INTRODUCTION

Ginseng (Panax ginseng C. A. Meyer) has been shown to have anti-stress effects in animal studies. However, most studies have only managed to detect altered levels of biomarkers or enzymes in blood or tissue, and the actual molecular mechanisms by which ginseng exerts these effects remain unknown. In this study, the anti-oxidative effect of Korean red ginseng (KRG) was examined in human SK-N-SH neuroblastoma cells. Incubation of SK-N-SH cells with the oxidative stressor hydrogen peroxide resulted in significant induction of cell death. In contrast, pre-treatment of cells with KRG decreased cell death significantly. To elucidate underlying mechanisms by which KRG inhibited cell death, the expression of apoptosis-related proteins was examined by Western blot analysis. KRG pre-treatment decreased the expression of the pro-apoptotic gene caspase-3, whereas it increased expression of the anti-apoptotic gene Bcl-2. Consistent with this, immunoblot analysis showed that pre-treatment of the SK-N-SH cells with KRG inhibited expression of the pro-inflammatory gene cyclooxygenase 2 (COX-2). RT-PCR analysis revealed that the repression of COX-2 expression by KRG pre-treatment occurred at the mRNA level. Taken together, our data indicate that KRG can protect against oxidative stress-induced neuronal cell death by repressing genes that mediate apoptosis and inflammation.

Keywords: Red ginseng, Apoptosis, Anti-oxidative stress, SK-N-SH cells

Anti-apoptotic Effects of Red Ginseng on Oxidative Stress Induced by Hydrogen Peroxide in SK-N-SH Cells

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Ginseng (Panax ginseng C. A. Meyer) has been used medicinally in East Asian countries. It is currently one of the most widely taken herbal products, used as an alternative-supplementary medicine with anti-cancer, anti-diabetic, and anti-inflammatory activities. The major active ingredients of ginseng are recognized as ginsenosides [1]. Korean red ginseng (KRG) is produced by steaming, and then drying, fresh ginseng. The steaming process hydrolyzes and converts ginsenosides into other types of ginsenosides, which include red ginseng-specific anti-cancer compounds (ginsenoside-Rh2, -Rb1, -Rb2, -Rs3, -Rs4, -Rg5), and the anti-metastasis and vasodilation compound ginsenoside-Rg3. The anti-oxidative and anti-cancer activities of KRG seem to be superior to those of white ginseng [1]. Stress decreases physical and mental tolerances and disturbs homeostasis, and subsequently causes diseases or aggravates existing illness. Of the many types of stresses, oxidative stress induces numerous diseases and contributes to aging and brain damage.

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Therefore, reduction of stress is important for the prevention of diseases and improvement of quality of life. Although many studies have demonstrated the reduction of stress or its physiological indicators in animals by administration of ginseng or several of its selected components, these studies are limited to describing the levels of specific factors that are altered, such as hormones and enzymes in blood or tissue. On a treadmill running test, ginseng administration significantly increased endurance time to exhaustion, and concomitantly increased the basal level of ACTH and corticosteroids [2]. Administration of ginseng extract to human subjects significantly increased catalease and superoxide dismutase levels after prolonged running and decreased malondialdehyde levels [3]. Ginseng extract administration was also shown to prevent oxidative damage to rat muscle in response to intense exercise [4]. Ginsenoside Rg1 was shown to decrease brain damage caused by the oxidative stress-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [5]. Ginseng suppressed adrenal gland weight gain and gastric ulcer formation, and was more effective against long-term stress than short-term stress. Moreover, ginseng effectively maintained levels of plasma glucose, triglycerides, cholesterol, creatine kinase, and corticosterone after exposure to stress [6]. After immobilization stress, administration of ginseng total saponin, Rg3, and Rb1 was shown to cause a decrease in polyamine component putrescine, a stress indicator [7], and administration of ginseng total saponin, ginsenoside Rb2, Rg1, and Rd decreased plasma levels of IL-6, which was induced by immobilization stress [8]. Ginseng total saponin was also shown to protect against oxidative stress induced by cyclophosphamide in mouse bone marrow cells and peripheral lymphocytes [9]. Consistent with anti-stress effects observed in the swim test [10,11] or cold stress swim test [10,11], these reports demonstrate classical anti-stress responses.

Despite these findings, a limited number of studies have been performed in brain and neuronal cells. Ginseng extract or ginsenosides have been shown to protect astrocytes grown in primary culture [12,13]. In other studies, ginseng total saponin protected human neuroblastoma cells from cyclosporine A-induced calcineurin inhibition and tau hyperphosphorylation [14], and improved depression caused by forced swim and chronic stress [15]. Ginsenoside Rb1 was shown to repress cell death induced by 6-hydroxydopamine in SH-SY5Y neuroblastoma cells [16], and protected dopaminergic cells from oxidative stress [17,18]. Other ginsenosides have been shown to be protective: for neuronal cells exposed to kainic acid- and glutamate-induced excitotoxicity [17,19], for PC12 pheochromocytoma cells exposed to glutamate-induced neurotoxicity [20], and for hippocampal neurons exposed to oxygen-glucose depleted condition [21]. However, the mechanism by which red ginseng decreases or modulates apoptosis induced by oxidative stress in brain remains unknown. Therefore, we investigated the antioxidant and anti-apoptosis activity of KRG in neuronal cells.

**MATERIALS AND METHODS**

**Mice, eukaryotic cells, antibodies, and primers**

Four week old male ICR mice were purchased from Orient (Seoul, Korea), and kept for 1 week in the animal room at the College of Pharmacy, Sungkyunkwan University, for adaptation. Human neuroblastoma SK-N-SH cells (HTB-11; American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 media (Lonza, Walkersville, MD, USA) containing 10% fetal bovine serum, 1% 10,000 U penicillin/mL, 10,000 μg streptomycin/mL, 1 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarbonate, 2 mM L-glutamine at 37°C, 5% CO₂. Prior to experiments, cells were seeded on Petri-dishes and incubated for more than 12 hours. Antibodies raised against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Caspase-3 (Cell Signaling Technology, Danvers, MA, USA), and NF-κB p50 (Upstate Biotechnology, Lake Placid, NY, USA) were purchased.

**Red ginseng extract treatment**

Red ginseng extract (KRG extract from Korea Ginseng Corporation, Daejeon, Korea) was diluted with sterile phosphate buffered saline (PBS) to make 10 mg/mL of stock solution. Prior to experiment, the KRG stock was further diluted to 1 mg/mL with RPMI 1640 media and filtered through 0.22 μm filter (Nalgene, Rochester, NY, USA).

**MTT assay**

Cell viability was determined by the MTT assay as described [22]. Briefly, 1x10⁴ SK-N-SH cells in 100 μL of RPMI 1640 media were cultured in 96 well plates overnight. After overnight incubation, the cells were treated with 1 mg/mL of KRG (for 48 h) (cells in RPMI 1640 media alone were used as a control). After treatment with H₂O₂ (0.5 mM for 1, 2 h), cells were washed with PBS and incubated with 100 μL of RPMI 1640...
and 25 μL of MTT solution (2 mg/mL) followed by incubation at 37°C, 5% CO2 for 4 hours. The supernatant was removed and 150 μL of dimethyl sulfoxide (DMSO) was added into the well. Subsequently, purple formazan was dissolved completely in DMSO for 15 minutes, and optical density at 540 nm was determined by ELISA reader. Three samples were used for each condition.

**Western blot analysis**

Cells treated with KRG and H2O2 were washed with PBS, re-suspended in 1 mL of PBS, and harvested by centrifugation at 12,000 rpm, 4°C for 15 minutes. The cell pellet was re-suspended in 200 μL of lysis buffer (Tris 0.6 g, NP-40 1 mL, NaCl 0.88 g, sodium deoxycholate 0.5 g, sodium dodecyl sulfate (SDS) 0.01 g/100 mL, and 20 μL of 100 mM phenylmethylsulphonyl fluoride) and subjected to sonication. The sonicated sample was centrifuged at 12,000 rpm, 4°C for 15 minutes, and supernatant was harvested for protein determination by the Bradford assay. For Western blot analysis, 30-40 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis on 10% or 15% gels. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). To block non-specific binding of proteins to membrane, the membrane was treated with blocking buffer (5% skim milk in 20 mM Tris 0.6 g, NaCl 0.88 g, NP-40 1 mL, and 20 μL of 100 mM phenylmethylsulphonyl fluoride) and incubated with secondary antibody (HRP-conjugated anti-IgG antibody) for 1 h at room temperature. Subsequently, proteins were incubated with primary antibody for >5 h with shaking, followed by washing with TTBS three times. Subsequently, proteins were incubated with secondary antibody (HRP-conjugated anti-IgG antibody) for 1 hour at room temperature. The membrane was washed with TTBS three times, and incubated with secondary antibody (HRP-conjugated anti-IgG antibody) for 1 hour at room temperature. Subsequently, the membrane was washed with TTBS three times, and exposed to detection reagent (TMB; Sigma-Aldrich, St Louis, MO, USA); the amount of each protein was quantitated by the band intensity.

**mRNA level determination**

To isolate total RNA, 1.5×10⁶ SK-N-SH cells were plated in a 60 m culture dish and treated with KRG and H2O2. After treatment, the cells were washed with PBS and 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to dissolve the cells. Subsequently, total RNA was isolated according to the manufacturer’s suggestion. The amount of isolated RNA was determined by UV spectrophotometric absorbance at 260 nm. One μg of isolated RNA was converted to cDNA using the M-MLV Reverse Transcriptase kit (ReGene Biotech, Cheongwon, Korea) according to the manufacturer’s suggestion. **PCR was performed using a thermal cycler (GeneAmp PCR system 2700; Applied Biosystems, Foster City, CA, USA), with β-actin serving as a control.** PCR was performed for 35 cycles with pre-denaturation at 95°C for 10 minutes, and denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 75°C for 30 seconds followed by preservation at 4°C.

**Agarose gel electrophoresis**

To analyze nucleic acid, electrophoresis was performed using 1% agarose gel with 1 Kb Plus Marker (Bioneer, Daejeon, Korea) as a molecular weight marker.

**Statistical analysis**

The results are expressed as the mean±standard deviation. The statistical significance of differences between two groups was assessed using the Dunnett’s t-test in the ANOVA program. Probability values of less than 0.05 were considered significant (*p<0.05, **p< 0.01, ***p<0.001).

**RESULTS AND DISCUSSION**

**Effect of KRG on cell viability**

The effect of KRG on the viability of neuronal cells has not been previously reported. We therefore tested various concentrations of KRG on SK-N-SH cells to determine if a certain concentration would affect cell viability. Incubation of various concentrations of KRG with SK-N-SH cells for 24 hours revealed that cell viability was not affected by <1 mg/mL of KRG, but was decreased by >5 mg/mL of KRG (Fig. 1).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’-3’</th>
<th>Size (b.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2 (RT-PCR)</td>
<td>Forward: act cac ctc tgt tgt gtc att c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: ttt gat tag tac tgt agg gtt gat aat g</td>
<td>582</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: tgg aat cct gtg gca ttc atg aaa c</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Reverse: taa aac gca gca ctc tca tca cgt g</td>
<td></td>
</tr>
</tbody>
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COX-2, cyclooxygenase 2.
Therefore, 1 mg/mL of KRG was used in subsequent experiments.

**Anti-oxidative effect of KRG**

Due to the ability of KRG to scavenge free radicals and thereby potentially interfere with the oxidative stress effect of H$_2$O$_2$, cells pre-treated with KRG were washed with new media prior to addition of hydrogen peroxide. Moreover, a preliminary experiment showed that a 48 hour pre-treatment with KRG was more protective than 24 hour pre-treatment against H$_2$O$_2$ induced damage. The ability of KRG to modulate H$_2$O$_2$-mediated toxicity was examined. When SK-N-SH cells were exposed to 0.5 mM H$_2$O$_2$ alone, cell viability was decreased, as compared to the untreated control (Fig. 2). In contrast, when the cells were pre-treated for 48 hours with 1 mg/mL KRG, the viability of the cells was significantly higher than the non-pre-treated control group (35% and 28%, after 1 hour and 2 hours of exposure to H$_2$O$_2$, respectively) (Fig. 2).

**Expression of caspase-3 and Bcl-2 genes after oxidative stress**

The increase in SK-N-SH cell viability by pre-treatment with KRG before exposure to H$_2$O$_2$ suggests that KRG might modulate expression of cell death-related genes. To examine this possibility, expression levels of the pro-apoptotic protein caspase-3, the anti-apoptotic protein Bcl-2, and inflammation-related protein NF-κB were determined by Western blot analysis. Results showed that after exposure to H$_2$O$_2$, expression levels of caspase-3 were increased by 27.6%, and NF-κB and Bcl-2 genes were increased by 1.75% and 1.26%, respectively, compared to untreated SK-N-SH cells. However, pre-treatment with KRG resulted in a 14.4% decrease in caspase-3 expression, even after exposure to oxidative stress; NF-κB and Bcl-2 levels were induced by 33.57% and 60.8%, respectively (Fig. 3). To confirm inhibition of caspase-3 expression by KRG, the experiment was repeated twice and the same results were observed each time (data not shown).

**NF-κB gene expression after oxidative stress**

To confirm the anti-oxidative stress effect of KRG, time-dependent modulation of NF-κB protein expression by KRG was determined by Western blot. After exposure of SK-N-SH cells to 0.5 mM H$_2$O$_2$, for 1 hour or 2 hours, expression of NF-κB protein was increased by 79% or 169.8%, respectively, compared to untreated SK-N-SH cells. However, pre-treatment with 1 mg/mL of KRG for 48 hours limited NF-κB induction to 23.4% or 31.2% at the 1 hour and 2 hours points, respectively (Fig. 4). These results demonstrate that pre-treatment with KRG significantly repressed (by three-fold) the induction of NF-κB expression by exposure to oxidative stress (Fig. 4). It must be noted that our Western blot analysis demonstrates changes in NF-κB expression levels, not NF-κB activity. Further experiments are required to determine how KRG treatment induces NF-κB levels.

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**Fig. 1.** Effects of Korean red ginseng (KRG) on cytotoxicity of SK-N-SH cells. SK-N-SH cells (5x10$^4$ cells) were pre-treated with KRG extract (0.01, 0.1, 0.5, 1, and 5 mg/mL) for 24 hours. Cytotoxicity of the SK-N-SH cells was determined by MTT assay. **p<0.01; O.D, optical density; RG, red ginseng.

**Fig. 2.** Effects of Korean red ginseng (KRG) pre-treatment on the viability of H$_2$O$_2$-exposed SK-N-SH cells. SK-N-SH cells (5x10$^4$ cells) were pre-treated with 1 mg/mL KRG extract for 48 hours; cells were then washed with fresh media and exposed to 0.5 mM H$_2$O$_2$ for 1 or 2 hours. Cytotoxicity was determined using the MTT assay. *p<0.05; ***p<0.001.**
Effect of KRG on cyclooxygenase 2 gene expression after oxidative stress

The inflammatory response to stress exposure can affect behavior, mood, memory, and cell survival [23]. Moreover, cells exposed to stress activate neutrophils and subsequently secrete myeloperoxidase, which is responsible for protein oxidation [24]. To determine whether the increased resistance to oxidative stress by KRG is due to repression of the cyclooxygenase (COX-2) gene, COX-2 mRNA levels were examined by RT-PCR. Results showed that COX-2 gene expression was not affected by KRG treatment alone (Fig. 5A). COX-2 expression was induced after 30 minutes of exposure to H$_2$O$_2$, and remained induced over a 2 hour incubation with H$_2$O$_2$ (Fig. 5B). In contrast, KRG pre-treatment significantly repressed this induction of COX-2; levels of COX-2 mRNA were 60% and 70% less in the co-treatment groups (at 1 hour and 2 hours, respectively), compared to cells that were exposed to H$_2$O$_2$ alone (Fig. 5B), demonstrating that KRG did not affect expression of COX-2 under normal conditions, but repressed expression only when the cells are exposed to oxidative stress.

Oxidative stress is associated with various diseases as well as aging [25]. Among the organs of the body, the brain contains large amounts of oxidizable substrates such as neurotransmitters and polyunsaturated fatty acids; thus, the brain is one of the organs most sensitive to oxidative stress [26]. The brain consumes oxygen constantly to catalyze metabolic processes that generate large amounts of energy; these processes generate reactive oxygen species, which can cause oxidative stress and concomitant neuronal damage and cell death [26].
Therefore, prevention of oxidative stress in the brain is important for increasing quality of life and longevity. However, the mechanisms underlying neuronal cell death have not been clearly elucidated.

Stress causes production of reactive oxygen species, which subsequently induces cell death. A previous study has shown that cold swim stress induced an increase in serum lipid peroxide and nitrate levels; these changes led to disruption of anti-oxidative responses and induction of NO production and neutrophil infiltration, and resulted in vacuolization and cell death in the rat liver [27]. Moreover, as the immobilization stress time was lengthened, expression levels of stress/cell death-related and muscle-specific genes were induced [28], and chronic immobilization stress induced cell death in rat testicular germ cells [29]. It has also been shown that once oxidative stress is induced, the balance of anti-oxidative responses is disrupted, and this imbalance leads to DNA damage, an increase in calcium and iron release from cells, damage of ion transport in membranes, and lipid peroxidation; the overall result is severely abnormal metabolism [30].

Our results show that oxidative stress can increase cell death and induce expression of pro-inflammatory genes. We demonstrate that pre-treatment of neuronal cells with KRG can reverse this process, probably by decreasing the expression of pro-inflammatory and pro-inflammatory genes (caspase-3 and COX-2, respectively) and increasing the expression of anti-inflammatory genes such as Bcl-2. However, how and why NF-κB expression is induced by KRG should be investigated further. These studies support the importance of further investigation into how KRG modulates signaling pathways that promote apoptosis and/or anti-inflammatory activity.

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REFERENCES

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