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Inhibitory Effect of Fermented Red Ginseng against Passive Cutaneous Anaphylaxis Reaction and Scratching behaviors in Mice

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Abtract : To evaluate the antiatopic effect of Korea red ginseng (RG, steamed root of *Panax ginseng* CA Meyer, Family Araliaceae) fermented by *Bifidobacterium longum* H-1 (FRG), its inhibitory effect on passive cutaneous anaphylaxis (PCA) reaction and itching in mice was measured. FRG and its ingredient saponin fraction (FSF) potently inhibited PCA reaction and scratching behaviors. FRG at a dose of 200 mg/kg and FSF at a dose of 50 mg/kg significantly inhibited the scratching frequency by 45% and 47%, respectively. FRG and FSF also inhibited the degranulation and protein expression of tumor-necrosis factor-α and interleukin-4 of RBL-2H3 cells induced by IgE-complex. However, polysaccharide fraction of FRG (FPF) weakly inhibited it, compared with FSF. The inhibitory effect of FRG against PCA reaction and scratching behaviors more potently inhibited than that of RG. Based on these findings, FRG can improve allergic skin disorders atopic dermatitis by the regulation of TNF-α, and IL-4 produced by mast cells and basophils and its degranulation.

Key words : fermented red ginseng, scratching behaviors, passive cutaneous anaphylaxis, atopic dermatitis, allergy.

INTRODUCTION

Allergic diseases such as atopic dermatitis, asthma, allergic rhinitis, and food allergy afflict up to 20% of the human population in most countries[1]. The etiology of allergy reactivity is based on IgE-medicated pharmacological processes of a variety of cell populations such as mast cell and basophils[2]. Degradation of mast cells and basophils with antigen-crosslinked IgE releases histamine, prostaglandins, leukotrines and cytokines affecting lymphocytes, macrophages, eosinophils and neutrophils. Finally cytokine-induced reaction causes tissue damages by allergic diseases, such as allergic rhinitis, atopic dermatitis, asthma and food allergies[3-5]. However, improving these diseases is very difficult. Therefore, herbal medicines have been advanced for allergic diseases, and their effectiveness has received increasing attention[6].

Red ginseng (RG, the steamed root of *Panax ginseng* C.A. Meyer, family Araliaceae) is frequently used as a traditional medicine taken orally in Korea, China, Japan and Asian countries. The major components of ginseng are ginsenosides and polysaccharides.[7-8] Many kinds of saponins, such as ginsenosides Rb1, Rb2, Rc and Rf, have been isolated. However, RG contains genuine saponins, ginsenosides Rg3 and Rh2[9,10]. Ginsenosides Rg3 and Rh2 were produced from protopanaxadiol ginsenosides by steaming to prepare RG[11]. When RG is fermented by *Bifidobacterium* H-1, ginsenoside Rg3 is transformed to ginsenoside Rh2, which is a representative constituent in fermented RG (FRG)[12,13]. These ginsenosides have been reported to show various biological activities including anti-inflammatory activity, antiallergic, endothelium-independent aorta relaxation and anti-tumor effects.[14-17] Compared to ginsenoside Rg3, ginsenoside Rh2 exhibits potent cytotoxicity against tumor cells, antiallergic effect against mast cells and antiinflammatory activity in microglial cells[13,18,19]. Sugiyama *et al*. reported that ginsenoside Rg3 suppressed histamine release from mast cells caused due to stimulation with compound 48/80 *in vitro*[20]. We reported that red ginseng exhibited antiallergic effect[21]. We also reported the antiallergic and anti-inflammatory effect of RG and ginsenoside Rh1[22,23], antiallergic and passive cutaneous anaphylaxis reaction (PCA)-inhibitory effects of compound K[23] and the ginsenoside Rh2 more potently inhibited the PCA reaction[6].

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However, antiatopic effects, such as PCA reaction scratching behavior reactions, of FRG and its ingredients saponins fraction (FSF) and polysaccharide fraction (FPF) have not been thoroughly studied.

Therefore, the present study is to investigate the inhibitory effect of FRG, FSF and FPF on passive cutaneous anaphylaxis reaction and itching in mice.

MATERIALS AND METHODS

Materials

p-Nitrophenyl-N-acetyl-β-D-glucosaminide, Freund's complete adjuvant, anti-dinitrophenol (DNP)-IgE, DNP-human serum albumin (HSA), Evans blue, trichloroacetic acid, betamethasone, and azelastine were purchased from Sigma Chemical Co. (U.S.A). FRG water extract was prepared according to the previous method of Trinh *et al.*[15]. The FSF and FPF were isolated according to the previous reported methods[15,16].

Animals

The male ICR mice (20-25 g) were supplied from the Orient Co., Ltd, a branch of Charles River Laboratories (Seoul, Korea). All animals were housed in wire cages, maintained at 20-22°C and 50 ± 10% humidity, fed standard laboratory chow (the Orient Co., Ltd), and allowed water *ad libitum*. All procedures relating to the animals and their care conformed to the international guidelines: 'Principles of Laboratory Animals Care' (NIH publication no. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

Passive Cutaneous Anaphylaxis (PCA) Reaction

An IgE-dependent cutaneous reaction was measured according to the previous method of Choo *et al.*[24] The male ICR mice (25-30 g) were injected intradermally with 10 μg of anti-DNP IgE into each of two dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each mouse received an injection of 200 μl of 3% Evans blue PBS containing 200 μg of DNP-HSA *via* the tail vein. The test agents were administered 1 h prior to DNP-HSA injection. Thirty min after DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed for measurement of the pigment area. After extraction with 1 ml of 1.0 N KOH and 4 ml of a mixture of acetone and 0.6 N phosphoric acid (13:5), the amount of dye was determined colorimetrically (the absorbance at 620 nm).

Assay of scratching behavior frequency

The scratching behavioral experiment in male mice was performed according to the method of Sugimoto *et al.*[25] Briefly, the mice were placed in acrylic cages (22 × 22 × 24cm) and allowed to acclimatize for about 10 min. The rostral part of the skin on the back of the mice was clipped, and 300 μg/50 μl of histamine in ICR mice then intradermally injected into each mouse. Immediately after the intradermal injection, the mice (one animal/cage) were placed back in the same cage, and the scratching behavior was recorded using an 8-mm video camera (SV-K80, Samsung, Seoul, Korea). The scratching frequency of the injected site with the hind paws was counted for 60 min. The test agents were orally administered 1 h before the scratching agent.

Assay of inhibitory activity against β-hexosaminidase release of RBL-2H3 cells

The inhibitory activity of test agents against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to Choo *et al.*[24]. RBL-2H3 cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum and L-glutamine. Before the experiment, cells were dispensed into 24-well plates at a concentration of 5×10^5 cells per well, and using a medium containing 0.5 μg/ml of mouse monoclonal IgE, the cells were sensitized by incubation overnight at 37°C in 5% CO_2. They were then washed with 500 ml of Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH) and incubated in 160 μl of Siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA for additional 10 min at 37°C. The cells were exposed to 40 *l* of test agents for 20 min, treated with 20l of antigen (DNP-HSA, 1 μg/ml) for 10 min at 37°C to activate cells and to evoke allergic reactions. The reaction was stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged at 2000 rpm for 10 min and 25 μl aliquots of the supernatant were transferred to 96 well plates and incubated with 25 μl of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) for 1 h at 37°C. The reaction was stopped by adding 200 μl of 0.1 M Na_2CO_3/$NaHCO_3$. Absorbance was measured by using an ELISA reader at 405 nm.

Enzyme-Linked Immunosorbent Assay (ELISA) of IL-4 and TNF-α in RBL-2H3 Cells Stimulated by IgE-antigen Complex

RBL-2H3 cells (5×10^5 cells), previously cultured in DMEM, were treated with 0.5 μg/ml of mouse monoclonal IgE to sensitize the cells. The cells (1.8 ml) were

exposed to 0.2 ml of the test agents (dissolved in 0.5% dimethyl sulfoxide) for 4 h, followed by treatment with 0.2 ml DNP-HSA (1 μg/ml) for 40 min at 37°C. The supernatant (50 μl) was transferred to 96-well ELISA plates, and the IL-4 and TNF-α concentrations then determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistics

All the data were expressed as the mean ± standard deviation, and statistical significance was analyzed by one way ANOVA followed by Student-Newman-Keuls test.

RESULTS

Inhibition of FRG on PCA reaction and scratching behaviors

To evaluate the antiallergic effect of FRG, the inhibitory effect of FRG against mouse PCA reaction induced by the intradermal injection of anti-DNP-HSA and DNP-HSA was investigated (Table 1). FRG extract potently inhibited PCA reaction, and at doses of 100 and 200 mg/kg inhibited PCA reaction by 30 and 45%, respectively. Therefore, we fractionated FSF and FPF from FRG and investigated their PCA reaction-inhibitory effect. The FSF potently inhibited the PCA reaction, but the FPF weakly exhibited the PCA reaction-inhibitory effect.

Next, we measured the inhibitory effects of FRG and its ingredients on compound 48/80-induced scratching behaviors in mice. FRG and its FSF potently inhibited scratching behaviors (Table 2). However, FPF did not inhibit it. FRG at a dose of 200 mg/kg and FSF at a dose of 50 mg/kg significantly inhibited the scratching frequency by 48% and 47%, respectively.

Table 1. Inhibitory effects of fermented red ginseng (FRG) and its ingredients on passive cutaneous anaphylaxis reaction in mice

Agent	Dose (mg/kg)	Inhibition[a)] (%)
FRG	100	30±4[c]
	200	45±6[c,d]
FSF	25	32±6[c,d]
	50	47±7[c,d]
FPF	25	14±1[b]
	50	24±4[c]
Azelastine	10	71±19[e]

[a)] The amounts of extravasated Evan blue from the dorsal skin (1 × 1cm) of the control stimulated with the IgE-antigen complex and vehicle-treated groups were 23±3 and 8±2 μg, respectively. Each experiment consisted of six observations. All inhibitory values indicate mean ± S.D.
[b,c,d,e]Those with the same letter are not significantly different in each column (P<0.05).

Inhibitory activity of FRG and its ingredients against degranulation of RBL-2H3 cells

To understand the inhibitory mechanism of FRG against PCA reaction and scratching behaviors, their inhibitory effect against β-hexosaminidase release (degranulation) of

Table 2. Inhibitory effects of FRG and its ingredients on the scratching behaviors induced by histamine in mice

Agent	Dose (mg/kg)	Inhibition[a)] (%)
FRG	100	36±5[d]
	200	48±6[e]
FSF	25	32±4[d]
	50	47±6[e]
FPF	25	-[b)]
	50	12±6[c]
Azelastine	10	62±5[f]

All agents were administered p.o. prior to compound histamine. Each experiment consisted of six observations. All inhibitory values indicate mean ± S.D.
[a)] Scratching behavior frequency numbers of normal control, which was treated with saline alone, and control group, which was treated with compound 48/80 histamine and saline, for 1 h were 235±21 and 3±2, respectively.
[b)]Not determined.
[c,d,e,f]Those with the same letter are not significantly different in each column (P < 0.05).

Table 3. Inhibitory effects of FRG and its ingredients on the β-hexosaminidase release from RBL 2H3 cells induced by IgE-antigen complex

Agent	IC_{50} (mg/ml)
FRG	> 0.2 (22)
FSF	0.19
FPF	> 0.2 (11)
Azelastine	0.02

RBL-2H3 cells, which were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine, were dispensed into 24 well plates, at a concentration of 5×10^5 cells per well, and sensitized using 0.5 μg/ml of mouse monoclonal IgE. They were then washed with 500 μl of siraganian buffer, exposed to test agents for 20 min, and treated with 20 μl of antigen (DNP-HSA, 1 μg/ml) for 10 min at 37°C. The release of β-hexosaminidase from RBL-2H3 cells was measured according to the method of Choo *et al.*[18)]
The values in parenthesis indicate degranuation-inhibitory percent at a dose of 0.2 mg/ml.

RBL-2H3 cells induced by IgE-antigen complex was investigated (Table 3). FRG and FSF inhibited the degranulation of RBL-2H3 cells. However, FPF did not inhibit it. Among them, SF most potently inhibited it.

Inhibition of FRG and its ingredients on cytokine production of RBL-2H3 cells

The effects of FRG and its ingredients against TNF-α and IL-4 protein expressions were also measured in RBL-2H3 cells stimulated by IgE-antigen complex by the analysis of ELISA analysis (Fig. 1). FRG and its ingredients reduced protein expression of TNF-α and IL-4, showing most strong reduction in case of FSF.

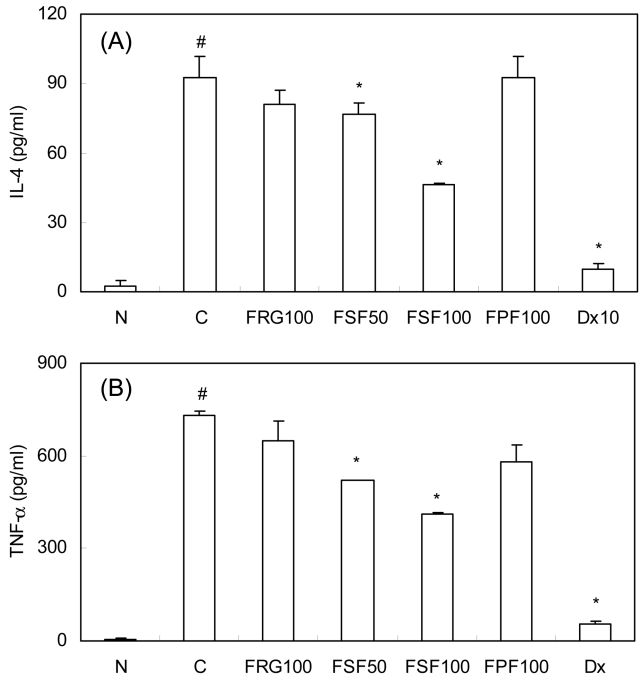

Fig. 1. Effect of red ginseng and its ingredients on the protein expression of TNF-α and IL-4 in RBL-2H3 cells induced by IgE-antigen complex. RBL-2H3 cells (5×10^5 cells) were treated with 0.5 μg/mL of mouse monoclonal IgE for 1 h, then exposed to 0.2 mL of the test agents (N, normal; C, vehicle alone; FRG100, 100 μg/ml FRG; FSF50, 50 μg/ml FSF; FSF100, 100 μg/ml FSF; FPF100, 100 μg/ml FP.F; D×10, 10 μM dexamethasone) for 20 min, followed by treatment with 0.2 mL dinitrophenol-human serum albumin (DNP-HSA, 1 μg/mL) for 4 h at 37°C, and the performance of ELISA for IL-4 (A) and TNF-α (B). Then normal group (N) was treated with the vehicle alone, while the control group (C) was treated with the vehicle and IgE-antigen complex. Values represent the mean ± S.D. for duplicate experiments. Inhibition values indicate the mean ± S.D. (n=3).
#Significantly different, compared with the normal ($P < 0.05$).
*Significantly different, compared with the control ($P < 0.05$).

DISCUSSION

Red ginseng is prepared by the steaming and drying of fresh ginseng. The major components of fresh ginseng are ginsenosides Rb1, Rb2, Rc, Rf, Rg1 and Re and those of RG are genuine saponins, ginsenosides Rg3 and Rh2 transformed from ginsenoside Rb1, Rb2, Rc, *etc*[9,10]. Fermented red ginseng also contains ginsenoside Rg3 and Rh2. However, the ratio of these ginsenosides Rg3 and Rh2 between red ginseng and fermented red ginseng is different, because the ginsenoside Rg3 in red ginseng is transformed to ginsenoside Rh2 by the fermentation of lactic acid bacteria. Fermentation produces beneficial products for humans. Therefore, fermentation has been used for their manufacture on an industrial scale. These processes are performed by beneficial and healthful microbes. These microbes transform some components of foods as well as convert sugars to alcohol and lactic acid. For example, lactic acid bacterial fermentation of ginseng produces lactic acid as well as compound K, which is transformed from ginsenoside Rb1, Rb2 and Rc and exhibits the potent cytotoxicity against tumor cells[26].

When RG is fermented by *Bifidobacterium* H-1, ginsenoside Rg3 is transformed to ginsenoside Rh2, which exhibits potent cytotoxicity against tumor cells, antiallergic effect against mast cells and antiinflammatory activity in microglial cells[13,18,19]. We also reported that the anti-allergic and anti-inflammatory effect of RG[22,23,27], and the ginsenoside Rh2 more potently inhibited the PCA reaction[14]. However, antiatopic effect such as PCA reaction scratching behavior reactions, of FRG has not been thoroughly studied. Therefore, in the present study, the antiallergic effect of FRG and its ingredients FSF and FPF was investigated. FRG and its PSF potently inhibited PCA reaction induced by IgE-antigen complex and compound 48/80-induced scratching behaviors. These inhibitory activities were more potent than those of red ginseng. FSF inhibited the release of β-hexosaminidase from RBL-2H3 cells. The previous study, the inhibitory effects of ginsenoside Rh2 against PCA reaction and scratching behaviors were more potent than those of ginsenoside Rg3. This result suggests that the potent antiallergic effect of FRG may be due to the higher content of ginsenoside Rh2 in fermented red ginseng than in red ginseng. The previous studies reported that ginsenoside Rh1, compound K and ginsenoside Rh2 showed more potent membrane stabilizing effect than those of disodium cromoglycate[14,15,24].

These results suggest that the inhibitory action of these ginsenosides on the release of β-hexosaminidase may be due to protection of the cytolytic response by antigen-IgE and these ginsenosides after all showed the most potent inhibitory activity on PCA reaction. FRG and its FSF also potently inhibited the scratching behaviors (itching) induced by histamine. Itching, which provokes the desire to scratch, can be local or widespread and associated with atopic dermatitis, uriticaria or systemic disorders (cholestasis, uraemia). Many endogenous chemical agents, like amines, proteases, growth factors, neuropeptides, opioids, ecosanoids and cytokines, can act as pruritogens[28,29]. Scratching can cause skin lesions and contribute to severe psychological disturbances[30], and therefore, inhibition of this response is consistently beneficial for improving the quality of life.

FRG, particularly FSF, significantly inhibited protein expression of proinflammatory TNF-α and IgE-inducing IL-4 in RBL-2H3 cells induced by IgE-antigen complex. However, these inhibitions are not inconsistent with *in vivo* antiallergic effects. These findings suggest that FRG can improve allergic skin disorders atopic dermatitis and contact dermatitis by the regulation of TNF-α and IL-4 production in mast cells and basophils as well as their membrane stabilization. Therefore, we believe that FRG can show extensive antiallergic effect and its FSF can be a candidate for the therapeutic agent for allergy.

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