Immunostimulatory Effect of Fermented Red Ginseng in the Mouse Model

Sang-Yong Park1*, Ho-Bin Kim2*, Jeong-Hoon Kim2, Joo-Mi Lee2, Sang-Rae Kim2, Heon-Sub Shin1, and Tae-Hoo Yi1

1Department of Oriental Medicinal Materials & Processing, Kyung Hee University, Gyeonggi 446-701, Korea
2Woongjin Food Co., Ltd., Seoul 100-705, Korea

ABSTRACT: In this study, Woongjin fermented red ginseng extract (WFRG) was evaluated for its potential ability to act as an adjuvant for the immune response of mice. For the in vitro study, macrophages were treated with serial concentrations (1 μg/mL, 10 μg/mL, and 100 μg/mL) of WFRG. For in vivo studies, mice were administered different concentrations (10 mg/kg/day, 100 mg/kg/day, and 200 mg/kg/day) of WFRG orally for 21 days. In vitro, the production of nitric oxide and TNF-α by RAW 264.7 cells increased in a dose-dependent manner. In vivo, WFRG enhanced the proliferation of splenocytes induced by two mitogens (i.e., concanavalin A and lipopolysaccharide [LPS]) and increased LPS-induced production of TNF-α and IL-6, but not IL-1β. In conclusion, WFRG has the potential to modulate immune function and should be further investigated as an immunostimulatory agent.

Keywords: fermented red ginseng, immune responses, saponin, cytokine, anti-cancer

INTRODUCTION

Korean ginseng is recognized worldwide as a natural health food, and its consumption is increasing with the global trend of preference for natural products (1). There are two kinds of Korean ginsengs: white ginseng (Panax ginseng C.A. Meyer), which is processed by air-drying, and red ginseng (Panax ginseng C.A. Meyer Radix Rubra), which is produced by steaming raw ginseng at 98 ∼ 100°C for 2 ∼ 3 h. During the steaming process, ginsenosides undergo chemical changes which confer distinct physiological activities (2).

Red ginseng contains various pharmaceutical components, including ginsenosides (saponins), polyacetylenes, polyphenolic compounds, and acidic polysaccharides (3). The main ginsenosides are glycosides, which contain an aglycone with a dammarane skeleton and include protopanaxadiol-type saponins, such as ginsenosides Rb1, Rb2, Rc, and Rd and protopanaxatriol-type saponins, such as ginsenosides Re and Rg1, which constitute over 80% of the total ginsenosides (4). Ginsenosides are an important class of physiologically active compounds that are found in many herbs. Ginsenosides possess anti-inflammatory activities (5) and anti-tumor activities, including an inhibitory effect on tumor-induced angiogenesis and a protective effect against tumor invasion and metastasis (6).

However, the biological activities of ginsenosides are closely linked to their sugar chains; modification of these chains may markedly change the biological activity of a ginsenoside (7,8). Recent work indicates that, when taken orally, ginsenosides are metabolized (e.g., deglycosylated) by human intestinal bacteria. Deglycosylated ginsenoside metabolites are more readily absorbed into the bloodstream and act as active compounds (9,10). For example, the protopanaxadiol-type saponins, including Rb1 and Rb2, are metabolized to compound K by human intestinal bacteria (11,12).

The immune response in mammalian cells may be influenced by several essential nutrients that modify immune system functions. During the last decade, an upsurge of information has surfaced concerning the immunological role of plant-derived materials used in traditional medicine (13). WFRG may be able to modulate the immune system through several mechanisms, including induction of lymphocyte proliferation (14), cytokine synthesis (15,16), and increase of macrophage phagocytosis (17,18). Therefore, we used immune sys-
tem cells (i.e., macrophages) to investigate the immunological role of WFRG \textit{in vitro}.

In this study, we elucidated the influence of WFRG on the immune response by assessing WFRG's effect on splenocyte proliferative ability and cytokine production during the immune response \textit{in vitro} and \textit{in vivo} models.

\section*{MATERIALS AND METHODS}

\subsection*{Preparation and analysis of WFRG}

Red ginseng extract (RG) and Woongjin fermented red ginseng extract (WFRG) were purchased from Woong-Jin Foods R&D Center (Seoul, Korea). WFRG was produced by fermentation with \textit{Lactobacillus paracasei} subsp. \textit{paracasei} that had been isolated from red ginseng Makgeolli.

Thin layer chromatography and HPLC were used to determine the composition of RG and WFRG. HPLC conditions were as follows: a C18 (250×4.6 mm, ID 5 μm) column was used for separation, the mobile phase was acetonitrile and distilled water (gradient shown in Table 1), the sample injection volume was 20 μL, the flow rate was 1.6 mL/min, and the UV detection wavelength was 203 nm.

\subsection*{Nitric oxide detection in the RAW 264.7 monocyte macrophage cell line}

The RAW 264.7 murine macrophage cell line was obtained from Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco’s modified Eagle’s medium (PAA Laboratories GmbH, Pasching, Austria) containing 10% fetal bovine serum (PAA Laboratories GmbH) and 1% penicillin-streptomycin (PAA Laboratories GmbH) at 37°C in a 5% CO2 incubator. RAW 264.7 cells were subcultured twice a week, and the medium was changed every 2 days. For experiments, cells were cultured in the presence of various concentrations (1 μg/mL, 10 μg/mL, and 100 μg/mL) of RG and WFRG. Stock solution of RG and WFRG were prepared in DMSO (Sigma-Aldrich, St. Louis, MO, USA). Control cells were incubated for 24 h in the absence of RG and WFRG.

NO production by RAW 264.7 cells was measured on aliquots of culture medium using the Griess reagent system (Promega, Fitchburg, WI, USA). Cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated for 24 h. RG or WFRG was added, and the incubation was continued for an additional 24 h. Supernatant aliquots (50 μL) were transferred in triplicate into a 96-well plate, 50 μL of a sulfanilamide solution was added to each well, and the plates were incubated for 10 min at room temperature. Then, an N-(1-naphthyl)ethylene diamine dihydrochloride solution was added to each well, and the plates were incubated for an additional 10 min at room temperature. Absorbance was measured at 540 nm using an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell viability was measured using a standard MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay. RAW 264.7 cells were seeded in 48-well plates at density of 5×10^4 cells/well and then incubated for 24 h. Culture media was removed and replaced with media containing appropriate concentrations RG or WFRG. After incubation for 24 h, the treatment media was removed, 5 mg/mL MTT reagent (Sigma-Aldrich) was added, and the cells were incubated for 4 h. After incubation, the MTT reagent was removed, and DMSO was added to solubilize the formazan product generated by the mitochondrial activity of live cells. One hour later, the absorbance at 570 nm was measured with an ELISA microplate reader (Bio-Rad Laboratories).

Detection of TNF-α in the RAW 264.7 monocyte macrophage cell line

A TNF-α ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to assess the production of pro-inflammatory cytokines by RAW 264.7 cells. Cells were seeded in 24-well plates, various concentrations of RG and WFRG were added to each well, and the cells were incubated for 24 h. Culture media was then collected for measurement of TNF-α according to the ELISA kit manufacturer’s instructions. The absorbance was measured at 450 nm using an ELISA microplate reader (Bio-Rad Laboratories).

\subsection*{Phagocytosis assay}

A Cytoselect 96-well phagocytosis assay kit was used, according to the manufacturer’s instructions (Cell Biolabs, San Diego, CA, USA), to measure phagocytic activity. Briefly, the RAW 264.7 cells were plated at 80% confluence in 100 μL of culture medium in 96-well plates. Macrophages were plated 1 day prior to the assay and incubated overnight (37°C, 5% CO2). The RAW 264.7 cells

\begin{table}[h]
\centering
\caption{The composition of the mobile phase for HPLC analysis}
\begin{tabular}{|c|c|c|}
\hline
Time (min) & Eluent A & Eluent B \\
\hline
15 & 83 & 17 \\
25 & 80 & 20 \\
45 & 71 & 29 \\
55 & 71 & 29 \\
75 & 40 & 60 \\
80 & 40 & 60 \\
90 & 10 & 90 \\
100 & 10 & 90 \\
102 & 83 & 17 \\
107 & 83 & 17 \\
\hline
\end{tabular}
\end{table}

Eluent A, 100% D.W.; Eluent B, acetonitrile.
were plated 1 h prior to the assay (5×10^4 cells/well). Control wells were treated with 2 μM of cytochalasin D, which blocks phagocytosis by interacting with actin microfilaments, for 1 h prior to the assay. Sheep erythrocytes (Cappel Laboratore Inc., Cochranville, PA, USA) were opsonized with IgG as directed. Opsonized or non-opsonized erythrocytes (10 μL) were added to the macrophages and incubated for 1 h at 37°C. Non-phagocytosed erythrocytes were washed, lysed with a hypotonic buffer, and a proprietary erythrocyte substrate solution was added. The extent of phagocytosis was determined by measuring the absorbance at 620 nm.

Cancer cell culture
Human lung carcinoma (A549), human colorectal cancer (HT-29), and human gastric cancer (AGS-1) cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). A549, HT-29, and AGS-1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO2 at 37°C.

Treatment and laboratory animals
We purchased RG and WFRG from Woongjin Food Co. (Seoul, Korea). The animals were 24–26 g, 7-week-old, male C57BL/6 mice (DBL Co., Ltd., Chungbuk, Korea). The mice were housed in the laboratory at 22±2°C and 40–60% humidity with a 12-h light/12-hour dark cycle. The mice had free access to solid food and water and were allowed to aclimatize to the laboratory setting for 1 week before the experiment. The animals were divided into 5 groups: 1) NC: normal control group; 2) RGE: orally administered 200 mg RG/kg body weight (bw); 3-5) WFRGE: orally administered WFRG at 50 mg WFRG/kg bw, 100 mg WFRG/kg bw, or 200 mg WFRG/kg bw. For the RGE and WFRGE treatment groups, RG and WFRG were dissolved in distilled water and administered orally on alternate days for 3 weeks. All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals issued by Kyung Hee University.

Measurement of splenocyte proliferation
Splenocytes were separated using the method of Wei et al. (14). To measure splenocyte proliferation, splenocyte suspensions from each treatment group were diluted and plated onto 96-well plates (Corning Inc., Tewksbury, MA, USA). Concanavalin A (Con A; 5 μg/mL) and lipopolysaccharide (LPS) (15 μg/mL; Sigma-Aldrich) were used as mitogens. For the MTT assay, optical density was added. The extent of phagocytosis was determined by measuring the absorbance at 620 nm.

Measurement of ginsenoside concentration in mouse serum
The concentration of ginsenosides in mouse serum was analyzed using an LC-MS/MS-based technique developed by Jin et al. (7). An Agilent 1100 Series LC System (Agilent Technologies, Santa Clara, CA, USA) was used for ion detection. An API 4000 Q-trap Mass Spectrometer (Varian Medical Systems, Inc., Palo Alto, CA, USA) was used for a mass spectrometer system. An API 4000 Q-trap Mass Spectrometer System was used to for ion detection. Analysis conditions are shown in Table 2.

Table 2. LC-MS/MS analysis conditions

<table>
<thead>
<tr>
<th>Analysis method</th>
<th>LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector Column</td>
<td>API 4000 Q-trap Mass Spectrometer</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Luna C18 (2.0×100 mm ID, 3 μm)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>10 mM ammonium acetate/methanol/ acetonitrile (5/47.5/47.5, v/v/v)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>MS/MS parameter</td>
<td>10 μL</td>
</tr>
<tr>
<td>K</td>
<td>Compound K: 621.494 → 161.0</td>
</tr>
<tr>
<td>Internal standard (digoxin): 779.395 → 649.500</td>
<td></td>
</tr>
</tbody>
</table>
Immunostimulatory Effect of Fermented Red Ginseng

Fig. 1. TLC and HPLC Analysis of the WFRG. (A), TLC analysis of WFRG: S, saponin standard; RG, red ginseng; WFRG, Woongjin fermented red ginseng; (B), HPLC analysis of RG; (C), HPLC analysis of WFRG.

Statistical analysis
Data are expressed as mean±standard deviation (SD). SigmaPlot 2001 software (Systat Software Inc., San Jose, CA, USA) was used to determine differences among groups using independent two-tailed Student's t-tests. Statistical significance was set at \( P<0.05 \).

RESULTS

Changes in ginsenosides of WFRG
The concentrations of total ginsenoside and ginsenoside metabolites were greater in WFRG than in RG (Fig. 1). The concentrations of major ginsenosides, such as Rb1 and Rd, changed slightly during fermentation. In addition, ginsenoside metabolites that are easily absorbed by the small intestine (i.e., F2, CK) were significantly higher in WFRG than in RG. WFRG transformed ginsenoside Rb1 to metabolites 1 and 2, as shown in Fig. 1A. This is thought to be the case because the TLC RF values of metabolite products 1 and 2 were similar to those of ginsenoside F2 and compound K. This result suggests that Rb1 was converted by an enzyme. Reaction products 1 and 2 were analyzed using HPLC. As shown in Fig. 1B and Fig 1C, the concentrations of ginsenoside Rb1, and the concentrations of the decomposition products, ginsenosides F2 and compound K, exhibit regular changes with WFRG.

Effect of WFRG on RAW 264.7 cell viability
To determine the optimal concentration of RG and WFRG for all experiments, the effect of WFRG on the viability of RAW 264.7 cells was investigated by MTT assay. As demonstrated in Fig. 2, treatment with RG and WFRG at concentrations between 1 \( \mu \)g/mL and 100 \( \mu \)g/mL did not significantly reduce cell viability.

These findings are in agreement with previous studies that reported that WFRG promotes the proliferation of macrophages in vitro (19), and that the proliferation of macrophages enhances immune activity (20). For this reason, a concentration of 100 \( \mu \)g WFRG/mL was used in all subsequent experiments.

Effect of WFRG on NO production by RAW 264.7 cells
Following stimulation with LPS, macrophages produce NO. Excessive production of NO during inflammation increases blood vessel permeability, resulting in tissue injury, nerve damage, and edema (21). To assess the effect of RG and WFRG on NO production by RAW 264.7 cells, we used the Griess reagent method to measure the
TNF-α in the media of LPS-stimulated, WFRG-treated cells was 96% higher than that of unstimulated, untreated control cells and 34% higher than that of LPS-stimulated, untreated control cells. The concentration of TNF-α in the media of LPS-stimulated, RG-treated cells was 70% higher than that of unstimulated, untreated control cells and 18% higher than that of LPS-stimulated, untreated control cells. These results also indicate that WFRG has greater immune stimulating effects than RG.

**Effects of WFRG on phagocytosis**

Phagocytosis, the primary function of macrophages, leads to a diverse range of antimicrobial and cytotoxic responses, including respiratory burst generation, immune mediator secretion, and antigen presentation (26). In this study, we examined the effects of RG and WFRG on macrophage phagocytosis. When RAW 264.7 cells were treated with various concentrations of WFRG (1 ∼ 100 μg/mL) for 24 h, phagocytic activity was significantly increased (Fig. 5). However, RG (100 μg/mL) had no effect on the phagocytic activity of macrophages. These results suggest that WFRG and RG have different effects on phagocytosis by macrophages.

**In vitro cytotoxicity against human cancer cell lines**

An MIT assay was used to evaluate the cytotoxicity of RG and WFRG on human cancer cell lines (i.e., A549-1, HT-29, AGS-1). Cells were treated for 2 days with 1 μg/mL, 10 μg/mL, or 100 μg/mL of RG or WFRG. Cell survival was lower in cells receiving the 100 μg/mL WFRG treatment, as shown in Fig. 6. Forty-eight hours of treatment with 100 μg/mL of WFRG was associated
Immunostimulatory Effect of Fermented Red Ginseng

Effect of orally administered WFRG on splenocyte proliferation
The effects of 21 days of oral administration of RG or WFRG on splenocyte proliferation are shown in Fig 7. In the experiment in which splenocyte proliferation was induced by Con A (selectively induces T cell proliferation), the splenocyte proliferation after 21 days of treatment was 38% higher in the Con A treated, 200 ng WFRG/kg bw group than in the Con A treated, control group. Splenocyte proliferation was higher in the experiment in which splenocyte proliferation was induced by LPS activation (selectively induces B cell proliferation) than when splenocyte proliferation was stimulated by Con A, but the difference was not significant. Regardless of the induction agent used (i.e., Con A or LPS), splenocyte proliferation was highest in the WFRG-treated group.

Effects of orally administered WFRG on cytokine release by mouse peritoneal cells
It is well established that WFRG is an immunomodulatory agent that regulates immunological functions. Hence, we planned to study whether WFRG treatment could modulate cytokine (i.e., TNF-α, IL-1β, and IL-6) expression at the protein level in treated murine peritoneal macrophages. We observed that, relative to the control group, treatment with WFRG increased the release of TNF-α (Fig. 8), IL-1β (Fig. 9), and IL-6 (Fig. 10) from macrophages. The significant increase in the release of TNF-α, IL-1β, and IL-6 from macrophages might play a pivotal role in triggering an enhanced immune response. TNF-α and IL-6 protein levels were significantly higher (52% and 212%, respectively) in the 200 mg WFRG/kg bw group than in the vehicle treated group (control) or the RG treated group. However, IL-1β protein levels were not affected by treatment with RG or WFRG.

Mouse serum ginsenoside level
Fig. 11 presents RG and WFRG administration-induced changes in serum compound K concentrations. Serum compound K concentrations were higher after WFRG administration than after RG administration. This result means that the active compound of WFRG is more readily absorbed by the body.
Fig. 8. TNF-α secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD. **P<0.01; significantly different from the control (without LPS). ***P<0.001; significantly different from the LPS treated control.

Fig. 9. IL-1β secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD of quadruplicate experiments.

Fig. 10. IL-6 secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD. *P<0.05, **P<0.01; significantly different from the LPS treated control.

Fig. 11. Compound-K level in mouse serum of orally administered WFRG for 21 days. The data represents the mean±SD. ***P<0.001; significantly different from the control (vehicle treatment).

DISCUSSION

The prevalence of a variety of immune disorders has been gradually increasing in developed countries. The present results indicate that WFRG contains immunomodulators that are active in vitro and in vivo. Our results show that oral administration of WFRG stimulates immune-related cytokine release and mouse splenocyte proliferation.

LPS is present in the outer cell membrane of gram negative bacteria. LPS stimulates the release of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, by macrophages and monocytes (27). One important role of macrophages is the phagocytosis of pathogens and necrotic cellular debris (28). NO is generated by macrophages as part of the human immune response. The immune system may regulate the armamentarium of phagocytes that play a role in inflammation and immune responses with NO. NO has anti-cancer and anti-microbial effects, but excessive secretion can kill normal cells and promote inflammation. In this study, treatment of cells with WFRG suppressed the LPS-stimulated production of NO to a statistically significant extent. And, the expression of TNF-α in LPS-treated cells was increased by WFRG. Moreover, TNF-α is one of the cytokines produced by mononuclear phagocytes; it is toxic to cancer cells and has an anti-viral effect (29). In this results inference that WFRG has anti-cancer effects by regulation of phagocytes.

In the in vivo experiment described here, the immunomodulative effects of WFRG were studied by determining the impact of WFRG on mouse splenocyte proliferation. Previous studies indicate that the size or proliferation of splenocytes can be an indicator of immune function. In our ex vivo study, a 200 mg/kg bw dose of WFRG enhanced splenocyte proliferation.

We also investigated the immune-related cytokine production by mouse peritoneal macrophages. Our results, WFRG treatment of peritoneal macrophages was increased in the release of TNF-α, IL-1β, and IL-6, relative to the control group. Commonly, IL-1β is initially secreted to activate T cells, and IL-6 is then secreted and participates in B cell and T cell differentiation (30). According to this results suggested that the WFRG was...
regulated immune response by T cell and B cell differentiation. The WFRG of higher concentrations were overexpression of TNF-α, IL-1β, and IL-6. In this reason the WFRG of higher concentrations seem to have harmful effects. But, these results show that when the LPS treatment. In the USA and Asia, RG was categorized as a food and considered to be safe for the vast majority of people to take. Previous reported that 2,100 mg/kg dose of fermented red ginseng had the prevention effect of diabetes in human (31). In this study used that WFRG concentration is a low dose. Because, we suggested that there are no side effects. Moreover, WFRG suppresses tumor cell proliferation. Compound K levels were higher after WFRG administration than after RG administration. This result suggests that the active compound of WFRG is better absorbed by the body. These results suggest that WFRG has a possible anti-cancer immune response.

According to the results of our experiment, WFRG has moderated the immune response by the increased the release of immune-related cytokines. And, it was suppressed cancer cell proliferation. In addition, the WFRG treatment helped restore cytokine levels in mice. However, the effective WFRG dose may vary with physiological parameters.

In conclusion, the results of this study confirm that WFRG increases immune function, relieves inflammation, and enhances active compound absorptivity in mice. We anticipate that these findings will be used as a basis for developing functional food products that contain WFRG.

ACKNOWLEDGEMENTS

This research was supported by the Industrialization Support Program for Woongjin Food Co., Ltd.

AUTHOR DISCLOSURE STATEMENT

The technologies described in this article were transferred to the Woongjin Food Co., Ltd. for the further development of fermented red ginseng extract as an immunomodulatory supplement.

REFERENCES


