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RESEARCH NOTE

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Effect of Red Ginseng and Its Representative Constituents, Ginsenosides Rg3 and Rh2, on Dextran Sulfate Sodium-induced Colitis in Mice

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Abstract To evaluate the anticolitic effect of red ginseng (RG, the steamed root of *Panax ginseng* C.A. Meyer, Araliaceae), RG and its representative constituents, ginsenosides Rg3 and Rh2, were orally administered to dextran sulfate sodium (DSS)-induced colitic mice and inflammatory markers investigated. RG and its constituents, ginsenosides Rg3 and Rh2, inhibited colon shortening and myeloperoxidase activity induced by DSS. The ginsenosides Rg3 and Rh2 inhibited mRNA expression of interleukin (IL)-1β as well as protein levels of IL-1β and IL-6. These ginsenosides also inhibited the activation of a transcription nuclear factor (NF)-κB. Ginsenoside Rh2 was a more potent inhibitor than ginsenoside Rg3. The anticolitic effects of these ginsenosides were comparable with sulfasalazine.

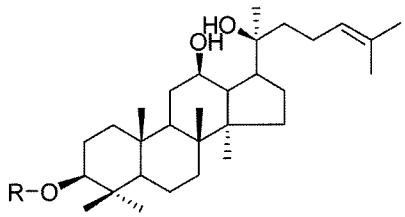
Keywords: colitis, red ginseng, ginsenoside Rg3, ginsenoside Rh2, interlukin (IL)-1β, nuclear factor (NF)-κB

Introduction

Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae) is used as an herbal medicine and functional substance for tonic, tumors, inflammation, and stress in Korea, Japan, and China. Its main constituents are ginsenosides Rb1, Rb2, and Rg1, which are glycosides containing an aglycone with a dammarane skeleton (1). When it is steamed, red ginseng (RG) is called. Of its ginsenosides, protopanaxadiol ginsenosides Rb1, Rb2, Rc, and Rd are transformed to ginsenoside Rg3 and/or ginsenoside Rh2. The ginsenosides Rg3 and Rh2 (Fig. 1) are representative saponins in RG (2-6). These ginsenosides have anti-inflammatory, antiallergic, and antitumor effects (7-11).

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn disease, are characterized by chronic and relapsed inflammation of the gut, but their aetiology remains unknown (12). Recently, many studies suggest that IBD involve a dysregulation of the intestinal immune response to genetic and non-genetic environmental factors, such as intestinal microflora (12,13). The proinflammatory and immunoregulatory cytokines, such as interlukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8 are important markers for IBD therapy as well (14,15). Sulfasalazine is used for the treatment of ulcerative colitis. Its efficacy and side effects, such as gastrointestinal disturbances and anorexia, are also a major clinical problem (16). Therefore, Chinese herbal medicines are receiving increased attention as alternative treatments for IBD (17). However, anticolitic effects of RG have not been studied.

Therefore, we investigated whether RG and its component ginsenosides, Rg3 and Rh2, influenced inflammatory molecular markers in dextran sulfate sodium (DSS)-induced colitic mice.



	R
Ginsenoside Rh2	-D-glc
Ginsenoside Rg3	-D-glc (1→2)-β-D-glc

Fig. 1. Structures of ginsenosides Rg3 and Rh2.

Materials and Methods

Materials Dextran sulfate sodium (DSS), sulfasalazine, hexadecyl trimethyl ammonium bromide, and tetramethyl benzidine were purchased from Sigma-Aldrich (St. Louis, MO, USA). A protease inhibitor cocktail was purchased from Boehringer/Roche (Mannheim, Germany).

RG extracts (61°Bx, 13% total saponin) were donated by KuAn Industries Co., (Seoul, Korea). The contents of ginsenosides Rg3 and Rh2 were 0.55 and 0.03%, respectively. Ginsenosides Rg3 (purity, >95%) and Rh2 (purity, 95%) were prepared according to our previous method (7,11).

Animals Male ICR mice (24-28 g) were supplied by Jung-Ang Lab. Animal, Inc. (Seoul, Korea). All animals were housed in wire cages at 20-22°C and 50±10%

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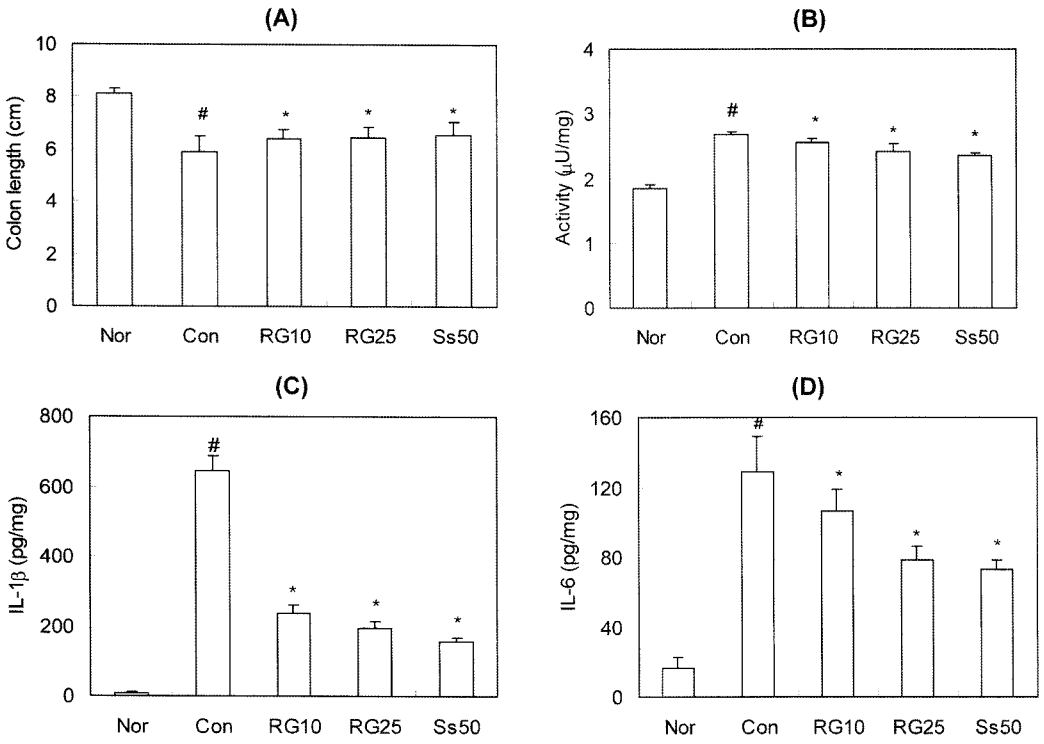

Fig. 2. Red ginseng inhibited DSS-induced (A) shortening, (B) myeloperoxidase activity, and (C) IL-1β and (D) IL6 induction. DSS, except in the normal group (Nor, normal group treated vehicle alone), were orally treated in control (Con), RG and sulfasalazine groups. RG (RG10, 10 mg/kg of red ginseng; RG25, 25 mg/kg of red ginseng) or sulfasalazine (50 mg/kg), except in the normal and control groups, was orally administered from 3 day prior to the DSS treatment to 1 day before killed. The mice were anesthetized with ether and killed. Enzyme activity values are the mean±SD (n=10). #,*Significantly different from normal and control group, respectively (p<0.05).

humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea), and allowed water *ad libitum*. All procedures relating to the animals and their care conformed to international guidelines as outlined in 'principles of laboratory animals care' (NIH Publication no. 85-23 revised 1985, and Kyung Hee University 2006).

Preparation of experimental colitic mice DSS-induced colitic mice were prepared according to the method of Fukata *et al.* (18). Each group is consisted of 7 mice. RG (10 and 25 mg/kg), Rg3 (5 and 10 mg/kg), Rh2 (5 and 10 mg/kg), and sulfasalazine (50 mg/kg) were orally administered to the mice once a day for 3 days. Thereafter, these agents were simultaneously administered with 4% DSS dissolved in water instead of tap water *ad libitum* once a day for 7 days. The control group was given 4% DSS. The normal group was treated with water alone. The mice were anesthetized with ether and sacrificed on the 7th day after DSS treatment, and then the colons were obtained.

Assay of myeloperoxidase activity in colonic mucosa The colonic mucosa were collected, homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5% hexadecyl trimethyl ammonium bromide, and then centrifuged for 30 min at 20,000×g and 4°C. An aliquot (50 μL) of the supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2, incubated at 37°C, and then the absorbance measured by time-scanned spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/mL of peroxide/min at 37°C and was expressed as μmol/min/mg protein (19). Protein content was assayed using the Bradford method (20).

Reverse transcription-polymerase chain reaction (RT-PCR) Colon tissue extract for RT-PCR analysis was performed by the method of Shin *et al.* (21). Total RNA was extracted by using TRI reagent according to the manufacturer's instructions, and treated with RNase-free DNase. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm and stored at −70°C until RT-PCR analysis. The RT-PCR was performed with AccPower® RT/PCR Premix (Bioneer, Seoul, Korea). The primers were designed as described by UniSTS database: IL-1α, forward primer 5'- ATGGCAAC TGTCCCTGAACT-3 and reverse primer 5'-GTCGTTGC TTGTCTCTCCTT-3' (product size 508 bp); TNF-α, forward primer 5'-GATTTTATTTGTTTAAAAGCAGATA TC-3' and reverse primer 5'-CATCCTAAGTCTACACAG GATCT-3' (product size 206 bp); glyceraldehydes-3-phosphate dehydrogenase (GAPDH), forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' (product size 452 bp). The RT-PCR products were electrophoresed on 2% agarose gel in Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide and photographed under ultraviolet (UV) light. The GAPDH gene was used as an internal control.

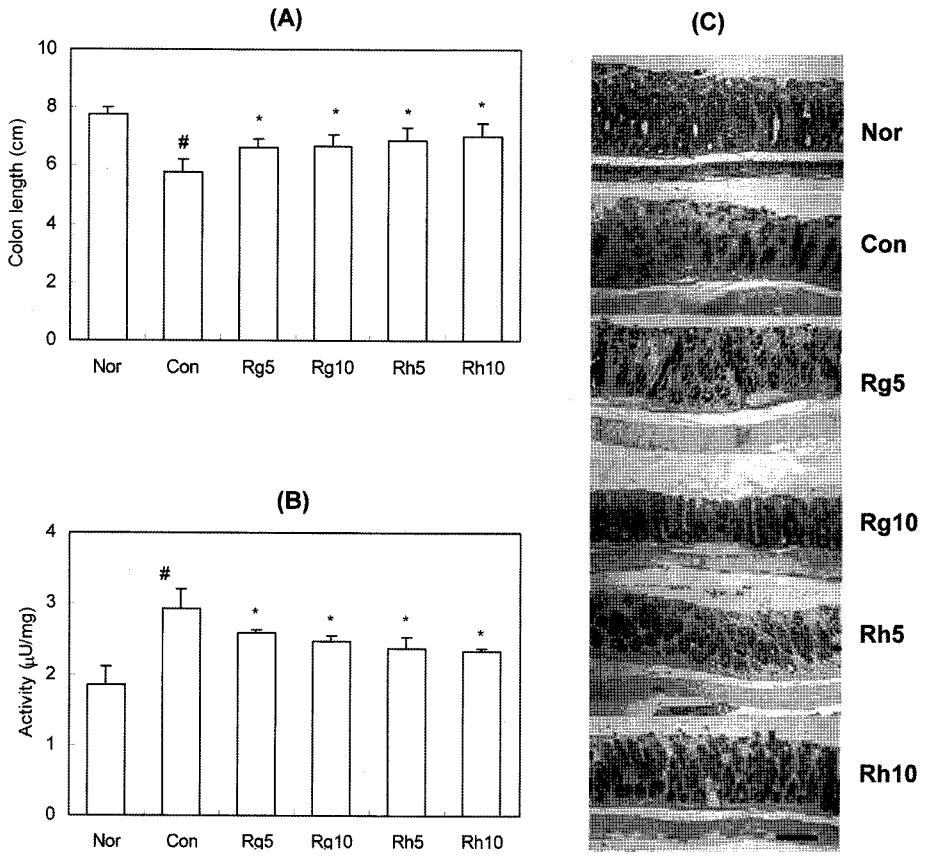

Fig. 3. Ginsenosides Rg3 and Rh2 inhibited the colon length (A), colonic myeloperoxidase activity (B) and colon histological photograph (C) in DSS-induced colitic mice. DSS, except in the normal group (Nor, normal group treated vehicle alone), were orally treated in control (Con), and ginsenoside groups. Ginsenosides (Rg5, 5 mg/kg of ginsenoside Rg3; Rg10, 10 mg/kg of ginsenoside Rg3; Rh5, 5 mg/kg of ginsenoside Rh2; Rh10, 10 mg/kg of ginsenoside Rh2) except in the normal and control groups, were orally administered from 3 day prior to the DSS treatment to 1 day before killed. The mice were anesthetized with ether and killed. Enzyme activity values are the mean±SD (n=10). $^{\#,*}$Significantly different from normal and control group, respectively (p<0.05). Scale bar in (C) is 0.1 mm.

Enzyme-linked immunosorbent assay (ELISA) and immunoblot For the ELISA of IL-1β and IL-6, colons were homogenized in 1 mL ice-cold lysis buffer (radioimmunoprecipitation assay, RIPA) containing 1% a protease inhibitor cocktail and 1% phosphatase inhibitor cocktail). The lysate was centrifuged (15,000×g, 4°C) for 15 min, and IL-1β and IL-6 concentrations for the supernatant were determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, USA) (22).

The immunoblot of pp65 (phospho-NF-κB), p65 (NF-κB), and β-actin were performed according to the previously reported method (21). Immunodetection was carried out using an enhanced chemiluminescence detection kit.

Statistical analysis All data are expressed as the mean± standard deviation (SD), with statistical significance analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls tests (p<0.05).

Results and Discussion

Anti-colitic effect of RG It was first evaluated whether RG inhibited colitis induced by DSS administration. Treatment with DSS for more than 10 days caused mice to have severe bloody diarrhea and die. Body weight loss and severe inflammation, manifested by shortened, thickened, and erythematous colons, were present by the 7th day of oral DSS administration alone (Fig. 2). RG treatment inhibited colon shortening and thickening, as well as the severe inflammation induced by DSS. RG also inhibited DSS-induced increases in myeloperoxidase activity, an inflammatory marker, in the colon. DSS increased protein levels of the proinflammatory cytokines, IL-1β and IL-6, in the colon, and treatment with RG inhibited this induction. Rg3 at a dose of 25 mg/kg inhibited cytokine expression of IL-1β and IL-6 by 71 and 43%, respectviely. Overall, the RG showed the potent inhibition of DSS-induced colitis, comparable with that of sulfasalazine.

Anti-colitic effect of ginsenosides Rg3 and Rh2 It was also investigated whether the representative constituents, ginsenosides Rg3 and Rh2, isolated from RG inhibited colitis induced by DSS. Treatment with the component ginsenosides, Rg3 and Rh2, also protected the colon from shortening and thickening as well as severe inflammation, repressed myeloperoxidase activity (Fig. 3). The treatment with ginsenosides Rg3 and Rh2 also inhibited mRNA expression of an inflammatory marker IL-1β except TNF-α as well as protein levels of the proinflammatory cytokines, IL-1β and IL-6 (Fig. 4). These ginsenosides at a

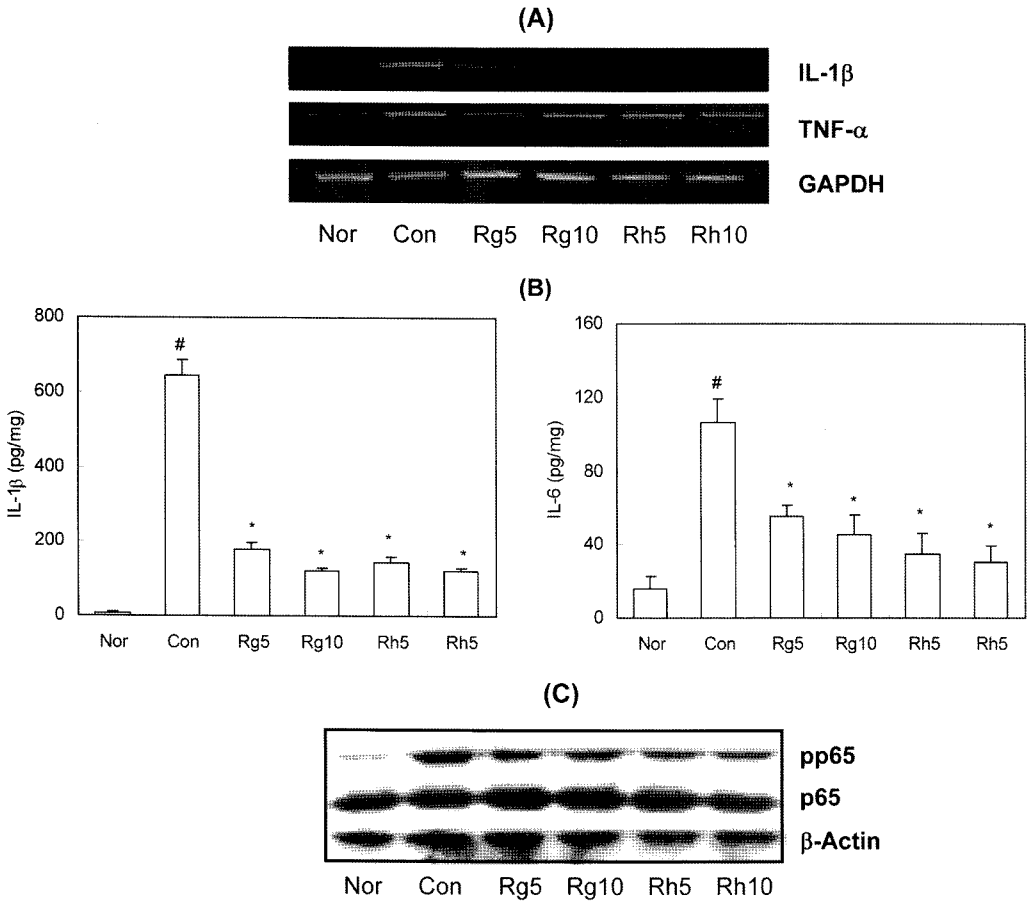

Fig. 4. Ginsenosides Rg3 and Rh2 inhibited DSS-induced IL-1β and TNF-α mRNA expression (A), IL-1β and IL-6 protein expression (B) and NF-κB activation (C) in mice. DSS, except in the normal group (Nor, normal group treated vehicle alone), were orally treated in control (Con), and ginsenoside groups. Ginsenosides (Rg5, 5 mg/kg of ginsenoside Rg3; Rg10, 10 mg/kg of ginsenoside Rg3; Rh5, 5 mg/kg of ginsenoside Rh2; Rh10, 10 mg/kg of ginsenoside Rh2) except in the normal and control groups, were orally administered from 3 day prior to the DSS treatment to 1 day before killed. The mice were anesthetized with ether and killed. Enzyme activity values are the mean±SD (n=10). #,*Significantly different from normal and control group, respectively (p<0.05).

dose of 10 mg/kg inhibited protein expression levels of IL-1β expression by 81 and 82%, respectively, and IL-6 by 67 and 81%, respectively. These ginsenosides also inhibited the activation of NF-κB, which is known to regulate the expressions of IL-1β and IL-6. Of them, ginsenoside Rh2 more potently inhibited it than ginsenoside Rg3.

IBD is a severe form of intestinal inflammation, the pathogenesis of which remains to be clearly understood. IBD does not significantly developed or progress in germ-free animals (22), suggesting that intestinal microflora may contribute to initiating and perpetuating colonic inflammation. It is thought that the disease might be due to complex mucosal immune responses to resident enteric bacteria (23,24). Therefore, the antibiotic sulfasalazine has been used can to treat colitis. The innate immune system recognizes the presence of specific bacterial antigens through pattern recognition receptors (25-27). TLR-4 is one of an extensive family of pattern recognition receptors and compelling research has been shown that lipopolysaccharide (LPS), which is the constituent of Gram-negative bacteria, binds to TLR-4. The triggering of TLR-4 complex signaling by LPS activates transcription factor NF-κB and results in a cascade of events that leads to the secretion of proinflammatory mediators from monocytes and dendritic cells, which leads ultimately to activation of the acquired immune response (28,29). NF-κB is one of the most important transcription factors for the induction of genes mediating innate and adaptive immunity (30), and is also the key transcription factor for proinflammatory responses in IBD (31). Therefore, we investigated the effect of RG and its constituents, which is known to inhibit NF-κB activation in macrophage cells, against the colitis in mice.

In this study, DSS induced diarrhea and shortening of the colon, as well as myeloperoxidase, IL-1β, IL-6, and COX-2, like the previously reported (12,13). DSS also activated the transcription factor NF-κB, like the previous reports (32). Oral administration of RG, Rg3, and Rh2 inhibited colon shortening, intestinal epithelial myeloperoxidase activity, and expression of IL-1β and IL-6 as well as the their transcription factor NF-κB activation, like the previous studies that Rg3 and Rh2 inhibit NF-κB activation (33,34), providing a potential mechanism for the inhibition of cytokines induction. These findings suggest that the RG and its constituents Rg3 and Rh2 may improve colitis by the regulation of the inflammatory cytokine expression via the activation of transcription factor NF-κB and have a

different mechanism of action than sulfasalazine.

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