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Article

Suppressive Effect of Aqueous Extract of Red-Ginseng on the Herbicide-induced DNA Damage and Hemolysis

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The effects of aqueous extracts of red ginseng on the damage of DNA and erythrocyte by herbicides were evaluated using comet assay and hemolysis assay. Notably, the oxidative DNA damage and erythrocyte hemolysis by 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) were significantly suppressed by red ginseng treatment. Moreover, red ginseng could suppress significantly paraquat-induced oxidative DNA damage and hemolysis. These suppressive effects of red ginseng on the herbicide-induced damages might be due to the antioxidant components.

Key words: 2,4-D, paraquat, red ginseng, 2,4,5-T

Phenoxyherbicide 2,4-D (2,4-dichlorophenoxyacetic acid) has been used in agriculture for the control of aquatic vegetation. The oral LD_{50} of 2,4-D ranges from 375 to 666 mg/kg in the rat; 370 mg/kg in the mouse; and 320 to 1,000 mg/kg in the guinea pig, showing that 2,4-D is only moderately toxic. Several studies suggested an association of 2,4-D exposure with cancer, while other studies all negative. There remains considerable controversy about the carcinogenic effects of 2,4-D in humans [Ibrahim *et al.*, 1991], while same conclusions have been drawn for 2,4-D's potential to cause adverse effects on reproduction, teratogenic effects [Tuschl and Schwab, 2003] and mutagenic effects [Zetterberg, 1978].

Phenoxyherbicide 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) itself is also moderately toxic, with oral LD_{50} of 389 mg/kg in mouse and 500 mg/kg in rat. However, 2,4,5-T was found to contain the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is regarded as one of the most toxic compounds known to human. Thus, the EPA terminated all uses of 2,4,5-T in 1985, and 2,4,5-T has largely been replaced by dicamba and triclopyr. Apart from agricultural uses, 2,4,5-T was a major ingredient of 'Agent Orange', a herbicide blend used by the U.S. military in Vietnam between January 1965 and April 1970 as a defoliant. 'Agent Orange' was equal parts of 2,4,5-T and 2,4-D, and most diseases associated with the use of 'Agent Orange' were found to be associated with a contaminant TCDD in the 2,4,5-T component of the defoliant. According to Vietnamese Ministry of Foreign Affairs, 4.8 million Vietnamese people were exposed to 'Agent Orange', resulting in 400,000 deaths and disabilities, and 500,000 children born with birth defects [Schecter *et al.*, 2009]. The National Toxicology Program has classified TCDD as known to be a human carcinogen [Huff *et al.*, 1990], frequently associated with soft-tissue sarcoma, non-Hodgkin's lymphoma, Hodgkin's disease [Bertazzi *et al.*, 2001], chronic lymphocytic leukemia, prostate cancer, lung cancer, soft tissue sarcoma and liver cancer as well as increased rates of nerve, digestive, skin and respiratory disorders [Pesatori *et al.*, 2003].

Paraquat (N,N-dimethyl-4,4-bipyridinium dichloride, PQ) has been one of the most widely used herbicides in the world, held the largest share of the global herbicide market. Paraquat is known to be highly toxic to animals and has serious and irreversible delayed effects if absorbed. Paraquat has been known to exert its effects by elevating intracellular levels of superoxide [Ranjbar *et al.*, 2002]. It has been previously demonstrated that oxidative stress and nitrosative stress participate in PQ-induced cell death [Ortiz-Ortiz *et al.*, 2010]. US EPA has classified paraquat as a possible human carcinogen. Paraquat can lead to a disease called Paraquat lung, and paraquat-induced toxicity in rats has also been linked to Parkinson's-like neurological degenerative mechanisms [Ossowska *et al.*, 2006].

Red ginseng is a ginseng (*Panax ginseng* C.A. Meyer) that has been heated through either steaming or sun-drying. It has been exhibited multiple pharmacological efficacies [Hong *et al.*, 2002], although their precise mechanisms of actions remain still elusive. Red ginseng has been known to reduce the incidence of cancer [Shin *et al.*, 2000; Suh *et al.*, 2007], show potentially

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beneficial effects on HIV-1-Infection [Cho *et al.*, 2001; Cho *et al.*, 2004]. The main molecular components responsible for the action of ginseng are the ginsenoside, which are also known as ginseng saponins [Kim and Nah, 2005]. Falcarinol, a seventeen-carbon diyne fatty alcohol was isolated from red ginseng, thought to have potent anticancer properties on primary mammary epithelial cells of breast cancer [Hansen *et al.*, 2003]. Other acetylenic fatty alcohols named panaxacol, panaxydol, panaxytriol showed antibiotic properties [Yang *et al.*, 2010]. However, the information on the antioxidative activity with suppressive effects against various herbicide-induced DNA damage and hemolysis is not available. In this study, suppressive effects of red ginseng on the oxidative DNA damage and hemolysis by 2,4-D, 2,4,5-T and paraquat were evaluated.

Materials and Methods

Red ginseng preparation. Powdered red ginseng (*Panax ginseng* C.A. Meyer) was supplied from NT&BT Co. Ltd (Chungnam, Korea). Red ginseng powder was dissolved in PBS (Phosphate-buffered saline), and centrifuged at 3,000 rpm for 5 min, and then the supernatant was used in this investigation.

Treatment of lymphocytes. A 400 μL of fresh whole blood from rats was added to 600 μL of PBS and layered onto 400 μL of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,450 rpm for 5 min at room temperature, the lymphocytes were collected from the layer just above the Histopaque 1077 boundary. After washing the lymphocytes with PBS, the lymphocyte was suspended in PBS.

Paraquat was dissolved in PBS, and 2,4-D and 2,4,5-T were dissolved in DMSO. To investigate the ability of red ginseng to inhibit oxidative DNA damage, the lymphocytes in were pre-incubated with various concentrations of red ginseng for 30 min at 37°C in the dark, and then treated with 50 μM paraquat, 2,4-D or 2,4,5-T for 5 min on ice for pre-treatment of lymphocytes with red ginseng. The total experiment volume was 1 mL. PBS or DMSO-treated sample was used as a negative control.

Determination of DNA damage by comet assay. The alkaline comet assay was performed according to Singh *et al.* [1988] with slight modifications, as reported previously [Park *et al.*, 2005]. The lymphocytes were mixed with 75 μL of 0.7% low-melting-point agarose and added to slides precoated with 1.0% normal melting-point agarose. After the agarose solidified, the slides were covered with 100 μL of 0.7% low-melting-point agarose and immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were placed in an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 20 min to allow the DNA to unwind. Electrophoresis was performed at 25 V/300 mA for 20 min at 4°C. The slides were washed with neutralizing buffer (0.4 M Tris·HCl pH 7.5) three times for 5 min at 4°C, and then treated with ethanol for 5 min.

Image analysis. The slides were stained with ethidium bromide (20 μg/mL) and cover slipped. Measurements were made by image analysis using Komet 5.5 software (Kinetic Imaging, Liverpool, UK) and fluorescence microscopy (Leica, Wetzlar, Germany). To quantify DNA damage in the comet assay, the olive tail moment was calculated as: (Tail.mean-Head.mean)×Tail% DNA/100 [Sul *et al.*, 2008]. A total of 150 randomly captured comets were examined from each slide.

Hemolysis assay. 100 L of erythrocyte suspension (2×10^8 cells/mL) and water-soluble fraction of red ginseng at different final doses (0.6, 0.7, 0.8, 0.9 and 1 mg/mL) were pre-incubated together before herbicide was added. Upon treating 2,4-D, 2,4,5-T or paraquat at final doses of 7 mM, the final 1 mL reaction mixture was incubated for 1 h at the 37°C. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm, as previously reported [Katsu *et al.*, 1989]. The absorbance of the control group was used as the blank.

Statistical analysis. The comet assay data are the means of three determinations and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The mean values of DNA damage (olive tail moment) for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Erythrocyte damage was measured using Duncan's multiple range test. $P<0.05$ was considered significant.

Antioxidant activity. Following an established methodology [Murakami *et al.*, 2000], the antioxidant activity of red ginseng powder was expressed as scavenging ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. 500 μM DPPH solution in ethanol and 100 μL sample at different final doses (20, 60, 100, 140, 180 and 200 μg) were mixed in a 96 well plate and allowed to react at 37°C for 30 min. The final reaction volume was 200 μL. Absorbance at 520 nm was measured, and the percent of activity was calculated.

Results and Discussion

The comet assay (single-cell gel electrophoresis assay) is a well-established genotoxicity test and biomonitoring method for estimating oxidative DNA damage at the individual cell level, both in blood and in cells [Hartwig, 2002]. The comet assay is a very sensitive and rapid tool to detect subtle damages of DNA, including DNA single-strand breaks, double-strand breaks, or cross-links in virtually any cell type. It has been widely used to detect primary DNA damage in human and animal cells exposed to various toxicants or occupational exposure through detecting

oxidative DNA damage induced by reactive oxygen species (ROS) [Lee *et al.*, 2007; Garaj-Vrhovac *et al.*, 2009].

Herbicide-induced DNA damages were evaluated with comet assay, and their suppressions by red ginseng were examined as shown in Fig. 1. Lymphocytes treated with 2,4-D showed notable DNA damage, determined by the olive tail moment in a comet assay (Fig. 1(A)). The olive tail moment at 50 M 2,4-D was about 34.46±0.57, compared with 5.87±0.39 in the DMSO-treated control, indicating an approximately 5-fold increase in DNA damage with 50 M 2,4-D. The addition of red ginseng inhibited the oxidative DNA damage caused by 2,4-D as demonstrated by the reduction of the olive tail moment. The olive tail moment of 3 g/mL red ginseng cells was approximately 19.97±1.19, more enhanced suppressvie effects were also seen at 5 and 8 g/mL of red ginseng. The olive tail moment at 5 g/mL and 8 g/mL red ginseng were approximately 16.70±1.25 and 10.38±0.16, respectively.

The olive tail moment at 50 M 2,4,5-T was about 37.37±2.45, compared with 9.73±0.35 in the DMSO-treated control, indicating an approximately 4-fold increase in DNA damage as shown in Fig. 1(B). Upon treating red ginseng, the oxidative DNA damage by 2,4,5-T was suppressed as demonstrated by the reduction of the olive tail moment in a comet assay. The olive tail moment of 3 g/mL red ginseng cells was approximately 22.26±2.35, and the protective effects were also seen at 5 and 8 g/mL of red ginseng. These result might suggest that antioxidative components with radical scavenger activity in red ginseng could suppress the oxidative DNA damage by phenoxyherbicide. However, the extent of protection by red ginseng on the 2,4,5-T-induced hemolysis was much weaker than that on the 2,4-D-induced hemolysis. In considering the fact that Agent Orange was equal parts of 2,4,5-T and 2,4-D, red ginseng seems to be helpful to suppress the oxidative DNA damage by Agent Orange, although large-scale in vivo experiment should be performed.

Figure 1(C) shows that red ginseng could inhibit paraquat-induced oxidative DNA damage demonstrated by comet assay. Red ginseng protected the lymphocyte from DNA damage induced by 50 M paraquat. The olive tail moment at 3 g/mL red ginseng was approximately 22.80±1.46, and those at 5 and 8 g/mL of red ginseng were approximately 19.85±6.81 and 17.44±4.90, respectively. The result also indicated that red ginseng could suppress the oxidative DNA damage by paraquat.

Figure 2 shows the protective effect of red ginseng on erythrocytes damage under 2,4-D, 2,4,5-T and paraquat treatment, evaluated with hemolysis assay. Erythrocyte membrane is composed of a lipid bilayer primarily with proteins embedded in, which keeps the membrane in dynamic equilibrium between fluidity and solidity. The erythrocyte membrane is vulnerable to oxidative damage due to the high poly-unsaturated fatty acid

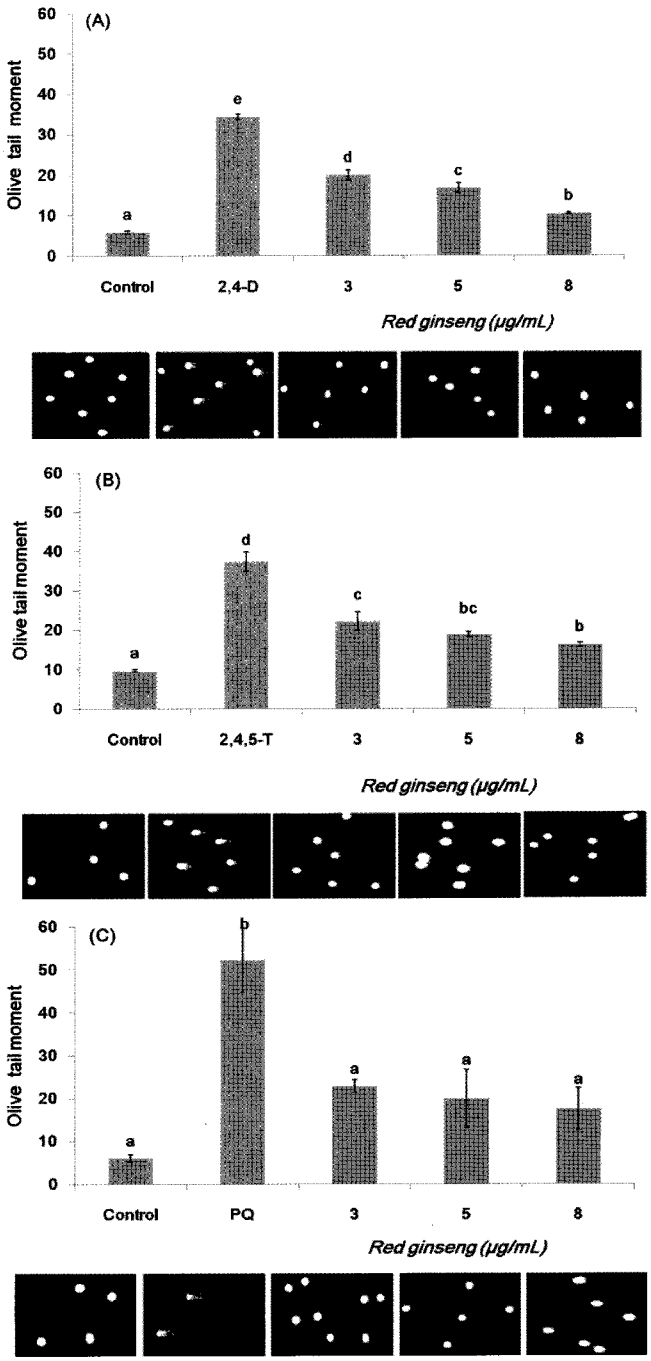

Fig. 1. Effect of red ginseng on the (A) 2,4-D (2,4-dichlorophenoxyacetic acid), (B) 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) and (C) PQ (paraquat)-induced oxidative DNA damage in lymphocytes. Values not sharing the same letter are significantly different from one another ($p<0.05$).

content in the membrane. Thus, the erythrocyte toxicity has been used for new chemical research [Li *et al.*, 2008]. The erythrocyte was damaged at 7 mM 2,4-D and 2,4,5-T, with release of content hemoglobin into surrounding fluid [Hebbel *et al.*, 1990]. However, 2,4-D and 2,4,5-T -induced hemolysis was reduced by red ginseng treatment demonstrated by decrease in the absorbance at 540 nm (Fig. 2(A) and 2(B)). The result indicates the hemoprotective effect of red ginseng against phenoxyherbicide

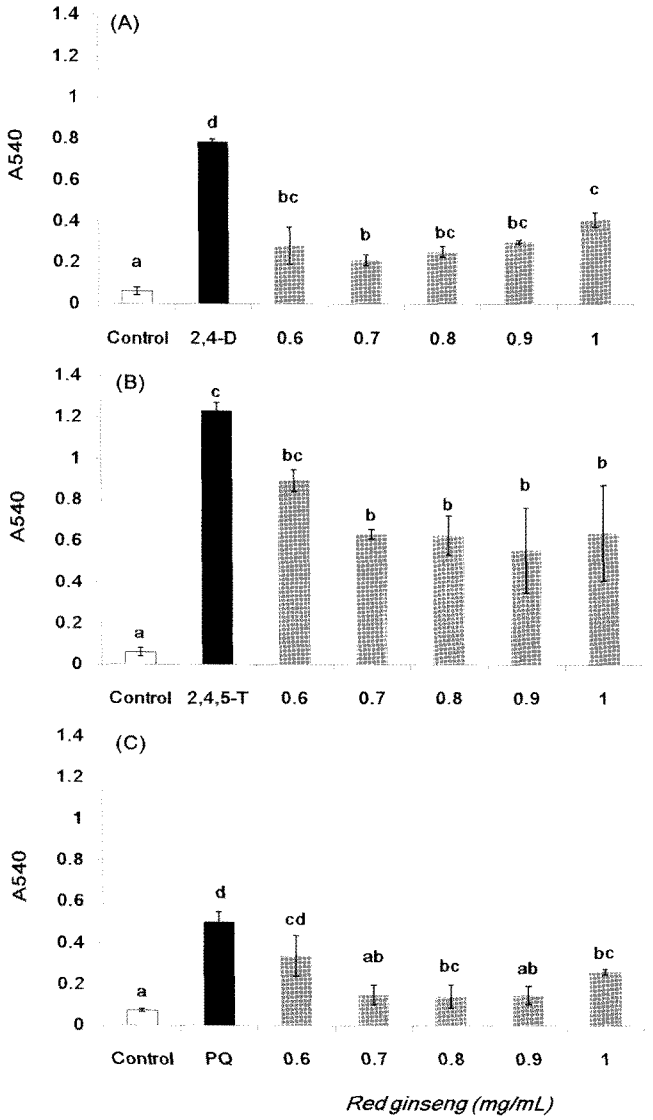

Fig. 2. Effect of red ginseng on the (A) 2,4-D (2,4-dichlorophenoxyacetic acid), (B) 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) and (C) PQ (paraquat)-induced erythrocyte damage. Values not sharing the same letter are significantly different from one another ($p<0.05$).

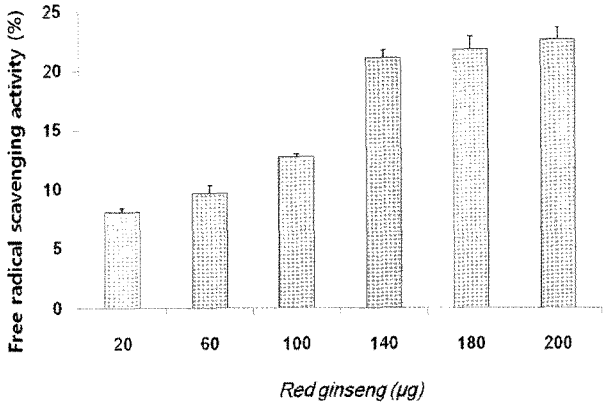

Fig. 3. Free radical scavenging activity of red ginseng by DPPH assay.

such as 2,4-D and 2,4,5-T. Red ginseng could also suppress the hemolysis induced by paraguat as shown in Fig. 2(C). Thus, red ginseng could possess hemoprotective effect against oxidative herbicide of paraquat. Similar protective effect of red ginseng against phenanthrene was also seen in our previous report [Seo and Lee, 2009]. Antioxidant components, named ginsenoside, polyphenol and flavonoid were suggested to be responsible to suppress the cell damage by phenanthrene [Seo and Lee, 2009].

In order to examine the radical scavenging ability of red ginseng, electron-transfer reaction based-DPPH assay was carried out (Fig. 3). The result showed that water-soluble red ginseng fraction could suppress the DPPH radical. However, about 60 g of large amount of water-soluble red ginseng fraction was needed to scavenge approximately 10% of 500 μM DPPH radical. Similar dose ranges of medicinal herb extracts were reported to be needed to scavenge DPPH radical [Pongtip and Siripen, 2009]. The DPPH assay is technically simple to evaluate the free radical scavenging ability. However, the reaction of DPPH with eugenol was reported to be reversible, and this would result in low reading for samples containing eugenol and other phenols [Huang *et al.*, 2005]. Therefore, the possibility that much lower value of radical scavenging ability could be read by DPPH assay than real endogenous radical scavenging ability could not be ruled out in this experiment. We have used three different assays to measure the suppressive effect of red ginseng on the herbicide-induced DNA and erythrocyte damages. These assays should not directly compromise their value in guiding clinical research, because the detection limits of the assays are markedly different. Many studies have applied diverse in vitro assays to examine the impact of antioxidant consumption on reducing oxidative stress markers. The synergistic effects of such a combination assays will allow us to investigate the impact of aqueous extract of red ginseng in reducing ROS-induced oxidative stress and the implication for disease prevention in this investigation.

There are six major ROS causing oxidative damage in the human body. These species are superoxide anion, hydrogen peroxide, peroxyl radicals, hydroxyl radical, singlet oxygen, and peroxynitrite. Therefore, the beneficial effects of red ginseng in this study, which might suppress herbicide-induced DNA and erythrocyte damage through counteracting the assault of ROS, seem to be due to the diverse antioxidants, while more studies needs to understand the action mechanism and exact components responsible for the antioxidant effect of red ginseng.

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