Antiinflammatory effect of roasted licorice extract on type II collagen-induced arthritis

Chan-Kwon Jeong

The Graduate School

Yonsei University

Department of Dental Science

Antiinflammatory effect of roasted licorice extract on type II collagen-induced arthritis

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Chan-Kwon Jeong

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This certifies that the dissertation thesis
of Chan-Kwon Jeong is approved

Thesis Supervisor: **Prof. Jong-Hoon Choi**

Thesis Committee Member: **Prof. Hyung-Joon Ahn**

Thesis Committee Member: **Prof. Dong-Min Shin**

Thesis Committee Member: **Prof. Won-Yoon Chung**

Thesis Committee Member: **Prof. Jae-Ho Lee**

The Graduate School

Yonsei Univeristy

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감사의 글

박사학위 논문은 저의 부족함을 많은 주위의 도움과 사랑이 보상해주어 나올 수 있었습니다.

좁은 세상을 나와, 더 넓고 높은 세상을 배울 수 있도록 허락해주신 김종열 교수님, 학교로 돌아와 낯설고 어설픈 대학원 생활을 즐겁고 보람 있게 해나갈 수 있도록 많은 배려와 이해를 보여주신 최종훈 교수님, 또한 김성택 교수님, 안형준 교수님 외 여러 선생님들의 친절한 배려로, 너무나 소중한 시간들이었습니다.

본 논문을 위한 실험, 연구에서 깊은 지식과 뛰어난 통찰력으로 물심양면으로 도와주신 정원윤 교수님, 바쁜 시간 속에서도 여러 실험을 도와주신 김기림 연구원, 본인의 시간을 쪼개어 도와주신 최영찬 선생님에게도 큰 감사를 드립니다.

어려서부터 희생과 사랑으로 저에게 항상 편안함과 존경심을 느끼게 해주신 부모님에게 감히 감사의 글로 표현하기는 너무나 부족함을 느낍니다. 저에게 일에 대한 열정과 세상에 대한 겸손함을 배우게 해주신 아버님, 자식에 대한 무조건적인 사랑과 기다림을 보여주신 어머님을 생각하면, 저도 제 아들에게 그 반에 반만이라도 되는 사랑을 전하는 것이 가능할지 고개가 숙여집니다. 치과개원 후 저의 임상에 대한 부족함을 채우고자 하는 여러 과정으로 인해 가족과 많은 시간을 보내지 못함으로써 많은 실망을 주었지만, 이를 끈기 있게 지켜봐주며 응원과 질책을 해주었던 사랑하는 나의 아내와 사랑하는 나의 아들 지우는 앞으로 제가 항상 보답해야 할 가족이라 생각합니다.

젊을 때 생각하고 믿었던 옳은 치과의 길이 과연 옳은 것인가 하는 의문이 들 때에 다시 한번 초심으로 돌아가서 주위 모든 사람들에게서 배움을 찾음으로써 힘을 내어 나아갈 수 있도록 도와주신, 주변 여러 선생님들과 연우 치과를 함께 하며 힘들 때나 기쁠 때나 같이 울고 웃었던 이동호 원장님과 치과 스태프 선생님들에도 무한한 감사를 드립니다.

이 논문이 제가 앞으로 살아갈 치과인생에서 큰 디딤돌이 되도록 앞으로 스스로를 더 갈고 닦을 것을 다짐하며, 감사의 글을 마칩니다.

2008년 12월

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ABSTRACT

Antiinflammatory effect of roasted licorice extract on type II collagen-induced arthritis

Chan Kwon Jeong, D.D.S., M.S.D.

Department of Oral Medicine/Dental Science,

The Graduate School, Yonsei university

(Directed by Prof. **Jong-Hoon Choi**, D.D.S., M.S.D., Ph.D.)

Licorice is an esteemed crude drug in both the Orient and Occident that is originated from the dried roots of several *Glycyrrhiza* species. In Chinese traditional medicine, licorice has been used in the treatment of various ailments ranging from tuberculosis to peptic ulcers. A recent study and my preliminary data showed that antiinflammatory activity of roasted licorice extract (rLE) was more potent than that of licorice extract (LE) in lipopolysaccharide (LPS)-treated Raw264.7 murine macrophages and in a 12-*O*-tetradecanoylphorbol 13-acetate-treated mouse ear edema model. rLE showed more inhibitory activity than LE on TPA-induced mouse ear edema. To determine anti-arthritic activity of LE and rLE, we investigated their effects using a type II collagen (CII)-induced arthritis (CIA) mouse model, which is a useful model for studying inflammation, autoimmune arthritis, cartilage destruction and bone

erosion. Oral administration of LE and rLE reduced the clinical arthritis score and paw swelling in the CIA model, and inhibited joint space narrowing and the histological arthritis, representing the severity of synovial hyperplasia, infiltration of inflammatory cells, pannus formation and erosion of cartilage and bone. Orally administered LE and rLE decreased the serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and down-regulated matrix metalloproteinase (MMP)-3 expression in joints. Cell proliferation by CII stimulation and cytokine secretion by CII or LPS stimulation were suppressed in spleen cells isolated from CIA mice orally administered with these extracts. In liver and kidney tissues of CIA mice, the increased malondialdehyde level was inhibited and the decreased antioxidant reduced glutathione and catalase levels were recovered by oral administration of LE and rLE. No difference was between anti-arthritic activities of LE and rLE. Taken together, both LE and rLE have beneficial effects against arthritis including rheumatoid arthritis and osteoarthritis.

Key words: licorice, roasted licorice, collagen-induced arthritis, TNF-α, IL-1β, MMP-3, malondialdenyde, reduced glutathione, catalase

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I. INTRODUCTION

Human rheumatoid arthritis (RA) is an autoimmune disease associated with painful joints that affects about 1% of the population worldwide, but for which no effective cure is available(Lee DM and Weinblatt ME, 1995; Feldmann M, 2001). It is chronic joint inflammation causing hyperplasia of synovial tissues and structural damage to cartilage, bone, and ligaments(Tak PP *et al*., 1997). Most autoimmune diseases involve the development of autoimmunity to autologous proteins and have been probed for autoreactive T cells and antibodies. The high incidences of anti-type II collagen (CII) antibodies and CII-specific T cells indicate that CII is one of the major autoantigens of human RA(Kim HY et al.,1999; Kim WU *et al*., 2000). In particular, CII has been considered as a potential source of biomarkers in osteoarthritis because CII is the most abundant protein component of cartilage and relatively

specific for hyaline cartilage. Moreover, damage to CII meshwork is a critical event in the pathology of osteoarthritis(Henrotin Y et al., 2007).

Rheumatoid synovial tissue is central to the pathogenesis of RA and represents hyperplasia by the accumulation and proliferation of inflammatory cells including B cells, T cells, macrophages and synovial fibroblasts(Tak PP et al., 1997). Infiltrating immune cells produce a variety of cytokines, predominantly tumor necrosis factor (TNF)-α and interleukin (IL)-1β which play an important role in attracting and activating other inflammatory cells and the associated bone and cartilage damage(Feldmann M et al., 1997). Both cytokines affect an imbalance between Th1 and Th2 cells and stimulate the expression of COX-2 and adhesion molecules, the activation of leukocytes, the production of NO, IL-6, and chemokines, and the synthesis of matrix metalloproteinases (MMPs)(Muller-Ladner U et al., 1997).

Human temporomandibular joint (TMJ) disorders classify as a low-inflammotory arthritic disorder. The most common joint pathology affecting the TMJ is degenerative joint diseases, known as osteoarthrosis and osteoarthritis (OA)(Tanaka E et al., 1997). However, the ratio of TMJ involvement in RA has changed from 4.7 to 84% in different studies depending on the use of different criteria for clinical and radiological evaluation(Goupille P et al., 1990; Goupille P et al., 1992; Bayar N et al., 2002; Voog U et al., 2003; Celiker R et al., 1995). The pathological process is characterized by deterioration and abrasion of articular cartilage and local thickening and remodeling of the underlying bone. In chondrocytes of articular cartilage, osteoarthritic cartilage and mandibular condylar cartilage of mechanically induced TMJ-osteoarthrosis, vascular endothelial growth factor (VEGF) expression increased(Forsythe JA et al., 1996; Wong M et al., 2003; Tanaka E et al., 2005), and VEGF controls the production of MMPs and tissue inhibitor of MMPs (TIMPs)(Pufe T et al., 2004). Reduction of TIMPs and induction of MMPs result in degradation of extracellular matrix components, such as collagens and proteoglycans, leading to cartilage destruction. In addition, VEGF expression in synovial tissues and the TMJ disc is involved in the development of inflammatory changes in

the TMJ as a reaction to the cytokines(Leonardi R et al., 2003; Sato J et al., 2003). Of these cytokines, TNF-α and IL-1 and IL-6, play critical roles in the pathogenesis of osteoarthrosis, like RA. With overloading, the increase in intra-articular pressure, when it exceeds the capillary perfusion pressure, will cause temporary hypoxia, which is corrected by re-oxygenation on cessation of degradation by the overloading. This hypoxia-reperfusion cycle has been reported to release reactive oxidative radical species non-enzymatically(Grootveld M et al., 1991). The produced reactive oxidative radical species in synovial joints resulted in inhibition of the biosynthesis and degradation of hyaluronic acid. Furthermore, the degradation of hyaluronic acid may lead to cartilage destruction by the enhanced expression of MMPs.

Available therapies currently used to treat RA are based on immunosuppressive agents that inhibit the inflammatory component of RA, and have the potential to delay cartilage and bone damage(Finckh A et al., 2006), Non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs) and corticosteroids are widely used to have multiple effects including normalization of the immune system and reduction of inflammatory mediators(Smolen JS et al., 2003; Henderson NK et al., 1996). However, these drugs are undesirable for the continued treatments to prevent joint damage because of poor efficacy, delayed onset of action, long-term side effects and toxicity(Scott DL et al., 2005).

In the present study, I found that roasted licorice extract (rLE) showed more potent inhibition than licorice extract (LE) in ear edema of mice treated with phorbol ester, which induces acute inflammation representing the increased TNF-α and IL-1β(Lee DY et al., 2008). Furthermore, I investigated the anti-arthritic effects of LE and rLE using a CII-induced mouse arthritis model, which has been widely accepted as a valid animal model of human rheumatoid arthritis(Cho YG et al., 2007).

II. MATERIALS AND METHODS

1. Reagents

LE and rLE were generously provided by Professor Jung Han Yoon Park at Department of Food Science and Nutrition, Hallym University (Chunchon, Korea). Kits for enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-1β were purchased from R&D systems (Minneapolis, MN, USA). 5-Bromo-2'-deoxy-uridine (BrdU) cell proliferation ELISA kit was obtained from Roche Diagnostics (Mannheim, Germany). Goat polyclonal anti-MMP-3 antibodies and normal mouse immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Polink-1 HRP goat for 3,3-diaminobenzidine (DAB) kit, consisting of polymeric horseradish peroxidase (HRP)-linked anti-goat antibody, chromogen and substrate, was obtained from Life Science Division (Mukilteo, WA, USA). Bovine collagen type II (CII), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were obtained from Chondrex (Redmond, WA, USA). Harris's hematoxylin was purchased from Merck (Darmstadt, Germany). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL, USA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic-antimycotic mixture containing penicillin and streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), dimethyl sulfoxide, bovine serum albumin (BSA), eosin, Mayer's hematoxylin, lipopolysaccharide (LPS) from *Escherichia coli*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phenylmethylsulfonylfluoride (PMSF), sodium dodesyl sulfate (SDS), thiobarbituric acid (TBA), sulfanilamide, Triton X-100, 2-mercaptoethanol, N-(1-naphtyl) ethylenediamine dihydrochloride, red blood cell (RBC) lysing buffer, and all other chemicals were purchased from Sigma Chemical. (St. Louis, MO, USA).

2. Animals

Female ICR mice (5 weeks old) for a mouse ear edema test and male DBA/1J mice (7-9 weeks old) for collagen-induced arthritis (CIA) study were obtained from Central Lab. Animal (Seoul, Korea) and the Jackson Laboratory (Bar Harbor, ME, USA), respectively. All mice were permitted free access to a normal standard chow diet (Daejong Inc., Seoul, Korea) and tap water, and housed under conditions of 20-22℃, with a relative humidity of 55 ± 5% and a 12-h light/12-dark cycle. Animal studies were conducted in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea.

3. TPA-induced mouse ear edema test

The right ear of each male ICR mouse (5 per group) were topically treated with various doses of LE and rLE in 50 μl of vehicle (DMSO-acetone: 15-85, v/v) 30 min prior to the application of 5 nmol TPA in 50 μl vehicle. The left ears were treated with vehicle alone. The control group received vehicle only on both ears. Four hours later, the mice were sacrificed by cervical dislocation, and the ears were removed. Right and left ear punches 6 mm in diameter were taken from each mouse. Edema was indicated as the increase in weight of the right ear punch over that of the left.

4. Induction of CIA and administration of extracts

DBA/1J mice were intradermally injected with 100 μg CII emulsified in CFA (1:1, w/v) to the tail base. On day 21, all mice were boosted by an intradermal injection with 100 μg CII in IFA (1:1, w/v). CIA mice were divided into three group, each containing 5 mice, and were orally administered with vehicle (PBS containing 1% DMSO), LE or rLE (10 mg/kg body

weight) once daily from day 25 to day 45, respectively. Normal group was neither immunized with CII nor treated with extracts.

5. Assessment of CIA severity

The development of arthritis was evaluated by macroscopic scoring system and swelling of paws every other day from day 25 to day 45. Paw swelling was assessed by measuring the thickness of two hind paws with an electric digital caliper. The clinical scoring system was classified 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, deformity and/or ankylosis. The macroscopic arthritis score of each mouse was presented as the sum of the each score of the four limbs, the maximum score being 16. On day 45, all mice were sacrificed by anesthesia after serum collection. Then, hind paws and knee joints for histological examination, spleens for measurement of cytokine production, and livers and kidneys for detection of oxidative stress were collected after

6. Histopathologic and immunohistochemical analysis

The joints from control and CIA mice were fixed in 10% buffered formalin solution, decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA), embedded in paraffin, and longitudinally cut into 5 μm serial sections. The sections were then stained with hematoxylin and eosin. Histological evaluation was performed independently and blindly using the scoring system as follows: 0, no inflammation; 1, hyperplasia of the synovial tissue; 2, infiltration of the inflammatory cells; 3, pannus formation and erosion of cartilage; 4, extensive erosion of cartilage and bone.

The sections were incubated with 3% H_2O_2 in absolute methanol for 10 min at room temperature, followed by incubation with 3% BSA in PBS for 30 min. Tissue sections were

incubated for 2 h with goat polyclonal anti-MMP-3 antibody. For the negative control staining, normal mouse IgG was used instead of the primary antibody. After washing three times with PBS containing 0.05% Twin-20, the sections were incubated with polymeric HRP-linked anti-goat antibody at room temperature for 20 min, and then reacted with 0.02% DAB as chromogen. All sections were counterstained with hematoxylin.

7. Preparation of single-cell suspensions from spleens

Spleens were removed from the control and CIA mice (3 per group) to prepare single-cell suspensions. The spleen tissues were washed with cold PBS and dissociated into a single-cell suspension using cell strainers. The red blood cells were lysed with RBC lysing buffer and single-cell suspensions were then washed three times with PBS. Suspensions of spleen cells were cultured with RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS and counted by hemacytometer.

8. Measurement of cytokine levels in blood serum and conditioned medium of spleen cells

Blood samples were collected from all mice by the cardiac punctures before sacrificing mice on day 45. Blood was clotted for 2 h at room temperature, and centrifuged at 2,000 x g for 20 min at 4℃ to obtain serum. Single-cell suspensions were adjusted to at a density of 2×10^6 cells/well, and added in triplicate into a 24-well plate. The cells were then stimulated with 50 μg/ml denatured-CII or 5 μg/ml LPS for 48 h at 37 ℃ in a 5 % CO_2-humidified atmosphere. The conditioned media were collected. The levels of TNF-α and IL-1β in blood serum and the conditioned medium of spleen cells were assayed using commercially available ELIZA kits according to manufacturer's protocols, respectively.

9. Spleen cell proliferation assay

Spleen single-cell suspensions (2×10^5 cells/well) were added into a 96-well plate, and stimulated with 50 μg/ml denatured-CII for 72 h at 37 ℃ in 5 %-CO_2 humidified atmosphere. Cell proliferation was measured using a BrdU cell proliferation ELISA kit in accordance with manufacturer's protocol.

10. 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 PMSF, and then centrifuged at 800 x g for 10 min at 4℃. The supernatants were collected to determine the levels of malondialdehyde (MDA), reduced glutathione (GSH) and catalase, an antioxidant enzyme. Total protein concentrations were measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) and BSA was used as a standard.

11. Determination of lipid peroxidation

MDA levels in liver and kidney tissues were measured as an indicator of lipid peroxidation as previously described. 0.2 ml homogenates were added to the reaction mixture containing 0.2 ml 8.1% SDS, 1.5 ml 20% acetic acid (pH 3.5), 1.5 ml 0.8% TBA, and distilled water. The mixture (4.0 ml) was incubated for 1 h at 95℃, cooled with tap water, mixed with 5 ml *n*-butyl alcohol-pyridine (15:1, v/v), and then centrifuged at 4,000 rpm for 10 min. The absorbance or the organic layer was measured at 532 nm using a spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). A standard curve was established using 1,1,3,3-tetramethoxypropane to calculate the MDA levels in homogenates, and MDA level was expressed as nmol /mg protein.

12. Determination of GSH level

To determine GSH level, 0.1 ml homogenates was mixed with 50 µl 5% 5-sulfasalicylic acid buffer, incubated for 10 min at 4℃, and then centrifuged at 10,000 x g for 10 min. 10 µl supernatant was added 150 µl reaction mixture, consisting of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1.5 mg/ml DTNB and 6 unit/ml glutathione reductase. The mixture was incubated for 5 min at room temperature and 50 µl 0.16 mg/ml NADPH was then added to the mixture to start the reaction. Absorbance was immediately measured using a spectrophotometer and GSH level was expressed as nmol /mg protein.

13. Catalase activity

To determination of CAT activity, homogenates were added to an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 1mM EDTA, and centrifuged at 10,000 x g for 15 min. 20 µl supernatant was mixed with 0.1ml 100 mM potassium phosphate (pH 7.0), 30 µl methanol, and 20 µl 0.035M H_2O_2 to initiate the reaction. After 20 min of incubation at room temperature, 30 µl 10 M KOH was added to each mixture to terminate the reaction, followed by the addition of 30 µl 3.4 mM Purpald (chromogen) in 0.5M hydrochloric acid. The mixture was incubated by shaking for 10 min at room temperature. 10 µl 13 mM potassium periodate was added and incubated again for 5 min at room temperature. The absorbance was measured at 540 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). A standard curve was established using formaldehyde and CAT activity was expressed as units (U/mg protein). One unit (U) of CAT was defined as the amount of enzyme that results in the formation of 1 nmol of formaldehyde per min at 25℃.

14. Statistical analysis

Data were expressed as mean ± standard deviation (SD), and analyzed via One-way ANOVA with multiple comparisons followed by Student *t*-test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Effects of LE and rLE on TPA-induced mouse ear edema

To evaluate the inhibitory activity of LE and rLE on TPA-induced acute inflammation, topically applied TPA for 4 h substantially caused ear edema, and pretreatment with these extracts 30 min prior to TPA application suppressed mouse ear edema in a dose-related manner, as shown in Figure 1. rLE showed more potent activity than LE at the same doses.

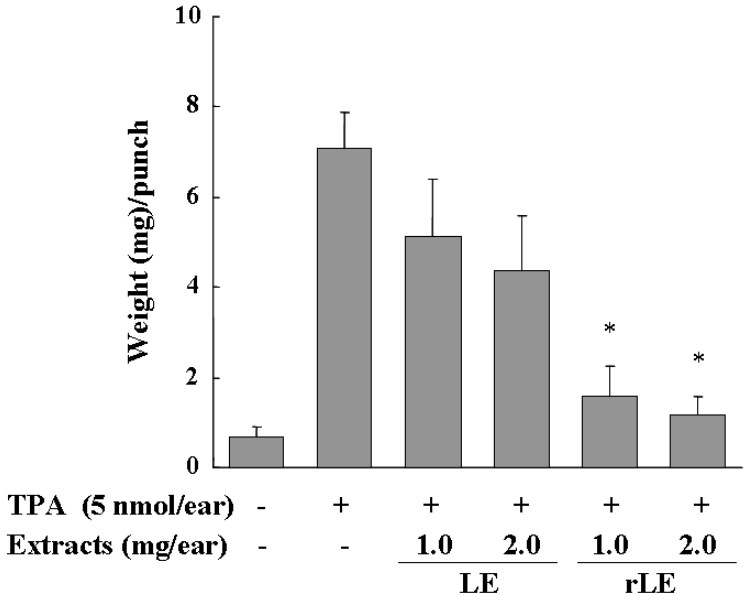

Figure 1. Inhibitory effects of LE and rLE on TPA-induced mouse ear edema. The right ear of each male ICR mouse was topically treated with the indicated doses of LE and rLE in 50 μl of vehicle (DMSO-acetone: 15-85, v/v) 30 min prior to the application of 5 nmol TPA in 50 μl vehicle. The left ears were 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 ± S.E of 5 mice per group. Experiments were repeated twice independently. *P <0.001 *versus* TPA alone-treated group.

2. Effects of LE and rLE on progression of CIA in mice

DBA/1J mice were immunized with CII/CFA, and then boosted with CII in IFA on day 21 after primary immunization. The mice were orally administered with LE and rLE once daily from day 25, when the first clinical signs of disease, such as redness or swelling, were begin to observe. The administration of LE and rLE at 10 mg/kg BW to the immunized mice significantly reduced the progression of arthritis and also inhibited the increase in paw swelling as compared to the vehicle-treated immunized mice (Figure 2). LE and rLE did not show the significant difference in their inhibition on CIA induction.

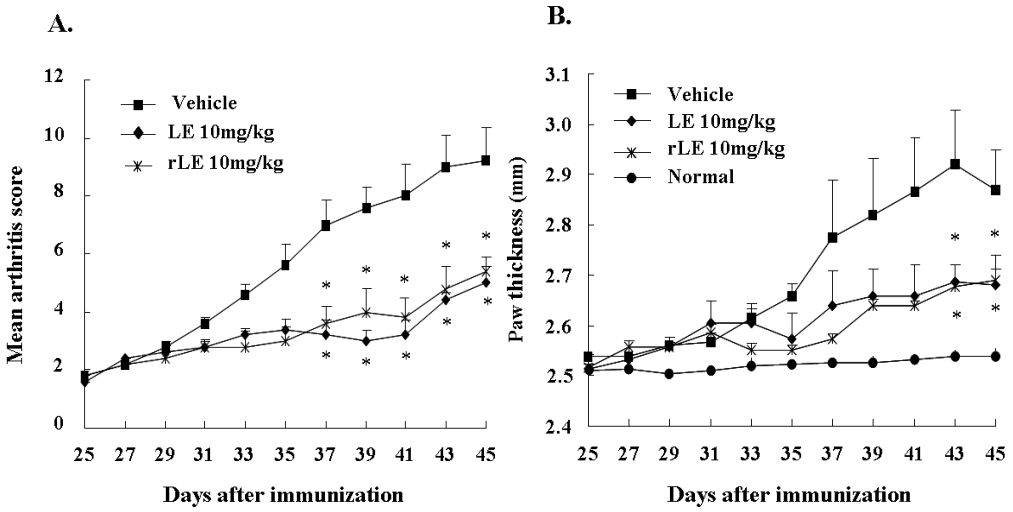

Figure 2. Effects of LE and rLE on progression of CIA in mice. LE and rLE at 10 mg/kg were orally administered to the mice immunized with CII once daily from day 25 to day 45. The severity of arthritis was evaluated by clinical arthritis score (A) and hind paw thickness (B). Data are expressed as mean±SEM of 5 mice per group. $^{*}P < 0.05$ *versus* vehicle-treated CIA group

3. Effects of LE and rLE on the histopathological changes in the joints of CIA mice

In order to investigate the effect of LE and rLE on the histopathological changes, the ankle and knee joint tissues of mice were stained with hematoxylin and eosin. As shown in Figure 3A, histological examination of the joints of the vehicle-treated mice revealed marked pathologic changes, including CIA-characteristic synovial hyperplasia, infiltration of inflammatory cells into the joint cavity, and extensive pannus formation. The destruction of articular cartilage and subchondral bone was observed in the tissues of vehicle-treated CIA mice as compared with the joints of normal mice. Semiquantitative analysis of the histopathological features by scoring on a scale of 0-4 was indicated in Figure 3B. Pathological events demonstrated in vehicle-treated CIA mice were substantially reduced in the joints of LE- and rLE-treated mice.

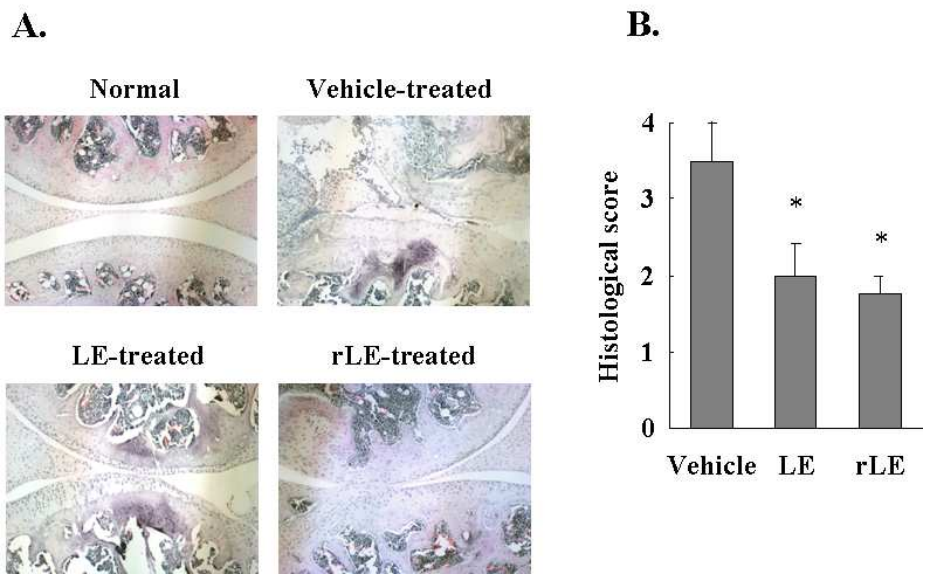

Figure 3. Effects of LE and rLE on the histopathological changes in the joints of CIA mice. A. Histopathological features were examined on H&E-stained joint tissues from normal and CIA mice treated with vehicle, LE, or rLE (A). Histopathologic findings was quantified by scoring evaluated on a 0-4 scale as described in Materials and methods (B). Data is expressed as mean±SEM of 5 mice per group. * $P < 0.05$ *versus* vehicle-treated CIA mice.

4. Effects of LE and rLE on TNF-α and IL-1β levels in serum of CIA mice

To investigate effect of LE and rLE on the production of pro-inflammatory cytokines closely linked to joint inflammation and arthritis progression, I measured levels of TNF-α and IL-1β in the sera of normal and CIA mice treated with vehicle, LE or rLE on day 45. While the levels of TNF-α and IL-1β were significantly increased in the sera of vehicle-treated CIA mice, orally administrated LE and rLE lowered CIA-induced levels of serum TNF-α and IL-1β (Figure 4). No significant differences in serum levels of these cytokines were detected between LE-treated and rLE-treated mice. These results suggest that the inhibitory effects LE

and rLE on CIA severity is closely related to the reduced cytokine levels in mice treated with these extracts.

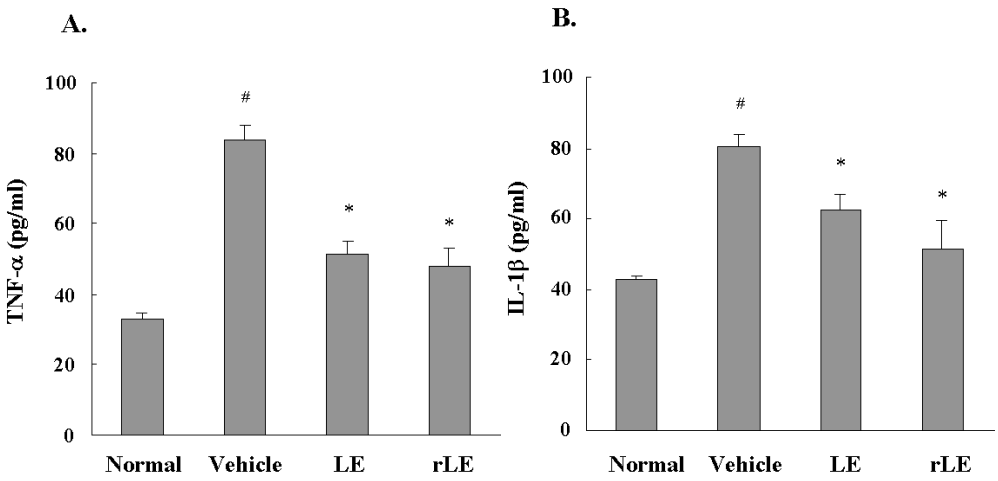

Figure 4. Effects of LE and rLE on the serum levels of TNF-α and IL-1β in CIA mice. The serum levels of TNF-α (A) and IL-1β (B) were measured by ELISA assay on 45 day. Data are expressed as mean±SEM of 5 mice per group. $^{\#}$ $P < 0.01$ *versus* normal mice, * $P < 0.01$ *versus* vehicle-treated mice.

5. Effects of LE and rLE on MMP-3 expression in the joints of CIA mice

I examined whether treatment with LE and rLE could reduce the expression of MMP-3 which is considered to be a key factor in the pathological destruction of cartilage. Immunohistochemical analysis for MMP-3 was performed in the knee joint sections obtained from the normal and vehicle or extracts-treated CIA mice. The knee joint specimens from vehicle-treated CIA mice were strongly stained for MMP-3 at localization of chondrocytes in

inflammatory articular cartilage (Figure. 5). In contrast, positive staining for MMP-3 was reduced in joint tissue sections of CIA mice treated with LE and rLE.

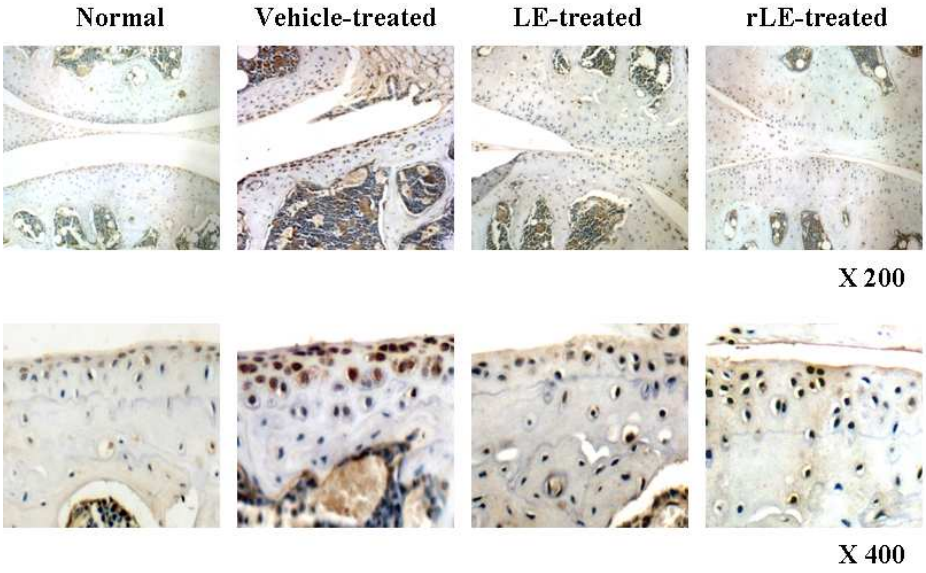

Figure 5. Effects of LE and rLE on MMP-3 expression in the joints of CIA mice. On day 45, knee joint tissues of normal and vehicle, LE or rLE-treated CIA mice were collected and subjected to immunohistochemical staining for MMP-3.

6. Effects of LE and rLE on pro-inflammatory immune response in spleen cells of CIA mice

The spleen is significantly enlarged in CIA mice as compared with normal mice. Re-stimulation of collagen triggers exacerbated immune response in spleen of CIA mice. To determine whether treatment with LE and rLE affected cell-mediated immunity to collagen *in vivo*, I first investigated their effects on CII-specific proliferation of spleen cells from CIA mice treated with vehicle or extracts on day 45. The proliferation of spleen cells from vehicle-treated CIA mice was increased by stimulation with CII for 72 h, but spleen cells from LE-

and rLE-treated CIA mice exhibited significantly less proliferation than those from vehicle-treated CIA mice (Figure. 6).

Next, I examined CII- and LPS-induced cytokine levels in spleen cells from CIA mice treated with vehicle, LE and rLE. The secretion of TNF-α and IL-1β was elevated in both CII- and LPS-stimulated spleen cells of vehicle-treated CIA mice, but were reduced in stimulated spleen cells of LE-or rLE-treated CIA mice (Figure. 7).

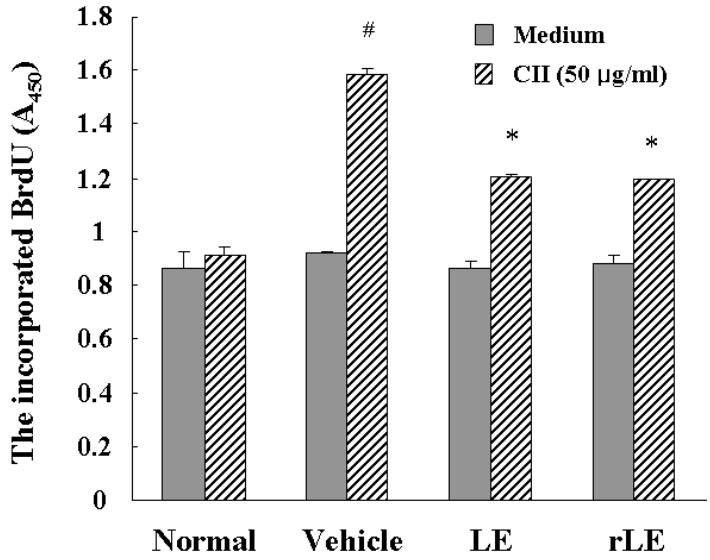

Figure 6. *In vitro* CII-stimulated proliferation of spleen cells from CIA mice treated with vehicle, LE or rLE. Single-cell suspensions from spleens were obtained from normal and CIA mice treated with vehicle, LE or rLE on day 45, and were then stimulated with 50 μg/ml CII for 72 h. Cell proliferation was determined using BrdU cell proliferation ELISA kit. Data is expressed as mean±SEM of 3 mice per group. $^{\#}$ $P < 0.01$ *versus* normal mice, * $P < 0.01$ *versus* vehicle-treated CIA mice.

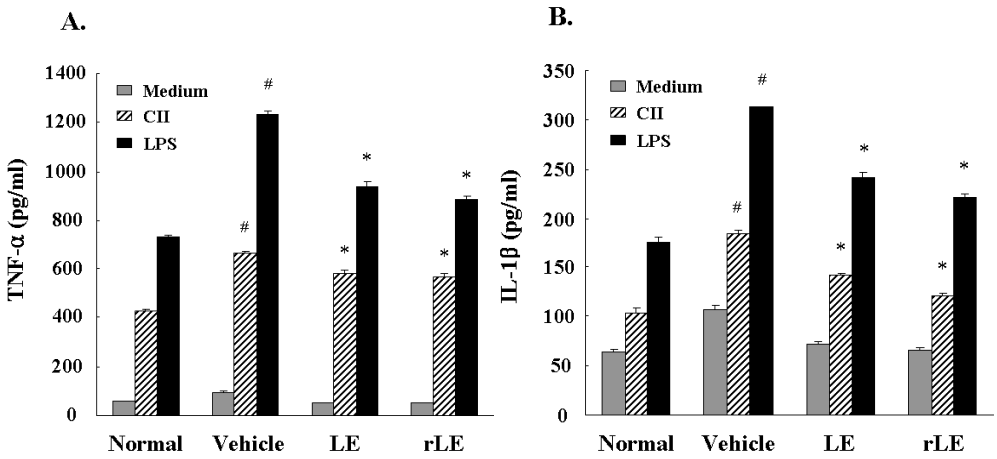

Figure 7. Cytokine production in CII- or LPS-stimulated spleen cells from CIA mice treated with vehicle, LE or rLE. Single-cell suspensions from spleens of normal and CIA mice treated with vehicle, LE or rLE, and were then stimulated with 50 μg/ml CII or 5 μg/ml LPS for 48 h. The levels of TNF-α (A) and IL-1β (B) in conditioned media were measured using each specific ELISA kit. Data is expressed as mean±SEM of 3 mice per group. $^{\#}$ $P < 0.01$ *versus* normal mice, * $P < 0.01$ *versus* vehicle-treated CIA mice.

7. Effects of LE and rLE on tissue oxidative damages in CIA mice

To study effects of LE and rLE on oxidative damage in CIA, MDA level as an indicator of lipid peroxidation and GSH content were measured in homogenates of liver and kidney tissues from normal and CIA mice treated with vehicle, LE or rLE. MDA levels were elevated in liver and kidney tissues of vehicle-treated CIA mice, but inhibited in those of LE or rLE-treated CIA mice (Figure 8A). In addition, the decreased tissue GSH (Figure 8B) levels and catalase activities (Figure 8C) in vehicle-treated CIA mice recovered by oral administration of LE or rLE. Thus, LE and rLE treatment may protect against oxidative damages in tissues of CIA mice.

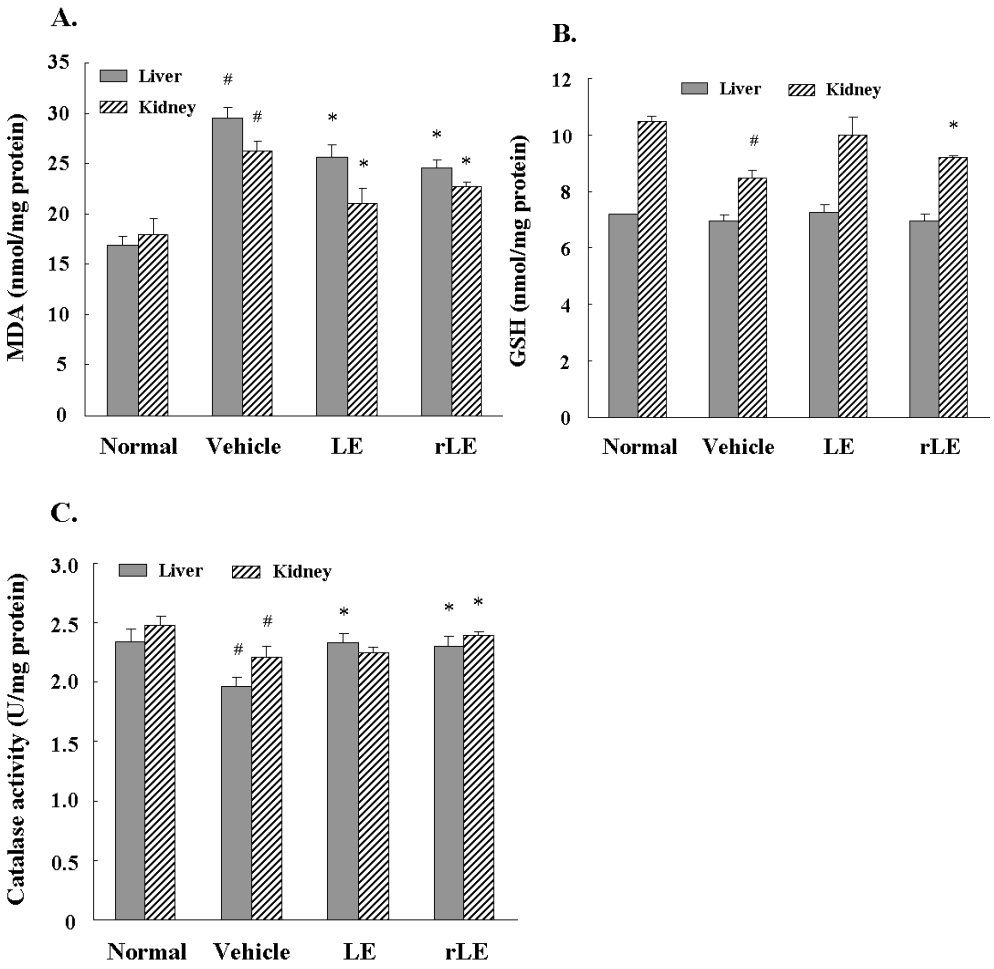

Figure 8. Effects of LE and rLE on oxidative damages in liver and kidney tissues of CIA mice. Liver and kidney tissues were removed from normal and all CIA mice on day 45, and homogenized in 1.15% KCl buffer. MDA (A) and GSH (**B)** levels, and catalase activity (C) were determined as described in Materials and methods. Data is expressed as mean ± SEM of 5 mice per group. $^{\#}$ $P < 0.01$ *versus* normal mice, * $P < 0.05$ *versus* vehicle-treated mice.

Ⅳ. DISCUSSION

The present study was designed to determine anti-arthritic activity of LE and rLE. I investigated arthritis score, paw swelling, the levels of inflammatory cytokines in serum and spleen cells, the proliferation of immune cells, histopathological changes, and tissue oxidative damages in CIA mice with or without oral administration of extracts.

Licorice is an esteemed crude drug in both the Orient and Occident that is originated from the dried roots of several *Glycyrrhiza* species(Hatano T et al., 1991; Hayashi H et al., 2000). In Chinese traditional medicine, licorice remains one of the most commonly prescribed herbs and has been used in the treatment of various ailments ranging from tuberculosis to peptic ulcers(Huang KC et al., 1993). The chemical constituents of licorice include glycyrrhizin and its aglycone, glycyrrhetinic acid(*Alternative Medicine Review*, 2005), as well as species-specific flavonoids such as liquiritin, isoliquiritin and their corresponding aglycones, licochalcone A, glabridin, glabrol, glabrene, hispaglabridin A and hispaglabridin B(Kiso Y et al., 1984; Vaya J et al., 1997). These flavonoids exhibit antioxidative(Fuhrman B et al., 1997), superoxide scavenging(Haraguchi H et al., 1998) and anticarcinogenic activities(Jung JI et al., 2006). High-performance liquid chromatographic analysis indicated that roasted licorice contained more licochalcone A and less glycyrrhizin and isoliquiritigenin (data not shown). A recent study showed that antiinflammatory activity of rLE was more potent than that of LE in lipopolysaccharide-treated Raw264.7 murine macrophages(Kim JK et al., 2006). In this study, rLE showed stronger inhibition on TPA-induced acute inflammation in a mouse ear edema model than LE. TPA has been known to induce the inflammatory responses characterized by the development of edema, hyperplasia, induction of proinflammatory cytokines and enzymes, and enhanced release of reactive oxygen species (ROS) (Weyand CM et al., 2000).

RA is closely involved in chronic inflammatory and destructive events such as joint pain and swelling, synovial hyperplasia by inflammatory cells infiltrated in the synovium and the synovial fluid, pannus formation, and finally joint malformation by destruction of articular cartilage and erosion of subchondral bone(Joosten LA et al., 1999). Long-used standard treatments, such as DMARDs and NSAIDs, have well-known, but have significant toxic side effects. Therefore, developing new agents without any adverse effects is required, and plant-derived components can be a good source in creating the new therapeutic and preventive agents.

Although CIA model does not reflect completely human RA, it is a useful model for studying inflammation, autoimmune arthritis, destruction of cartilage and bone erosion. My data indicated that oral administration of LE and rLE reduced the clinical arthritis score and paw swelling in the CIA model, and inhibited joint space narrowing and the histological arthritis representing the severity of synovial hyperplasia, infiltration of inflammatory cells, pannus formation and erosion of cartilage and bone. Therefore, LE and rLE possess the potential as novel anti-arthritic agents.

As in human RA, the binding and accumulation of anti-CII antibodies in the articular region of CII-immunized mice may initiate inflammatory responses by activating the complement cascade. The activation of complement cascade, especially C5a (a cleavage product of C5), recruits neutrophils and macrophages, and the recruited inflammatory cells secrete chemotoxic materials and proinflammatory cytokines, such as IL-1β, TNF-α, IL-8, IL-6, MIP-1α, nitric oxide, and prostaglandin E2(Lee DY et al., 2008). Of these, TNF-α drives early joint inflammation and the inflammatory cell infiltration in the synovial tissue, while IL-1β plays a pivotal role in the mediation of cartilage degradation and bone erosion(A. Murakami et al., 2000). In particular, TNF-α emerged as a valid therapeutic target for RA.

In both RA and OA, inflammatory cytokines such as TNF-α and IL-1β stimulate the production of MMPs, enzymes that can degrade all components of the extracellular matrix. The collagenases, MMP-1 and MMP-13, have predominant roles in RA and OA. MMP-1 is produced primarily by the synovial cells that line the joints, and MMP-13 is a product of the chondrocytes that reside in the cartilage. MMP-13 also degrades the proteoglycan molecule, aggrecan, giving it a dual role in matrix destruction. Expression of other MMPs such as MMP-1, MMP-3 and MMP-9, is also elevated in arthritis and these enzymes degrade non-collagen matrix components of the joints. Therefore, controlling MMPs activity is required for treatment of arthritis. In this study, oral administration of LE and rLE decreased the serum levels of TNF-α and IL-1β in the CIA mice. Immunohistochemical analysis demonstrated that LE and rLE down-regulated MMP-3 expression in joints of the CIA mice. These results suggest that LE and rLE may ameliorate CIA by blocking the increases in TNF-α and IL-1β levels of CIA mice, and cytokine-mediated MMP-3 expression in the joints of CIA mice.

CIA causes a proinflammatory immune response, thereby rendering it an excellent model to evaluate functional T cell response *in vivo*. Although the pathways that drive cytokine release in RA synovium still remains unclear, T cells regulate by direct release of IL-17 or via cell contact with synovium macrophages(Mclnnes IB et al., 2007). In this study to estimate the effects of these extracts on CII-specific T cell response, cell proliferation was investigated with spleen cells isolated from normal mice and CIA mice administered with vehicle, LE or rLE. Spleen is the major site of adaptive immune response to blood-borne antigens, and contains phagocytes and lymphocytes. The increase in CII-stimulated proliferation in spleen cells from vehicle-treated CIA mice was significantly suppressed in spleen cells from CIA mice treated with LE or rLE. In addition, CII- or LPS-stimulated TNF-α and IL-1β levels were also reduced in spleen cells derived from CIA mice treated with LE or rLE. LPS is one of the most potent activators of macrophages known. These results suggest that LE and rLE

alleviate CIA by reducing the immune responses of spleen cells and the resultant cytokine production.

Reactive oxygen species (ROS) released from the infiltrated inflammatory cells are implicated in breakdown of cartilage and bone in RA(Ostrakhovitch EA et al., 2001). TNF-α overproduction is considered to be the main contributor to increased ROS release in patients with RA. Moreover, chronic inflammatory conditions are reduced a body's anti-oxidant capacity by a variety of endogenous ROS scavenging proteins, enzymes and chemical compounds(Halliwell B et al., 1990). Increased ROS levels results in tissue damages through the interaction with cellular components including DNA, proteins, and lipids. Therefore, controlling ROS levels is important for reducing CIA severity and joint damages. In this study, I investigated whether LE and rLE could block oxidative stress in the CIA mice. The decomposition of peroxidized lipid produces many end-products including MDA. Elevated level of MDA has been observed in the serum and synovial fluid of RA patients(Ostrakhovitch EA et al., 2001). My data indicated that oral administration of LE or rLE inhibited the increase in MDA level and recovered the reduced GSH level and catalase activity in liver and kidney tissues of CIA mice. These results suggest that LE and rLE can prevent tissue damages by blocking oxidative stress in CIA mice.

V. CONCLUSION

Topical application of LE and rLE onto each mouse ear inhibited TPA-induced acute inflammation, and rLE showed more potent activity than LE. Furthermore, oral administration of LE and rLE effectively interfered with the inflammatory response and destruction of cartilage and bone in the CIA mouse model. These extracts ameliorated the severity of arthritis and histopathological changes in CIA mice, correlating with reduced serum levels of TNF-α and IL-1β as well as the lowered MMP-3 expression in the joints. LE and rLE administration inhibited the activation of CII-specific T cells and LPS-stimulated macrophages in spleens of CIA mice. LE and rLE also reduced oxidative stress-mediated tissue damages by preventing the increase in lipid peroxidation and the decreases in GSH level and antioxidant enzyme catalase activity in liver and kidney tissues of CIA mice. Contrary to my expectations, anti-arthritic activity of rLE was not higher than that of LE. Taken together, LE and rLE may have beneficial effects against chronic inflammation including RA and OA.

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ABSTRACT (IN KOREAN)

콜라겐으로 유도된 생쥐 관절염 모델에서 구운 감초 추출물의 염증 억제 효과

< 지도 교수 : 최 종 훈>

연세대학교 대학원 치의학과

정 찬 권

감초는 *Glycyrrhiza* 속에 속하는 식물의 뿌리를 말린 것으로 중국에서는 결핵에서부터 위궤양 치료에까지 널리 사용되고 있는 전통 생약이다. 최근, 고온에서 구운 감초의 추출물이 굽지 않은 감초의 추출물보다 항염증 효과가 더 뛰어나다는 보고가 있었으며, 예비 실험에서도 12-*O*-tetradecanoylphorbol 13-acetate 로 유도한 생쥐 귀 부종에 대한 구운 감초 추출물의 항염증 효과가 일반 감초 추출물보다 더 뛰어난 것을 확인하였다. 이를 바탕으로 이형 콜라겐으로 유도한 생쥐 관절염 모델을 사용하여 감초 및 구운 감초 추출물의 관절염 억제 효과를 조사하였다. 일차로 *Mycobacterium tuberculosis* 를 포함하는 CFA 를 우형 제 2 형 콜라겐 (type II collagen ; CII) 과 혼합하여 DBA/1J 생쥐 꼬리 근저에 피내 주사하여 면역화하였다. 21 일째에 IFA 에 혼합한 CII 를 추가 접종하여 CIA 를 유도하였다. 관절염의 증상이 발병한 날부터 21 일

동안 매일 감초 및 구운 감초 추출물을 몸무게 kg 당 10mg 의 용량으로 CIA 가 유도된 생쥐에 경구 투여하였다. 각 추출물을 투여하지 않은 양성 대조군과 비교하여 추출물을 구강 투여한 군에서, 관절염 지수와 paw swelling 이 유의적으로 감소되었으며 조직병리학적 변화도 억제되었다. 또한 정상 생쥐의 혈청과 비교하여 CIA 가 유도된 생쥐의 혈청에서 TNF-α 와 IL-1β 의 수준이 올라가지만, 각 추출물을 구강 투여한 군에서는 이들 사이토카인의 증가가 현저히 억제되었고, 관절에서 MMP-3 의 발현이 감소되었다. CIA 생쥐에서 비장 세포를 분리한 후, 주로 T 세포를 자극하는 CII 로 비장세포를 자극한 결과 비장 세포의 증식뿐만 아니라 TNF-α와 IL-1β 수준이 현저히 증가하였으나, 각 추출물을 투여한 CIA 생쥐에서 분리된 비장세포의 증식 및 사이토카인의 증가는 억제되었다. 비장세포를 LPS 로 처리하여 주로 대식세포를 자극한 경우에도 추출물을 투여한 군에서는 TNF-α 와 IL-1β 수준의 증가가 억제되었다. CIA 가 유도된 생쥐의 간과 신장에서 산화적 스트레스로 인한 과산화지질의 생성이 증가하였으며, 내인성 항산화물질인 환원형 글루타치온의 양과 항산화효소인 catalase 의 활성이 감소되었으나, 각 추출물을 구강 투여한 CIA 생쥐의 간과 신장에서는 과산화지질의 생성이 억제되고, 환원형 글루타치온 수준 및 항산화효소인 카탈라제의 활성이 회복되었다. 구운 감초 추출물의 CIA 억제효과는 일반 감초 추출물의 억제 효과와 유사하였다. 이러한 결과들은 구운 감초 추출물과 일반 감초 추출물이 류마티스 관절염과 같은 자가면역 만성 염증성 질환을 억제할 수 있다는 것을 나타낸다.

핵심되는 말: 감초, 구운 감초, 콜라겐-유발 관절염, TNF-α, IL-1β, MMP-3, 과산화지질, 환원형 글루타치온, 카탈라제