A Comparative Study of the Effects of Whole Red Ginseng Extract and Polysaccharide and Saponin Fractions on Influenza A (H1N1) Virus Infection

Sun Young Yin, Hyoung Jin Kim, and Hong-Jin Kim*

College of Pharmacy, Chung-Ang University; 221 Hukus-Dong, Dongjak-Gu, Seoul 156–736, South Korea.
Received February 7, 2013; accepted March 25, 2013

Total extracts of ginseng (the root of *Panax ginseng* C. A. MEYER) and saponin and polysaccharide fractions have been the main products used to investigate novel effects of ginseng over the last five decades. However, the differences if any between the pharmacological effects of total extract and saponin and polysaccharide fractions are largely unknown. In this study, we compared their effects on influenza A virus infection. Mice received total extract of Korean red ginseng (RG), and polysaccharide and saponin fractions of Korean RG, orally for 14 d prior to influenza A virus infection. Seventy eight percent of mice infected with 2× the 50% lethal dose (LD<sub>50</sub>) of virus survived when administered the polysaccharide fraction, compared to 67%, 56% and 17% when administered total extract, saponin fraction and phosphate-buffered saline (PBS), respectively. Moreover, body weight loss in mice given the polysaccharide fraction was significantly reduced while there was mild reduction in body weight loss in that receiving saponin fraction or total extract when mice were infected with 0.2× or 0.5×LD<sub>50</sub> of virus. We also confirmed that the polysaccharide fraction was most effective in reducing the accumulation of tumor necrosis factor alpha (TNF-α)/inducible nitric oxide synthase (iNOS)-producing dendritic cells (tipDCs) in the mouse lungs. Our results indicate that the polysaccharides of RG have a pronounced beneficial effect on the symptoms of influenza virus infection.

Key words red ginseng; polysaccharide; saponin; influenza virus; tumor necrosis factor alpha/inducible nitric oxide synthase-producing dendritic cell

Ginseng (the root of *Panax ginseng* C. A. MEYER) is a novel pharmacological agent which has been used as a traditional medicine in Asia. Red ginseng (RG) is *Panax ginseng* that has been boiled in water, and crushed and dried, and is one of the most widely used forms of ginseng.1) Ginseng contains biologically active materials such as saponins, polysaccharides, nitrogenous substances, amino acids, peptides, phytosterol, essential oils, organic acids, vitamins and minerals.2) It is known to improve physical functions and host resistance against infectious agents.2,3) It is one of the most widely used medicinal plants worldwide, with products commercially available in roots, tablets and capsules, liquid extracts, carbonated drinks and teas.4)

Influenza viruses are RNA viruses belonging to the Orthomyxoviridae family, and cause respiratory infectious diseases. Two large glycoproteins, hemagglutinin (HA) and neuraminidase (NA), protrude as spikes on their surface.5) Influenza viruses have no proofreading system for newly synthesized RNA,6) and their error-prone replication results in antigenic drift, allowing infection of new hosts and quick evasion of protective immunity.7,8) Therefore, vaccination only has a protective effect when the prevalent strains match the strains contained in the vaccine.9) Mutation of influenza virus allowing it to move from one species to another has often threatened human beings. The Spanish flu in 1918 caused 50 million deaths, and swine flu in 2009 was responsible for 0.2 million deaths.10,11) It is thought that the high mortality and morbidity of influenza virus infections are due to excessive immune responses causing sepsis.12,13) Therefore, measures that modulate the excessive immune responses to influenza virus infection might reduce its severity.

Many of the pharmacological actions of ginseng are due to its polysaccharides and saponins. The polysaccharide fraction, obtained from the water-soluble fraction of ginseng, has anticancer and anti-hyperlipidemic effects and immunomodulatory effects such as macrophage activation,2,14–16) while the saponin fraction, known as ginsenoside, has anticancer, antineoplastic and immunomodulatory effects.2,17) In addition, non-polysaccharide and non-saponin constituents also have pharmacological actions.17)

Total extracts, and the saponin and polysaccharide fractions of ginseng, have been used as the main products in investigations of novel functions of ginseng.2) However few studies have compared their pharmacological effects. In this study, we compared the actions of orally administered total extract, polysaccharide and saponin, and investigated their immunomodulatory effects on influenza virus infection.

MATERIALS AND METHODS

**RG Total Extract** Korean Red Ginseng Extract Gold, which is a commercial product of Korean RG extract, was obtained from Geumsan Korean Ginseng Nonghyup: (Geumsan Ginseng Agricultural Corporative Association, lot number 60611005, Geumsan, South Korea). The total extract of RG was obtained by hot water extraction of Korean RG and confirmed to have 4.5 mg of saponins (Rg1+Rb1) per gram. HPLC profile of the RG total extract for analyzing saponins is presented in Supplemental information 1. It was confirmed that RG total extract contains 8 mg of carbohydrate per gram (Supplementary information 2). Therefore, RG total extract contains 0.45% saponins (Rg1+Rb1) and 0.8% carbohydrate although data about total carbohydrate and Rg1+Rb1 contents do not reflect the contents of polysaccharide and total saponin contained in RG total extract accurately.

**Preparation of RG Polysaccharide** RG polysaccharide

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: hongjink@cau.ac.kr © 2013 The Pharmaceutical Society of Japan
was purified as described previously.\textsuperscript{19} The RG total extract (110 g) was dialyzed against distilled water (DW) for 48–72 h and clarified by centrifugation. The soluble fraction was mixed with 4 volumes of 80% ethanol and kept overnight at 4°C to precipitate the RG polysaccharides. The precipitate was collected by centrifugation and dried in a vacuum drying oven. The crude RG polysaccharide was dissolved in DW and loaded on a diethylaminoethyl cellulose (DEAE) sepharose CL-6B column (8 mL resin, GE Healthcare, U.S.A.). After the column was washed with five column volumes of DW, RG polysaccharides were successively eluted by addition of DW containing 0.25, 0.5 and 0.75 mM NaCl. The eluted RG polysaccharide was dialyzed against DW, and carbohydrate content of the eluate was analyzed by phenol–sulfuric acid method.\textsuperscript{19} It was confirmed that the polysaccharide finally recovered is almost 100% pure (Supplemental information 2).

**Preparation of RG Saponin** RG saponin was purified as described previously.\textsuperscript{20} The RG extract (10 g) was dialyzed against DW for 72 h and precipitated with 70% of saturated ammonium sulfate. The precipitate was collected by centrifugation and dialyzed against DW to remove salts. The salt-free solution was freeze-dried and extracted with 99% methanol in a water bath under reflux, and the methanol fraction was concentrated under reduced pressure. Diethyl ether was added to the concentrated fraction and it was kept overnight at 4°C to allow precipitation of the saponin. The precipitated saponin was recovered by centrifugation, dried overnight and resuspended in phosphate buffered saline (PBS). The saponin finally recovered was confirmed by thin layer chromatography (TLC) analysis (Supplemental information 3). TLC analysis was performed as described.\textsuperscript{20}

**Virus Influenza A/PR/8/34 virus (H1N1 subtype) was propagated in 11-d-old fertilized chicken eggs at 37°C for 48 h. Egg allantoic fluid was clarified by centrifugation at 682 × g for 10 min and filtered using a 0.2 μm syringe filter.\textsuperscript{21} The 50% lethal dose (LD\textsubscript{50}) of the influenza A virus stock was determined as described.\textsuperscript{22}

**Animals** Five-week-old female BALB/c mice were purchased from Orient Bio (Orient Bio Inc., South Korea) and acclimatized for 1 week. All animal experiments were performed in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals and with the Guidelines for Animal Experiments of Chung-Ang University, and all experiments were approved by the university committee for Animal Experiments.

**Measurement of Survival Rates Following Influenza A Virus Infection** Mice were divided into five groups (PBS, oseltamivir, total extract, polysaccharide, saponin), each consisting of four to six mice. PBS, oseltamivir, total extract, polysaccharide and saponin were directly given into digestive tracts of mice by oral feedings for 14 d prior to virus challenge. Two of the groups (PBS and oseltamivir) received PBS (200 μL/d) orally, and three (RG extract, RG polysaccharide and RG saponin) received the corresponding sample (250 μg/ kg/d) orally. PBS and oseltamivir were used as negative and positive control, respectively. The amounts administered were on a dry weight basis. The mice were intraperitoneally anesthetized with zoletil (50 mg/kg; Zoletil 50, Virbac S.A, France) and xylazine (Rompun, Bayer, Germany) and exposed to 50 μL of virus by intranasal instillation. Following infection the experimental groups continued to be administered the appropriate factors for 3 d, and the oseltamivir group received oseltamivir (Tamiflu, 10 mg/kg/d, Roche, Switzerland) for 7 d.\textsuperscript{23,24} The body weight of each mouse on the day before virus challenge was set at 100% and monitored for 14 d after infection.

**Measurement of Survival Rates Following Influenza A Virus Challenge** Mice received total RG extract, polysaccharide and saponin for 14 d were intranasally infected with influenza A virus (2 ×(ld\textsubscript{50})). BALF cells were collected on day 1 post infection and washed with staining buffer (1% fetal bovine serum in PBS). Thereafter they were stained with allopurinol (APC)-labeled anti-Ly6C antibody (ebioscience, U.S.A.) and phycoerythrin (PE)-labeled anti-CD11b antibody (ebioscience). The stained cells were washed with staining buffer and examined using a FACS caliber flow cytometer (Becton Dickinson, U.S.A.). Ten thousand events were acquired for scoring CD11b\textsuperscript{+} and Ly6C\textsuperscript{+} cells.

**Statistical Analysis** The statistical significance of differences between groups was determined by two-tailed Student’s t-tests. p-Values less than 0.05 were considered statistically significant.

**RESULTS**

**Comparison of Body Weight Changes Following Influenza A Virus Challenge** Mice received total RG extract, saponin and polysaccharide or PBS for 14 d prior to virus infection and were challenged with 0.1 ×, 0.2 × or 0.5 × ld\textsubscript{50} of influenza A virus as described in the legend to Fig. 1. In virus challenge with 0.1 ×, 0.2 × and 0.5 × ld\textsubscript{50} of influenza A virus, 83 to 100% of mice survived (Supplemental information 4). Data in Fig. 2 are means ± standard errors of the mean (S.E.M.) of body weights of mice that survived. We couldn’t find effects of total extract, saponin and polysaccharide for increasing the survival rates in the virus challenges because there were little differences in survival rates between mouse groups (Supplemental information 4). Meanwhile, body weight losses were not observed in the oseltamivir group while significant losses were observed in the PBS group (Fig. 2). Interestingly, there was little loss of body weight in the mice exposed to 0.1 × ld\textsubscript{50} of virus and given polysaccharide (Figs. 2A, D). Moreover, administration of polysaccharide significantly reduced the body weight losses when mice were exposed to 0.2 × and 0.5 × ld\textsubscript{50} of virus (Figs. 2B, C, E, F). There was no significant difference in ameliorating effect between total extract and saponin group although body weight loss was reduced in the mice receiving total extract or saponin (Fig. 2).

Oseltamivir group was designed to positive control group. According to our experiments, 3-d oseltamivir regimen
showed increased morbidity of mice in the lethal influenza virus infection (data not shown). In mouse experiment, 5- to 8-d regimen is required to sustain its effect on lethal influenza virus infection. Mice of oseltamivir group received oseltamivir for 7 d while those of other groups received corresponding sample for 3 d after the virus challenge in this study. Therefore, we think that the difference of ameliorating effects between oseltamivir and RG preparations (total extract, saponin and polysaccharide) may be reduced if 3-day regimen is applied to oseltamivir group.

Survivals of Mice Following Influenza A Virus Challenge Mice were challenged with 2×LD₅₀ of influenza A virus after oral administration of polysaccharide, saponin or total extract for 14 d (Fig. 1). 67% and 56% of mice given total extract and saponin survived, respectively, while 17% of those given PBS survived (Fig. 3). At the same time, 78% of the mice administered polysaccharide survived.

Recruitment of TipDCs by Mice Following Influenza A Virus Infection TipDCs are a subset of DCs described as TNF-α/iNOS-producing DCs. Higher levels of tipDCs in the lung following influenza virus infection are associated with excessive inflammatory responses and are thought to be pivotal in exacerbating the symptoms of infection. To compare the inflammatory responses of the different groups we analyzed tipDC levels by flow cytometry (Fig. 4). Figure 4A shows representative plots of flow cytometry analyses. The mean percentages of tipDCs in the PBS, oseltamivir, polysaccharide, saponin and total extract group were 25, 12, 6, 18 and 15%, respectively (Fig. 4B).

In conclusion, orally administered RG polysaccharide ameliorates the symptoms and reduces tipDC accumulation more effectively than RG extract or RG saponin following influenza A virus infection.

DISCUSSION

Total extract and polysaccharide and saponin fractions of ginseng are known to have diverse pharmacological effects and have been the main products used to investigate novel effects of ginseng. However, chemical characteristic of polysaccharide is different from that of saponin (Supplemental information 3). Moreover, commercially available RG extract contains small proportions of saponin and carbohydrate (Supplemental information 2 and 3), indicating that the chemical constituents of total extract, saponin and polysaccharide are different. Therefore, we hypothesized that pharmacological functions of RG total extract, RG polysaccharide and RG saponin are different. In this study, we compared the effect of orally administered total extract of RG, and saponin and polysaccharide fractions on influenza virus infection. RG polysaccharide was the most effective in ameliorating the symptoms of influenza virus infection, although saponin and total RG extract also had some effect (Figs. 2, 3).

Previously, many studies have been focused on the effect of saponin fraction of ginseng, and the saponin fraction has been believed to have a pivotal role on the pharmacological function of ginseng. When compared to the effect of Ginseng saponin, less attention has been paid to the effect of Ginseng polysaccharide. However, accumulated results indicate that polysaccharide fraction also has important role for the phar-
macological function of ginseng. 14–16) TipDCs are considered to have both positive and negative effects: their accumulation in the lung following influenza virus infection increases the number of virus-specific effector CD8 T cells, which are required for viral clearance, but it also enhances immunopathology. 25) Therefore, it has been assumed that appropriate control of tipDC accumulation in the lung could lessen the symptoms of infection. Recently, it was suggested that tipDC accumulation can be reduced by interleukin-10, which is known to modulate inflammatory responses.27) However it remains unclear whether the accumulation of tipDCs can be effectively regulated by pharmacological agents. Our results suggest that RG polysaccharide can prevent excessive tipDC recruitment (Fig. 4). Moreover, the mortality and morbidity of the mice decreased (Figs. 2, 3) with decreasing lung tipDC levels (Fig. 4), supporting the idea that there is correlation connection between tipDC levels and influenza-related symptoms. 25,28)

As shown in Fig. 4B, mean tipDC levels recruited in PBS, oseltamivir, polysaccharide, saponin and total extract group were 25, 12, 6, 18 and 15%, respectively. Therefore, polysaccharide group showed significantly reduced tipDC level when compared to other groups, and the level of polysaccharide group was lower than that of oseltamivir group. It has been known that oseltamivir inhibits neuraminidase activity of influenza virus and oral administration of that markedly reduces propagation of virus in the lung. 23) Therefore, it is thought that the reduced lung tipDC levels of oseltamivir group are resulted from the reduced viral activity. Meanwhile, morbidities of RG total extract, RG saponin and RG polysaccharide group are distinct from those of oseltamivir group: body weights of mice administered with RG total extract, RG saponin and RG polysaccharide significantly reduced after virus challenge while those with oseltamivir didn’t (Fig. 2). Also, previous report indicated that orally administered RG extract has no effect on the reduction of influenza virus activity. 21) Unlike the function of oseltamivir, therefore, orally administered RG components are thought to have little effects on the inhibition of influenza virus activity. Taken all together, it seems that orally administered RG polysaccharide downregulates the
inflammatory responses induced by influenza virus but not inhibit the function of virus directly while oseltamivir inhibits the function of virus directly.

In contrast with our results, Javanovski et al. found that administration of RG polysaccharide was not effective in reducing arterial stiffness whereas RG root and RG saponin were effective.29) Previously, we found that oral administration of total extract of RG prior to vaccination enhanced anti-influenza virus antibody responses, and that the mice receiving total extract of RG gave better antibody responses than those receiving RG saponin.30) In the same situation we have found that oral administration of RG polysaccharide has little or no effect on antibody responses (data not shown). Taken together our observations indicate that RG polysaccharide specifically ameliorates the symptoms of influenza virus infection. It is not surprising that ginseng has multiple actions as it contains different kinds of components. We anticipate that further comparative study of the effects of various ginseng fractions on specific diseases will provide novel insights and reveal novel effects.

**Acknowledgements** This research was supported by a Grant from Geumsan-gun Province in South Korea. We thank Man Jin Choi for help conducting the experiments.

**REFERENCES**


