

Red Ginseng Saponin Extract Attenuates Murine Collagen-Induced Arthritis by Reducing Pro-inflammatory Responses and Matrix Metalloproteinase-3 Expression

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Received August 25, 2009; accepted January 14, 2010; published online January 19, 2010

Ginseng, the root of *Panax ginseng* C. A. MEYER, has been used as a food product and medicinal ingredient. In this study, we assessed the anti-arthritic effects of red ginseng saponin extract (RGSE), including ginsenosides Rg3, Rk1 and Rg5 as major components, on a murine type II collagen (CII)-induced arthritis (CIA), which is a valid animal model of human arthritis. Oral administration of RGSE at 10 mg/kg reduced the clinical arthritis score and paw swelling in the CIA mice, and inhibited joint space narrowing and histological arthritis, illustrating the severity of synovial hyperplasia, inflammatory cell infiltration, pannus formation, and erosion of cartilage. RGSE inhibited the expression of matrix metalloproteinase-3 and nitrotyrosine formation, and recovered the expression of superoxide dismutase in the joints of the CIA mice. Orally administered RGSE also reduced the levels of serum tumor necrosis factor- α and interleukin-1 β in the CIA mice. CII- or lipopolysaccharide-stimulated cytokine production, in addition to CII-specific proliferation, was reduced in the spleen cells of the RGSE-treated CIA mice, as compared with those from vehicle-treated CIA mice. Furthermore, RGSE administration protected against CIA-induced oxidative tissue damage by restoring the increased malondialdehyde levels and the decreased glutathione levels and catalase activities almost to control levels. Therefore, RGSE may be a beneficial supplement which can improve human arthritis.

Key words *Panax ginseng*; ginsenoside; anti-arthritic; cytokine; matrix metalloproteinase-3

Ginseng, the root of *Panax ginseng* C. A. MEYER (Araliaceae), has been frequently used for several centuries in Asian countries as a food product and medicinal ingredient. In addition, it is one of the highest-selling herbal supplements in the United States, and the use of ginseng continues to increase.^{1,2)} The two kinds of ginseng, air-dried white ginseng and steamed red ginseng, harbor a variety of active components, including ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids, and its diverse pharmacological effects have been observed in the central nervous system and cardiovascular, endocrine, and immune systems.³⁾ In particular, ginsenosides, unique saponins contained in *Panax* species, are believed to be responsible for the majority of pharmacological actions of ginseng, which include antineoplastic, antistress, anti-inflammatory and antioxidant activities.^{4–6)} Furthermore, ginseng and ginsenosides have been reported to exhibit good efficacy in the treatment of Alzheimer's disease,⁷⁾ and have also been associated with improved glucose and insulin regulation in type 2 diabetes in clinical trials,⁸⁾ attenuated gastrointestinal injury after cardiopulmonary bypass in patients with congenital heart disease,⁹⁾ and prolonged life span in patients with non-small cell lung cancer after operation.¹⁰⁾

Rheumatoid arthritis (RA) is a systemic autoimmune disease that afflicts numerous joints throughout the body, whereas osteoarthritis (OA) develops in a small number of joints, generally as the result of chronic overuse or injury. Human RA affects approximately 1% of the population worldwide, and OA is noticeably increasing as the result of

the combination of an aging population and growing levels of obesity.^{11,12)} The irreversible destruction of the cartilage, tendon, and bone that comprise the synovial joints is the hallmark of both RA and OA. The pathogenesis of RA is closely associated with hyperplasia via the accumulation and proliferation of inflammatory cells, including B cells, T cells, macrophages, and synovial fibroblasts.¹³⁾ Additionally, recent studies have demonstrated the involvement of macrophages in OA-related pathology,¹⁴⁾ and have reported that macrophages mediate the formation of osteophytes and fibrosis in the early stages of OA.¹⁵⁾ In both RA and OA, infiltrating immune cells generate pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β ,¹⁶⁾ and these cytokines stimulate the production of matrix metalloproteinases (MMPs), enzymes which are capable of degrading all extracellular matrix components,^{17,18)} as well as reactive oxygen species.¹⁹⁾ The expression of MMPs in the synovial membranes, cartilage, tendon, and bone of the synovial joints is correlated with tissue destruction.

Non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressive disease-modifying anti-rheumatic drugs and corticosteroids have been utilized extensively for the treatment of RA by normalizing the immune system and reducing inflammatory mediators.^{20,21)} NSAIDs are also recommended for the management of knee and/or hip OA.¹²⁾ However, these drugs are generally undesirable for prolonged treatments due to poor efficacy, delayed onset of action, long-term side effects, and toxicity.²²⁾ Successful therapeutic targeting using cytokine antagonists evidences substantial effi-

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cacy, but entails high cost, parenteral administration, and hypersensitivity to medications and infections.^{22,23)} Therefore, it is necessary to develop preventive and therapeutic agents without side effects and toxicity, and oriental medicinal plants with anti-inflammatory activity may prove to be excellent candidates in this regard.

In this study, we evaluated the anti-arthritic effects of saponin extract derived from red ginseng, primarily including Rg3, Rk1, and Rg5, using a collagen-induced mouse arthritis model, which has been widely accepted as a favorable animal model of human inflammatory arthritis.²⁴⁾

MATERIALS AND METHODS

Preparation of Saponin Extract from Korean Red Ginseng Dried roots of *P. ginseng* were steamed at 98–100 °C for 2–3 h to produce red ginseng. The red ginseng was then extracted three times, each time with 3 volumes (v/w) of 70% ethanol for 3 h at 70 °C, followed by evaporation *in vacuo* to obtain the crude ginseng extract. The crude ginseng extract was then subjected to adsorption chromatography using Diaion HP-20 (Mitsubishi Chemicals) as a resin for saponin fractionation. In brief, the crude ginseng extract was loaded onto a HP-20 open column and sequentially eluted with water, 30% ethanol, 60% ethanol, and 95% ethanol. The fraction was concentrated *via* evaporation and freeze-dried to generate red ginseng saponin extract (RGSE) powder. The 95% ethanol eluted fraction was enriched with less polar red-ginseng-unique saponins, including Rg3, Rk1, and Rg5 in high-performance liquid chromatographic (HPLC) analysis.²⁵⁾

Animals Five-week-old female ICR mice for the ear edema test and 7–9-week-old male DBA/1J mice for the collagen-induced arthritis (CIA) model were acquired from Central Laboratory Animals (Seoul, Korea) and the Jackson Laboratory (Bar Harbor, ME, U.S.A.), respectively. All mice were permitted *ad libitum* access to a normal standard chow diet (Daejong, Seoul, Korea) and tap water, and housed under conditions of 20–22 °C, with a relative humidity of 55±5% and a 12-h light/12-dark cycle. All animal studies were conducted in accordance with the guidelines and regulations for the use and care of animals established by Yonsei University (Seoul, Korea).

12-O-Tetradecanoylphorbol-13-acetate (TPA)-Induced Mouse Ear Edema Test The right ear of each ICR mouse (5 per group) was treated topically with 5 nmol TPA in 50 µl vehicle [dimethyl sulfoxide (DMSO)–acetone: 15–85, v/v], 30 min after oral administration of RGSE or each ginsenoside (10, 50 mg/kg) in phosphate-buffered saline (PBS) containing 1% DMSO. The left ears were treated with vehicle alone. The control mice received vehicle only in both ears. Four hours later, the mice were sacrificed *via* cervical dislocation, and the ears were removed. Right and left ear punches 6 mm in diameter were obtained from each mouse. Edema was indicated as the increase in weight of the right ear punch over that of the left.

Induction of CIA DBA/1J mice were intradermally injected at the base of the tail with 100 µg of bovine type II (CII; Chondrex, Redmond, WA, U.S.A.) emulsified with an equal volume of Freund's complete adjuvant (CFA; Chondrex). On day 21 after primary immunization, the mice were

boosted with 100 µg of CII in Freund's incomplete adjuvant (IFA; Chondrex). RGSE was dissolved in PBS containing 1% DMSO. The CIA mice were divided into the vehicle group and RGSE group with oral administration at 10 mg/kg once daily from day 26 to day 46, each containing 6 mice matched with regard to clinical arthritis score and body weight. The control mice were neither immunized with CII nor treated with RGSE.

Macroscopic Scoring in CIA Mice Mice were monitored for signs of arthritis for which severity scores were derived as follows: 0, no signs of arthritis; 1, swelling and/or redness in only one joint; 2, swelling and/or redness in more than one joint; 3, swelling and/or redness in the entire paw; 4, severe swelling of the entire paw with deformity and/or ankylosis. The macroscopic arthritis score of each mouse was presented as the sum of each score of the four limbs, with the maximum score being 16. Paw swelling was evaluated by measuring the thickness of the two hind paws with an electric digital caliper every other day beginning on day 25. On day 45, all mice were sacrificed *via* anesthesia after serum collection. Hind paws and knee joints for histological examination, spleens for measurement of cytokine production, and livers and kidneys for the detection of oxidative stress were also removed from the control and CIA mice.

Histopathologic and Immunohistochemical Analysis The joints from the control and CIA mice were fixed in 10% buffered formalin solution, decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, U.S.A.), embedded in paraffin, and longitudinally cut into 5 µm serial sections. The sections were then stained with hematoxylin and eosin. For immunohistochemical analysis, the sections were incubated with 3% hydrogen peroxide in absolute methanol for 10 min at room temperature, followed by 30 min of incubation with 3% bovine serum albumin (BSA) in PBS. The tissue sections were incubated for 2 h with a specific antibody against MMP-3 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), nitrotyrosine (Millipore, Billerica, MA, U.S.A.), and superoxide dismutase (SOD; Abcam, Cambridge, U.K.). After washing three times with PBS containing 0.05% Twin-20, the sections were incubated for 20 min with horseradish peroxidase-linked secondary antibody at room temperature, and then allowed to react with 0.02% 3,3'-diaminobenzidine as chromogen (Life Science Division, Mukileto, WA, U.S.A.). All sections were counterstained with hematoxylin.

Preparation of Single-Cell Suspensions from Spleens Spleens were removed from the controls and all CIA mice (3 per group), washed with cold PBS, and dissociated into single cells using cell strainers. The red blood cells were lysed with red blood cell lysing buffer and the single-cell suspensions were then washed three times in PBS. The suspended spleen cells were counted with a hemocytometer, and cultured with RPMI 1640 medium containing 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS).

Measurement of Cytokine Levels in Blood Serum and Conditioned Medium of Spleen Cells Blood samples were collected from all mice *via* cardiac punctures prior to sacrificing the mice on day 45. Blood was clotted for 2 h at room temperature, and centrifuged for 20 min at 2000×g at 4 °C to obtain serum. Single-cell suspensions at a density of

2×10^6 cells/well were added in triplicate to 24-well plates. The cells were then stimulated with 50 $\mu\text{g/ml}$ denatured-CII or 5 $\mu\text{g/ml}$ lipopolysaccharide (LPS) for 48 h at 37 °C in a 5% CO_2 -humidified atmosphere. The conditioned media were collected, and the levels of TNF- α and IL-1 β in blood serum and the conditioned medium of spleen cells were assayed with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) in accordance with the manufacturer's recommendations.

Measurement of Spleen Cell Proliferation Spleen single-cell suspensions (2×10^5 cells/well) were added to 96-well plates, and stimulated with 50 $\mu\text{g/ml}$ denatured-CII for 72 h at 37 °C in 5%- CO_2 humidified atmosphere. Cell proliferation was measured with a 5-bromo-2'-deoxyuridine (BrdU) cell proliferation ELISA kit in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

Preparation of Tissue Homogenates The isolated liver and kidney tissues were immediately washed with ice-cold saline to remove the blood. Tissues (100 mg) were homogenized in 1 ml cold 1.15% KCl buffer (pH 7.4) containing 100 mM phenylmethylsulfonyl fluoride, then centrifuged for 10 min at $800 \times g$ at 4 °C. The supernatants were collected to determine the levels of malondialdehyde (MDA) and reduced glutathione (GSH) and catalase activity. The total protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) and BSA was utilized as a standard.

Determination of Lipid Peroxidation To evaluate MDA levels in the liver and kidney tissues, 0.2 ml homogenates were added to the reaction mixture containing 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5), 1.5 ml 0.8% thiobarbituric acid, and distilled water. The mixture (4.0 ml) was incubated for 1 h at 95 °C, cooled with tap water, mixed with 5 ml *n*-butanol-pyridine (15:1, v/v), and then centrifuged for 10 min at $2000 \times g$. The absorbance of the organic layer was determined at 532 nm using a spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.). A standard curve was established using 1,1,3,3-tetramethoxypropane to calculate the MDA levels in the homogenates, and the MDA level was expressed as nmol/mg protein.

Determination of GSH Level To determine the GSH level, 0.1 ml of homogenates were mixed with 50 μl of 5% 5-sulfasalicylic acid buffer, incubated for 10 min at 4 °C, then centrifuged for 10 min at $10000 \times g$. 10 μl of supernatant was added to the 150 μl reaction mixture, consisting of 100 mM potassium phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM 5,5-dithiobis(2-nitrobenzoic acid) and 6 unit/ml of glutathione reductase. The mixture was then incubated for 5 min at room temperature and 50 μl of 0.2 mM nicotinamide adenine dinucleotide phosphate (reduced form; NADPH) was added to the mixture to start the reaction. Absorbance was measured immediately at 412 nm using a spectrophotometer and the GSH level was expressed as nmol/mg protein.

Determination of Catalase Activity To determine the catalase activity, homogenates were added to an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and centrifuged for 15 min at $10000 \times g$.

Twenty μl of supernatant was mixed with 0.1 ml 100 mM potassium phosphate (pH 7.0), 30 μl methanol, and 20 μl 0.035 M H_2O_2 to initiate the reaction. After 20 min of incubation at room temperature, 30 μl of 10 M KOH was added to each mixture to terminate the reaction, followed by the addition of 30 μl of 3.4 mM purpald (chromogen) in 0.5 M hydrochloric acid. The mixture was incubated with shaking for 10 min at room temperature. Ten μl of 13 mM potassium periodate was added and the mixture were incubated for an additional 5 min at room temperature. The absorbance was measured at 540 nm in a microplate reader (Bio-Rad, Hercules, CA, U.S.A.). A standard curve was established using formaldehyde and the catalase activity was expressed as units/mg protein. One unit (U) of catalase was defined as the amount of enzyme resulting in the formation of 1 nmol of formaldehyde per min at 25 °C.

Statistical Analysis Data were expressed as the means \pm standard error (S.E.), and analyzed *via* one-way ANOVA with multiple comparisons followed by Tukey's test. *p* values of less than 0.05 were considered to be statistically significant.

RESULTS

Effect of RGSE on TPA-Induced Mouse Ear Edema

To evaluate the anti-inflammatory activity of RGSE and major ginsenosides in RGSE, mice were orally administered with RGSE, Rg3, Rg5, or Rk1 30 min prior to the application of TPA. TPA is a potent, acute inflammatory agent, and this property has been attributed to increased TNF- α and IL-1 β activity.^{26,27} Topical application of TPA alone for 4 h caused substantial ear edema, but pretreatment with RGSE suppressed TPA-induced mouse ear edema in a dose-dependent fashion (Fig. 1A). In addition, oral administration of Rg3, Rg5, and Rk1 blocked the induction of ear edema in TPA-treated mice, respectively (Fig. 1B). Anti-inflammatory activity of Rg3 was more potent than those of Rg5 and Rk1.

Effect of RGSE on CIA Progression and Histopathological Changes

DBA/1J mice were immunized with CII/CFA, then boosted with CII in IFA on day 21 after primary immunization. Vehicle or RGSE at 10 mg/kg was administered to CII-immunized mice by oral gavage once daily from day 26 after primary immunization, when the clinical signs of CIA first appeared. Vehicle-treated immunized mice displayed significant increases in clinical arthritis scores and the thickness of hind paws as compared with control mice, but CIA progression and severity were prevented in the RGSE-treated mice (Fig. 2A). In our histopathological evaluation by H&E staining, the knee joints of the vehicle-treated CIA mice evidenced notable synovial hyperplasia, inflammatory cell infiltration into the joint cavity, and partial bone destruction. In the joint tissues of the CIA mice treated with RGSE, these pathological events were reduced substantially (Fig. 2B). Furthermore, immunohistochemical analysis demonstrated that the joint specimens of vehicle-treated CIA mice were strongly stained for MMP-3 and nitrotyrosine in the inflammatory articular cartilage, but little significant positive staining for MMP-3 and nitrotyrosine was noted in the joint tissues of the RGSE-treated CIA mice (Fig. 2B). MMP-3 is considered to be a key factor in the pathological destruction of cartilage, and nitrotyrosine is an index of the nitro-

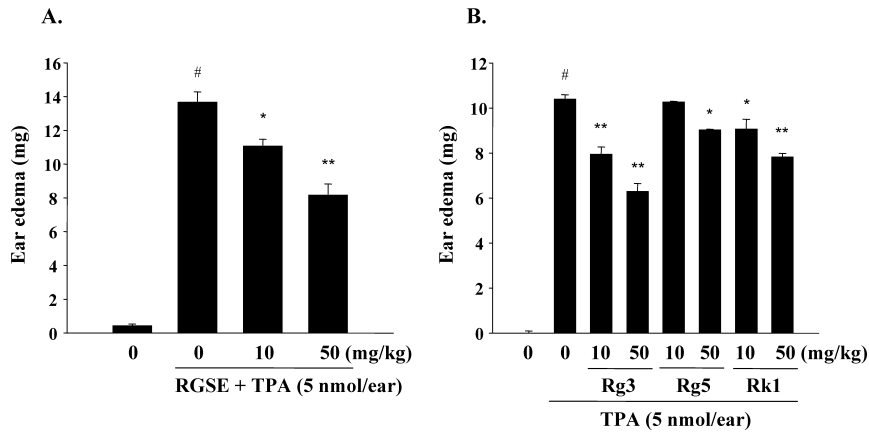


Fig. 1. Effect of RGSE and Major Ginsenosides in RGSE on TPA-Induced Mouse Ear Edema

Each ICR mouse were administrated by oral gavage with the indicated doses of RGSE, Rg3, Rg5, and Rk1 30 min prior to the application of 5 nmol TPA in 50 μ l of vehicle onto the right ear. The left ears were treated with vehicle alone. Four hours later, edema was measured as the increase in the weight of the right ear punch over that of the left. Data are expressed as means \pm S.E. of 5 mice per group. Experiments were independently repeated twice. [#] $p < 0.0001$ versus vehicle-treated group, ^{*} $p < 0.05$, ^{**} $p < 0.001$ versus TPA alone-treated group.

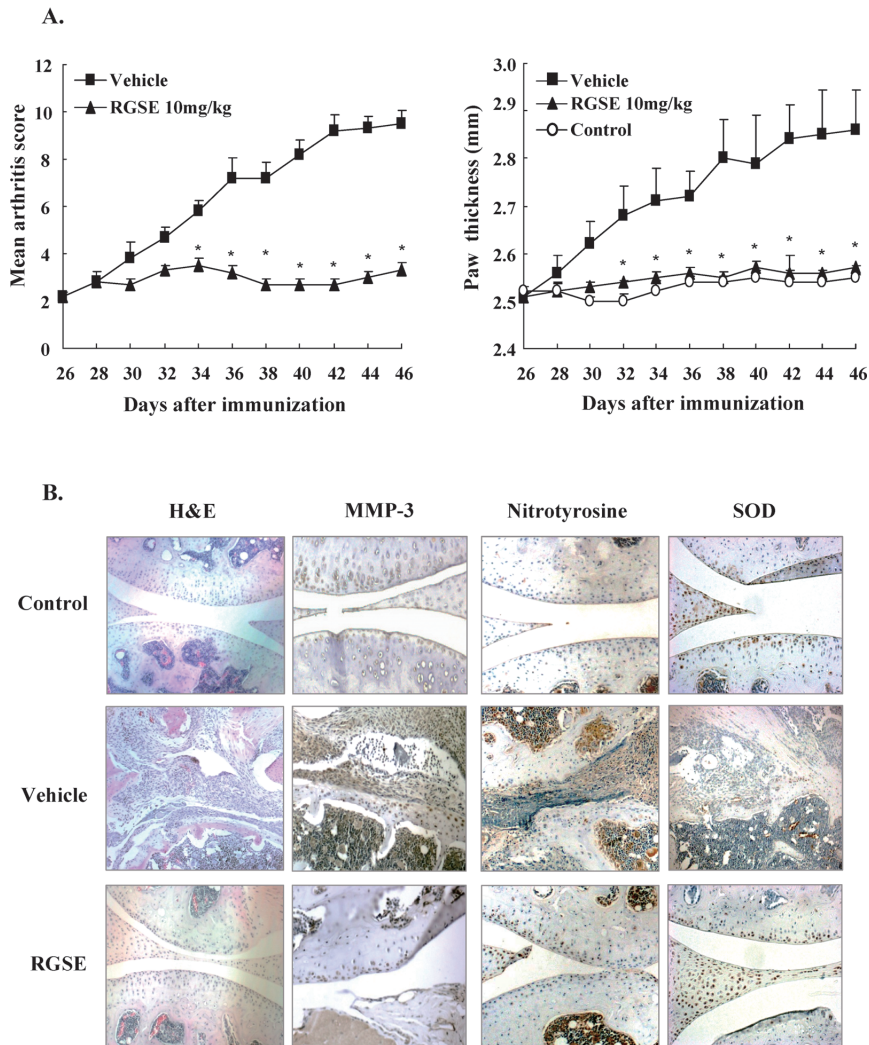


Fig. 2. Effect of RGSE on the Progression and Severity of CIA in Mice

(A) RGSE at 10 mg/kg was administered orally to CIA-immunized mice once daily from day 26 to day 46. The severity of arthritis was evaluated by clinical arthritis score and hind paw thickness. Data are expressed as means \pm S.E. of 5 mice per group. ^{*} $p < 0.05$, versus vehicle-treated CIA mice. (B) On day 46, joint tissues from control mice and CIA mice treated with vehicle and RGSE were stained with H&E for histopathological examination ($\times 100$ original magnification), and immunohistochemically stained for MMP-3, nitrotyrosine, and SOD ($\times 200$ original magnification).

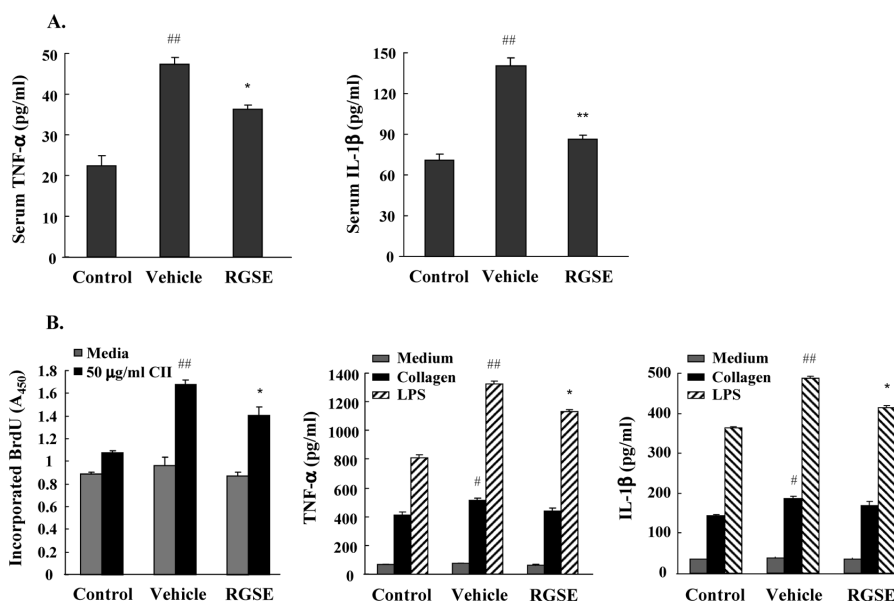


Fig. 3. Effect of RGSE on the Serum TNF- α and IL-1 β Levels and Pro-Inflammatory Immune Responses of Spleen Cells in CIA Mice

(A) The serum levels of TNF- α and IL-1 β were measured *via* ELISA assay on day 46. (B) Single-cell suspensions from spleens were acquired from the control mice and CIA mice treated with vehicle and RGSE on day 46. The proliferation of spleen cells stimulated with 50 μ g/ml of denatured CII for 72 h was evaluated with a BrdU cell proliferation ELISA kit, and the TNF- α and IL-1 β levels were measured in the conditioned media of spleen cells stimulated with 50 μ g/ml of denatured CII or 5 μ g/ml LPS for 48 h, using each specific ELISA kit. Data are expressed as means \pm S.E. of 6 mice per group. # p <0.05, ## p <0.001 versus control mice, * p <0.05, ** p <0.001 versus vehicle-treated CIA mice.

sylation of tissue proteins by highly reactive peroxynitrite generated from oxygen-derived free radicals and nitric oxide (NO).¹⁹ In contrast, an antioxidant enzyme SOD was negligibly observed in the joints of vehicle-treated CIA mice, but positive staining for SOD was presented in the joints of RGSE-treated CIA mice. The antioxidant enzyme SOD converts superoxide anion to hydrogen peroxide, thereby preventing the formation of peroxynitrite. These results indicate that RGSE may inhibit the induction of CIA by reducing inflammation in synovial tissues and by blocking the expression of MMP-3.

Effect of RGSE on Serum TNF- α and IL-1 β Levels and Pro-inflammatory Immune Response of Spleens in CIA Mice In order to determine the effects of RGSE on the production of pro-inflammatory cytokines closely associated with joint inflammation and arthritis progression, we measured the serum levels of TNF- α and IL-1 β in the vehicle or RGSE-treated CIA mice on day 46. TNF- α and IL-1 β levels were increased significantly in the sera of the vehicle-treated CIA mice, but the increase in serum TNF- α and IL-1 β levels was inhibited in the CIA mice treated with RGSE at 10 mg/kg (Fig. 3A). In order to determine whether treatment with RGSE *in vivo* affected cell-mediated immunity to collagen, spleen cells were isolated from all mice on day 46, then stimulated with denatured CII. Whereas the collagen-stimulated spleen cells from vehicle-treated CIA mice proliferated significantly as compared with the control mice, an inhibition in collagen-induced cell proliferation was noted in the spleen cells from the RGSE-treated CIA mice (Fig. 3B). Moreover, the levels of TNF- α and IL-1 β were elevated in the conditioned media of denatured CII- or LPS-stimulated spleen cells from vehicle-treated CIA mice, but LPS-stimulated elevation in cytokine levels were significantly inhibited in spleen cells from the RGSE-treated CIA mice (Fig. 3B). These results indicate that RGSE may attenuate CIA *via* the

inhibition of inflammatory responses such as collagen-specific proliferation and the LPS-stimulated secretion of TNF- α and IL-1 β in the immune cells of spleens.

Effect of RGSE on CIA-Induced Oxidative Damages in the Liver and Kidney Tissues To assess the effects of RGSE on CIA-induced oxidative tissue damage, MDA and GSH levels as well as catalase activity were measured in the liver and kidney tissues of CIA mice. MDA levels, which are regarded as a good indicator of lipid peroxidation, were increased in the tissue homogenates from vehicle-treated CIA mice, but RGSE treatment at 10 mg/kg suppressed the increase in CIA-induced MDA levels (Fig. 4A). The reduced GSH level (Fig. 4B) and catalase activity (Fig. 4C) in the liver and kidney tissues of vehicle-treated CIA mice were also recovered in the liver and kidney tissues of RGSE-treated CIA mice. These results show that the administration of RGSE suppresses CIA-induced oxidative tissue damages.

DISCUSSION

RA is classified as a high-inflammatory disorder, but OA is a low-inflammatory degenerative joint disease. However, the secretion of pro-inflammatory cytokines by immune cells, MMP expression, pannus formation, and oxidative stress have been observed in the inflamed synovial tissues of both RA and OA.²⁸ Although a CIA model is not completely reflective of human arthritis, it is a useful model for the study of inflammation, autoimmune arthritis, cartilage destruction and bone erosion.²⁴ The current study was designed to assess the anti-arthritis effects of RGSE using a murine CIA model. HPLC analysis demonstrated that RGSE contained 80.6 mg/g Rg3, 85.8 mg/g Rk1, 72.9 mg/g Rg5, and 17.1 mg/g other ginsenosides (data not shown). Previous studies demonstrated the anti-arthritis effects of Rb1 and Ro.^{29,30} However, *in vivo* anti-arthritis activity of Rg3, Rk1, and Rg5 still re-

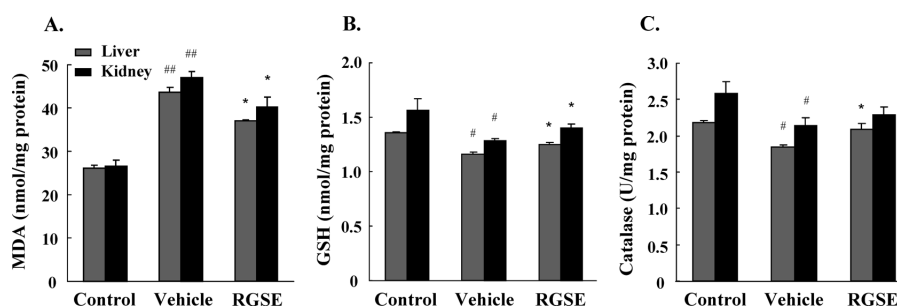


Fig. 4. Effect of RGSE on Oxidative Tissue Damages in CIA Mice

Liver and kidney tissues were collected from all mice on day 46, homogenized in 1.15% KCl buffer, and centrifuged. MDA (A) and GSH levels (B) and catalase activities (C) in the homogenates were measured as described in Materials and Methods. Data are expressed as means \pm S.E. of 6 mice per group. # p <0.05, ## p <0.005 versus control mice, * p <0.05 versus vehicle-treated CIA mice.

mains unknown.

As a result of mouse ear edema test to estimate the anti-inflammatory effect of RGSE, orally administered RGSE markedly suppressed TPA-induced acute inflammation—acute inflammation in this case is characterized by the development of edema, hyperplasia, the induction of pro-inflammatory cytokines and enzymes, and enhanced release of reactive oxygen species (ROS).²⁶⁾ Among major ginsenosides in RGSE, Rg3 noticeably inhibited TPA-induced mouse ear edema. We subsequently determined the anti-arthritis activity of RGSE. Oral administration of RGSE to CIA mice reduced the clinical arthritis score and paw swelling, and inhibited joint space narrowing and histopathological arthritis. MMP-3 expression in the joints of the CIA mice was also reduced substantially by the administration of RGSE. Despite low oral absorption and bioavailability of Rg3 and other ginsenosides^{31–33)}, RGSE at 10 mg/kg was concluded to possess a potent anti-arthritis activity, decreasing inflammation in synovial tissues and blocking the destruction of cartilage and bone *via* the expression of MMP-3. Rg3, rather than other ginsenosides, may contribute to anti-arthritis activity of RGSE.

As in human RA and OA, the recruited inflammatory cells, such as neutrophils, macrophages, and T cells, secrete a variety of pro-inflammatory cytokines in murine CIA.²⁴⁾ Among them, TNF- α drives early joint inflammation and inflammatory cell infiltration in the synovial tissue, whereas IL-1 β performs a pivotal function in the mediation of cartilage degradation and bone erosion.³⁴⁾ Additionally, TNF- α and IL-1 β overproduction is considered to be the principal contributors to increased ROS release in patients with joint diseases. On the other hand, these chronic inflammatory conditions reduce a body's anti-oxidant capacity by affecting a variety of endogenous ROS scavenging enzymes and chemical compounds. The resultant oxidative stress induces chondrocyte apoptosis, cartilage matrix breakdown, and tissue damage in both RA and OA.¹⁹⁾ In this study, oral administration of RGSE reduced the serum levels of TNF- α and IL-1 β in the CIA mice. RGSE administered to CIA mice also suppressed the CII-stimulated proliferation of immune cells (primarily T cells) in the spleen, as well as TNF- α and IL-1 β secretion in LPS-stimulated spleen cells (primarily macrophages), as compared with vehicle-treated CIA mice.

We further attempted to determine whether RGSE could block oxidative stress in the CIA mice. In both RA and OA, TNF- α and IL-1 β are thought to be the main contributors to

the increased ROS release and NO production. In particular, superoxide anion and peroxynitrite cause cartilage damage.¹⁹⁾ The increased nitrotyrosine formation and the reduced SOD expression in joint cartilage can serve as markers of oxidative tissue injury during chronic inflammation. In this study, strongly detected nitrotyrosine in the joints of vehicle-treated CIA mice did not observe in the joints of RGSE-treated CIA mice, whereas the reduced SOD level in the joints of vehicle-treated CIA mice was substantially recovered by RGSE treatment. In addition, the increased levels of MDA as well as the reduced GSH levels and catalase activity in liver and kidney tissues from vehicle-treated CIA mice were restored almost to control levels in those of RGSE-treated CIA mice. The decomposition of peroxidized lipids in cell membranes generates MDA, and thus an increase in MDA levels can be considered as the indicative of oxidative tissue damage. Actually, elevated MDA levels were observed in the synovial fluid of RA patients.³⁵⁾ The depletion of GSH, an intracellular antioxidant, results in increased sensitivity of the organ to oxidative stress, thereby higher MDA levels in CIA mice may be attributable to the reduced levels of GSH as compared with the control mice. Therefore, the anti-arthritis activity of RGSE may be ascribable to the reduction of pro-inflammatory immune responses and the blockage of oxidative stress in CIA mice.

In conclusion, oral administration of RGSE inhibited murine CIA by effectively reducing TNF- α and IL-1 β levels, T-cell proliferation, MMP-3 expression, and oxidative damages, which play critical roles in the progression and severity of RA and OA. Therefore, RGSE may be a beneficial supplement for the prevention and treatment of both RA and OA.

Acknowledgements This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (R13-2003-013-03002-0).

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