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Protective Effect of Fermented Red Ginseng on a Transient Focal Ischemic Rats

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Red ginseng and fermented red ginseng were prepared, and their composition of ginsenosides and antiischemic effect were investigated. When ginseng was steamed at 98-100°C for 4 h and dried for 5 h at 60°C, and extracted with alcohol, its main components were ginsenoside Rg_3> ginsenoside Rb_1 > ginsenoside Rb_2. When the ginseng was suspended in water and fermented for 5 days by previously cultured *Bifidobacterium* H-1 and freeze-dried (fermented red ginseng), its main components were compound K > ginsenoside Rg_3 ≥ ginsenoside Rh_2. Orally administered red ginseng extract did not protect ischemia-reperfusion brain injury. However, fermented red ginseng significantly protected ischemica-reperfusion brain injury. These results suggest that ginsenoside Rh2 and compound K, which was found to be at a higher content in fermented red ginseng than red ginseng, may improve ischemic brain injury.

Key words: Ginseng, Intestinal bacteria, Fermented ginseng, Ischemia

INTRODUCTION

An inflammatory reaction in the brain, due to an intervention or decrease in blood circulation, causes an ischemic stroke, and this occurs in about 80% of brain stroke patients (Bartosik-Psujek *et al.*, 2003; Barber *et al.*, 2003). The damage to the brain neuronal cells due to an inflammatory reaction causes the release of excessive excitational neuronal transmitters, production of free radicals, inhibition of protein synthesis, abnormal expression of genes, and the activation of immune responses. However, there have been no therapeutically effective agents developed to protect against such damage to the neuronal cells of the brain.

Ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae) is frequently used as a crude substance, and it is taken orally as a traditional medicine in Asian countries. The major components of ginseng are the ginsenosides Rb_1, Rb_2, and Rc, which are glycosides with a dammarane skeleton (Shibata *et al.*, 1963; Tanaka *et al.*, 1972). When ginseng is streamed at 98-100°C, it is called a red ginseng, whose main component is ginsenoside Rg_3 as well as ginsenoside Rb_1 (Kitakawa *et al.*, 1983).

These ginsenosides have been reported to show various biological activities, which include anti-inflammatory activity (Wu *et al.*, 1992) and antitumor effects (Sato *et al.*, 1994; Moxhizuki *et al.*, 1995). In order to explain these pharmacological actions, it is thought that ginseng saponins must be metabolized by human intestinal bacteria after being taken orally (Akao *et al.*, 1998a; Akao *et al.*, 1998b; Kanaoka *et al.*, 1992; Kanaoka *et al.*, 1994). Ginsenosides Rb_1, Rb_2, and Rc are transformed to 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901, compound K) by human intestinal bacteria. This transformed IH-901 induces an anti-metastatic effect by blocking tumor invasion (Wakabayashi *et al.*, 1998). Ginsenoside Rg_3, which is a main component of red ginseng or acid-treated ginseng, was metabolized to ginsenoside Rh_2 by human intestinal bacteria (Bae *et al.*, 2002). The transformed ginsenoside Rh_2 showed a more potent cytotoxic activity than ginsenoside Rg_3 and a higher level of protection against ischemia brain injury (Bae *et al.*, 2002; Park *et al.*, 2004).

Therefore, we isolated bifidobacteria hydrolyzing ginsenoside Rg_3, which is a main component in red ginseng, from human intestinal microflora, fermented red ginseng by bifidobacteria, and investigated their antiischemic effects on the ischemic brain injury induced by a transient focal ischemic rat model.

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MATERIALS AND METHODS

Materials

The 2,3,4-triphenyltetrazolium chloride (TTC), hexadecyltrimethyl ammonium bromide, and *o*-dianisidine were purchased from Sigma Chem. Co. (MI, U.S.A.). The isoflurane was purchased from Choongwae Pharm. Co., Ltd (Korea), and the general anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Japan). All other chemicals were of analytical reagent grade. 20(*S*)-Ginsenoside Rg_3 and 20(*S*)-ginsenoside Rh2 were prepared by the previously reported method (Bae *et al.*, 2002).

Isolation of *Bifidobacterium* H-1 from human intestinal microflora

Ten grams of fresh feces were suspended with 100 mL of saline and were centrifuged at 200 g for 5 min, and the precipitate was discarded. The supernatant was centrifuged at 5000 g for 20 min. The precipitate was washed twice with saline before used. Among the human feces tested, potent Rg_3-hydrolyzing fresh feces were anaerobically diluted 10^3 to 10^7-fold. Two hundred microliters of the diluted fecal suspension were inoculated in GAM agar plates. The plates were anaerobically incubated at 37°C for 3 days. More than 200 colonies isolated from several plates, or bacteria previously isolated from human intestinal bacteria, were cultured in 50 mL of tryptic soy broth containing 0.01% sodium thioglycolate and 0.1% ascorbic acid. Then, each cultured cell was collected at 5000 rpm for 10 min and was washed twice with saline. The ginsenoside Rg_3-hydrolyzing activities of these collected cells were measured according to the assay method below. The most potent ginsenoside Rg_3-hydrolzing *Bifidobacterium* was named as H-1 and was identified according to the Bergey's manual (Scardovi, 1984). H-1 is a gram-positive rod that produced fructose 6-phosphate phosphoketolase but did not produce gas, indole, and nitrate reduction enzymes. H-1 utilized glucose, fructose, and raffinose, but it did not use sorbitol (Table I).

Assay of metabolic activity of ginsenosides by human intestinal bacteria

The reaction mixture containing 100 μL of 0.1 mM of each ginsenoside (or 0.1% acid-treated ginseng) and 100 μL of fecal suspension (or bacterial suspension cultured in TS broth) was incubated for 24 h at 37°C. The reaction mixture was extracted with BuOH, evaporated, and assayed by TLC and HPLC systems. TLC was performed as follows: TLC plates, silica gel $60F_{254}$ (Merck Co., USA); developing solvent, $CHCl_3$-MeOH-H_2O (65:35:10 v/v, lower phase). The plates were stained by spraying with MeOH-H_2SO_4 (95:5 v/v), followed by heating. The stained TLCs were then analyzed by a TLC scanner (Shimadzu model CS-9301PC, Japan). HPLC was performed as follows: HPLC column, Lichrosorb NH_2 (25×0.4 cm, 5 mm, Merck Co.); elution solvent, mixtures of solvent A (acetonitrile / water / isopropanol = 80:5:15) and solvent B (acetonitrile / water / isopropanol = 80:20:15)-gradient profile of solvent A to solvent B from 75:25 to 0:100 for 51 min and from 0:100 for 51-57 min; detector, ELSD (Altech., U.S.A.). Each isolated bacterium was cultured in 50 mL of GAM broth and was collected at 5000 g for 30 min. Each collected bacterial pellet was suspended in a 50 mM phosphate buffer and was used as a crude enzyme solution.

Preparation of red ginseng and fermented red ginseng

The red ginseng extract was prepared as follows. Fresh ginseng (1 kg) was steamed at 98-100°C for 4 h and dried for 5 h at 60°C. Then, it was extracted with 60% ethyl alcohol at 70°C (freeze-dried extract weight, 39 g). Fermented red ginseng was prepared as follows. Red ginseng extract (15 g) was suspended in water, fermented for 5 days by previously cultured Bifidobacterium H-1 (30 g), wet weight, and freeze-dried. Each extract was suspended in water, extracted with BuOH (1 L) twice, and evaporated (dried extract weight, 3.1 g). The BuOH extracts were used as samples for antiischemic assay in a transient focal ischemic rat model.

Animals

The male SD rats (270-280 g) were purchased form the Charles River Branch of Biogenomics Co., Ltd. (Seoul, Korea). The rats were housed, 5 per cage, allowed free access to water and food, and maintained at a constant temperature (22 ± 2°C) and humidity (55 ± 10%), with a 12-h light/dark cycle (light on 07:30-19:30 h). All procedures related to the animals and their care conformed to the International Guidelines; Principles of Laboratory Animals Care (NIH publication no. 85 - 23, revised 1985).

Transient cerebral ischemia and morphometric measurement of infarct volume

The rats were anesthetized in a chamber containing a mixture of N_2O and O_2 (70:30) and 2.5% isoflurane. The middle cerebral artery (MCA) was occluded according to the modified method of Nagasawa and Kogure (1989). The neck skin of the rats were incised, the right common carotid artery was exposed, and a 17-mm-long nylon thread, with a rounded tip (coated with silicon), was inserted from the bifurcation to the right MCA. After surgery, the rats were allowed to recover from the anesthesia. 2 h after the MCA occlusion, the thread was removed under reanesthesia to allow complete reperfusion of the ischemic

area. Neurological deficits, which were characterized by severe left-sided hemiparesis and right Horners syndrome, were used as criteria for evaluating the ischemic insult. The rats exhibiting no behavioral deficits before reperfusion were excluded from the experiment. The body temperature of the rats was maintained at 37 ± 0.5°C throughout surgery using a heating pad (Biomed S.L., Spain). The testing agents were either dissolve in water or dispersed, if insoluble, in an aqueous suspension of 5% v/v tween 80 and were administered orally or intraperitoneally. The rats of the control group were given an oral vehicle alone. The agent samples were administered 2 h before the reperfusion was completed. After reperfusion for 22 h, the rats were decapitated, and their brains were carefully removed. The brains were sectioned coronally with a brain slicer at 2 mm intervals from the frontal pole. Slices were incubated for 60 min in a solution of 2% 2, 3, 4-triphenyltetrazolium chloride (TTC) at 37°C and were fixed by immersion in 10% neutral-buffered formalin. The area of infarction (mm^2) of each section was determined using a computerized image analysis system (Multiscan, Fullerton, CA), and the total lesion volume (mm^3) was calculated by summing the infarct areas of each section (seven slices in all) and multiplying the result with the distance between the sections.

Statistical analysis

All values are expressed as the mean ± S.E.M. The data were analyzed by a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test. Statistical significance was set at $P<0.05$. A Students *t*-test was carried out on the infarct area data for comparisons with the control group.

RESULTS AND DISCUSSION

Fermentation of red ginseng by human intestinal bifidobacteria

The ginsenoside contents of red ginseng, which was prepared by steaming at 98-100°C for 4 h and dried at 60°C for 5 h, and the ginsenoside contents of fermented red ginseng, which was fermented with *Bifidobacterium* H-1, were investigated.

When ginseng was incubated under steaming conditions at 98-100°C, the amount of ginsenoside Rg_3 was significantly increased compared to that of untreated ginseng. The constituents of red ginseng were Rg_3 > ginsenoside Rb_1 > ginsenoside Rb_2. This result was supported by previous studies of Kitagawa *et al.* (1983). When red ginseng was incubated with human intestinal microflora, ginsenoside Rg_3, ginsenoside Rb_1, and ginsenoside Rb_2 were decreased, and compound K and ginsenoside Rh_2 were increased. The constituents of fermented red ginseng were compound K > ginsenoside Rg_3 ≥ ginsenoside Rh_2. The previous report of Bae *et al.* supports that ginsenoside Rg_3 was metabolized to ginsenoside Rh_2 by human intestinal bacteria (Bae *et al.*, 2002).

Therefore, ginsenoside Rg_3-hydrolyzing lactic acid bacteria were screened from human intestinal microflora, and then, H-1 was isolated, which was gram-positive, rod-shaped, non-gas-forming and fructose 6-phosphate phosphoketolase-productive. Comparing the sequences to those from the GenBank databases, the sequence of *Bifidobacterim* H-1 16S rDNA exhibited a 98% homology with that of *Bifidobacterium longum*, which is a representative probiotic (Data not shown).

When red ginseng was incubated with *Bifidobacterium* H-1, ginsenoside Rh_2 and compound K were significantly produced. These findings and previous reports suggest that compound K originated from the biotransformation of ginsenosides Rb_1, Rb_2, and Rc in the red ginseng through H-1, and ginsenoside Rh_2 originated from that the biotransformation of ginsenoside Rg_3 (Kanaoka *et al.*, 1994; Bae *et al.*, 2000; Bae *et al.*, 2002).

Inhibitory effect of red ginseng and fermented red ginseng on brain injury induced by transient focal in the ischemia-reperfusion rat model

The inhibitory effect of red ginseng and fermented red ginseng against brain ischemic injury in rat was investigated (Fig. 1). The occlusion of the MCA resulted in extensive and reproducible hemispheric swelling and

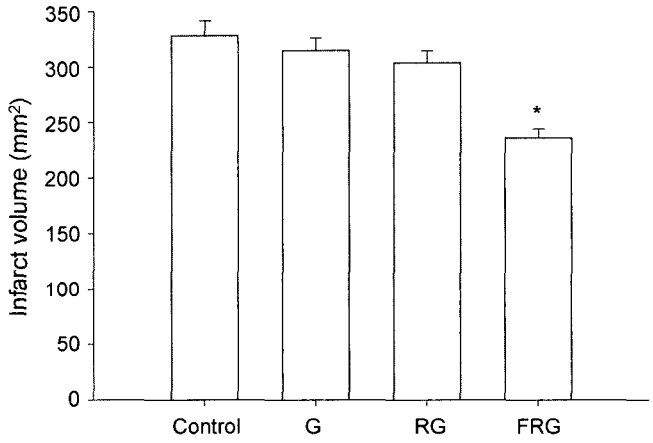

Fig. 1. Effect of red ginseng and fermented ginseng on the rat ischemia-reperfusion model. Measurements were made 2 h after MCA occlusion followed by 22 h of reperfusion. The edema rate in the infarcted area was calculated. Vehicle and samples were administered, promptly prior to reperfusion. Vehicle for control group (n=10), ginseng (100 mg/kg, n=5) for G, red ginseng (100 mg/kg, n=5) for RG, and fermented red ginseng (100 mg/kg, n=5) for FRG group were orally administered, promptly prior to reperfusion. Values are the mean ± S.E.M. *Significantly different compared with vehicle controls (ANOVA followed by Student-Newman-Keuls test) ($p<0.05$).

Table I. Utilization of various carbohydrates by H-1

Carbohydrate	Utilization[a)]
Amygdalin	-
Arabinose	+
Cellobiose	-
Dextrin	-
Esculin	+/-
Fructose	+
Galactose	+
Gluconate	-
Glucose	+
Glycogen	-
Inositol	-
Inulin	+/-
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melezitose	-
Melibiose	+
Raffinose	+
Ribose	+/-
Salicin	+
Sorbitol	-
Starch	-
Sucrose	+
Trehalose	-
Xylose	+

[a)] +, used; -, not used; +/-, 40-60% used.

Table II. Protopanaxadiol ginsenoside contents of ginseng treated with steam and *Bifidobacterium* H-1

	Content[a)] (%)		
	Ginseng[b)]	Red ginseng	Fermented red ginseng
Ginsenoside Rb1	15.1	5.1	1.7
Ginsenoside Rb2	8.2	3.5	2.1
Ginsenoside Rc	9.5	3.2	1.9
Ginsenoside Rd	3.5	1.2	2.1
Ginsenoside Rg3	<1	6.8	2.5
Ginsenoside Rh2	<1	<1	2.2
Compound K	<1	<1	2.8
Protopanaxadiol	<1	<1	<1

[a)] The content of each ginsenoside in BuOH extracts was indicated.
[b)] The concentrate of each ginseng (0.5 g) was suspended in 10 mL of distilled water, extracted with 20 mL of BuOH, and assayed by TLC and HPLC systems.

focal infarction throughout the cortical and subcortical structures. A large infarct (total infarct volume 393.3 ± 38.1 mm^3) was significantly induced after 120 min of ischemia/22 h of reperfusion in the control animals. The ginseng and red ginseng extract-treated groups did not show a reduction of the infarct area in all regions. However, the fermented red ginseng-treated group showed reductions of the infarct area in all regions, especially in the 3rd (AP 0.2 mm based on the bregma) and 4th (AP 1.8 mm based on the bregma) sections (data not shown), and the total infarct volume of this group was significantly lowered than in that of the vehicle-treated controls ($p< 0.05$).

In the previous study, we reported that ginsenoside Rh_2 potently inhibited the synthesis of PGE2, and orally administered ginsenoside Rh_2 significantly decreased a large infarct of the rat brain induced by MCA occlusion, although Yuan *et al.* (1998) reported that American ginseng showed GABAergic effects in rats. Based on these findings, the antiischemic action of fermented red ginseng may have originated from the effect of ginsenoside Rh_2 and compound K, which were main components in fermented ginseng. These results suggest that the orally administered red ginsengs main component, ginsenoside Rg_3, could be metabolized to ginsenoside Rh_2 by the intestinal bacteria. Also, the metabolite ginsenoside Rh_2 can inhibit ischemic brain injury caused by inflammatory reactions. Intestinal microflora of most humans exhibited ginsenoside Rg_3-hydrolzing activity. However, the hydrolyzing activity is significantly varied.

Therefore, fermented red ginseng can possibly improve ischemic brain injury more effectively than ginseng or red ginseng.

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