Immunostimulatory Effect of Fermented Red Ginseng in the Mouse Model

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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 *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 in *in vitro* and *in vivo* models.

MATERIALS AND METHODS

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 *Lactobacillus paracasei* subsp. *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.

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% CO_2 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)ethylenediamine 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 were 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).

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% confluency 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% CO_2). The RAW 264.7 cells

Table 1. The composition of the mobile phase for HPLC analysis

Time (min)	Mobile phase	
	Eluent A	Eluent B
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

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 Laboratoies 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% CO_2 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 acclimatize 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 measured at 540 nm using an enzyme-linked immunoassay (ELISA) reader. The proliferation index was calculated as follows:

$$\text{proliferation index} = \frac{\text{optical density in the sample well}}{\text{optical density in the control well}}$$

Peritoneal cells

After being anaesthetized, the animals were exsanguinated, and their peritoneal cavities were washed with 5 mL of sterile RPMI medium without fetal bovine serum, penicillin, or streptomycin. The resulting cell suspensions were centrifuged for 10 min at 1,000 rpm and 4°C. The resulting peritoneal cell pellet was re-suspended in 10 mL of sterile RPMI medium without glutamine, transferred to 24-well polystyrene culture plates (cell density: $1×10^9$ cells per well), and incubated for 2 h. Non-adherent cells were removed by three vigorous washes with RPMI medium without glutamine. The entire procedure was executed under aseptic conditions with sterile materials.

Detection of cytokine production by mouse peritoneal cells

To assess the production of pro-inflammatory cytokines by mouse peritoneal cells, we used ELISA kits (R&D Systems) to measure IL-1β, TNF-α, and IL-6 concentrations in cell culture supernatants. Cells were seeded into 24-well plates, treated with various concentrations of RG or WFRG, and incubated for 24 h. Culture supernatants were then collected for ELISA measurement of IL-1β, TNF-α, and IL-6 concentrations, according to the manufacturer's instructions. For all targets, the final absorbance was read at 450 nm using an ELISA microplate reader.

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) with a Phenomenex® Luna® C18 column (2.0×100 mm ID, 3 μm; Phenomenex Inc., Torrance, CA, USA) was used for chromatographic separation, and an API 4000 Q-trap Mass Spectrometry System (Varian Medical Systems, Inc., Palo Alto, CA, USA) was used to for ion detection. Analysis conditions are shown in Table 2.

Table 2. LC-MS/MS analysis conditions

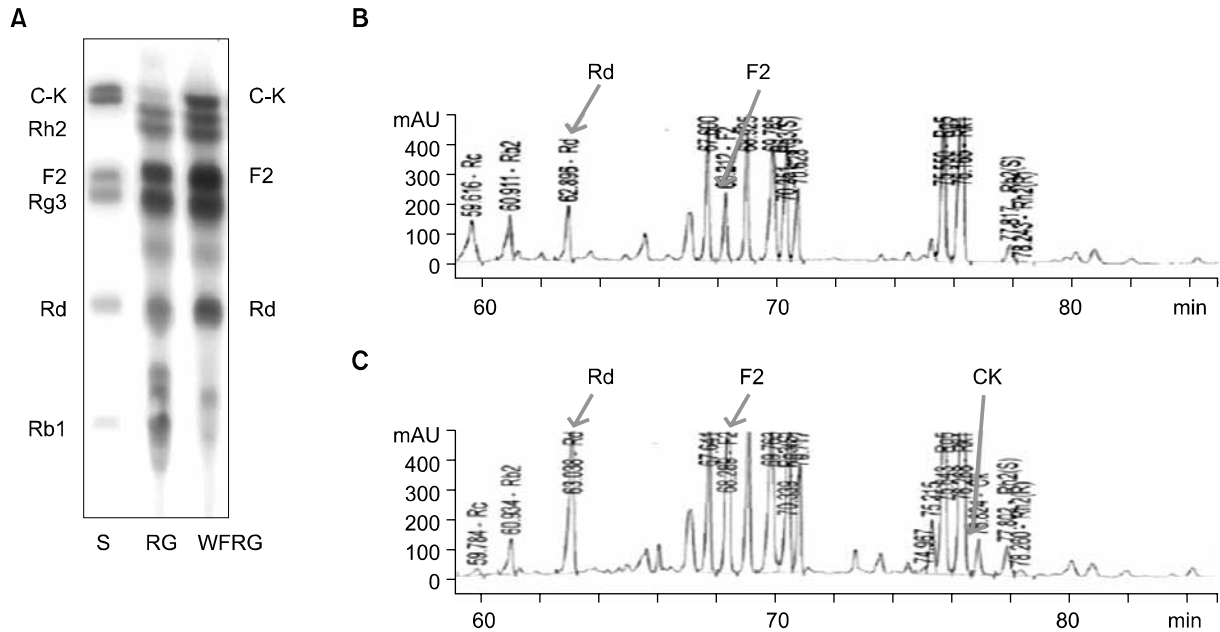| Analysis method	LC-MS/MS
Detector	API 4000 Q-trap Mass Spectrometer
Column	Luna C18 (2.0×100 mm ID, 3 μm)
Mobile phase	10 mM ammonium acetate/methanol/ acetonitrile (5/47.5/47.5, v/v/v)
Flow rate	0.5 mL/min
Injection volume	10 μL
MS/MS parameter	MRM mode
	Compound K: 621.494 → 161.0
	Internal standard (digoxin): 779.395 → 649.500

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
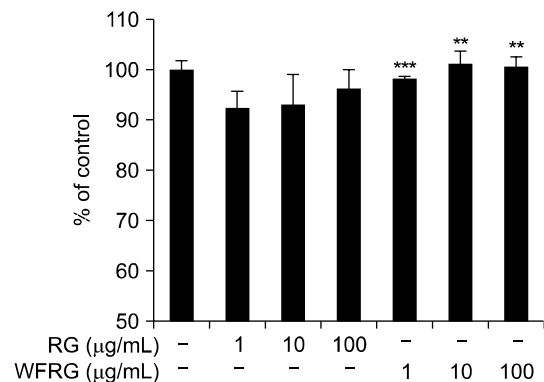

Fig. 2. The survival rate (%) of RAW 264.7 cells compared to the control as determined by MTT assay following treatment with the RG or WFRG. $^{**}P<0.01$, $^{***}P<0.001$ compared with the (−) control group.

assay. As demonstrated in Fig. 2, treatment with RG and WFRG at concentrations between 1 μg/mL and 100 μg/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 μg 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
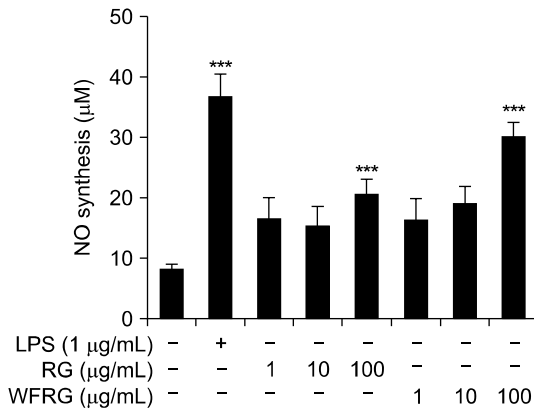
Fig. 3. Effect of RG or WFRG on NO production in RAW 264.7 cells. RAW 264.7 cells were treated with 1~100 μg/mL RG or WFRG for 24 h. Nitrite levels in the culture supernatants were measured with Griess reagent. ***P<0.001 for LPS, RG or WFRG versus the untreated control.
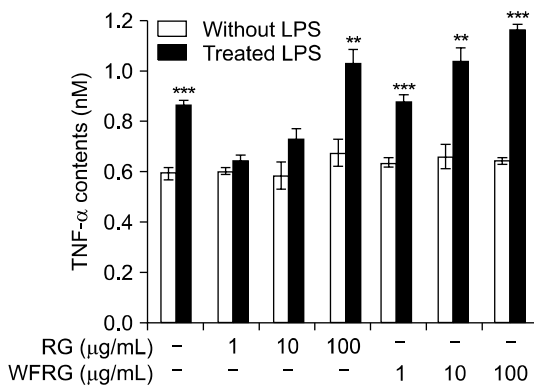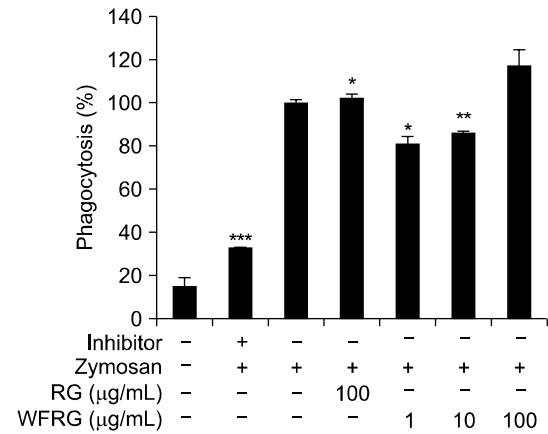

Fig. 5. Effects of RG and WFRG on phagocytosis of RAW 264.7 cells. RAW 264.7 cells were treated with RG and WFRG for 24 h. Wild type, DMEM (10% FBS); I-CON, DMEM (10% FBS)+inhibitor (2 μM / 24 h)+zymosan; P-CON, zymosan; RG, RG+zymosan; WFRG, WFRG+zymosan. Formazan formation was measured at 620 nm. The data represents the mean±SD of quadruplicate experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the P-CON.

Fig. 4. Effect of WFRG on TNF-α expression. TNF-α production was measured by ELISA (protein level). The cells were treated for 24 h. LPS (1 μg/mL) were use as positive control. Data are presented as mean±SD of three independent experiments performed in triplicate. **P<0.01, ***P<0.001 compared with the control group (without LPS).

concentration of nitrite in the culture medium. As shown in Fig. 3, LPS treatment significantly increased NO production compared to unstimulated cells. Treatment of cells with 100 μg WFRG/mL suppressed LPS-stimulated production of NO to a statistically significant extent.

Effect of WFRG on TNF-α production
Tumor necrosis factor-α (TNF-α) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulates the acute phase reaction. TNF-α is produced chiefly by activated macrophages, although it can be produced by other cell types as well (22). The primary responsibility of TNF-α is to regulate immune cells. It is able to induce the production of other cytokines, including IL-1β (23), IL-6 (24), and IL-8. These results indicate that TNF-α stimulates macrophage phagocytosis (25). As shown in Fig 4, the concentration of

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 MTT 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
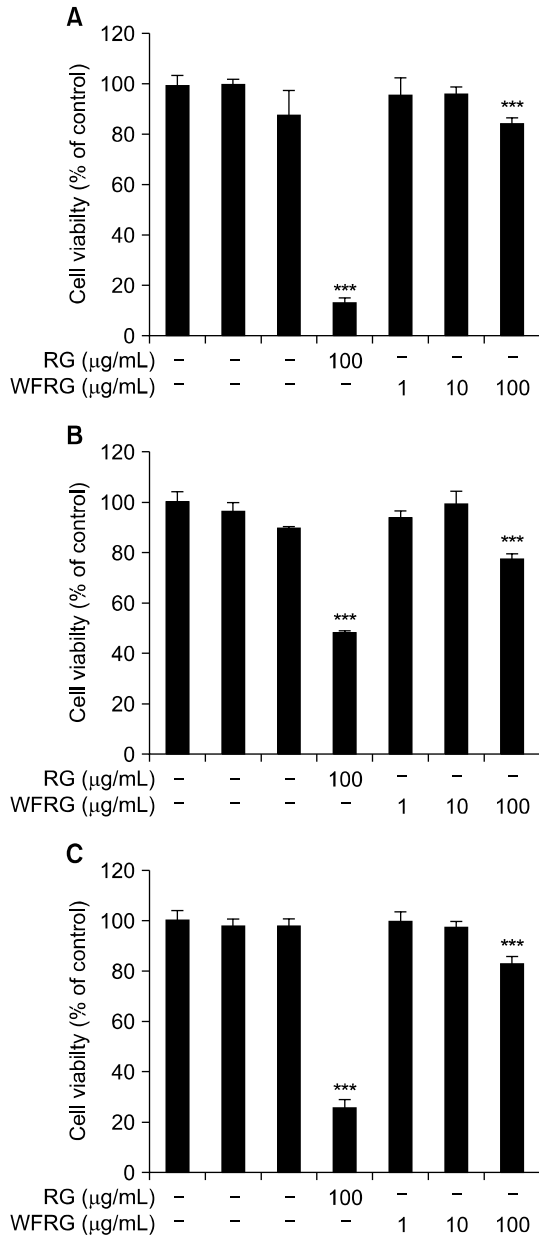
Fig. 6. Inhibition ratio of growth of (A) A549-1, (B) HT-29, (C) AGS-1 in adding different concentration fucoidan and nanoparticle of fucoidan. The data represents the mean±SD of triplicate experiments. ***$P<0.001$ compared with the (-) control group.

with growth inhibition ratios of 80%, 60%, and 75% for the A549-1, HT-29, and AGS-1 cell lines, respectively. The results of this experiment show that WFRG has better anticancer activity.

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
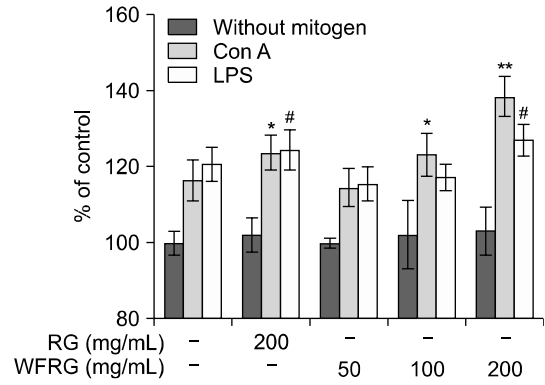

Fig. 7. Proliferation of splenocytes in mouse orally administered WFRG treated for 21 days. The data represents the mean±SD. *$P<0.05$, **$P<0.01$; significantly different from the LPS treated control. #$P<0.05$; significantly different from the CON-A treated control.

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.
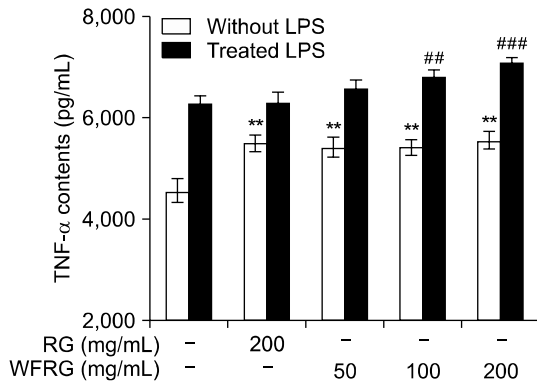
Fig. 8. TNF-α secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD. **$P<0.01$; significantly different from the control (without LPS). ##$P<0.01$, ###$P<0.001$; significantly different from the LPS treated control.
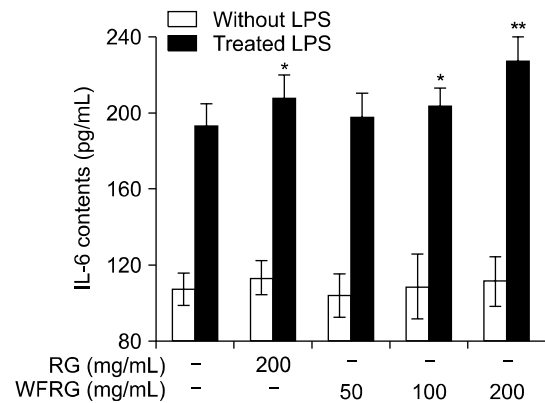

Fig. 10. IL-6 secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD. *$P<0.05$, **$P<0.01$; significantly different from the LPS treated control.
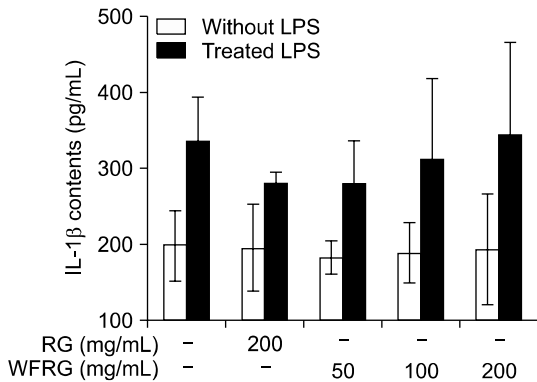

Fig. 9. IL-1β secretion in peritoneal cells of mice orally administered WFRG for 21 days. The data represents the mean±SD of quadruplicate experiments.
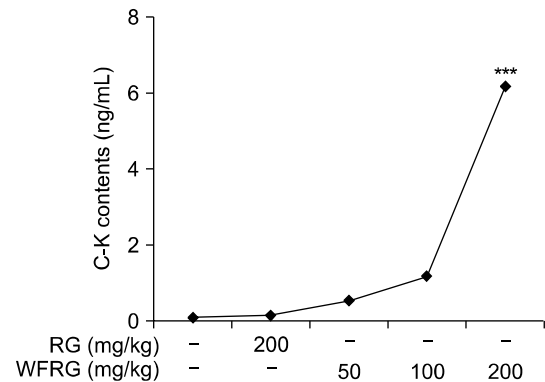

Fig. 11. Compound-K level in mouse serum of orally administered WFRG for 21 days. The data represents the mean±SD. ***$P<0.001$; significantly different from the control (vehicle treatment).

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.

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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

1. Kim CT, Kim BS, Kim MJ, Park BH, Kwon S, Maeng HY, Kwak J, Chun J, Cho YJ, Kim N, Kim CJ, Maeng JS. 2012. Draft genome sequencing of *Bacillus* sp. strain M2-6, isolated from the roots of Korean ginseng, *Panax ginseng* C.A. Meyer, after high-hydrostatic-pressure processing. *J Bacteriol* 194: 7003-7004.

2. Shehzad O, Kim HP, Kim YS. 2013. State-of-the-art separation of ginsenosides from Korean white and red ginseng by countercurrent chromatography. *Anal Bioanal Chem* 405: 4523-4530.

3. Cho E, Cho SH. 2013. Effects of Korean red ginseng extract on the prevention of atopic dermatitis and its mechanism on early lesions in a murine model. *J Ethnopharmacol* 145: 294-302.

4. Park HM, Kim SJ, Mun AR, Go HK, Kim GB, Kim SZ, Jang SI, Lee SJ, Kim JS, Kang HS. 2012. Korean red ginseng and its primary ginsenosides inhibit ethanol-induced oxidative injury by suppression of the MAPK pathway in TIB-73 cells. *J Ethnopharmacol* 141: 1071-1076.

5. Fu BD, Bi WY, He CL, Zhu W, Shen HQ, Yi PF, Wang L, Wang DC, Wei XB. 2012. Sulfated derivatives of 20(S)-ginsenoside Rh2 and their inhibitory effects on LPS-induced inflammatory cytokines and mediators. *Fitoterapia* 84: 303-307.

6. Kim YJ, Kwon HC, Ko H, Park JH, Kim HY, Yoo JH, Yang HO. 2008. Anti-tumor activity of the ginsenoside Rk1 in human hepatocellular carcinoma cells through inhibition of telomerase activity and induction of apoptosis. *Biol Pharm Bull* 31: 826-830.

7. Jin H, Seo JH, Uhm YK, Jung CY, Lee SK, Yim SV. 2012. Pharmacokinetic comparison of ginsenoside metabolite IH-901 from fermented and non-fermented ginseng in healthy Korean volunteers. *J Ethnopharmacol* 139: 664-667.

8. Niu T, Smith DL, Yang Z, Gao S, Yin T, Jiang ZH, You M, Gibbs RA, Petrosino JF, Hu M. 2013. Bioactivity and bioavailability of ginsenosides are dependent on the glycosidase activities of the A/J mouse intestinal microbiome defined by pyrosequencing. *Pharm Res* 30: 836-846.

9. Wang L, An DS, Kim SG, Jin FX, Kim SC, Lee ST, Im WT. 2012. *Ramlibacter ginsenosidimutans* sp. nov., with ginsenoside-converting activity. *J Microbiol Biotechnol* 22: 311-315.

10. Hou JG, Xue JJ, Sun MQ, Wang CY, Liu L, Zhang DL, Lee MR, Gu LJ, Wang CL, Wang YB, Zheng Y, Li W, Sung CK. 2012. Highly selective microbial transformation of major ginsenoside Rb1 to gypenoside LXXV by *Esteya vermicola* CNU120806. *J Appl Microbiol* 113: 807-814.

11. Wang CZ, Du GJ, Zhang Z, Wen XD, Calway T, Zhen Z, Musch MW, Bissonnette M, Chang EB, Yuan CS. 2012. Ginsenoside compound K, not Rb1, possesses potential chemopreventive activities in human colorectal cancer. *Int J Oncol* 40: 1970-1976.

12. Wu L, Jin Y, Yin C, Bai L. 2012. Co-transformation of *Panax* major ginsenosides Rb$_1$ and Rg$_1$ to minor ginsenosides C-K and F$_1$ by *Cladosporium cladosporioides*. *J Ind Microbiol Biotechnol* 39: 521-527.

13. Kim MC, Lee GH, Kim SJ, Chung WS, Kim SS, Ko SG, Um JY. 2012. Immune-enhancing effect of Danggwibohyeoltang, an extract from *Astragali Radix* and *Angelicae gigantis Radix*, *in vitro* and *in vivo*. *Immunopharmacol Immunotoxicol* 34: 66-73.

14. Wei X, Chen J, Su F, Su X, Hu T, Hu S. 2012. Stereospecificity of ginsenoside Rg3 in promotion of the immune response to ovalbumin in mice. *Int Immunol* 24: 465-471.

15. Cavalcanti YV, Brelaz MC, Neves JK, Ferraz JC, Pereira VR. 2012. Role of TNF-alpha, IFN-gamma, and IL-10 in the development of pulmonary tuberculosis. *Pulm Med* 2012: 745483.

16. Goyal N, Shukla G. 2013. Probiotic *Lactobacillus rhamnosus* GG modulates the mucosal immune response in *Giardia intestinalis*-infected BALB/c mice. *Dig Dis Sci* 58: 1218-1225.

17. Rodrigues AA, Clemente TM, Dos Santos MA, Machado FC, Gomes RG, Moreira HH, Cruz MC, Brígido PC, Dos Santos PC, Martins FA, Bahia D, Maricato JT, Janini LM, Reboredo EH, Mortara RA, da Silva CV. 2012. A recombinant protein

based on *Trypanosoma cruzi* P21 enhances phagocytosis. *PLoS One* 7: e51384.

18. Adolph S, Fuhrmann H, Schumann J. 2012. Unsaturated fatty acids promote the phagocytosis of *P. aeruginosa* and *R. equi* by RAW264.7 macrophages. *Curr Microbiol* 65: 649-655.

19. Kwon OK, Ahn KS, Park JW, Jang HY, Joung H, Lee HK, Oh SR. 2012. Ethanol extract of *Elaeocarpus petiolatus* inhibits lipopolysaccharide-induced inflammation in macrophage cells. *Inflammation* 35: 535-544.

20. Won DP, Lee JS, Kwon DS, Lee KE, Shin WC, Hong EK. 2011. Immunostimulating activity by polysaccharides isolated from fruiting body of *Inonotus obliquus*. *Mol Cells* 31: 165-173.

21. Qureshi AA, Guan XQ, Reis JC, Papasian CJ, Jabre S, Morrison DC, Qureshi N. 2012. Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent proteasome inhibitor. *Lipids Health Dis* 11: 76.

22. Di Rosa M, Malaguarnera G, De Gregorio C, Palumbo M, Nunnari G, Malaguarnera L. 2012. Immuno-modulatory effects of vitamin D3 in human monocyte and macrophages. *Cell Immunol* 280: 36-43.

23. Dhib-Jalbut S, Sumandeep S, Valenzuela R, Ito K, Patel P, Rametta M. 2013. Immune response during interferon beta-1b treatment in patients with multiple sclerosis who experienced relapses and those who were relapse-free in the START study. *J Neuroimmunol* 254: 131-140.

24. Hoge J, Yan I, Jänner N, Schumacher V, Chalaris A, Steinmetz OM, Engel DR, Scheller J, Rose-John S, Mittrücker HW. 2013. IL-6 controls the innate immune response against listeria monocytogenes via classical IL-6 signaling. *J Immunol* 190: 703-711.

25. Jiao L, Wan D, Zhang X, Li B, Zhao H, Liu S. 2012. Characterization and immunostimulating effects on murine peritoneal macrophages of oligosaccharide isolated from *Panax ginseng* C.A. Meyer. *J Ethnopharmacol* 144: 490-496.

26. Wu HW, Yun KM, Han DW, Xu RL, Zhao YC. 2012. Effects of glycine on phagocytosis and secretion by Kupffer cells *in vitro*. *World J Gastroenterol* 18: 2576-2581.

27. Maeda R, Ida T, Ihara H, Sakamoto T. 2012. Immunostimulatory activity of polysaccharides isolated from *Caulerpa lentillifera* on macrophage cells. *Biosci Biotechnol Biochem* 76: 501-505.

28. Kenarova B, Neychev H, Hadjiivanova C, Petkov VD. 1990. Immunomodulating activity of ginsenoside Rg_1 from *Panax ginseng*. *Jpn J Pharmacol* 54: 447-454.

29. Kwon JY, Hong SH, Park SD, Ahn SG, Yoon JH, Kwon BM, Kim SA. 2012. 2'-Benzoyloxycinnamaldehyde inhibits nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 cells via regulation of AP-1 pathway. *Eur J Pharmacol* 696: 179-186.

30. Im SA, Kim GW, Lee CK. 2003. Immunomodulatory activity of *Salicornia herbacea* L. components. *Nat Prod Sci* 9: 273-277.

31. Lee KJ, Lee SY, Ji GE. 2013. Diabetes-ameliorating effects of fermented red ginseng and causal effects on hormonal interactions: testing the hypothesis by multiple group path analysis. *J Med Food* 16: 383-395.