KDE (100 µg/ml) significantly suppressed the cell damage induced by glutamate at a concentration of 100 µM (BWE, 13.3 ± 1.9%; KDE, 11.0 ± 0.8%, p < 0.01). At the concentration of glutamate 300 µM, KDE significantly suppressed glutamate-cell damage while BWE did not (KDE 100 µg/ml, 13.6 ± 0.5%, p < 0.05, Fig. 3A).

**Protective Effects of BWE and KDE against Kainate-Induced Cell Damage**

Kainate (1–3 mM) dose dependently induced...
cell damage in a primary culture of hippocampal neurons (1 mM, 19.0 ± 0.6%; 3 mM, 22.5 ± 0.6%). BWE and KDE (100 µg/ml) significantly inhibited the cell damage induced by kainate at a concentration of 1 mM (BWE, 14.9 ± 0.3%, p < 0.05; KDE, 11.7 ± 0.4%, p < 0.01, Fig. 3B). KDE was more potent than BWE. At a concentration of kainate 3 mM, both BWE and KDE significantly suppressed kainate-induced cell damage (BWE 100 µg/ml, 13.7 ± 0.4%, p < 0.05; KDE 100 µg/ml, 12.4 ± 1.3%, p < 0.01).

Protective Effects of BWE and KDE against β-Amyloid-Induced Cell Damage

β-Amyloid (10–20 µM) dose dependently induced cell damage in primary culture of hippocampal neurons (10 µM, 21.7 ± 0.9%; 20 µM, 24.0 ± 0.4%). BWE and KDE (100 µg/ml) significantly suppressed the cell damage induced by β-amyloid at a concentration of 10 µM (BWE, 9.5 ± 0.2%, p < 0.01; KDE, 11.1 ± 0.4%, p < 0.01). At a concentration of β-amyloid 20 µM, BWE significantly inhibited the cell damage induced by β-amyloid while KDE did not (BWE 100 µg/ml, 10.7 ± 0.7%, p < 0.01; KDE 100 µg/ml, 20.6 ± 0.8%, Fig. 3C). BWE was more potent than KDE.

Protective Effects of BWE and KDE against Scopolamine-Induced Spatial Memory Impairment

The eight-arm radial maze task was performed 30 min after scopolamine (0.5 mg/kg i.p.) injection. Scopolamine impaired spatial memory (vehicle, correct choices, 7.9 ± 0.1, error, 0.1 ± 0.1; scopolamine, correct choices, 5.3 ± 0.4, errors, 10.3 ± 1.8, p < 0.001, Fig. 4), and neither BWE nor KDE at doses of 100–300 mg/kg improved the spatial memory impairment induced by scopolamine. BWE (600 mg/kg) tended to increase the number of correct choices, but significantly decreased the increase in errors induced by scopolamine (BWE 600 mg/kg, correct choices, 3.6 ± 0.2, p < 0.05, Fig. 4A). KDE (600 mg/kg) did not improve spatial memory impairment (Fig. 4B).

DISCUSSION

The results of the present experiments confirmed that KDE has greater DPPH radical-scavenging activity than BWE. Both KDE and BWE inhibited the cell damage induced by glutamate, kainate, and β-amyloid(25-35) in primary cultures of hippocampal neurons. BWE showed weaker neuroprotective activity than KDE against the cellular damage induced by glutamate, but stronger activity against β-amyloid(25-35). BWE, but not KDE, improved the spatial memory impairment induced by scopolamine in the eight-arm radial maze. These results suggest that
there are differential effects between the flavonoid in BWE and phenylethanoid glycoside in KDE on neuroprotection in primary cultured hippocampal neurons and spatial memory impairment in the eight-arm radial maze.

In acteoside, isoacteoside, and ligupurpuroside structures of KDE (Fig. 1) there are plural rhamnosyl or glucosyl connections with caffeoyl and 3,4-hydroxyphenylethyl moieties, and they are vulnerable to the loss of a proton or an electron due to resonance delocalization. The observed antioxidant activities of acteoside, isoacteoside, and ligupurpuroside A are likely attributable to their proton-donating capacities. It has been determined that the caffeoyl groups at C-4’ of glucose tend to enhance the inhibitory effects on the hemolysis of red blood cells induced by free radicals, but variation in the number of sugar units demonstrated no apparent effect. In the structure of catechin, epicatechin, rutin and quercetin, there are mainly three structural groups that determine the free radical-scavenging and/or antioxidative potential of flavonoids: a catechol moiety of the B ring; the 2,3-double bond in conjugation with a 4-oxo function of a carbonyl group in the C ring (quercetin and rutin); and the additional presence of hydroxy groups at the 3 and 5 positions in the A ring. These structures determine the different actions of BWE and KDE in inhibiting the neurotoxicity induced by glutamate, kainate, and \( \beta \)-amyloid.

It has been reported that acteoside scavenges DPPH radicals more efficiently than \( \alpha \)-tocopherol, vitamin C, and resveratrol. It was also reported that rutin, quercetin, and catechin scavenge DPPH radical, and both quercetin and rutin are less potent than vitamin C. The scavenging effects of buckwheat hull extract on DPPH radicals is a slightly less potent than green tea extract, and the antioxidants of acteoside, isoacteoside, and ligupurpuroside A were comparable to those of (–)-epicatechin gallate from green tea in the oxidation of human low-density lipoprotein. Therefore previous reports support our finding that KDE has greater DPPH radical-scavenging activity than BWE.

Glutamate and kainate are excitatory amino acids causing neuronal death by activating \( N \)-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors relating to both acute and chronic neurologic disease. Excess glutamate induces \( \text{Ca}^{2+} \) influx into the postsynapse through NMDA and non-NMDA channels, a profound collapse of the mitochondrial potential, synthase activity and production of free radicals. Kainate has a structure analogous to that of glutamate and has a high affinity for the non-NMDA receptor of glutamate. Kainate causes the influx of \( \text{Ca}^{2+} \) into the postsynapse and induces convulsions and the production of free radicals. It has been reported that the inhibitory effects of quercetin on kainate-induced neurotoxicity are greater than those on NMDA-induced neurotoxicity. It was also reported that (–)-epigallocatechin gallate (EGCG), a catechin from tea, prevented the neurotoxicity induced by the AMPA receptor via the inhibition of \( \text{Ca}^{2+} \) influx. In the present experiment, BWE 100 \( \mu \text{g/ml} \) did not scavenge DPPH radicals,
but KDE 100 µg/ml exhibited potent DPPH radical-scavenging activity. These results suggest that the neuroprotective effects of BWE are less related to their free radical-scavenging activity, but the neuroprotective effects of KDE are related to the free radical-scavenging activity in primary cultures of hippocampal neurons. The neuroprotective effects of BWE against the kainate-induced neurotoxicity were greater than those against glutamate-induced neurotoxicity, suggesting that the neuroprotective activity of flavonoids may be mainly related to the non-NMDA receptor, but the neuroprotective action of phenylethanoid glycoside may be related to both the NMDA receptor and the non-NMDA receptor.

β-Amyloid is the characteristic neuropathologic feature of the senile plaques in Alzheimer’s disease. It is known that aggregated β-amyloid induces lipid peroxidation, protein oxidation, and the formation of reactive oxygen species and reactive nitrogen species, suggesting that the cell damage induced by β-amyloid is related to oxidative stress. Antioxidants, including catechin, epicatechin, and quercetin, were reported to have a neuroprotective effect against β-amyloid toxicity in vitro. It is still unclear whether phenylethanoid glycoside protects against β-amyloid in addition to its antioxidant effect. On the other hand, we studied the effects of rutin, quercetin, catechin, and rutin on spatial memory impairment induced by repeated cerebral ischemia in rats (unpublished results). Rutin and quercetin, but not catechin and EGCG, improved the spatial memory impairment and neuronal damage induced by repeated cerebral ischemia. This result shows that the antioxidants did not improve spatial memory impairment. Moreover, catechin, a BWE constituent, was reported to inhibit soluble tyrosine kinase activity and increase the particulate activity to act on the signal transduction pathway involving protein phosphorylation against β-amyloid toxicity in PC12 cell growth. These results suggest that the greater inhibition by BWE, but not KDE, against β-amyloid damage may not only act as an antioxidant but also affect the signal pathway.

It is known that β-amyloid-impaired pyruvate dehydrogenase (PDH) activity causes an inadequate supply of acetyl-CoA owing to PDH impairment and results in reduced acetylcholine synthesis, a characteristic of Alzheimer’s disease. Therefore we investigated whether BWE and KDE could improve the spatial memory impairment induced by scopolamine in the eight-arm radial maze, which is related to cholinergic dysfunction. The results demonstrated that BWE slightly improved spatial memory impairment, suggesting that the neuroprotective effects of BWE may be related to cholinergic neurons. It was reported that EGCG facilitated cholinergic ganglionic transmission in the myenteric plexus of the guinea pig small intestine.

In conclusion, both BWE and KDE act as antioxidants. Moreover, BWE exhibited a greater inhibition of β-amyloid-induced neurotoxicity and scopolamine-induced impairment of spatial memory, while KDE exhibited stronger inhibition of DPPH radical and glutamate-induced neurotoxicity. These results suggest that BWP may be more effective in Alzheimer’s disease, which is related to cholinergic dysfunction, while KDP may be more effective in cerebral ischemia, which is related to glutaminergic dysfunction.

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