

A comparison of antioxidant activity of Korean White and Red Ginsengs on H₂O₂-induced oxidative stress in HepG2 hepatoma cells

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The aim of this study was to determine and compare the preventive effect of Korean White Ginseng and Red Ginseng on oxidative stress in H₂O₂-treated HepG2 cells. The roots of ginseng were extracted with 70% methanol and partitioned with butanol to obtain saponin fractions, which have been known as bioactive constituents of ginseng. 2',7'-Dichlorofluorescein diacetate (DCF-DA) assay and malondialdehyde (MDA) content were measured for evaluating intracellular reactive oxygen species (ROS) generation. Also, mRNA expressions and activities of antioxidant enzymes were analyzed to determine the antioxidant activity of saponin or non-saponin fractions of ginsengs. According to DCF-DA assay, H₂O₂-induced MDA release and ROS generation were significantly reduced by treatment with saponin fractions of white and red ginseng roots. Also, saponin fractions increased effectively intracellular antioxidant enzyme activities including catalase, glutathione peroxidase and superoxide dismutase in H₂O₂-treated HepG2 hepatoma cells. In general, red ginseng was more effective than white ginseng for reducing oxidative stress. These results indicate that administration of red ginseng may certainly contribute relatively stronger than white ginseng to prevent from damaging liver function by oxidative stress.

Keywords: *Panax ginseng*, Oxidative stress, HepG2 hepatoma cell, Antioxidant enzyme

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radical have been recognized to be one of the factors involved in the mechanisms of a variety of diseases including cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, reperfusion injury [1]. ROS are largely generated from mitochondrial energy metabolism through oxidative phosphorylation and mostly removed by endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT). The liver is particularly

susceptible to toxic and oxidative substances because the portal vein brings blood to this organ after intestinal absorption. Hepatic aerobic metabolism under normal conditions involves a steady-state production of ROS, which are balanced by a similar rate of their consumption by antioxidants [2]. However, production of excessive oxidative stress can lead to hepatic disease (fatty liver, hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma) which is a major cause of illness and death in the world [3]. Hepatotoxicity by oxidative stress may be achieved through a direct attack of ROS on essential molecule

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with loss of their biological function and cell viability [4]. In this respect, functional foods which have antioxidant property may be effective and useful for reducing the risk of oxidative damage.

Ginseng is one of the most widely used as a natural tonic and acclaimed medicinal herbs in the world. Some researchers have demonstrated ginseng roots show anti-oxidative [5], antidiabetic [6], neuroprotective [7], anti-tumor [8], and hepatoprotective [9] effects. Two different types of ginseng (white and red) have mainly been used in commercial. Steamed and dried (red) ginseng has been recognized as being appreciably more biologically active than simply dried (white) ginseng in some notable respects.

Difference in biological effects of red and white ginsengs is due to the chemical changes of ginsenosides after the steaming process [10]. Original ginsenosides under steaming are partially converted to deglycosylated derivatives that have enhanced anti-cancer activity (e.g., ginsenoside Rg3, Rg5, and Rh2) [11]. It has been known that red ginseng is long-term preservable and has different bioactivities when compared to white ginseng. Additionally, red ginseng has been demonstrated protective effects on hepatotoxicity induced by carbon tetrachloride (CCl₄), high fat diets, and alcohol in animal models [12-14]. Due to these benefits of red ginseng, its consumption has been growing in East Asia.

Although some researchers showed that ginseng roots were considerably effective to improve hepatic damages due to toxic substances, comparative understanding between red and white ginsengs on hepatoprotective effect is not clearly elucidated. Therefore, our study examined that pharmacological differences between Korean White and Red Ginsengs on hydrogen peroxide (H₂O₂)-induced oxidative stress in HepG2 hepatoma cells.

MATERIALS AND METHODS

Preparation of saponin fractions

White ginseng (4-year) and red ginseng (6-year) roots were purchased respectively from Chungbuk Ginseng Nonghyup (Jeungpyeong, Korea) and Korea Ginseng Corporation (Daejeon, Korea) in 2011. Roots of ginseng (100 g) were extracted with 70% methanol three times at 80°C in a water bath under reflux system for 4 h. The methanol extract was filtered and concentrated *in vacuo* to dryness. The extracts of white (32.8 g) and red (35.2 g) ginsengs were suspended in water and partitioned with butanol three times to obtain crude saponin fractions. Therefore, we obtained crude saponin fractions from

roots of white ginseng and red ginseng, respectively, 8.6 g (saponin fraction of white ginseng, WGS) and 10.4 g (saponin fraction of red ginseng, RGS).

Cell culture

HepG2 hepatoma cells were obtained from American Type Culture Collections (Rockville, MD, USA) and maintained in Dulbecco's-modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. The cells were cultured in 96-well plate (1×10⁴ cells/well) containing DMEM overnight. The next day, cells were incubated with vehicle or ginseng samples at 37°C for 24 h. After addition of 10 µL MTT solution (5 mg/mL), the cells were subsequently incubated at 37°C for 4 h. The formazan crystals formed in the viable cells were dissolved in 100 µL dimethylsulfoxide after the medium of the wells was removed. The optical density was read at 570 nm using a microplate reader (Tecan, Mannedorf, Switzerland). All experiments were carried out in triplicate and repeated twice independently.

Determination of intracellular reactive oxygen species

Intracellular ROS were determined by the 2',7'-dichloro-fluorescein diacetate (DCF-DA; Sigma-aldrich, MO, USA) assay using a microplate reader. For the assay, HepG2 hepatoma cells were seeded in 96-well plate at a rate of 1×10⁴ cells per well and pretreated with saponin or non-saponin fractions of white and red ginsengs (50 µg/mL or 100 µg/mL). After 18 h, the cells were incubated with 20 µM DCF-DA in serum-free media for 45 min and were washed with Krebs Henseleit buffer (KHB). Next, the cells were treated 200 µM hydrogen peroxide in KHB for 30 min. Fluorescence was detected by excitation at 485 nm and emission at 535 nm using a Victor 3 instrument (Perkin-Elmer, Boston, MA, USA).

Assessment of lipid peroxidation

The process of lipid peroxidation is well defined to cause cellular damage. The thiobarbituric acid-reactive substance (TBARS) assay is a well-established assay for monitoring and screening lipid peroxidation. One of the major secondary products of lipid peroxidation is

malondialdehyde (MDA). MDA forms a 1:2 adduct with thiobarbituric acid, and can be measured colorimetrically or fluorometrically using an MDA equivalent standard. We detected the appearance of MDA in HepG2 cells using the OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction of antioxidant enzymes

Total RNA was extracted from HepG2 hematoma cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from total RNA (1 µg) using cDNA synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's protocol. The polymerase chain reaction (PCR) was performed using 2 µL cDNA in a total volume of 25 µL reaction. Amplification of all the genes of interest was done with the sense and anti-sense gene-specific primers at a concentration of 12.5 µL PCR Green Master Mix (Promega, Madison, WI, USA). The PCR conditions were summarized as follows. After initial denaturation at 95°C for 2 min, denaturation at 95°C for 40 s, annealing at 58°C for 50 s, extension at 72°C for 40 s, final extension at 72°C for 5 min and 30 amplification cycles were performed. Gene-specific primers were purchased from Bioneer Co. (Daejeon, Korea) and the sequences are described as follows: GPx3 (sense: 5'- cat tga tgg gga gga gta ca -3', anti-sense: 5'- cct gtt ttc caa att ggt tg -3'), GPx4 (sense: 5'- gtg agg caa gac cga agt aa -3', anti-sense: 5'- cgc aga tct tgc tga aca ta -3'), GR (sense: 5'- ccc aca ata gag gtc agt gg -3', anti-sense: 5'- caa tgt aac ctg cac caa ca -3'), CAT (sense: 5'- act tct gga gcc tac gtc ct -3', anti-sense: 5'- cgc atc ttc aac aga aag gt -3'), SOD2 (sense: 5'- tt tct gga caa acc tca gc -3', anti-sense: 5'- tgg aca agc agc aat ttg ta -3'), GAPDH (5'- ctg acc tgc cgt cta gaa aa -3', anti-sense: 5'- gag ctt gac aaa gtg gtc gt -3'). The electrophoresis analysis of PCR products was performed in 1.5% agarose gel containing 0.5 mg/mL ethidium bromide. The gels were photographed using a gel-documentation system (Kodak, Rochester, NY, USA) and the relative intensities of bands were analysed by using an NIH ImageJ 1.41o package.

Antioxidant enzyme activities

Cell homogenate was prepared in Tris-HCl buffer (0.1M; pH 7.4), centrifuged (2,500 rpm for 10 min at 4°C) to pellet the cell debris and the clear supernatant used for biochemical assays. The activity of GPx (EC.1.11.1.9) was determined by the method of Rotruck

et al. [15] using hydrogen peroxide as substrate in the presence of reduced glutathione. One unit of enzyme activity has been defined as µmol of glutathione utilized/min/mg protein. The activity of CAT (EC.1.11.1.6) was measured by the method of Sinha [16]. The principle of this method is that dichromate in acetic acid is reduced to chromic acid, when heated in the presence of H₂O₂, with the formation of perchloric acid as an unstable intermediate; the chromic acetate thus produced is measured at 570 nm. Unit of enzyme activity is expressed as µmol of H₂O₂ consumed/min/mg protein. SOD (EC 1.15.1.1) was assayed by the method of Marklund and Marklund [17]. The degree of inhibition of the auto-oxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Unit of enzyme activity is expressed as 50% inhibition of auto oxidation of pyrogallol/min/mg protein.

Statistical analysis

Each experiment was performed in triplicate and repeated three times. The data were presented as the mean±SD. Differences between the control and H₂O₂-treated groups were evaluated by an unpaired Student's

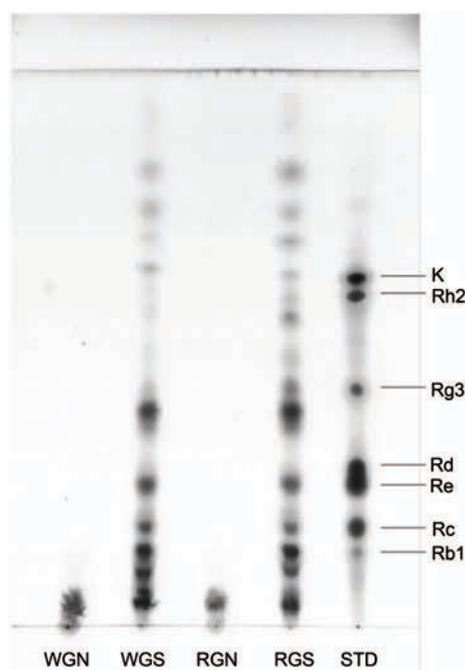


Fig. 1. Thin layer chromatography (TLC) profile of Korean White and Red Ginseng roots. Non-saponin (WGN) and saponin (WGS) fractions of white ginseng; non-saponin (RGN) and saponin (RGS) fractions of red ginseng; VitC, 10 µM vitamin C; vehicle, 0.1% dimethylsulfoxide. Rb1, Rc, Rd, Re, Rg3, Rh2, and K indicate ginseng saponins. TLC plate; silica gel 60, mobile phase; CHCl₃:MeOH:H₂O (65:35:10, v/v, lower phase), sprayed with 20% aqueous H₂SO₄ and heated at 105°C for 10 min. STD, ginsenoside standard.

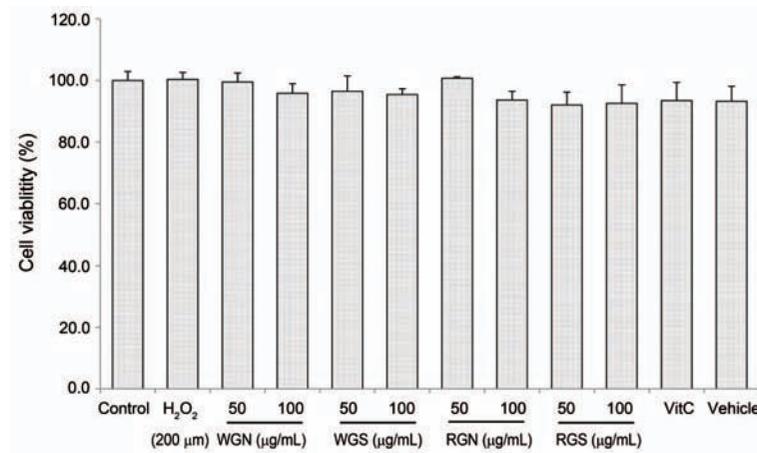


Fig. 2. Cell viability of HepG2 cells by treatment with saponin and non-saponin fractions of ginsengs. Non-saponin (WGN) and saponin (WGS) fractions of white ginseng; non-saponin (RGN) and saponin (RGS) fractions of red ginseng; VitC, 10 μM vitamin C; vehicle, 0.1% dimethylsulfoxide.

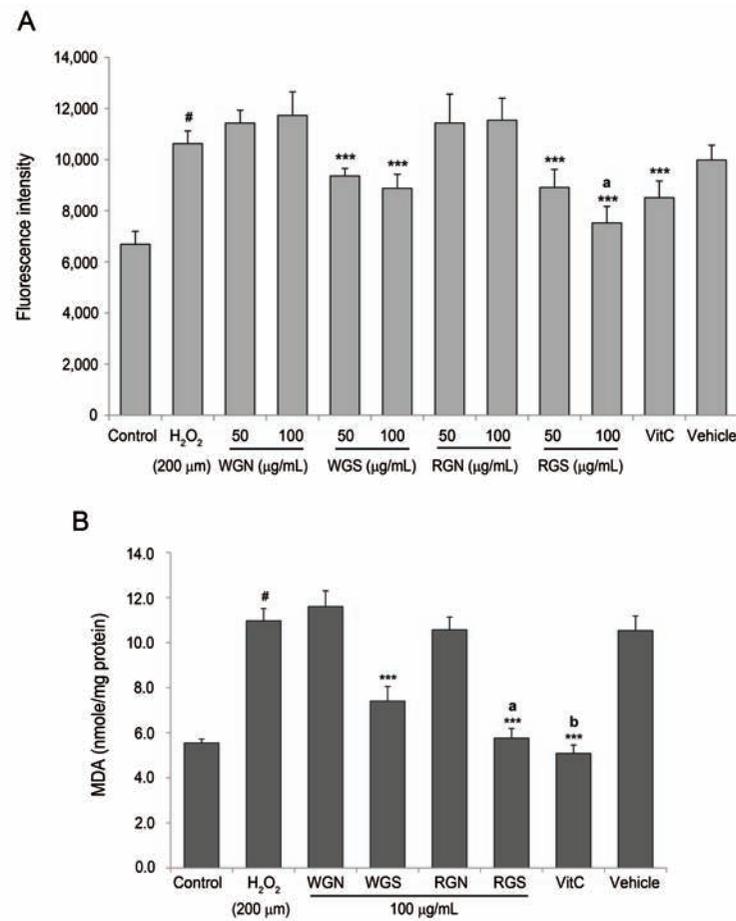


Fig. 3. Reactive oxygen species generation (A) and malondialdehyde (MDA) production (B) in H₂O₂-treated HepG2 cells. Non-saponin (WGN) and saponin (WGS) fractions of white ginseng; non-saponin (RGN) and saponin (RGS) fractions of red ginseng; VitC, 10 μM vitamin C; vehicle, 0.1% dimethylsulfoxide. [#]*p*<0.001 compared to control, ^{***}*p*<0.001 compared to 200 μM H₂O₂, ^a*p*<0.05 and ^b*p*<0.01 compared to WGS.

t-test. Statistical analyses between the H₂O₂-treated and ginseng-treated groups were performed using an ANO-

VA (analysis of variance) followed by Duncan's multiple range test for a post hoc comparison. Analyses were per-

formed using the SAS statistical software (SAS Institute, Cary, NC, USA). Difference was considered significant when *p*-values were below 0.05 (*p*<0.05).

RESULTS

Ginsenoside components

In order to determine saponin patterns of ginseng samples, we performed thin layer chromatography (TLC) with a mobile phase (CHCl₃:MeOH:D/W=65:35:10, lower phase). As a result, non-saponin fractions (non-saponin fractions of white ginseng [WGN] and red ginseng [RGN], respectively) not contained any ginsenosides on TLC profile (Fig. 1). In contrast, saponin fractions (WGS and RGS) of white and red ginseng roots possessed a variety of ginsenosides including Rb1, Rc, Re, Rg3, Rh2, and compound K. In addition, ginsenoside Rg3 and Rh2 in RGS existed much higher than WGS.

Cell viability

As shown in Fig. 2, cell viability was maintained

more than 90% after treatment of white ginseng and red ginseng at the concentration of 50 μg/mL or 100 μg/mL. Therefore, our ginseng samples not showed cell toxicity even the concentration of 100 μg/mL in H₂O₂-treated HepG2 cells.

Intracellular reactive oxygen species generation

We performed DCF-DA assay for measuring ROS production in HepG2 cells. As a result, H₂O₂-induced ROS generation was significantly increased up to 1.4-fold when compared with control group (Fig. 3A). In contrast, saponin fractions of white and red ginseng roots effectively reduced intracellular ROS production to more than 40% of H₂O₂-treated cells (*p*<0.01). In particular, 100 μg/mL RGS that reduced the ROS generation to almost control level was more potent than WGS (*p*<0.05).

Malondialdehyde production

To evaluate the possible consequences of oxidative stress, lipid peroxidation was measured as the formation of MDA in the homogenates of HepG2 cells. MDA

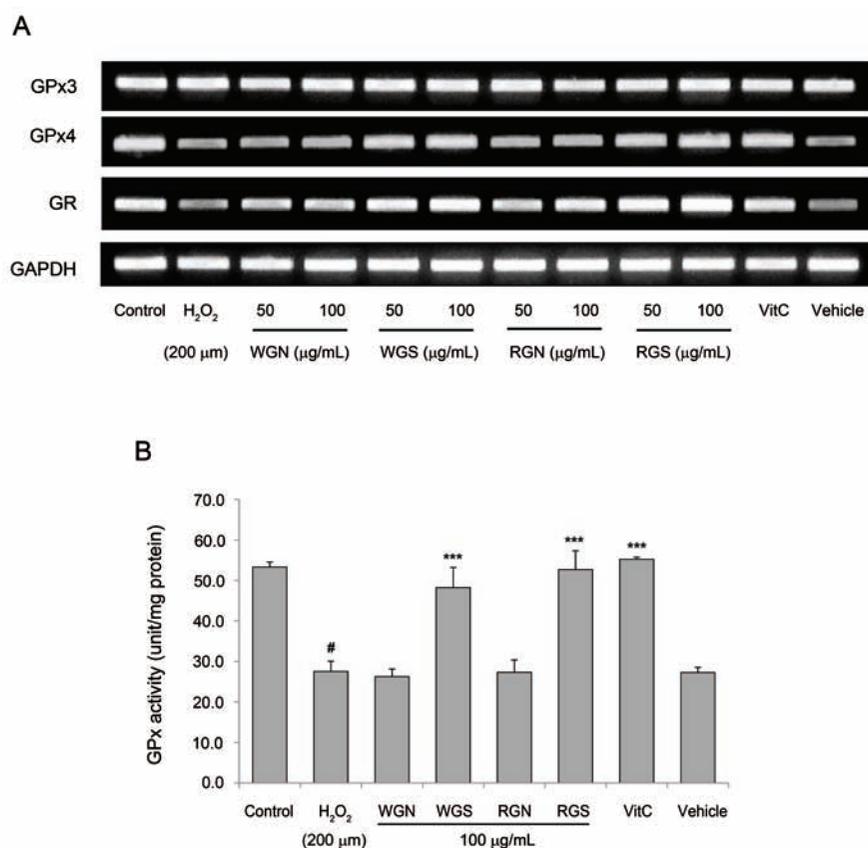


Fig. 4. Effect of saponin and non-saponin fractions of ginsengs on mRNA levels (A) and enzyme activities (B) of glutathione family in H₂O₂-treated HepG2 cells. Non-saponin (WGN) and saponin (WGS) fractions of white ginseng; non-saponin (RGN) and saponin (RGS) fractions of red ginseng; VitC, 10 μM vitamin C; vehicle, 0.1% dimethylsulfoxide. [#]*p*<0.001 compared to control, ^{***}*p*<0.001 compared to 200 μM H₂O₂. GPx, glutathione peroxidase; GR, glutathione reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

level in H_2O_2 -treated cells was significantly elevated as much as 2-fold when compared to the control ($p < 0.01$) (Fig. 3B). Consistent with ROS generation, saponin fractions of ginseng, in common with 10 μM vitamin C, definitely decreased the MDA production to almost control level at the concentration of 100 $\mu g/mL$ ($p < 0.01$). Interestingly, RGS was more effective than WGS in reducing ROS generation.

Enzyme activity of glutathione family

We evaluated whether ginseng extracts had an effect

on gene expressions or activities of glutathione-dependent enzymes. Gene expression of GPx3 was no change regardless of treatment of H_2O_2 and/or ginseng extracts, while GPx4 and GR were significantly decreased to almost 50% of control level (Fig. 4A). In contrast, saponin fractions of ginseng extracts (such as WGS and RGS) effectively improved the expressions of GPx4 and GR to more than 60% of H_2O_2 -treated group. Interestingly, gene expression of GR was significantly increased in both of saponin and non-saponin fractions of red ginseng. Consistent with RT-PCR results, enzyme activity of GPx was

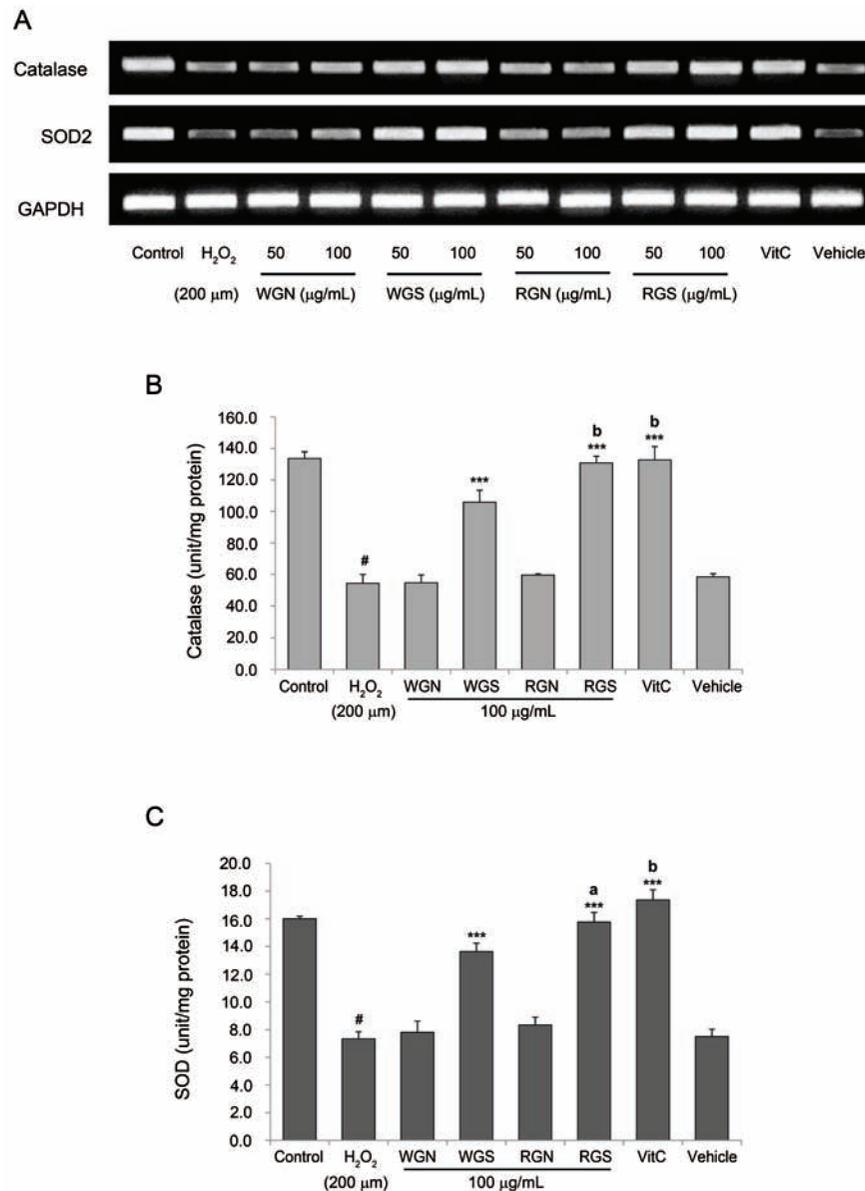


Fig. 5. Effect of saponin and non-saponin fractions of ginsengs on mRNA levels (A) and enzyme activities (B,C) of catalase and superoxide dismutase (SOD) in H_2O_2 -treated HepG2 cells. Non-saponin (WGN) and saponin (WGS) fractions of white ginseng; non-saponin (RGN) and saponin (RGS) fractions of red ginseng; VitC, 10 μM vitamin C; vehicle, 0.1% dimethylsulfoxide. [#] $p < 0.001$ compared to control, ^{***} $p < 0.001$ compared to 200 μM H_2O_2 , ^a $p < 0.05$ and ^b $p < 0.01$ compared to WGS. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

significantly decreased in H₂O₂-treated group, accounting for 27.5±2.5 unit/mg protein (Fig. 4B). In contrast, 100 µg/mL of WGS or RGS remarkably increased GPx activity to almost control level ($p<0.01$). However, significant difference between WGS and RGS was not shown.

Catalase and superoxide dismutase activities

To evaluate the protective effects against oxidative stress, SOD and CAT activities were analyzed because their functions are very closely related to scavenging of reactive oxygen radicals. CAT and SOD2 in HepG2 cells definitely reduced their gene expression by H₂O₂ (Fig. 5A). In contrast, saponin fractions of white and red ginsengs remarkably increased mRNA levels of CAT and SOD2. Meanwhile, enzyme activities of CAT and SOD significantly reduced more than 50% of control in oxidative stress by H₂O₂ exposure ($p<0.01$) (Fig. 5B, C). WGN and RGN not improved the reduced enzyme activities. On the other hand, WGS and RGS obviously showed antioxidant effect with the statistical significance through increasing enzyme activities of CAT and SOD ($p<0.01$). In addition, RGS was more effective than WGS in terms of SOD activities ($p<0.05$).

DISCUSSION

Ginseng as a good tonic has widely been used for increasing physical and mental energy at least 2000 years in East Asia. Two types of ginseng root (white and red) are mainly available in commercial. Red ginseng has been developed for long-term storage and widespread distribution. Chemical composition of ginseng is changed under steaming process for making red ginseng. Several studies have reported that white and ginseng roots show protective effects on liver damage by oxidative stress [18,19]. Additionally, red ginseng has been recognized as being appreciably more biologically active than white ginseng in some notable respects. However, the controversy with respect to scientific evidence on prominent effect of red ginseng remains to be elucidated in depth. Here we reported the differences in antioxidant activity of saponin and non-saponin fractions of Korean White and Red Ginsengs on oxidative stress by H₂O₂.

In this study, we obtained crude saponin fractions from Korean White and Red Ginseng roots using a partition chromatography with butanol. Crude saponin contents in ginseng extracts were 8.6% and 10.4%, respectively, white ginseng and red ginseng. Saponin is the major active component of ginseng and used as a marker for determining the quality of ginseng. As recently reported

by several researchers [20,21], our study showed that Korean Red Ginseng had much higher amounts of ginsenoside Rg3 and Rh2 than white ginseng.

It is well known that ginseng helps to reduce oxidative stress results from an imbalance between antioxidants and ROS, such as superoxide anions, H₂O₂, and hydroxyl radicals [22]. H₂O₂ has often been used as an experimental model to investigate the mechanism of cell injury by oxidative stress [19,23]. When HepG2 cells were treated with 200 µM H₂O₂, ROS formation was dose-dependently decreased by the pretreatment with WGS and RGS. Moreover, RGS was more potent than that of WGS at the same concentration of 100 µg/mL. These results imply that antioxidative effect of ginseng mostly results from radical scavenging activity of saponin components rather than non-saponin components.

Another target of the highly reactive free radicals is membrane lipids, commencing the self-perpetuating process of lipid peroxidation, which interrupts the function and stability of the membrane. The major reactive aldehyde resulting from the peroxidation of biological membranes is MDA which is used as an indicator of oxidative damage in tissue [24]. In our study, hepatic MDA concentrations were increased by 200 µM H₂O₂ treatment, but the elevation of lipid peroxidation were dose-dependently inhibited in both WGS and RGS. This data suggests that saponin components of ginseng may exert antioxidant effects by inhibiting hepatic lipid peroxidation

Antioxidant enzymes including GPx, GR, CAT, and SOD are regarded as the first line of the antioxidant defense system against ROS generation during oxidative stress [25]. GPx and CAT convert harmful H₂O₂ to harmless H₂O, and SOD eliminates superoxide anions by producing H₂O₂ [15,17]. In the present study, WGS and RGS showed antioxidant properties by up-regulating the gene expression of GPx3, GR, CAT, and mitochondrial SOD (SOD2) as well as by stimulating the enzyme activities. Recently, *Panax ginseng* was reported to prevent from oxidative stress with aging through enhancing intracellular antioxidant enzyme activities and decreasing vascular lipid peroxidation [26]. Our results are parallel to their study in that ginseng is helpful for reducing the ROS level and improving antioxidant enzymes activities. Meanwhile, RGS was generally more effective than WGS in reducing hepatic damage by oxidative stress. Especially, SOD activity was remarkably improved by treatment with RGS rather than WGS. Korean Red Ginseng has been reported to exhibit a variety of antioxidative and hepatoprotective action on EtOH-induced

oxidative injury in mouse hepatocytes [27]. According to several reports, ginsenoside Rg3 and Rh2, which are common in red ginseng, show antioxidant property to maintain cellular function against ROS *in vivo* as well as *in vitro* [27-29]. Therefore, relatively prominent antioxidant activity of red ginseng in our study is thought to be due to different chemical composition of ginsenosides from white ginseng. As some researchers have mentioned, the increased antioxidant activity of red ginseng may be because total phenolic compound content of ginseng was increased after heating process [5,30].

Many enzymes and proteins in antioxidant pathways highly coordinate regulation of gene expression and increase the complexity of redox signaling. Because of the widespread implications of ROS in almost all important biological functions, it is difficult to define all targets that are affected by redox signaling during ginseng treatment. Although our study has focused on several kinds of intracellular antioxidant enzyme, it is believed to be an evidential data that red ginseng is prominent at least in improving oxidative stress.

Taken together, the saponin fractions of white and red ginsengs seem to induce both gene expression and activity of the antioxidant enzymes. Our data strongly imply that saponin components of ginseng roots may participate in cellular protection not only directly as an antioxidant molecule but also indirectly as a stimulator of antioxidant enzymes. Furthermore, red ginseng rather than white ginseng may be useful as a functional biomaterial for the dietary supplement with strong antioxidant activity and hepatoprotective effect. However, our findings will require further investigation in animal experiment which will be carefully designed to optimal dose, duration, and administration method. Additionally, the extensive gene and protein expressions of a variety of enzymes should be measured to compare antioxidant activity of red ginseng with white ginseng.

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