Food Science and Biotechnology

Synergistic Effects of KH-red Ginseng/chlorella on the Endurance Capacity and Immune Enhancing in Forced Swimming Tested Mice

In-Young Choi[1], Ji-Hyun Lee[2], Tae-Hee Kang[1], Hyo-Jin An[1], Su-Jin Kim[1], Phil-Dong Moon[1], Na-Hyung Kim[1], Noh-Yil Myung[1], Deok-Chun Yang[3], In-Cheol Kang[1], Youngjin Choi[1], Min-Ho Kim[4], Jae-Young Um[1], Seung-Heon Hong[2], Hyung-Min Kim[1], and Hyun-Ja Jeong*

Biochip Research Center, Hoseo University, Asan, Chungnam 336-795, Korea

[1]*Department of Pharmacology, College of Oriental Medicine, Institute of Oriental Medicine, Kyung Hee University, Seoul 130-701, Korea*

[2]*Department of Pharmacology, College of Pharmacy, Wonkwang University, Iksan, Jeonbuk 540-749, Korea*

[3]*Department of Oriental Medicine Material and Processing, College of Life Science, Kyung Hee University, Suwon, Gyenggi 446-701, Korea*

[4]*NUMATE of Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea*

Abstract KH-red ginseng/chlorella (KH-RG/C) is the mixed material of the Korean red ginseng powder (*Panax ginseng*, 75%) and extract of *Chlorella vulgaris* (25%). To evaluate the effects of KH-RG/C on endurance capacity and immune regulation, the forced swimming test (FST) was conducted. The immobility time in the FST was significantly decreased in KH-RG/C treated group compared with the DW-treated group at the 3 and 10 days, respectively. In the analysis of the blood biochemical parameters, KH-RG/C treatment significantly increased the glucose level. However, the lactic dehydrogenase level decreased. Although KH-RG/C increased aspartate aminotransferase, it was not different significantly. And KH-RG/C had no affects in the alanine aminotransferase, and blood urea nitrogen levels. In splenocytes and macrophages, KH-RG/C also did not affect the interleukin (IL)-2, IL-4, and IL-12 production. These results suggest that KH-RG/C may influence to immune regulation through increasing the physical endurance capacity without effect in activation of immune cells.

Keywords: KH-red ginseng/chlorella, immune-enhancing effect, forced swimming test, cytokine

Introduction

KH-red ginseng/chlorella (KH-RG/C) composed of Korean red ginseng powder (KRGP, *Panax ginseng*) and extract of *Chlorella vulgaris* (CVE). KRGP is a product made by applying some processing to 6-year-old *P. ginseng* Radix. *P. ginseng* and its constituents have been shown to exhibit both anti-stress and antioxidant activity (1,2), and to exert various benefits relating to stress and the immune system (3). Ginseng has been well known to have a variety of ginsenosides that show diverse biological activities (4). Ginsenoside Rg3 is one of protopanaxadiol ginsenosides by steaming to prepare Korean red ginseng (5). Rg3 is well known as a potent ginsenoside which has anti-stress effect (6) and inhibitory effects on ovarian cancer (7), bladder cancer (8), and gastric cancer (9). *C. vulgaris* (CV) is a fresh water unicellular microscopic algae, widely used as a food supplement in the forms of tablets, capsules, extract liquid, or a food additive in Japan (10). Numerous human and animal experiments have documented various pharmacological effects of CV and CVE, which includes improvement in hypertension (11), lipid metabolism, enhancing anti-tumor (12), antibacterial activities (13), and promotion of dioxin excretion (14).

Fatigue, defined as a loss of force-generating capacity, may develop for a variety of reasons and involve both central and peripheral factors. Fatigue is the most frequent symptom experienced by children/adolescents with cancer (15). Fatigue patients were almost fallen immunity function. The forced swimming test (FST) has been used as anti-fatigue and endurance tests (16,17). Glucose (Glc), blood urea nitrogen (BUN), lactate dehydrogenase (LDH), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total protein (TP) are blood biochemical parameters related to fatigue. Energy for exercise is derived initially from the breakdown of glycogen and, later, from circulating glucose released by the liver and from nonesterified fatty acids (18). As is commonly known, glucose levels are decreased immediately after exercise. The blood urea nitrogen test is a routine test used primarily to evaluate renal function. Serum lactate dehydrogenase and creatine kinase are known to be accurate indicators of muscle damage. Total protein is a rough measure of serum protein. Protein measurements can reflect nutritional state, kidney disease, liver disease, and many other conditions (18). FST exposure produces a variety of time-dependent neurochemical, endocrine, and immune alterations in the rat (19). Therefore, it was analyzed that the blood biochemical parameters related to fatigue in blood after FST to investigate the immune-enhancing effect of KH-RG/C.

Cytokines play an important role in mucosal humoral and cell-mediated immunities. T-helper cells are divided into 2 subgroups, of T helper 1 cells (Th1) and T helper 2 cells (Th2), according to their cytokine production profiles (20). Th1 cells play a critical role in the regulation of cellular immunity through the secretion of interleukin-2 (IL-2) and interferon-γ (IFN-γ). On the other hand, Th2

cells regulate humoral immunity through the production of IL-4 and IL-10 (21,22). Various cytokines such as IL-2, IL-4, and IL-12 are related to immune reaction, directly or indirectly.

In the present study, the anti-immobility effect of KH-RG/C during the FST was examined. After the FST, the effects of KH-RG/C on the body weight, the energy source, the muscle damage, liver function, and renal function were estimated. In addition, it was evaluated that the effect of KH-RG/C on the production of IL-2, IL-4, and IL-12 in mouse splenocytes and macrophages for certifying the immune function of KH-RG/C.

Materials and Methods

Reagents A 20 (*R*)- and 20 (*S*)-ginsenoside-Rg3 (Rg3) isolated from red ginseng was kindly provided by Dr. Deok-Chun Yang (Department of Oriental Medicinal Material and Processing, College of Life Science, Kyung Hee University, Seoul, Korea). Absolute ethanol (EtOH) was purchased from Merck (Darmstadt, Germany). Fluoxetine hydrochloride (20 mg/capsule) was purchased from Myung In Pharm Co., Ltd. (Seoul, Korea). Thioglycollate (TG) and Concanvalin A (Con A) were purchased from Difco Laboratories (Detroit, MI, USA). Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), RPMI 1640 medium, 100 U/mL penicillin, and 100 mg/mL streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Avidin-peroxidase, 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Dulbecco's modified Eagle's medium (DMEM), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-mouse IL-2 (MAB702)/IL-4 (MAB404)/IL-12 (MAB419) antibody (Ab), biotinylated anti-mouse IL-2 (MAB702)/IL-4 (BAF404)/IL-12 (BAF419) Ab, and recombinant (r) mouse IL-2 (402-ML)/IL-4 (404-ML)/IL-12 (419-ML)/interferon (IFN)-γ (485-MI) were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of KH-RG/C KH-RG/C composed of KRGP (75%) and CVE (25%), respectively. KRGP (100%) was purchased from the Korea Ginseng Corp. (Seoul, Korea). It was decocted with distilled water (DW) for 3 hr. The decoction was filtered, lyophilized, and kept at 4°C. CVE was prepared to lyophilize the heat-treated water extracts of CV, supplied by Daesang Corp., WellLife (Seoul, Korea). KH-RG/C and Rg3 were dissolved in DW and EtOH, respectively.

Animals Male ICR strains of mice (4-week-old) were purchased from the Dae-Han Experimental Animal Center (Eumsung, Korea) and the animals were maintained in the College of Pharmacy, Wonkwang University. Food and water were provided *ad libitum* for 10 days. They were allowed at least 1 week to adapt to the laboratory environment before experiments. The mice were housed 7/cage in a laminar air-flow room maintained at a temperature of 22 ± 1 °C and relative humidity of $55 \pm 10\%$ throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines.

Treatment of KH-RG/C and reagents For *in vivo* study, 0.8, 8, and, 80 mg/kg KH-RG/C, 10 mg/kg Rg3, and 1 g/kg EtOH (solvent of Rg3) were administrated by oral for 10 days. Rg3 was used as active compound because KH-RG/C was mixed as 3:1 of KRGP and CVE. Rg3 is also one of unique active ingredient in Korean red ginseng (23). To compare the each immobility time, fluoxetine, which is a stronger antidepressant (24), was used as positive control. Because of the hepatic toxicity (24), fluoxetine was treated 1 hr before to get FST at 3 and 10 days. In *in vitro* study, the concentration of 0.8, 8, and 80 μg/mL KH-RG/C, 10 μg/mL Rg3, and 0.5% EtOH was used.

Forced swimming test (FST) During the 6 min in the FST, the duration of immobility was measured as previously described by Porsolt *et al.* (25). The apparatus consisted of 2 Plexiglas cylinders (height, 25-cm; diameter, 10-cm) placed side by side in a Makrolon cage filled with water (10 cm height) at 23-25°C. ICR mice (*n*=10/group) were dropped individually into Plexiglas cylinders. Two mice were tested simultaneously for a 6 min period inside vertical Plexiglas cylinders; a nontransparent screen placed between the 2 cylinders prevented the mice from seeing each other. The total duration of immobility, after a delay of 2 min, was measured during a period of 4 min. Each mouse was considered to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. DW-treated group used as a control group of KH-RG/C or fluoxetine-treated group. EtOH-treated group used as a control group of Rg3-treated group.

Preparation and ingredient analysis of blood serum Changes in several blood biochemical parameters in the mice were measured after the FST. Blood samples were collected by cardiac puncture under ether anesthesia after the last FST. After an anesthetization, blood was withdrawn into syringes from the heart. Then, serum was prepared by centrifugation at 900×g at 4°C for 10 min. The contents of Glc, TP, LDH, CK, and BUN were determined by an autoanalyzer (Hitachi 747; Hitachi, Tokyo, Japan). AST and ALT activity were determined with an ALT/GPT kit (Sigma-Aldrich) and AST/GOT kit (Sigma-Aldrich), respectively.

Splenocytes culture The ICR mice spleen was removed aseptically and teased into a single cell suspension in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 mg/mL), and 50 μM 2-ME (Sigma-Aldrich). Red blood cells were removed by lysis with 0.14 M Tris-buffered NH_4Cl. The remaining cells were washed 3 times in the culture medium (RPMI 1640 medium). The cells were then cultured in RPMI 1640 medium at 5×10^6 cells/dish in 60×15 mm petri dishes. Cells were stimulated with KH-RG/C (0.8, 8, and 80 mg/mL), Rg3 (10 mg/mL), and EtOH for 24 hr in the absence and presence of Con A.

Macrophage cultures TG-elicited macrophages were harvested in 3 days after intraperitoneal (i.p.) injection of 2.5 mL TG to mice and isolated. Using 8 mL of HBSS,

which contained 10 U/mL heparin, performed peritoneal lavage. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in either 96-well tissue culture plates (3×10^5 cells/well) incubated for 3 hr at 37°C in an atmosphere of 5% CO_2, washed 3 times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Cytokine assay The production of IL-2, IL-4, and IL-12 was assayed using a sandwich enzyme-linked immunosorbent assay (ELISA) following the instructions of the manufacturer (26). Briefly, 96-well ELISA plates (NUNC, Ros kilde, Denmark) were coated overnight at 4°C with anti-mouse IL-2, IL-4, and IL-12 monoclonal absorbance at 1.0 mg/mL in PBS at pH 7.4. The plates were washed in PBS containing 0.05% Tween-20 and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 1 hr. After additional washes, sample or standards of IL-2, IL-4, and IL-12 were added and incubated at 37°C for 2 hr. After washing the wells, avidin-peroxidase was added, and the plates were incubated for 20 min at 37°C. Wells were again washed, and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was run on each assay plate using recombinant IL-2, IL-4, and IL-12 in serial dilutions.

Statistical analysis The results were expressed as mean± standard error mean (SEM) of independent experiments, and statistical analyses were performed by one-way analysis of variance (ANOVA) with Tukey and Duncan post-hoc test to express the difference among the groups. All statistical analysis was done using the Statistical Package for Social Sciences (SPSS) statistical software (SPSS windows version 11.5). For all tests, $p < 0.05$ was considered to indicate statistical significance.

Results and Discussion

Effect of KH-RG/C on the body weight The body weight was evaluated at 1, 3, and 10 days after oral administration of KH-RG/C (0.8, 8, and 80 mg/kg), Rg3 (10 mg/kg, active compound of KH-RG/C), or 1 g/kg EtOH (solvent of Rg3) for 10 days. Fluoxetine (200 mg/kg, positive control) was treated 1 hr before to get FST at 3 and 10 days. The KH-RG/C or fluoxetine-treated groups did not affect on the body weight. However, the Rg3- or EtOH-treated group significantly decreased the body weight compared with the DW-treated group in ICR mice for 10 days (Fig. 1). These results were in accord with the report of Lieber and DeCarli (27) and they reported that a chronic intake of EtOH decreased the rate of weight gain, dietary intakes, and induced alcohol toxicity.

Effect of KH-RG/C on the FST The 4-week-old male ICR mice were divided into 7 groups of DW, KH-RG/C (0.8, 8, and 80 mg/kg), Rg3 (10 mg/kg), or fluoxetine (200 mg/kg) to match the swimming time in each group. Dose and duration of fluoxetine administration was determined in preliminary experiments (data not shown). DW, KH-RG/C, Rg3, or EtOH was administered orally for 10 days.
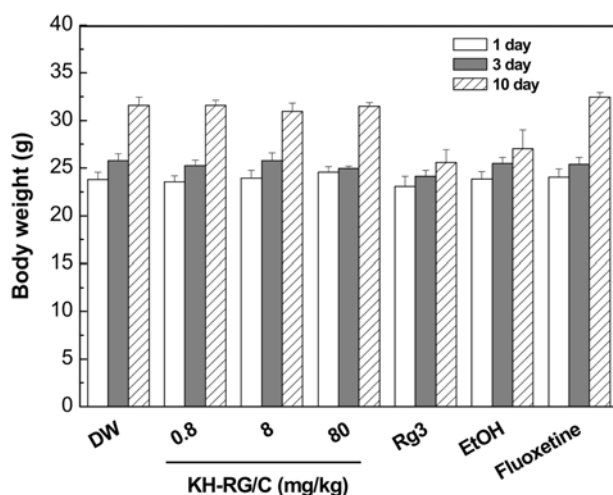

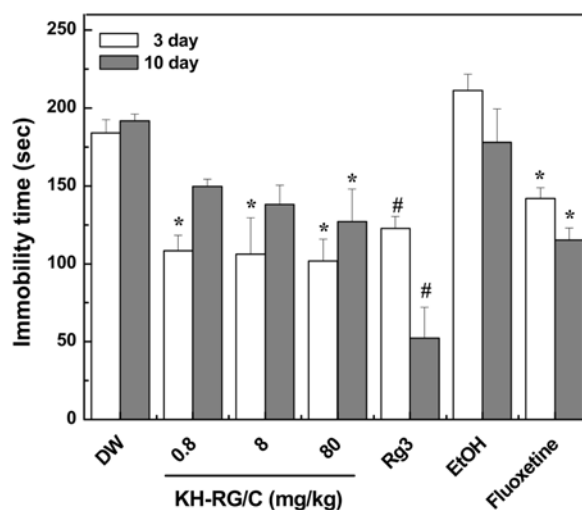**Fig. 1. Effect of KH-RG/C on the body weight.** Values are the mean±SEM.

Fig. 2. Effect of KH-RG/C on the immobility time in the FST. Values are the mean±SEM. Significant difference from the *DW-treated group and from #EtOH group at $p < 0.05$.

Especially, fluoxetine was orally administered to mice at the 3 and 10 days. The immobility time was measured at the 3 and 10 days after the administration of DW, KH-RG/C, Rg3, or fluoxetine. At the 3 and 10 days, the immobility time was significantly decreased in the administration group of the KH-RG/C, Rg3, or fluoxetine compared with the DW-treated group (Fig. 2). Previous studies reported that oral administration of CVE (100 mg/kg) and KRGP (1,000 mg/kg) decreased significantly the immobility time (28,29). But, CVE and KRGP concentration are very higher dose than that of KH-RG/C (0.8, 8, and 80 mg/kg). In addition, KH-RG/C reduced immobility time better than CVE and KRGP. Therefore, these results indicate that KH-RG/C has a synergistic effect on immobility time when CVE and KRGP was combined. Interestingly, an administration of KH-RG/C for 3 days decreased the immobility time more effective than that for 10 days; however Rg3 decreased the immobility time by time-dependant manner. Solvent of Rg3, EtOH also decreased the immobility time

Table 1. Effect of KH-RG/C on the glucose (Glc), total protein (TP), creatine kinase (CK), and lactase dehydrogenase (LDH) levels in mouse serum

Drugs (mg/kg)	Glc (mg/dL)	TP (g/dL)	CK (IU/L)	LDH (IU/L)
KH-RG/C				
DW	206.1±9.6[1]	5.4±0.1	171.5±25.3	978.2±91.8
0.8	247.0±11.7	5.3±0.1	162.7±14.2	1,242.8±167.2
8	216.8±6.8	5.2±0.1	212.8±22.6	936.5±107.9
80	296.3±11.3*	5.3±0.1	174.3±40.8	856.0±135.8
Rg3	192.4±17.1	4.9±0.1	155±19.9	1,304.0±295.4
EtOH	213.0±20.6	4.7±0.1	NA	NA
Fluoxetine	318.2±17.1*	5.4±0.1	258.9±49.3	1,235.3±60.2

[1]Values are the mean±SEM; NA, not applicable; *Significantly different from the DW-treated group ($p<0.05$).

by time-dependent manner. This result may be effect by EtOH. But, further investigation is necessary to more precisely clarify the regulatory mechanism of KH-RG/C and Rg3.

Effect of KH-RG/C on blood biomarker In general, the swimming exercise is known to induce biochemical changes in blood. The Glc, TP, LDH, and CK levels are blood biochemical changes related to fatigue. The effect of KH-RG/C on the energy source was investigated by checking the Glc and TP levels in mice serum. The Glc level was increased in 0.8 and 80 mg/kg. However it was observed significantly the increase in the 80 mg/kg KH-RG/C or fluoxetine-treated groups compared with the DW-treated group (Table 1). The decreased TP level may reflect inadequate nutrition status (30). The TP level was not changed in KH-RG/C (0.8, 8, and 80 mg/kg) or fluoxetine. In Rg3- and EtOH-treated group, TP level was decreased and it might reflect the result of EtOH, according to the reports EtOH inhibited the intake of nutrition (27). In the previous report, the Glc level was not changed in the CVE-treated group compared with the DW-treated group (28). Also, KRGP had no significant effect on the Glc level in healthy volunteer (29). However, the Glc level was significantly increased by oral administration of KH-RG/C (80 mg/kg). Probably, this result suggests that KH-RG/C may act as an energy source. The CK and LDH for the muscle damage were also evaluated. In the present study, the CK level was increased in the fluoxetine-treated group compared with the DW-treated group, but this difference was not statistically significant. The KH-RG/C-treated groups compared with the DW-treated group in the CK level did not show significant differences (Table 1). The LDH level tended to decrease by oral administration of KH-RG/C (0.8, 8, and 80 mg/kg) in a dose dependent manner (Table 1). Because 4 mice of the EtOH-treated group died, the observation of the CK and LDH in the EtOH-treated group was exccepted. These results indicate that KH-RG/C may act on the improvement of fatigue through the changes of the Glc and LDH levels. However, the further studies to clarify the detailed mechanisms involved in the anti-fatigue activities of KH-RG/C are necessary to support the present findings.

Effect of KH-RG/C on liver and renal function Liver function tests represent a broad range of normal functions

Table 2. Effect of KH-RG/C on liver and renal function[1]

Drugs (mg/kg)	AST (IU/L)	ALT (IU/L)	BUN (mg/mL)
KH-RG/C			
DW	94.8±2.6	34.3±0.9	29.4±0.6
0.8	122.3±5.2	34.2±3.0	33.0±0.6
8	108.3±10.6	28.0±0.7	33.2±1.4
80	115.8±15.1	32.4±1.9	32.8±1.0
Rg3	139.7±6.4	33.0±1.2	30.0±1.0
EtOH	NA	NA	26.4±0.6
Fluoxetine	260.1±8.7*	114.3±6.3*	23.0±0.6*

[1]AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.*Significantly different from the DW-treated group ($p<0.05$).

performed by the liver. Damage of the hepatic cells results in elevation in the AST and ALT levels. In the present study, the AST and ALT levels was evaluated except the EtOH-treated group. The AST level was increased in the KH-RG/C, Rg3, and fluoxetine-treated group compared with the DW-treated group. ALT level in the KH-RG/C-treated groups and Rg3-treated group were similar to those in the DW-treated group. However the AST and ALT levels in fluoxetine-treated group were statistically significant compared with the DW-treated group. In the previous study, CVE was not affected to the AST and ALT level in the serum (28) and Korean *P. ginseng* extract decreased in AST as the exposure time increased (5 and 16 days) (31). In the present study, KH-RG/C and Rg3 increased the AST level and these results indicate that KH-RG/C may affect liver function. However, there are lots of reports about the anti-hepatotoxic effects of CV, ginseng, KRGP, or Rg3 (31-33). Further study is needed to clarify the effects of KH-RG/C on liver function. Similarity, the BUN level was increased in the KH-RG/C-treated groups (0.8, 8, and 80 mg/kg) or Rg3-treated groups. However, this result was not significant compared with the DW-treated group (Table 2). On the other hand, this level was decreased in the EtOH- and fluoxetine-treated group compared with the DW-treated group and fluoxetine decreased the BUN level significantly. EtOH is well-known to induce hepatotoxicity. Four mice died in EtOH-treated group during the study. However, the Rg3-treated group kept alive all and did not have hepatotoxicity and nephrotoxicity. These results were
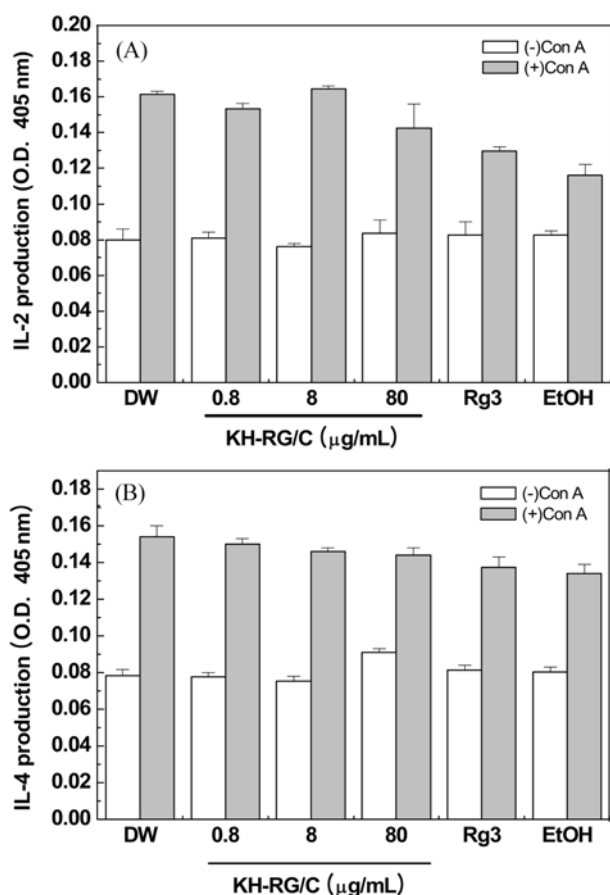
Fig. 4. Effect of KH-RG/C on IL-12 production in mouse peritoneal macrophages. The cells $(2.5 \times 10^5$ cells/well) were stimulated with KH-RG/C, Rg3, or EtOH in the absence and presence of rIFN-γ. Values are the mean$\pm$SEM of 3 independent experiments duplicate in each run.

Fig. 3. Effect of KH-RG/C on IL-2 (A) and IL-4 (B) production in mouse splenocytes. Mouse splenocytes $(5 \times 10^6$ cells/dish) were stimulated with KH-RG/C, Rg3, or EtOH in the absence and presence of Con A. The cells were incubated for 24 hr. Values are the mean$\pm$SEM of 3 independent experiments duplicate in each run.

in accordance with the literature. In the previous reports, Rg3 has neuroprotective, nephroprotective, hepatoprotective effects, and anti-cancer activites (34-36). Interestingly, the AST and ALT levels were increased by oral administration of fluoxetine, whereas the BUN level was decreased. The BUN level may be lower with liver disease, because of reduced hepatic synthesis (37). Fluoxetine-induced hepatotoxicity have been reported previously, but the mechanisms is unknown (38).

Effect of KH-RG/C on IL-2 and IL-4 production in mouse splenocytes An immune response can be broadly categorized into a cellular or humoral mediated response. The production of IL-2 and IFN-γ lead to Th1-type cellular response, while the production of IL-4 and IL-6 lead to Th2-type humoral immunity. Th1 cells play a role in inflammatory processes, macrophage activation, and delayed sensitivity, whereas Th2 cells help B cells to synthesize antibodies (39). To examine the effect of KH-RG/C on cytokine production in splenocytes, KH-RG/C or Rg3 were treated in mouse splenocytes on the absence and presence of Con A (5 mg/mL). The cells were incubated for 24 hr. The production of IL-2 and IL-4 were measured in the cell culture supernatants by ELISA (Fig. 3). KH-RG/C or Rg3
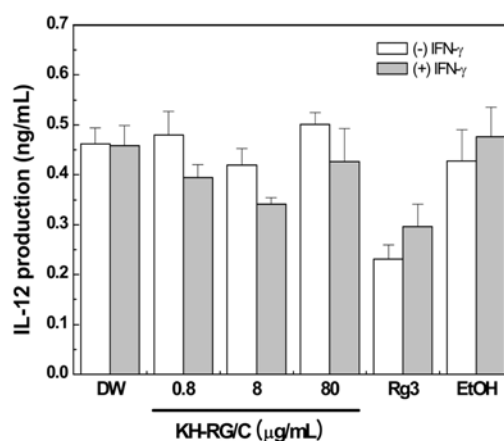
did not affect the IL-2 and IL-4 production in the absence and presence of Con A. In normal mice, CVE administration produces no effects in the levels of IFN-γ, IL-2, IL-4, and IL-10, whereas CVE administration in *Listeria monocytogenes* infected mice induced secretion of IFN-γ and IL-2. These results show that CVE is a biological response modifier (40). Therefore, to examine the effect of KH-RG/C on cytokine production in various experimental models was needed.

Effect of KH-RG/C on IL-12 production in mouse peritoneal macrophages The invasion of phagocytic cells by intracellular pathogens induces the copious production of IL-12. This cytokine selectively drives the differentiation of Th1 development and inhibit the Th2 responses (41). For observing the effect of KH-RG/C on IL-12 production in mouse peritoneal macrophages, KH-RG/C or Rg3 were treated in mouse peritoneal macrophages on the absence and presence of murine rIFN-γ $(1 \times 10^7$ U/mL). The cells were incubated for 24 hr without cell death (data not shown). KH-RG/C or Rg3 decreased IL-12 production without rIFN-γ also KH-RG/C in combination with rIFN-γ did not affect IL-12 production. However, Rg3 with rIFN-γ decreased IL-12 production compared with the EtOH-treated group on mouse peritoneal macrophages (Fig. 4). Further studies will be needed to elucidate the mechanism on IL-12 production of Rg3. In this study, KH-RG/C effected decrease of the IL-12 production or not. Therefore, it was thought that KH-RG/C had no effect on activation of splenocytes and macrophages.

These results showed that the duration of immobility in FST was shortened by administration of KH-RG/C. In general, the swimming exercise is known to induce biochemical changes in blood. In our earlier study, the immune-enhancing effect of several herbs such as pilopool and *P. ginseng* using a FST and human lymphocytes were established (16,42). These reports suggest that decrease of immobility time means promotion of immunity function. In our study, energy source, Glc level was significantly increased by KH-RG/C. But, previous studies reported that

CVE and KRGP were not changed Glc levels. In addition, KH-RG/C reduced immobility time better than CVE and KRGP. Therefore, these results indicate that KH-RG/C has a synergistic effect on immobility time. In this study, KH-RG/C had no effect on cytokine production from splenocytes and macrophages.

In conclusion, these results suggest that KH-RG/C may influence to immune regulation through increasing the physical endurance capacity without the direct activation of immune cells. Therefore, further studies will be needed to elucidate whether the immune cell activation in this experimental model is related to the immobility behavior.

Acknowledgments

References

1. Gillis CN. *Panax ginseng* pharmacology: A nitric oxide link? Biochem. Pharmacol. 54: 1-8 (1997)
2. Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: Multiple constituents and multiple actions. Biochem. Pharmacol. 58: 1685-1693 (1999)
3. Kiefer D, Pantuso T. *Panax ginseng*. Am. Fam. Physician 68: 1539-1542 (2003)
4. Ha YW, Lim SS, Ha IJ, Na YC, Seo JJ, Shin H, Son SH, Kim YS. Preparative isolation of four ginsenosides from KRGP (steam-treated *Panax ginseng* C.A. Meyer), by high-speed counter-current chromatography coupled with evaporative light scattering detection. J. Chromatogr. A 1151: 37-44 (2007)
5. Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK, Park JH. Steaming of ginseng at high temperature enhances biological activity. J. Nat. Prod. 63: 1702-1704 (2000)
6. Yoshikawa M, Morikawa T, Kashima Y, Ninomiya K, Matsuda H. Structures of new dammarane-type triterpene saponins from the flower buds of *Panax notoginseng* and hepatoprotective effects of principal ginseng saponins. J. Nat. Prod. 66: 922-927 (2003)
7. Xu TM, Xin Y, Cui MH, Jiang X, Gu LP. Inhibitory effect of ginsenoside Rg3 combined with cyclophosphamide on growth andangiogenesis of ovarian cancer. Chinese Med. J. -Peking 120: 584-588 (2007)
8. Chen JX, Peng HM, Pu SP, Guo YP. Inducement effect of ginsenoside Rg3 on apoptosis of human bladder transitional cell carcinoma cell line EJ. Zhongguo Zhong Yao Za Zhi 32: 1680-1684 (2007)
9. Chen ZJ, Cheng J, Huang YP, Han SL, Liu NX, Zhu GB, Yao JG. Effect of adjuvant chemotherapy of ginsenoside Rg3 combined with mitomycin C and tegafur in advanced gastric cancer. Zhonghua Wei Chang Wai Ke Za Zhi 10: 64-66 (2007)
10. Merchant RE, Andre CA. A review of recent clinical trials of the nutritional supplement *Chlorella pyrenoidosa* in the treatment of fibromyalgia, hypertension, and ulcerative colitis. Altern. Ther. Health M. 7: 79-91 (2001)
11. Cheng FC, Lin A, Feng JJ, Mizoguchi T, Takekoshi H, Kubota H, Kato Y, Naoki Y. Effects of chlorella on activities of protein tyrosine phosphatases, matrix metalloproteinases, caspases, cytokine release, B and T cell proliferations, and phorbol ester receptor binding. J. Med. Food 7: 146-152 (2004)
12. Shibata S, Oda K, Onodera-Masuoka N, Matsubara S, Kikuchi-Hayakawa H, Ishikawa F, Iwabuchi A, Sansawa H. Hypocholesterolemic effect of indigestible fraction of *Chlorella vulgularis* in cholesterol-fed rats. J. Nutr. Sci. Vitaminol. 47: 373-377 (2001)
13. Miyazawa Y, Murayama T, Ooya N, Wang LF, Tung YC,

Yamaguchi N. Immunomodulation by a unicellular green algae (*Chlorella pyrenoidosa*) in tumor-bearing mice. J. Ethnopharmacol. 24: 135-146 (1988)
14. Tanaka K, Koga T, Konishi F, Nakamura M, Mitsuyama M, Himeno K, Nomoto K. Augmentation of host defense by a unicellular green alfa, *Chlorella vulgaris*, to *Escherichia coli* infection. Infect. Immun. 53: 267-271 (1986)
15. Hockenberry MJ, Hooke MC, Gregurich M, McCarthy K. Carnitine plasma levels and fatigue in children/adolescents receiving cisplatin, ifosfamide, or doxorubicin. J. Pediat. Hematol. Onc. 31: 664-669 (2009)
16. Kumar A, Garg R. Protective effects of antidepressants against chronic fatigue syndrome-induced behavioral changes and biochemical alterations. Fundam. Clin. Pharmacol. 23: 89-95 (2009)
17. Ozturk N, Husnu Can Baser K, Aydin S, Ozturk Y, Calis I. Effects of *Gentiana lutea* ssp. Symphyandra on the central nervous system in mice. Phytother. Res. 7: 627-631 (2002)
18. Dorchy H. Sports and type I diabetes: Personal experience. Rev. Med. Brux. 23: A211-A217 (2002)
19. Connor TJ, Kelly JP, Leonard BE. Forced swim test-induced neurochemical endocrine, and immune changes in the rat. Pharmacol. Biochem. Be. 58: 961-967 (1997)
20. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348-2357 (1986)
21. Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A. A critical role for NF-kappa B in GATA3 expression and Th2 differentiation in allergic airway inflammation. Nat. Immunol. 2: 45-50 (2001)
22. Mullen AC, High FA, Hutchins AS, Lee HW, Villarino AV, Livingston DM, Kung AL, Yao TP, Yang SY, Reiner SL. Role of T-bet in commitment of Th1 cells before IL-12-dependent selection. Science 292: 1907-1910 (2001)
23. Luo YM, Cheng XJ, Yuan WX. Effects of ginseng root saponins and ginsenoside Rb1 on immunity in cold water swim stress mice and rats. Zhongguo Yao Li Xue Bao. 14: 401-404 (1993)
24. Cai Q, Benson MA, Talbot TJ, Devadas G, Swanson HJ, Olson JL, Kirchner JP. Acute hepatitis due to fluoxetine therapy. Mayo Clin. Proc. 74: 692-694 (1999)
25. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: A primary screening test for antidepressants. Arch. Int. Pharmacodyn. Ther. 229: 327-336 (1977)
26. Jeong HJ, Moon PD, Kim SJ, Seo JU, Kang TH, Kim JJ, Kang IC, Um JY, Kim HM, Hong SH. Activation of hypoxia-inducible factor-1 regulates human histidine decarboxylase expression. Cell. Mol. Life Sci. 66: 1309-1319 (2009)
27. Lieber CS, DeCarli LM. The feeding of ethanol in liquid diets. Alcohol Clin. Exp. Res. 10: 550-553 (1986)
28. An HJ, Choi HM, Park HS, Han JG, Lee EH, Park YS, Um JY, Hong SH, Kim HM. Oral administration of hot water extracts of *Chlorella vulgaris* increases physical stamina in mice. Ann. Nutr. Metab. 50: 380-386 (2006)
29. Yeon YS. The effect of red ginseng on stress and fatigue. MD thesis, University of Kyung Hee, Seoul, Korea (2008)
30. Koo HN, Um JY, Kim HM, Lee EH, Sung HJ, Kim IK, Jeong HJ, Hong SH. Effect of pilopool on forced swimming test in mice. Int. J. Neurosci. 118: 365-374 (2008)
31. Choi SJ, Sohn HO, Shin HJ, Hyun HC, Lee DW, Song YB, Lee SH, Lim HS, Lee CW, Moon JY. Protective effects of Korean *Panax ginseng* extracts against TCDD-induced toxicities in rat. J. Ginseng Rex. 32: 382-389 (2008)
32. Kim SJ, Baek SH, Lee JD, Kim US, Moon KH, Yim HB, Heo JW, Jeong SY. Effect of fed chlorella on the change of lipid components and enzyme activity in serum of rat by lead exposure. Korean J. Food Nutr. 14: 138-144 (2001)
33. Lee HU, Bae EA, Han MJ, Kim DH. Hepatoprotective effect of 20(*S*)-ginsenosides Rg3 and its metabolite 20(*S*)-ginsenoside Rh2 on *tert*-butyl hydroperoxide-induced liver injury. Biol. Pharm. Bull. 28: 1992-1994 (2005)
34. Kim JH, Cho SY, Lee JH, Jeong SM, Yoon IS, Lee BH, Lee JH, Pyo MK, Lee SM, Chung JM, Kim S, Rhim H, Oh JW, Nah SY. Neuroprotective effects of ginsenoside Rg3 against homocysteine-

induced excitotoxicity in rat hippocampus. Brain Res. 1136: 190-199 (2007)

35. Kang KS, Kim HY, Yamabe N, Park JH, Yokozawa T. Preventive effect of 20(*S*)-ginsenoside Rg3 against lipopolysaccharide-induced hepatic and renal injury in rats. Free Radical Res. 41: 1181-1188 (2007)

36. Yue PY, Mak NK, Cheng YK, Leung KW, Ng TB, Fan DT, Yeung HW, Wong RN. Pharmacogenomics and the Yin/Yang actions of ginseng: Anti-tumor, angiomodulating, and steroid-like activities of ginsenosides. Chin. Med. 2: 6 (2007)

37. Türkay C, Yönem O, Arikan O, Baskin E. Nitric oxide and renal functions in liver cirrhosis. Turk. J. Gastroenterol. 15: 73-76 (2004)

38. Cai Q, Benson MA, Talbot TJ, Devadas G, Swanson HJ, Olson JL, Kirchner JP. Acute hepatitis due to fluoxetine therapy. Mayo Clin. Proc. 74: 692-694 (1999)

39. Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol. Today 15: 19-26 (1994)

40. Queiroz ML, Bincoletto C, Valadares MC, Dantas DC, Santos LM. Effects of *Chlorella vulgaris* extract on cytokines production in *Listeria monocytogenes* infected mice. Immunopharm. Immunot. 24: 483-496 (2002)

41. Piccinni MP, Scaletti C, Maggi E, Romagnani S. Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. J. Neuroimmunol. 109: 30-33 (2000)

42. Shin HY, Jeong HJ, An HJ, Hong SH, Um JY, Shin TY, Kwon SJ, Jee SY, Seo BI, Shin SS, Yang DC, Kim HM. The effect of *Panax ginseng* on forced immobility time & immune function in mice. Indian J. Med. Res. 124: 199-206 (2006)