Enhanced Protective Effect of Ultrafine Particles of Red-Ginseng against Phenanthrene-induced Cell Damage

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Abstract : Phenanthrene, one of the polycyclic aromatic hydrocarbons, has been known to be toxic to the environment. In this investigation, the protective effect of red ginseng on phenanthrene-induced oxidative DNA damage was evaluated using Comet assay in A549 cells. Red ginseng's cytoprotective effect on phenanthrene-induced hemolysis was also investigated. This study's findings show that oxidative DNA damage and hemolysis were significantly prevented by red ginseng treatment. Notably, it was found that pulverizing red ginseng into ultra-fine particles even enhanced its protective effects against DNA damage and hemolysis. The results suggest that particle size reduction seems to effectively enhance red ginseng's pharmacological efficacies.

Key words : red ginseng, particle size reduction, cytoprotection, phenanthrene

INTRODUCTION

The revolution in nanotechnology gives advantages in diverse areas, and the resulting nanoparticles play important roles in a wide variety of industrial applications. Nanoparticles are already being used in medicines, cosmetics, food additives, disinfectants, fuel additives etc. Nanotechnology has also provided a theoretical and technical evidence for showing the increase in solubility and bioavailability of the bioactive components in drugs and foods.

The physico-chemical properties of nanoparticles are quite different from those of the same compounds on a larger scale.¹⁾ When the materials were reprocessed at the nano scale, the properties of the nano-sized materials were significantly different from their larger equivalents. Chemical reactivity, color, strength, conductivity, and magnetic properties of the nano-sized materials may all vary in extraordinary ways. Insoluble materials might easily dissolve at the nano scale, and the nano-sized particles could penetrate into the cellular membranes that their bulk particles normally cannot.²⁾

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a representative tonic in Korea. Although ginseng exhibits multiple pharmacological efficacies,³⁾ it's

precise mechanisms on the actions are still elusive. The main molecular components responsible for the action of ginseng are the ginsenoside, which are also known as ginseng saponins.⁴⁾ More than 30 different forms of ginseng saponins have been isolated and identified.⁵⁻⁷⁾ Ginsenosides are a class of steroid glycosides and triterpene saponins hence ginsenoside has a four-ring, steroid-like structure with sugar moieties attached.⁸⁾ Ginsenosides are often classified as three groups based on their structural differences: the Rb1 group, Rg1 group and oleanane group. The Rb1 group was characterized by the presence of protopanaxadiol presence, named Rb1, Rb2, Rc and Rg3. Rg1 group was classified by the presence of protopanaxatriol, named Rg1, Re, Rf, and Rg2.⁹⁾

Phenanthrene is one of the polycyclic aromatic hydrocarbon (PAH) composed of three fused benzene rings,¹⁰⁾ and they can exist in over 100 different combinations. PAHs are found naturally in the environment but they can be formed from the incomplete burning of the products like coal, oil, gas, and garbage.¹¹⁾ Phenanthrene, like most PAHs, is used to make dyes, plastics and pesticides, explosives and drugs.¹²⁾ A number of PAHs have caused tumors in laboratory animals by exposure through food, skin, and contaminated air. Other effects including damage to skin, body fluids and the immune system could also be seen.

The comet assay (single-cell gel electrophoresis assay) has been widely used to detect primary DNA damage in

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human and animal cells exposed to various environmental toxicants or occupational exposure.^{13,14} It is a well-established genotoxicity test for estimating oxidative DNA damage at the individual cell level, both in blood and in cells.¹⁵

In this study, the suppressive effects of red ginseng on the oxidative DNA damage and the hemolysis were evaluated. Notably, the enhancement of the protective effect of red ginseng was shown by reducing particle size of red ginseng.

MATERIALS AND METHODS

Cell culture and sample treatment

The A549 cell line (American type culture collection, Rockville, MD), human pulmonary epithelial cell line derived from a lung carcinoma, was used in this experiment. Mycoplasma-free cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and maintained at 37°C under an atmosphere of 5% CO₂, and harvested with 0.05% Trypsin/EDTA. Both of the coarse particles and ultrafine particles of red ginseng (*Panax ginseng* C.A. Meyer) were supplied from NT&BT Co. Ltd (Chungnam, Korea). Coarse particles and ultrafine particles of red ginseng were each dissolved in PBS at a concentration of 3, 5, and 8 µg/mL. A549 cells were pre-incubated with various concentrations of red ginseng for 1 h. And then phenanthrene dissolved in DMSO was added to the cells to investigate the effect of red ginseng to protect against phenanthrene-induced cell damage. PBS without an oxidative stimulus was used as a negative control.

Determination of DNA damage by comet assay

The alkaline comet assay using cultured cells was performed according to Singh *et al*¹⁶ with slight modifications. A549 cells (5.5×10^5 each) were seeded into 12 well plate (Becton Dickinson, USA). The cells were cultivated overnight and then pretreated with ginseng coarse particles or ultrafine particles for 1 h at 37°C. Cells were washed with PBS and then treated with phenanthrene dissolved in the media for 1 h. After washing with PBS, the cells were trypsinized with 250 µL of 0.05% Trypsin/EDTA. The cells were washed with PBS and then mixed with 75 µL of 0.7% low melting point agarose after adding 500 µL media. And then, the cells were added to slides precoated with 1.0% normal melting point agarose. After the agarose was 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 into an electrophoresis tank containing 300 mM NaOH and 10 mM Na₂EDTA (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 three times with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 5 min at 4°C and then treated with ethanol for a further 5 min before staining.

Image analysis

The slides were stained with ethidium bromide (20 µg/mL) and coverslipped. 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/100.¹⁷ A total of 150 randomly captured comets were examined from each slide.

MTT assay

The MTT assay was used to determine the cell viability. A549 cells were cultured in a 96-well plate (Corning Inc., Corning, NY) at a density of 2×10^4 cells per well. The cells were then treated with varying concentrations of coarse particles and ultrafine particles of red ginseng for 1 h. And then the cells were treated with 30 M phenanthrene, after which the plates were incubated at 37°C in the dark for 2 h following MTT treatment. After formation of formazan, 100 µL of DMSO was added and the absorbance was measured at 570 nm using ELISA reader.

Hemolysis assay

100 µL of erythrocyte suspension (5×10^7 cells/mL) was incubated with various concentrations of coarse particles and ultrafine particles of red ginseng for 1 h at 4°C. And then 0.9 mM phenanthrene was added to the mixtures for 3 h at the ice. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm, as previously reported.¹⁸ The absorbance of the control group was used as the blank.

Statistical analysis

The data for comet assay and hemolysis were the means of three determinations and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The mean values for DNA damage and erythrocyte

damage for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Phenanthrene-induced oxidative DNA damage

Nanotechnology is being introduced in food and medicine, because the solubility and bioavailability of bioactive components could be enhanced by particle size reduction of the bioactive components in food and medicine. The small size and high surface-volume ratio might endow the particles with increased active groups. Thus the small size and high reactive surface are totally different from their bulk particles. Korean red ginseng was pulverized into ultrafine particles to investigate whether the tiny particles reduced at almost nano grade might have increased cytoprotective effect against oxidative DNA damage and hemolysis. The protective effects of ultrafine particles were compared with those of coarse particles in this investigation.

Fig. 1 shows the scanning electron microscopy (SEM) images of ultrafine particles and coarse particles of red ginseng. The mean size of the ultrafine particles was in the 300-400 nm range, while that of the coarse particles was in the 100-150 μm range as determined by density analysis of the particles (data not shown).

The comet assay has been used in toxicity testing and biomonitoring, and it has proven to be a sensitive system for genotoxicity screening. Comet assay are also useful for detecting oxidative DNA damage induced by the generation of free radicals. In Fig. 2, A549 cells were treated with various concentrations of phenanthrene. The DNA damage, determined by the olive tail moment in a comet assay, increased in a dose-dependent manner. The olive tail moment at 30 M phenanthrene was about 47.94 ± 5.33 ,

compared with 6.24 ± 0.16 in the PBS-treated control, indicating an approximately 8-fold increase in DNA damage at 30 μM phenanthrene. Various oxidants such as hydrogen peroxide, paraquat and melamine have been reported to cause DNA strand breakage by generating hydroxyl radicals close to the DNA molecule.^{19,20} Paraquat (10 μM) showed an approximately 3-fold increase in DNA damage in comparison with the control,²⁰ while 10 μM phenanthrene induced approximately 4-fold more DNA damage than the control. These results indicate that phenanthrene-induced oxidative DNA damage is greater than paraquat-induced DNA damage.

Suppressive effect of red ginseng on phenanthrene-induced oxidative DNA damage

The addition of red ginseng inhibited the oxidative DNA damage caused by various concentrations of phenanthrene, as demonstrated by the reduction of the olive tail moment in a comet assay (Fig. 3). Coarse particles as well as ultrafine particles of red ginseng could protect the A549 cells from DNA damage induced by 30 μM phenanthrene. Notably, ultrafine particles could protect the cell much better than coarse particles as evaluated with olive tail moment. The olive tail moment of 3 $\mu\text{g/mL}$ ultrafine particle-pretreated cells was approximately 13.97 ± 0.19 , while that of the coarse particle-pretreated cells was approximately 21.96 ± 0.42 , indicating stronger protective effect of ultrafine particles than that of coarse particles. Same enhanced protective effects were also seen at 5 and 8 $\mu\text{g/mL}$ of ultrafine particles. The olive tail moment at 5 $\mu\text{g/mL}$ ultrafine particles was approximately 12.39 ± 4.11 , while that of the coarse particles was approximately 16.28 ± 4.24 . The olive tail moment at 8 $\mu\text{g/mL}$ ultrafine particles was approximately 11.57 ± 0.12 , while that of the coarse particles was approximately 14.35 ± 0.64 .

Much more antioxidant activity, ginsenosides, minerals,

Fig. 1. SEM images of coarse particles (A) and ultrafine particles (B) of red ginseng.

Fig. 2. Phenanthrene-induced oxidative DNA damage of A549 cells evaluated with comet assay. Values not sharing the same letter are significantly different from one another ($p < 0.05$) according to Duncan's multiple range test.

Fig. 3. Protective effect of ultrafine particles and coarse particles of red ginseng on phenanthrene -induced DNA damage in A549 cells. Values not sharing the same letter are significantly different from one another ($p < 0.05$) according to Duncan's multiple range test.

carbohydrates, lipids and proteins were reported to be detected in the ultrafine particles than in regular particles in our previous report.²¹⁾ Especially, the sum of all the saponins detected in ultrafine particles was about twice more than in regular particles.²¹⁾ Therefore, more antioxidative components including ginsenosides, polyphenols and flavonoids in ultrafine particle seem to suppress the

oxidative DNA damage more strongly than in coarse particles.

Suppressive effect of red ginseng on phenanthrene-induced hemolysis

Fig. 4 shows the information on erythrocytes morphology and stability under phenanthrene treatment. The

Fig. 4. Protective effect of ultrafine particles and coarse particles of red ginseng on phenanthrene-induced hemolysis. Values not sharing the same letter are significantly different from one another ($p < 0.05$) according to Duncan's multiple range test.

erythrocyte was not damaged at 30 μM phenanthrene that caused significant oxidative DNA damage by using comet assay, while the erythrocytes treated with 0.9 mM phenanthrene exhibited abnormal shapes in morphology. However, the numbers of abnormal shapes of erythrocytes were reduced when the erythrocytes were pretreated with red ginseng. Another result of the damage by phenanthrene was hemolysis, which happened when the content escaped from the inner to the outer membrane of erythrocyte²²⁾ upon treating phenanthrene. However, phenanthrene-induced hemolysis was reduced sharply under ultrafine particle treatment while slowly under coarse particles of red ginseng. The result indicates the enhanced cytoprotective effect of ultrafine particles of red ginseng than that of coarse particles. The reason for this difference may be related with the tiny size and high surface activity of ultrafine particles.

There is increasing evidence showing that a variety of diseases are associated with increased oxidative stress.²³⁾ Oxidative stress has been implicated in the pathogenesis of diverse diseases. The beneficial effects of red ginseng could be attributed to various ginsenosides, while the mechanism of actions of ginsenoside remains to be elucidated. In this examination, we examined the enhanced protective efficacies of ultrafine particles of red ginseng against DNA damage and hemolysis than those of the equivalent larger ones. These results suggest that nanotechnology including particle size reduction could be

applied to the development of enhanced medicinal foods primarily because the small size, high surface-volume ratio and enhanced absorption could endow them with increased reactive groups.

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