Reduction of Cisplatin-Induced Nephrotoxicity by Ginsenosides Isolated from Processed Ginseng in Cultured Renal Tubular Cells

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Ginsenosides, the unique constituents and secondary metabolites of the Panax species, have been known to be the pharmacologically active ingredients of ginseng. Recently, our research group has developed a new processed ginseng, called Sun ginseng (SG), which has an increased amount of the red ginseng unique ginsenosides (RGUG). In our previous studies, this new processed ginseng reduced cisplatin-induced nephrotoxicity more than white ginseng in both in vitro and in vivo systems. In this study, we isolated and characterized active principles through activity-guided fractionation. Ginsenosides Rh4 and Rk3 significantly reduced the cisplatin-induced nephrotoxicity in LLC-PK1 cells in a dose-dependent manner. The mechanisms of function and structure–activity relationships with other ginsenosides remain for further investigation.

Key words ginsenoside; cisplatin-induced nephrotoxicity; LLC-PK1; processed ginseng

Ginseng, the root of Panax ginseng C. A. MEYER (Araliaceae), is one of the most widely prescribed and intensively studied herbal medicines. Ginsenosides, the secondary metabolites and unique constituents in the Panax plants, are the pharmacologically active ingredients of ginseng. There are two traditional preparations of ginseng, white ginseng (WG) and red ginseng (RG), the dried root of ginseng and the steamed/dried root of ginseng, respectively. RG especially shows more enhanced pharmacologic activities than WG.1,2) These enhanced pharmacologic activities result from the red ginseng unique ginsenosides (RGUG) produced during processing. It is reported that unique ginsenosides in red ginseng are ginsenosides Rg3, Rg5, Rg6, Rh2, Rh3, Rh4, Rs3, and F4.1,2)

Recently, our research group has developed a new processed ginseng, called Sun ginseng (SG), by steaming WG at high temperature and pressure.3) HPLC-evaporative light scattering detector (ELSD) analysis of ginsenosides in SG revealed that SG had hundreds of times more RGUG than RG.4,5) A preparation containing SG extracts with specific standardization is now available as a functional food on the Korean market.

Cisplatin [cis-dichlorodiammine-platinum(II)] is an anti-neoplastic agent clinically used in the treatment of various solid tumors such as testis, ovary, urinary bladder, prostate, head, and neck cancer. However, despite its excellent anticancer activity, its clinical use is limited because of severe nephrotoxicity.4,5) The nephrotoxicity induced by cisplatin is characterized by morphologic destruction of intracellular organelles, such as cellular necrosis, loss of microvilli, alterations in the number and size of lysosomes, and mitochondrial vacuolization, followed by functional alterations including inhibition of protein synthesis, glutathione (GSH) depletion, lipid peroxidation, and mitochondrial damage.5) Cisplatin is preferentially uptaken and accumulated in cells of the renal proximal tubules, and intracellular cisplatin is thought to undergo aquation reactions in which the labile chloride ligands are replaced by water molecules, resulting in a positively charged and highly reactive electrophilic product.5) This toxic derivative is thought to cause the oxidative damage in renal proximal tubular cells, resulting in cell death.

From many reported in vitro and/or in vivo studies, there are several candidates of herbal origin that may reduce cisplatin nephrotoxicity. Quercetin, a common antioxidant bioflavonoid in fruit and vegetables, has potent cytoprotective effects against cisplatin nephrotoxicity in cultured renal proximal tubular epithelial cells (LLC-PK1).6) A tannin mixture fractionated from green tea increased the viability of cells exposed to cisplatin in a dose-dependent manner in LLC-PK1, in vitro and decreased blood levels of urea nitrogen and creatinine and urinary levels of protein and glucose in a rat model in vivo. Rats administrated the green tea tannin mixture were found to have increased activity of catalase in the renal tissue and decreased levels of malondialdehyde, suggesting that cisplatin nephrotoxicity involves reactive radicals and that green tea tannin may play a role as an antioxidant that scavenges the reactive radicals generated from cisplatin.7)

In our previous studies, ginsenoside mixtures of SG showed reducing effects on cisplatin induced nephrotoxicity in in vitro and in vivo models. Ginsenoside mixtures of SG scavenged free oxygen radicals generated from isolated neutrophils stimulated by cisplatin in an in vitro model. Rats administered the ginsenoside mixture of SG prior to cisplatin injection showed decreased blood levels of urea nitrogen and creatinine to the normal level and reduced side effects of cisplatin such as weight loss and decreased life span. However, the nephroprotective principles of SG against cisplatin-induced nephrotoxicity remain unclear. In this study, we evaluated the cytoprotective principles from SG on cisplatin-induced nephropathy in LLC-PK1 cells using activity-guided fractionation and isolation.

MATERIALS AND METHODS

General Analytical HPLC and semipreparative HPLC were performed on a Gilson chromatograph equipped with a Gilson pump 321, a Gilson UV/VIS-155 detector set at 210 nm, and a fraction collector FC204 (Gilson, Middleton, WI, U.S.A.). A Versamax Microplate reader (Molecular De-
Plant Material  
WG (*P. ginseng* C. A. Meyer, Araliaceae), the dried root of *P. ginseng* C. A. Meyer, was purchased at a local herbal market in Korea. SG and RG were kindly donated by Ginseng Science Inc. (Seoul, Korea). RG and SG were produced by steaming WG at 100 °C and 120 °C for 3 h, respectively.\(^1\) Each dried plant material (WG 50 g, SG 1 kg) was refluxed with MeOH for 3 h three times each. The organic solvent of methanolic extract of SG was removed under reduced pressure, and a portion of the residue was suspended with water and extracted with methylene chloride (CH\(_2\)Cl\(_2\)) and water-saturated n-butyl alcohol (n-BuOH) successively. Each organic or aqueous extract was evaporated under reduced pressure and stored in a refrigerator.

Activity-Guided Fractionation and Isolation  
A portion (43 g) of the CH\(_2\)Cl\(_2\) extract of SG was chromatographed on a silica gel column using a stepwise gradient elution of chloroform (CHCl\(_3\))/MeOH (40:1–5:1). Ten fractions were obtained. Fraction 9 showed the most potent protective activity in LLC-PK\(_1\) exposed to cisplatin. MeOH solution of fraction 9 was injected onto the semipreparative ODS column using a stepwise gradient elution of chloroform (CH\(_2\)Cl\(_2\)) and water-saturated n-butyl alcohol (n-BuOH) successively. Each organic or aqueous extract was evaporated under reduced pressure and stored in a refrigerator.

Cell Culture  
LLC-PK\(_1\) cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.), were cultured in a DMEM/F12 medium (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.) supplemented with 5% fetal bovine serum (Gibco BRL Life Technologies), 50 units/ml of penicillin G (Gibco BRL Life Technologies), and 50 µg/ml of streptomycin (Gibco BRL Life Technologies). Cell cultures were maintained at 37 °C in a humidified 95% air/5% CO\(_2\) incubator. The culture medium was changed every 2 to 3 d. Cells were grown to confluence in flasks (75 cm\(^2\)) over 6 or 7 d and then trypsinized and used for the experiments.

Experimental  
Methanolic extracts of WG and SG stock solutions (80 mg/ml), solvent (CH\(_3\)Cl\(_2\), n-BuOH, aqueous) extracts of SG stock solutions (16 mg/ml), chromatographic fractions of SG stock solutions (16 mg/ml), and ginsenoside stock solutions (10 mg/ml) were prepared in DMSO. Each stock solution was diluted with PBS (Gibco BRL Life Technologies) to corresponding concentrations in 5% DMSO in PBS and then serially diluted with 5% DMSO in PBS to make the test solution. Cisplatin stock solution (500 µM) was prepared with PBS. Confluent monolayers of LLC-PK\(_1\) cells in T-75 flasks were trypsinized and suspended in the culture medium and 190 µl of a single cell suspension containing 1×10\(^5\) cells/ml was seeded into 96-microtiter plates (NUNC, Roskilde, Denmark). Plates were incubated at 37 °C in a humidified 95% air/5% CO\(_2\) incubator for 2 h. After incubation, 10 µl of each test solution and 50 µl of cisplatin stock solution was added into each well. Plates were incubated at 37 °C in a humidified 95% air/5% CO\(_2\) incubator for 24 h, and then cell viability and LDH leakage were measured according to the procedure described below.

MTT Assay  
The MTT assay was performed using an MTT assay kit (CellTiter 96, Promega, Madison, WI, U.S.A.) according to the procedure described by the manufacturer with slight modifications. Briefly, after cells were exposed to cisplatin and/or test solutions for 24 h, premixed dye solutions were added to the wells and incubated for 4 h. The solubilization/stop solution was then added to the wells to solubilize the formazan products, and the absorbance at 570 nm was recorded. Cell viability was expressed as a percentage of the absorbance of wells without cisplatin.

Measurement of LDH Leakage  
Leakage of LDH from cells was measured using an LDH assay kit (CytoTox 96, Promega) according to the manufacturer’s procedure. After 24-h incubation with cisplatin and/or test compounds, plates were centrifuged and aliquots of culture media were transferred to fresh 96-well plates. The activity of LDH in the aliquots was assayed by the conversion of tetrazolium salt into a red formazan product. The absorbance at 492 nm was recorded. The extent of LDH leakage was expressed as percentages of the absorbance of wells treated with cisplatin alone.

Statistical Analysis  
The means and standard errors of means (S.E.M.) were calculated for all experiments. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan’s multiple-range test to determine whether means were significantly different from the control. In all cases, a p value of <0.05 (*) or <0.01 (**) was used to determine the significance.

RESULTS AND DISCUSSION  
In our previous investigations, we confirmed that SG showed protective effects against cisplatin-induced nephrotoxicity in vitro and in vivo. To reconfirm these effects of SG rather than WG in our in vitro system, each methanolic extract of WG and SG was added to cisplatin-exposed LLC-PK\(_1\) cells. Cells were treated with each methanolic extract of WG and SG at five different final concentrations (10, 20, 40, 80, 160 µg/ml) or with vehicle (DMSO), and 100 µM of cisplatin for 24 h. SG significantly increased the cell viability in

![Fig. 1. Chemical Structures of Ginsenoside Rh\(_4\) (A) and Rk\(_3\) (B)](image-url)
a concentration-dependent manner, suggesting that SG reduces cisplatin nephrotoxicity, while WG had no effects (Fig. 2). A scheme of the activity-guided fractionation and isolation of SG is shown in Fig. 3. Through the successive liquid–liquid extraction, the MeOH extract of SG was separated into the CH₂Cl₂, n-BuOH, and aqueous fractions. Each fraction was introduced in the cell system as described above at three different concentrations (8, 16, 32 mg/ml). The CH₂Cl₂ fraction significantly increased cell viability (Fig. 4). A portion of the CH₂Cl₂ extract of SG was chromatographed on a silica gel column using a stepwise gradient elution of CHCl₃/MeOH (40 : 1 → 5 : 1), and separated into 10 fractions. Fraction 9 showed the most potent protective activity in cells exposed to cisplatin (data not shown). Through the separation and purification of fraction 9 using semipreparative RP-HPLC, the active principles of SG were isolated and characterized as ginsenosides Rh₄ and Rk₃. Ginsenosides Rh₄ and Rk₃ are isomers distinguished by the position of the double bond; the former’s double bond is \( \Delta^{20-21} \) and the latter’s is \( \Delta^{20-22} \).

The effects of purified ginsenosides Rh₄ and Rk₃ on cisplatin nephrotoxicity were examined, based on metabolic activity assessed in the MTT assay and cell membrane rigidity and permeability in the LDH release assay. Each ginsenoside was added to cisplatin-exposed cells at various concentrations (5, 10, 20 mg/ml). Ginsenosides Rh₄ and Rk₃ significantly increased the metabolic activity of LLC-PK₁ cells exposed to cisplatin in a concentration-dependent manner, and decreased LDH leakage at 20 mg/ml and 5—20 mg/ml, respectively, suggesting that they reduce the cisplatin-induced nephrotoxicity in LLC-PK₁ cells (Fig. 5). The contents of ginsenosides Rh₄ and Rk₃ analyzed using the HPLC-ELSD method were 0.09% and 0.08% (w/w, dry weight of SG), respectively. Ginsenosides Rb₁, Rb₂, Rc, Rd, Re, Rg₃, Rg₅, and Rk₁ did not show any activity in this system (data not shown).

Cisplatin preferentially accumulates in cells of the S3 segment of the renal proximal tubules and is toxified to form a reactive metabolite intracellularly by hydration. The primary symptoms of cisplatin nephrotoxicity are inhibition of protein synthesis and intracellular GSH and protein-SH depletion, resulting in lipid peroxidation and mitochondrial damage. GSH and protein-SH form the major cellular oxidant defense system, which is a potent factor in the control of lipid peroxidation. From these pathomechanisms of cisplatin nephrotoxicity, it is clear that the nephrotoxicity of cisplatin involves reactive radicals. Thus the reasonable cellular-protective agents against cisplatin toxicity may have at least some antioxidant properties to prevent GSH depletion and/or scavenge the intracellular reactive oxygen species (ROS). Liu et al. examined the relationship between the structure of ginsenosides and their antioxidant or prooxidant activity. Various ginsenosides, propanaxadiol (PPD)-type ginsenosides (Rg₅, Rd, Rc, Rh₄, Rb₁, Rb₂), the triterpene dammarane backbone with hydroxyl or glycosides in C₃ and C₂₀, and

![Fig. 2. Effects of Methanolic Extracts of White Ginseng (WG) and Sun Ginseng (SG) on Cisplatin-Induced Toxicity in LLC-PK₁ Cells](image)

After cells were treated with 10—160 μg/ml of methanolic extracts of WG and SG, and 100 μM of cisplatin for 24 h, cell viability was measured using the MTT assay as described in Materials and Methods. Values are expressed as mean±S.E.M. of 5 independent experiments. ** Significant differences from vehicle control treated with cisplatin alone, using ANOVA followed by Duncan’s multiple-range test (p<0.01).

![Fig. 3. Scheme of Activity-Guided Fractionation and Isolation of Ginsenoside Rh₄ and Rk₃ from Sun Ginseng (SG)](image)

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protopanaxatriol (PPT)-type ginsenosides (Rg1, Rg2, Rh1, Re, R1), the sugar moieties in C6, and its aglycones (PPD, PPT) are antioxidants, i.e., the position of the triterpene dammarane made the ginsenosides in AAPH-induced hemolysis, while PPT increased hemolysis in a dose-dependent manner. Furthermore, ginsenoside Rg1 ameliorates cisplatin-induced nephrotoxicity by suppression of apoptosis through the reduction of DNA fragmentation and leads to restoration of renal function in vivo.11)

From the relationship between the structure of ginsenosides and their antioxidant or prooxidant activity discussed above, ginsenosides Rh2 and Rk3 may act as antioxidants during the oxidative stress involved in cell death because of its structural similarity to ginsenoside Rh1. Ginsenosides Rh4 and Rk3 have a glucose moiety in the 6-position like Rh1, while Rh4 and Rk3 have a double bond at A20—21 and A20—22, respectively, and Rh1 a hydroxyl in the 20-position. These slight differences in the 20- or 21-position may not affect the antioxidant activity of ginsenosides with glucose in the 6-position. Moreover, although cisplatin-induced toxicity in LLC-PK1 cells and AAPH-induced hemolysis in erythrocytes are not the same, there are critical similarities between the two in vitro systems. Cisplatin and AAPH act as radical generators, and the two cell systems were highly susceptible to reactive radicals. Cell death or damage in these systems resulted from oxidative stress. From this point of view, it is thought that the protective effect of ginsenosides against cisplatin toxicity might result from the antioxidant properties of Rh4 and Rk3. It is necessary to examine the protective action of ginsenosides in other ROS-mediated cell damage such as nephrotoxicity induced by cyclosporine A, 4-aminophenol, or paraquat.

In conclusion, this is the first report to evaluate the cytoprotective principles of the new processed ginseng (SG) using cisplatin-induced nephropathy in renal tubular epithelial cells using activity-guided fractionation. Ginsenosides Rh4 and Rk3, increased cell viability (5—20 μg/ml) decreased LDH leakage (5, 5—20 μg/ml, respectively) in a dose-dependent manner, reflecting reduced cisplatin nephrotoxicity in LLC-PK1 cells. The precise cytoprotective mechanisms are unclear, although Rh4 and Rk3 may play roles as antioxidants. Further study should be performed to apply Rh4 and Rk3 in in vivo experiments reflecting protection of the kidney as a whole to investigate the mechanism of protection and to evaluate the relationship between structure and protection with various well-known or new ginsenosides produced by processing.

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REFERENCES


