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Hydroponic-cultured Ginseng leaf extract attenuates murine collagen-induced arthritis by reducing pro-inflammatory responses

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Abstract

Purpose: We carried out the present study to demonstrate that oral treatment with Ginseng leaf protects SD rats from arthritis development and Ginseng leaf are an effective inhibitor of arthritis-induced proinflammatory cytokines.

Methods: Animal experimental groups were divided by six groups and CIA induced animal model group were fed on extract of Gingseng leaf (HGLE). The rats were evaluated daily for the clinical signs of arthritis, hind paw volume, and body weight. The plasma levels of cytokine(TNF-α, IL-1β, and IL-6), biochemical markers, and nitric oxide concentration were analyzed. In collected knee joint and medulla oblongata, we carried out histological analysis and monoclonal antibodies(NFκB, p38, iNOS, and Nrf2) of western bolt.

Conclusion: Our study has demonstrated the joint protective and anti-inflammatory effects of HGLE from RA animal model, which would be related to the inhibition of relevant signaling pathways such as NF-κB, and p38 controlling the production of inflammatory mediators.

Keywords: *hydroponic-cultured Ginseng Leaf, CIA animal model, Rheumatoid arthritis, TNF-α, IL-6, NF-κ B*

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder and systemic inflammatory disorder that result in painful joints [1]. According to a report by Centers for Disease Control in 2010, RA affects about 1% of the population worldwide, most commonly middle-aged women. The causes of RA are still not completely understood, but most evidence supports an autoimmune process [1, 2]. The progression of RA is associated with elevated levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β produced [3]. It also has been claimed that TNF-α is driving most of IL-1 production in the inflamed joints of RA patients [1, 2, 3]. Production of these TNF-α is controlled by three cell signaling, activation of NF-kappa B, activation of the MAPK pathways, and induction of death signaling [5]. TNF-α and IL-1β directly induce synthesis of enzymes such as matrix metalloproteinases (MMPs), which can lead to degradation of joint structures, especially the capsule around the joints [3, 4, 5].

Table 1. Bioactive material composition of Hydroponic-cultured Ginseng

Variable	**The result of analysis by UHPLC method**			
Ginsenoside (mg/g)	HGL	HGR	OGCG	SGCG
Rg1	3.26	0.92	0.24	0.44
Re	10.45	0.89	0.89	0.55
Rf	0.06	0.26	0.10	0.15
Rg2(S)	1.13	0.10	0.09	0.00
Rh1(R)	0.83	0.00	0.00	0.00
Rb1	0.02	0.30	0.23	0.29
F1	0.68	0.00	0.00	0.00
F2	2.23	0.03	0.00	0.00
Rh2(R)	0.06	0.00	0.00	0.00

Ginseng components were analyzed by using ultra high performance liquid chromatography. Re, Rf, Rg2, Rh1, Rb1, Rh2 F1, and F2 were a kind of ginsenoside. Abbreviation: HGL, Hydroponic Ginseng Leaf; HGR, Hydroponic Ginseng Root; OGCG, One-year old Ground Cultured Ginseng; SGCG, Six-year old Ground Cultured Ginseng Leaf

Ginseng, the root of *Panax ginseng* C.A Meyer is frequently used as a natural substance in Asian countries as food product as well as herb medicine. In oriental medicine practice, ginseng root is the most commonly used part of the plant. While ginseng leaf was less studied, a recent report indicates that ginseng leaf contains similar pharmacologically active ingredients more abundantly than ginseng root [6]. Table 1 lists bioactive material composition of Hydroponic-cultured Ginseng. According to the previous studies [7-9], Ginseng leaf extracts exhibit various pharmacological actions in cardiovascular system, central nervous system (CNS), and immune system. Ginseng leaf extracts also possess anti-hyperglycemic,

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anti-cancer, anti-fatigue, anti-obesity, anti-aging, and anti-oxidant activities [10-14]. Until now, there has been little and no research into anti-arthritic effect of ginseng leaf. Practically, growing and harvesting ginseng leaf have its fill of trouble. To solve these problems, we used Hydroponic-cultured Ginseng leaf extract (HGLE) which have quicker growing and easier harvest than the bare ground-cultured ginseng [15].

Recently TNF-α inhibitors and lots of medicine is developed and success rate of the treatment of RA is increasing. But these drugs have serious side effects that may outweigh their benefit and the price of protein based anti-TNF-α therapeutics drugs is relatively expensive. Hence, the development of inexpensive, orally bioavailable inhibitors of proinflammatory cytokines such as HGLE extract are highly desirable. In our study, we verified that oral administration of HGLE could suppress arthritis in CIA model. To describe the mechanism underlying the antiarthritic effect in terms of proinflammatory cytokine inhibitor, the effect of HGLE on regulation of kinase MARK pathways was investigated.

2. Materials and Methods

2.1. Animal Experiments

75 male Sprague-Dawley rats (aged at 4 weeks of 120 g averaged body weight) were purchased from central lab. Animal Inc (Seoul, Korea). Each animal was individually housed in a cage under standard laboratory conditions of 12/12 hours light/dark cycle at 25℃ and 60% humidity and was allowed to access food and water ad libitum for 2 weeks. All experiments were approved by the Ethics Committee of Dong-Eui University and were in accordance with the guidelines of the International Association for the Study of Pain (IASP).

Control group and experimental group were categorized as follows; Group Ⅰ: control group (n=12), GroupⅡ: induced CIA and water oral administration (n=12), Group Ⅲ:vehicle, induced CIA and HGLE (200 mg/kg $_{rat\ weight}$) extract oral administration (n=12), Group Ⅳ: induced CIA and HGLE (100 mg/kg $_{rat\ weight}$) extract oral administration (n=12), Group Ⅴ: induced CIA and HGLE (50 mg/kg $_{rat\ weight}$) extract oral administration (n=12), Group Ⅵ: positive control, induced CIA and leflunomide (3.5 mg/kg $_{rat\ weight}$) extract oral administration (n=12)

2.2. Chemicals and Drugs

Complete Freund Adjuvant (CFA), Rat type II Collagen, and leflunomide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). NfκB , p-NfκB, p38, p-p38, iNOS, and Nrf-2 monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Preparation of Hydroponic-cultured Ginseng Leaf Extract

Leaves of Ginseng, *Panax ginseng* C.A Meyer, were purchased from Oriental Bio. Co., in Busan. We rinsed its eaves clean, dried it in the shade for a week, chopped roots into small pieces by grinder, and chopped sources were lyophilized by freeze dryer. After filling 15 mL tube with 1 milligrams of lyophilized sample and 10 milligrams of 70% ethanol, tube whirled into rounded mixed machine for 18 hours. Only supernatant was collected, vaporized in 40℃ and also lyophilized. Yield of extracted material was 7.2%.

2.4. Acute toxicity test

The rat were subjected to toxicity test using the fixed-dose procedure, which is a sequential testing scheme that was proposed by the British Toxicology Society in1984 as an alternative for the assessment of acute toxicity via estimation of the Lethal Dose 50 (LD_{50}). The procedure is incorporated into the European Community Directive guidelines as the acute peroral toxicity test [17]. Briefly, an initial dose of 5, 50, 500, or 2000 mg per kg of body weight can be selected to evaluate the toxicity of the substance being investigated. Either 5 or 2000 mg per kg can serve as the starting dose. The procedure is terminated when either toxicity or death is observed.

2.5. Arthritis induction

Collagen type II solution (4 mg/ml) was prepared by dissolution in 0.1 M acetic acid. Collagen solution was emulsified with an equal volume of Complete Freund Adjuvant (CFA). A volume of 100 µl of final emulsion was injected subcutaneously at the base of the tail. After 7 days of the primary induction, the same amount of emulsion was injected at tail to stimulate booster effect [18].Time schedule as follows(Figure 1): 0~1 weeks - period of adaptation, 7 days –first injection for induction

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of RA(first immunization), 14 days – second injection for booster effect, 14~55 On day 21, respective dosages of HGLE were orally administered once daily for 41 days, measured rat weight and hind paw volume, finally sacrificed.

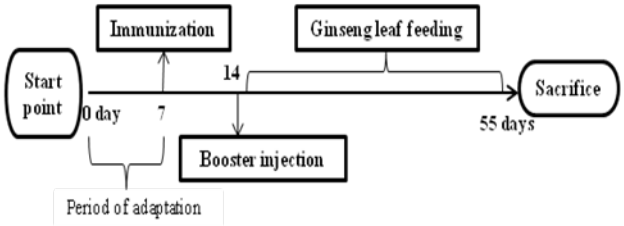

Figure 1. Experimental Time schedule

0~1 weeks -period of adaptation, 7 days –first injection for induction of RA(first immunization), 14 days – second injection for booster effect, 14~55 On day 21, respective dosages of HGLE were orally administered once daily for 41 days, measured rat weight and hind paw volume, finally sacrificed.

2.6. Arthritis Assessment-arthritic Score and Index, hind paw volume, and body weight

The rats were evaluated daily for the clinical signs of arthritis. This scoring system assigns numerical values to the digits and paws of each of the hind limbs as follows: 0=normal; 1=swelling or redness confined to 1 digit; 2=swelling or redness in 2 or more digits; 3=swelling or redness in entire paw; 4=severe arthritis of the entire paw and digits with difficulty in walking. The mean arthritis score for each rat was calculated by dividing the total scores for the group by the number of animals in the group. Hind paw edema of the rats was monitored per 4 day, using a dial thickness gauge. The arthritic index (AI) and CIA inhibition were calculated. Body weight was also measured per 4 day.

2.7. Hematological and Biochemical analyses

The 3 mL of blood was into EDTA tube for measuring biochemistry markers. The other blood was separated into serum for detections of biochemical variables. Hematological variables were analyzed by Auto Hematology Analyzer (BC-2800 ver., Shenzhen Mindary Bio-Medical Electronics Co., Ltd., Germany). The serum total protein(TP), total cholesterol (T-ch), triglyceride (TG), liver markers [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkalin phosphatase (ALP)], and renal markers [creatinine, uric acid, and blood urea nitrogen (BUN)] were analyzed by THOSHIBA 200FR (Toshiba medical system Co., Japan).

2.8. Measurement of Nitric Oxide Concentration and Cytokines in Plasma

NO was measured by incubating 100 μL of the plasma with 100 μL of the Griess reagent (Sigma Inc., USA) as per manufacturer's protocol. The absorbance was measured at 540 nm using ELISA microplate reader (Biorad, USA) and nitrite concentration was determined with a standard curve of sodium nitrite ($NaNO_2$). The plasma levels of TNF-α, IL-1β, and IL-6 were evaluated using the respective rat specific Enzyme-Linked Immunosorbent Assay (ELISA) kits as per the manufacturer's protocol (Bio-Rad., USA).

2.9. Tissue harvesting and Histological Analysis

On day 55, rat were deeply anesthetized with ether and then blood was collected and segments knee joint and medulla oblongata was dissected. The tissues were stored at -80 before using. The knee joint was excised postmortem, trimmed off skin and muscular parts, and fixed in 10% buffered formalin. Afterwards, the joints were placed in decalcifying solution for almost 10 d, embedded in paraffin, sectioned (10 μ) and stained with hematoxylin and eosin. The sections were observed under light microscope at 10× magnification.

2.10. Western blot

Segments of medulla and spleen were homogenized using homogenizer (Intron Biotechnology, Gyeonggi-do, Korea) with lysis buffer (PRO-PREP^{TM}, Protein Extraction Solution). Homogenized samples were centrifuged 13,000 rpm for ten minutes. Protein absorbance of supernatant was measured using X-ma spectrophotometer (Human Cor. Korea) at 595 nm. Extracting about each tissues, equal amounts of protein (50 μg) were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels in running buffer (25 mmol/L Tris, 0.25mol/L glycine, 0.1% sodium dodecyl sulfate, pH 8.3) at 90 V and then electroblotted to nitrocellulose membranes. NFκB, p38, iNOS, and Nrf2 were detected by monoclonal antibodies (Santa Cruz Biotech, Inc. Santa Cruz, CA). Membranes were blocked at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 and then incubated overnight at 4°c with the following primary antibodies:

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Histone monoclonal antibody(Cell Signaling Technology, INC. USA ; dilutions, each 1:2,000, 1:500, 1:20,000) Then the membranes were washed three times in Tween-20 and incubated with the corresponding secondary antibody (Santa Cruz Biochemicals; dilutions, each 1:8,000, 1:3,000, 1:10,000) conjugated to horseradish peroxidase at room temperature. Immunoreactive bands were visualized with the chemoluminescence kit (Santa Cruz Biochemicals) according to the manufacturer's instructions. Band intensities and molecular weight were quantified by using a Vision Works Image Software (UVP, Cambridge, UK).

2.11. Data analysis

Experimental values are presented as mean ± SD of triplicate cultures and representative of experiments performed on three occasions. Statistical significance was determined by Mann-Whitney *U* test or ANOVA with Bonferroni's post-hoc test using SPSS version 18. Values of $P < 0.05$ were considered statistically significant, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. In analysis of Nrf2, NOX4, NF-kappa B and p38, not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3. Results

3.1 Body Weight and Paw Edema

Although the starting weight for each group was more or less the same, there was a decided difference for all groups (figure 2.A). The average body weight of vehicle group began to show down trend between days 24 and 40 after immunization and vehicle group alone had the lower ending weight(144.21±9.8) than initial weight(147.51±8.1). The ending weight of control, HGLE 200 mg, and positive control groups was higher significant differences than those of vehicle group ($P < 0.01$) and the ending weight of HGLE 100 mg and HGLE 50 mg was higher significant differences than those of vehicle group ($P < 0.05$) (figure 2.A). As mentioned above, paw edema was quantified by measuring paw size using a digital caliper per 4 days. Arthritic animals began to show signs of arthritis between days 16 and 24 after immunization. With the exception of vehicle group, all rats showed no significant differences. Comparison between vehicle and HGLE treatment groups revealed a significantly higher effect of HGLE in reducing paw edema decrease ($P < 0.05$) (figure 2.B). Particularly paw volume of HGLE 200 mg treatment group was lower than those of vehicle group ($P < 0.01$).

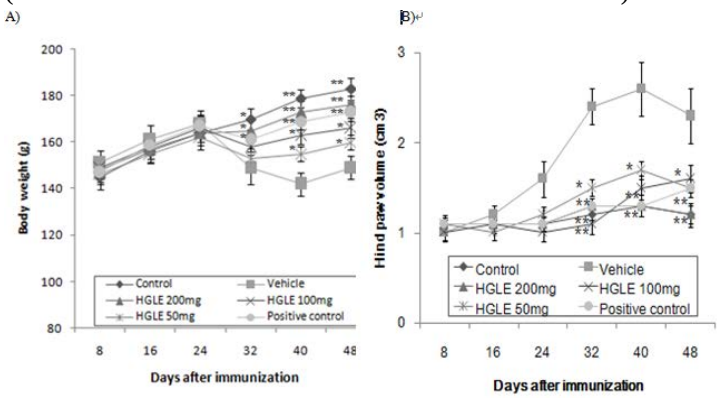

Figure 2. Clinical findings in rats with collagen-induced arthritis (CIA) A) The average body weight of SD B) The average paw edema of SD The average body weight of vehicle group began to show down trend between days 24 and 40 after immunization and vehicle group alone had the lower ending weight than initial weight(figure 2.A). Comparison between vehicle and HGLE treatment groups revealed a significantly higher effect of HGLE in reducing paw edema decrease ($P < 0.05$) (figure 2.B). ; * , $p<0.05$; **, $p<0.01$

3.2. Acute toxicity of HGLE in SD rat

If the estimated LD_{50} is less than 25 mg/kg body weight, the substance is classified as 'very toxic', as 'toxic' if it is between 25 and 200 mg/kg body weight, and as 'harmful' if it is between 200 and 2000 mg/kg body weight. Substances with an estimated LD_{50} larger than 2000 mg/kg body weight are termed 'unclassified' [18, 19]. Fourteen male SD rat were divided into two groups of 7. HGLE was administered at a single dose of 500 mg/kg to one group and at a dose of 2000 mg/kg to the other group. The rat were then observed over a period of two weeks to assess any changes in motor activity , reactivity, respiration rate, gait, and especially death. Rat given 2000 mg/kg body weight (n = 7) HGLE did not show any difference in their gross general behavior; no death or obvious weight changes were observed over the 14 days that the rat were observed. Thus, the LD_{50} of HGLE is more than 2000 mg/kg, which indicates that HGLE can be categorized as 'unclassified.' Tests on the liver and kidney function showed little blood biochemistry differences between the HGLE-treated and the control groups (table 3). Thus, HGLE had fewer adverse effects in our experimental animals.

Table 2. Hematological variables in the study groups

Variable	Group

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(10^3/)	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ
	Control	**Vehicle**	**HGLE 200mg**	**HGLE 100 mg**	**HGLE 50 mg**	**leflunomide**
T-WBC	4.16 ±0.45	6.06 ±0.32*	4.57 ±0.60	5.06 ±0.62+	4.95 ±0.58+	4.76 ±0.56+
Granul	1.30 ±1.31	1.87 ±0.55*	1.40 ±0.35	1.68 ±0.40+	1.65 ±0.60+	1.51 ±0.30+
Lymph	2.76 ±0.51	4.00 ±0.75*	3.06 ±0.82	3.23 ±0.82+	3.16 ±0.71+	3.14 ±0.72+
Mono	0.11 ±0.03	0.19 ±0.05*	0.11 ±0.10	0.15 ±0.02+	0.14 ±0.01+	0.11 ±0.03
RBC	6.98 ±1.12	8.59 ±0.99*	7.31 ±0.91	7.28 ±2.41	7.25 ±1.54	7.28 ±1.65
Platelet	478.9 ±8.6	528.67 ±10.98*	488.71 ±9.8	501.15 ±10.7	490.65 ±8.30	480.51 ±7.61
Ht (%)	42.99 ±3.12	48.28 ±8.46*	43.61 ±5.12	42.65 ±5.61	42.89 ±6.15	43.15 ±4.47

Group I, control rats (health rats); Group II, rats with collagen-induced arthritis (arthritis group); Group III, arthritis rats (arthritis +HGLE 200 mg/kg); Group III, arthritis rats (arthritis +HGLE 100 mg/kg); Group Ⅴ, arthritis rats (arthritis +HGLE 50 mg/kg);); Group Ⅵ, arthritis rats (arthritis + leflunomide 3.5 mg/kg) Data are expressed as mean±standard deviation (SD). *, ***P***<0.05 (compared with group III); +, ***P***<0.05 (compared with group I); Abbreviation: T-WBC, total white blood cells; Granul, granulocyte; Mono, monocyte; RBC, red blood cells; Ht, hematocrit

Table 3. Biochemical variables in the study groups

Variable	**Group**					
	Control	**Vehicle**	**HGLE 200mg**	**HGLE 100mg**	**HGLE 50 mg**	**leflunomide**
ALP (IU/L)	211.5 ±4.6	219.7 ±34.5	215.8 ±28.2	208.5 ±29.0	225.2 ±27.5	212.3 ±22.1
ALT (IU/L)	43.3 ±7.1	44.2 ±1.9	38.2 ±3.9	46.5 ±2.5	45.7 ±3.3	46.8 ±5.8
AST (IU/L)	82.6 ±19.4	88.1 ±9.4	79.0 ±6.8	75.4 ±4.9	78.0 ±7.8	81.5 ±9.0
BUN (mg/dL)	12.1 ±2.3	13.5 ±1.9	15.3 ±1.7	14.2 ±3.3	14.4 ±0.8	15.8 ±0.9
Uric acid (mg/dL)	2.6 ±0.3	2.3 ±0.2	2.6 ±0.5	2.8 ±0.5	2.2 ±0.6	2.1 ±0.4
Creatine (mg/dL)	0.7 ±0.05	* 1.95 ±0.04	0.78 ±0.11	0.74 ±0.05	0.68 ±0.6	0.71 ±0.15
Cholesterol (mg/dL)	65.6 ±4.6	68.5 ±4.1	68.4 ±8.4	71.4 ±9.2	69.0 ±5.2	71.5 ±4.6
TG (mg/dL)	60.3 ±9.3	62.8 ±7.8	63.2 ±13.1	68.0 ±4.3	69.1 ±11.3	65.4 ±8.8
TP (g/dL)	6.01 ±0.3	6.15 ±0.5	6.36 ±0.2	6.08 ±0.2	6.51 ±0.3	6.44 ±0.6

The creatine level of vehicle group was significant difference than the other groups (***P***<0.05) and there were no significant differences among groups except creatine (***P***>0.05).
Data are expressed as mean±SD. *, ***P***<0.05 (compared with control); Abbreviation: AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; TG, triglyceride; TP, total protein.

3.3. Hematological and biochemical variables

Hematological variables are summarized in Table 2. Total leukocyte, granulocyte, lymphocyte and monocyte counts in vehicle group were significantly higher than those of HGLE 200 mg (***P***<0.05). Those counts in HGLE 100 mg, HGLE 50 mg, and positive control were significantly higher than those of control (***P***<0.05). In RBC, Hematocrit, and Platelet, there are no significant differences among all groups. Biochemical variables are summarized in Table 3. The creatine level of vehicle group was significant difference than the other groups (***P***<0.05) and there were no significant differences among groups except creatine (***P***>0.05).

3.4. Hydroponic-cultured Ginseng leaf Extract Suppresses Collagen-Induced Arthritis

Results showed that administration of oral HGLE 200mg/kg $_{rat\ weight}$ once a day during 41days reduced the arthritic score and arthritis incidence almost completely compared to the oral administration of the vehicle (figure 3B). Although there were no differences among HGLE treated groups, it would seem that remedial effect of HGLE 50 mg group was lower than those of HGLE 200 mg group (***P***>0.05)(figure 3.A). The synovium of these rats appeared healthy and there was no evidence of inflammation. As shown in figure 3.B, HGLE 200 mg successfully suppressed the arthritic score in the CIA rat. Histological examination of the joints demonstrated that the paws and ankles of HGLE-treated rat had a lower degree of inflammation and cartilage damage compared with those of the vehicle-treated mice, as determined on day 48 after immunization(figure 3.C). The histological finding of the knee joint in control group was normal (Fig. 3.C.a). The joints of control group showed normal architecture with no swelling of joint space and there was the articulating surfaces and adequate gap were lined by a healthy lining of cartilage. In vehicle group, the articular capsule of knee was in disrepair as microscopic viewpoint and Changes observed included inflammation, cellular inflammation, synovial hyperplasia, erosion, and fibrosis. (figure 3.C.b). The inflammatory and damage degree were expressed as 1+ ~ 4+. Vehicle group (3+ to 4) showed severe inflammatory damage compared with control and HGLE 200 mg group (Fig 3.C.a, b, c).

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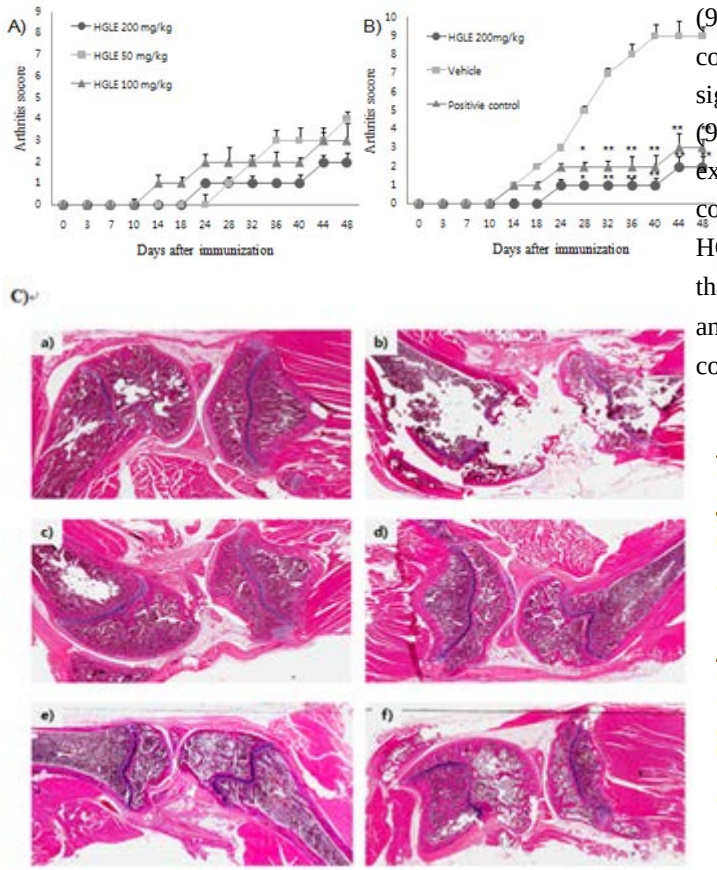

Figure 3. Hydroponic-cultured Ginseng Leaf Extract Suppresses Collagen-Induced Arthritis

Hydroponic-cultured Ginseng Leaf Extract (HGLE) was administered orally to Collagen-immunized rat once a day from day 10 to day 48. (A) The severity of arthritis was evaluated by clinical arthritis index score; CIA(vehicle), leflunomide(positive control), and HGLE administered orally at 200 mg/kg (B) The severity of arthritis among HGLE groups was evaluated by clinical arthritis index score. (C) On day 49, joint tissues from control rat and CIA rat treated with vehicle and HGLE were stained with H&E for histopathological examination (X 100 original magnification); a) Control, b) CIA model, not treated(vehicle), c)HGLE administered orally at 200 mg/kg, d) HGLE administered orally at 100 mg/kg, e) HGLE administered orally at 50 mg/kg, f) Positive control(leflunomide administered orally at 0.35 mg/kg)

3.5. HGLE reduces level of Inflammatory cytokine in CIA rat

As shown in figure 4.A, The serum IL-1β concentrations in vehicle group (175.05±18.16 pg/㎖) were significantly higher than those of HGLE 200 mg (88.31±9.61pg/㎖), HGLE 100 mg (101.35±8.11pg/㎖), and HGLE 50 mg (99.75±7.56pg/㎖) (***P***<0.05). The serum IL-6 concentrations in HGLE 200 mg (33.28±6.65 pg/㎖) were significantly lower than those of vehicle group (99.48±11.54 pg/㎖) (***P***<0.01) and those of the other experimental group (***P***<0.05). The serum TNF-α concentrations in HGLE 200 mg (37.51±4.61 pg/㎖) and HGLE 100 mg(41.58±6.69 pg/㎖) were significantly lower than those of vehicle group (145.09±5.28 pg/㎖) (***P***<0.01) and those of HGLE 50 mg (88.38±8.18 pg/㎖) and positive control(65.55±3.64 pg/㎖) (***P***<0.05).

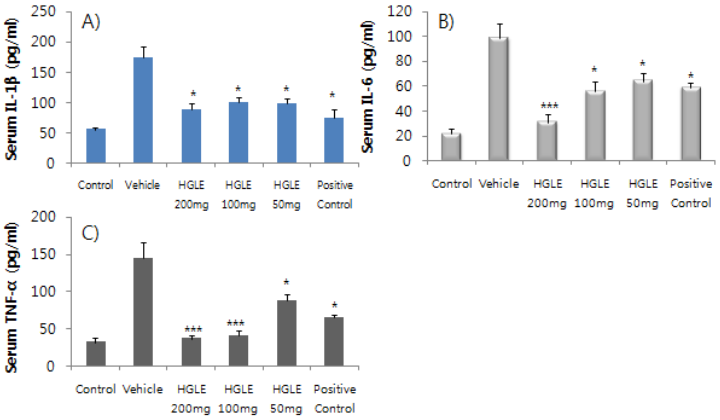

Figure 4. Hydroponic-cultured Ginseng leaf Extract reduces level Inflammatory cytokine

Serum proinflammatory cytokines concentrations in the study groups. A) Serum interlukin-1 beta (IL-1β) concentrations, B) Serum interlukin-6 (IL-6) concentrations C) Serum tumor necrosis factor- α (TNF-α) concentrations

3.6. HGLE Inhibits NO Production by Suppressing the Expression of iNOS in CIA rat

Vehicle group dramatically increased NO productions. However, HGLE treatment groups significantly inhibited the production of these inflammatory molecules in a dose-dependent manner (figure 5.A). In addition, HGLE 200 mg/kg treatment displayed stronger inhibition on NO production than leflunomide treatment. The expressions of inflammatory enzymes iNOS were also strongly induced by Collagen type II treatment while these over-expressions were suppressed by HGLE at protein levels (figure 5.B.). Although there seems to be a dose-dependent tendency by HGLE treatments in the expressions of NO, the expression levels of protein are not exactly same.

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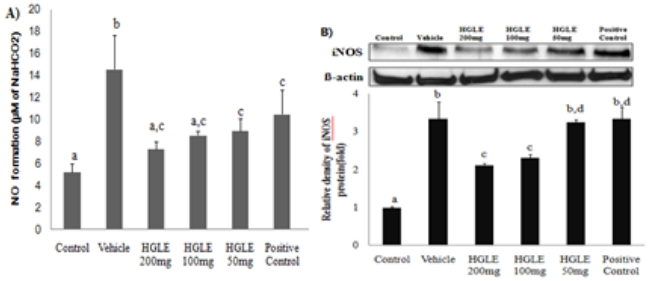

Figure 5. HGLE inhibits NO and iNOS expressions in CIA rat model

A) The nitrite concentration was measured by the Griess reaction

B) Expressions of inflammatory enzymes iNOS

Vehicle group dramatically increased NO productions. However, HGLE treatment groups significantly inhibited the production of these inflammatory molecules in a dose-dependent manner. Means not sharing a common letter were significantly different ($p < 0.05$) analyzed by ANOVA and Duncan's multiple range test.

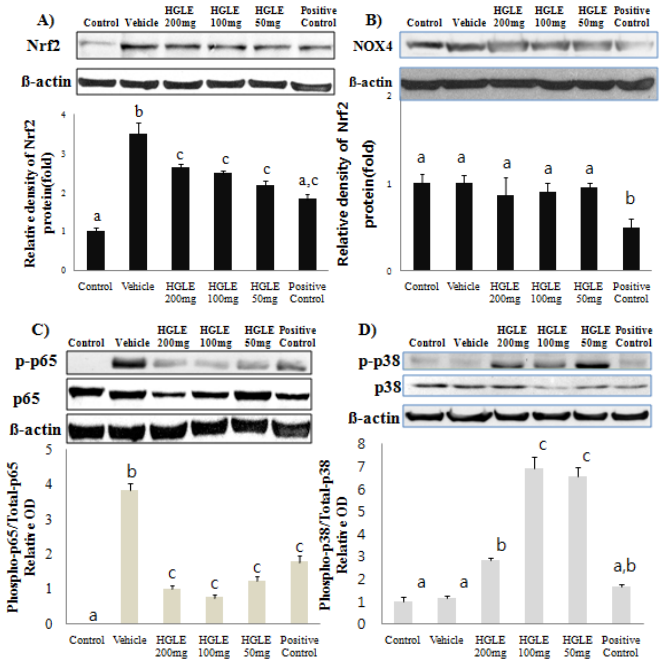

Figure 6. HGLE Inhibits Phosphorylation of NF-κ B in Medulla Oblongata

Protein expression of nrf2, NOX4, NF-κ B, and p38 was evaluated by Western blot analysis of Medulla Oblongata homogenates. The results show that arthritis strongly induced expression of Nrf2 and phosphorylation of NF-κB (p-p65), as observed in CIA vehicle rat with respect to naïve animals (figure 6.A, C). Means not sharing a common letter were significantly different ($p < 0.05$) analyzed by ANOVA and Duncan's multiple range test.

3.7. HGLE Inhibits Phosphorylation of NF-κ B in Medulla Oblongata

Protein expression of nrf2, NOX4, NF-κ B, and p38 was evaluated by Western blot analysis of Medulla Oblongata homogenates (figure 6). Figure 6 shows that arthritis strongly induced expression of Nrf2 and phosphorylation of NF-κB (p-p65), as observed in CIA vehicle rat with respect to naïve animals (figure 6.A, C). Treatment with HGLE 200 mg/kg and, to a lower extent, at 50 mg/kg, significantly reduced the expression of Nrf2 in arthritic rat (figure 6.A). Similarly, HGLE treatment groups significantly inhibited the production of phosphorylation of NF-κB (p-p65) in a dose dependent manner (figure 6.C). But, there were no significant differences of expression of NOX4 among six groups (figure 6.B) and phosphorylated p38 of treatment with HGLE 100 mg/kg and 50 mg/kg increased against those of control and vehicle (figure 6.D).

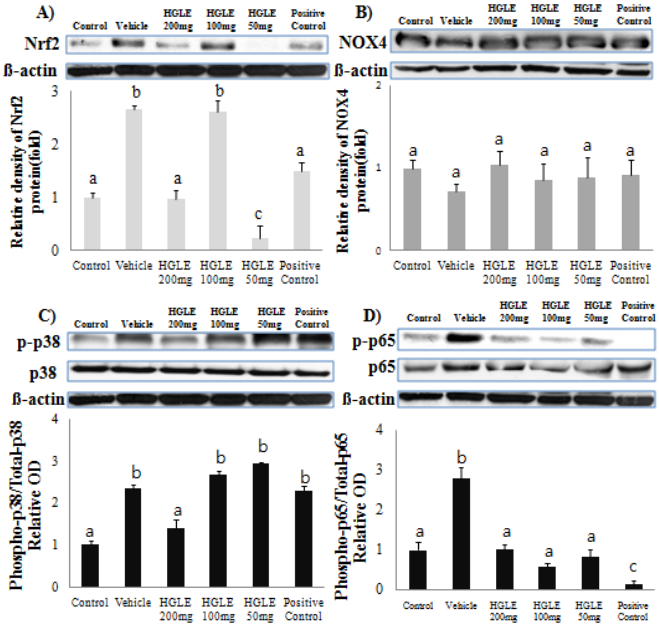

Figure 7. HGLE Inhibits Phosphorylation of NF-κB and p38 in knee joint

Protein expression of nrf2, NOX4, NF-κ B, and p38 was evaluated by Western blot analysis of knee joint homogenates. HGLE treatment in arthritic rat reduced phosphorylation of NF-κB in the same manner as Medulla Oblongata (figure 7.D) Means not sharing a common letter were significantly different ($p < 0.05$) analyzed by ANOVA and Duncan's multiple range test.

3.8. HGLE Inhibits Phosphorylation of NF-κB and p38 in knee joint

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Figure 7 also shows that arthritis strongly induced expression of Nrf2 and phosphorylation of NF-κB (p-p65), as observed in CIA vehicle rat with respect to control animals (figure 7.A, D). Treatment with HGLE 200 mg/kg reduced the expression of Nrf2 and p-p38 compared with vehicle group (figure 7.A, C) and HGLE treatment in arthritic rat reduced phosphorylation of NF-κB in the same manner as Medulla Oblongata (figure 7.D). But, there were no significant difference of expression of NOX4 among six groups (figure 7.B).

4. Discussion

The present study demonstrated that oral treatment with HGLE protects SD rats from arthritis development and HGLE are an effective inhibitor of arthritis-induced proinflammatory cytokines. We have identified that HGLE reduces level of proinflammatory cytokine, NO Production by Suppressing the Expression of iNOS, and Phosphorylation of NF-κ B, p 38 and Expression of Nrf2. To the best of our knowledge, this is the first study to identify anti-arthritic effect of HGLE in SD rat.

First of all, we conducted acute toxicity test of HGLE in SD rat to determine the fixed-dose [17-19]. The results were that the LD_{50} of HGLE is more than 2000 mg/kg, which indicates that HGLE can be categorized as 'unclassified'. Although levels of creatine in vehicle were higher than levels of creatine in other groups, Tests on the liver and kidney function of treatment with HGLE was no differences with those of control group apart from creatine. Considering low serum creatine activity has been found to be associated with muscle weakness in RA patient and CIA-rat [20, 21], HGLE had fewer adverse effects in our experimental animals.

A loss in weight is major symptom of inflammatory arthritis, where a gradual decrease in weight is observed as the disease progresses [22, 23]. HGLE-treated arthritis group experienced a significant recovery of body weight following the second immunization and losing weight of vehicle group was continued. Therefore HGLE supplement to arthritis rats may have inhibited the metabolic rate of arthritic rats. Inflammatory pain during the development of CIA is assessed by measuring paw edema [24, 25]. According to another study, CIA developed acutely in rats, with joint changes occurring much more rapidly than in human RA. This allowed for a more detailed observation of joint changes before and after treatment [26]. Our experimental results also showed acute CIA development within 20 days and the curative influence of HGLE for a short time. HGLE-treated arthritis group appeared to be effective in relieving RA, and this change of paw volume was similar to change of arthritis score. Especially at the oral doses of 200 and 100 mg/kg, HGLE revealed a strong antiarthritic property capable of reducing CIA paw edema and swelling as observed in arthritis score. Histologically, we noted significant attenuation in cellular infiltration of the synovium, synovial hyperplasia, and cartilage damage in HGLE-treated rat.

The progression of arthritis is associated with sustained production of proinflammatory cytokines. Proinflammatory cytokines induce further cytokines, chemokines, eicosanoids and reactive oxygen species (ROS) amplifying the inflammatory response [27, 32]. In particular, TNF-α suppresses the recruitment of osteoblasts [28], stimulates osteoclastogenesis [29], and inhibits the expression of matrix genes [30], whereas IL-6 increases osteoclast numbers in trabecular bone [31]. IL-1 mediates many inflammatory diseases by initiating and potentiating immune and inflammatory responses [32]. IL-1 is involved in several systemic autoinflammatory syndromes and in juvenile RA. It also plays a pathogenic role in inflammation and tissue destruction [33, 34]. In line with this study, proinflammatory cytokines are implicated in each phase of the pathogenesis of rheumatoid arthritis, by promoting autoimmunity, by maintaining chronic inflammatory synovitis and by driving the destruction of adjacent joint tissue [35]. Interestingly, it has been reported that there are different time-dependent roles for IL-1β and TNF in the various stages of collagen-induced arthritis [36]. As shown in figure 4, HGLE has strong effect on the downregulation of TNF-α, IL-6, and IL-1β production. Therefore, inhibition of proinflammatory cytokine production by HGLE would result in the reduction of the inflammatory response and tissue damage. In addition, Hydroponic-cultured Ginseng leaf extract-treated rat expressed remarkably lower levels of not only proinflammatory cytokines such as TNF-α, IL-1b, and IL-6, but also oxidative stress markers such as iNOS as demonstrated by western blot analysis (figure 5).

We investigated alternation of p38, NF-κB, nrf2, and NOX4 in medulla Oblongata and knee joint. Previous study reported that the Keap1/Nrf2 axis regulates RANKL-dependent osteoclastogenesis through regulation of

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intracellular ROS signaling by the expression of cytoprotective enzymes [37]. We hypothesized that Nrf2 activation would increase Nrf2-dependent expression of cytoprotective enzymes, and they would scavenge ROS, and therefore attenuate RANKL signaling and osteoclastogenesis. But the odd results of our experiment suggested that nrf2 expression of vehicle group was higher than those of the other groups. We suppose that HGLE would exercise no influence over nrf2 regulation in CIA rat, and if there is no correlation between HGLE and nrf2 expression, nrf2 upregulation of CIA rat may be a necessary result to maintain homeostasis. The mitogen-activated protein kinase (MAPK) signaling pathway has been strongly implicated in many of the processes that underlie the pathology of rheumatoid arthritis. MAPKs regulate the synthesis of chemokines, cytokines, and PGs involved in inflammation [38]. In addition, they mediate the induction of matrix metalloproteinases responsible for cartilage breakdown [39]. In particular, p38 are also involved in osteoclast differentiation and may contribute to bone destruction [40]. As shown in figure 6.D and 7.C, oral treatment of HGLE 200 mg/kg markedly lowers levels of p38 in knee joint, and HGLE 100 mg/kg treatment elevates level of p38 in medulla Oblongata. However, treatment of HGLE 200 mg/kg may be effective to reduced phosphorylation of p38 in knee joint, Considering previous studies have shown conflicting results [41, 42], more studies are necessary to determine relation between p38 and HGLE in medulla Oblongata. The NF-κB pathway is involved in the transcription of many inflammatory genes [43]. Cellular stimulation by proinflammatory cytokines induces the recruitment of costimulatory molecules, such as TNF receptor-associated factor (TRAF) leading to the activation of NF-κB-inducing kinase[42, 43]. This protein induces IκB kinase activation resulting in the phosphorylation of p65 [41-43], which is followed by ubiquitination and proteolytic degradation thus allowing the release of NF-κB to enter the nucleus and regulate gene transcription [40, 42]. Our results indicate that HGLE enhances the levels of the inhibitory protein phosphorylation of p65 leading to the reduction of NF-κB nuclear translocation. This mechanism may be responsible for the down-regulation of proinflammatory cytokines in the joint during the CIA process in the animals treated with HGLE. In addition, Proinflammatory cytokines could act as mediators of NF-κB activation [43]. As previously mentioned, HGLE reduced production of proinflammatory cytokines in CIA rat, suggesting that its mechanisms could be involved in the control of NF-κB translocation.

5. Conclusion

In this experimental rat model, we made novel observation that oral administration of HGLE may have preclusive and/or therapeutic effects on rheumatoid arthritis induced by Collagen type II solution. Our study also has demonstrated the joint protective and anti-inflammatory effects of HGLE from RA animal model, which would be related to the inhibition of relevant signaling pathways such as NF-κB, and p38 controlling the production of inflammatory mediators. There is a need for further clinical and experimental investigations regarding the efficacy of HGLE in humans.

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