



Research article

Protective effect of ginsenosides Rk3 and Rh4 on cisplatin-induced acute kidney injury *in vitro* and *in vivo*Seung-Hoon Baek^{1,2}, Byong-kyu Shin³, Nam Jae Kim⁴, Sun-Young Chang^{1,2}, Jeong Hill Park^{3,5,6,*}¹ College of Pharmacy, Ajou University, Suwon, Republic of Korea² Research Institute of Pharmaceutical Science and Technology (RIPST), Ajou University, Suwon, Republic of Korea³ College of Pharmacy, Seoul National University, Republic of Korea⁴ East–West Medical Research Institute, Kyung Hee University Medical Center, Seoul, Republic of Korea⁵ Institute of Green Bio Science and Technology, Seoul National University, Gangwon-do, Republic of Korea⁶ Faculty of Pharmacy, Ton Duc Thang University, HCMC, Viet Nam

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ABSTRACT

Background: Nephrotoxicity is the major side effect in cisplatin chemotherapy. Previously, we reported that the ginsenosides Rk3 and Rh4 reduced cisplatin toxicity on porcine renal proximal epithelial tubular cells (LLC-PK1). Here, we aimed to evaluate the protective effect of ginsenosides Rk3 and Rh4 on kidney function and elucidate their antioxidant effect using *in vitro* and *in vivo* models of cisplatin-induced acute renal failure. **Methods:** An enriched mixture of ginsenosides Rk3 and Rh4 (KG-KH; 49.3% and 43.1%, respectively) was purified from sun ginseng (heat processed *Panax ginseng*). Cytotoxicity was induced by treatment of 20 μM cisplatin to LLC-PK1 cells and rat model of acute renal failure was generated by single intraperitoneal injection of 5 mg/kg cisplatin. Protective effects were assessed by determining cell viability, reactive oxygen species generation, blood urea nitrogen, serum creatinine, antioxidant enzyme activity, and histopathological examination.

Results: The *in vitro* assay demonstrated that KG-KH (50 μg/mL) significantly increased cell viability (4.6-fold), superoxide dismutase activity (2.8-fold), and glutathione reductase activity (1.5-fold), but reduced reactive oxygen species generation (56%) compared to cisplatin control cells. KG-KH (6 mg/kg, *per os*) also significantly inhibited renal edema (87% kidney index) and dysfunction (71.4% blood urea nitrogen, 67.4% creatinine) compared to cisplatin control rats. Of note, KG-KH significantly recovered the kidney levels of catalase (1.2-fold) and superoxide dismutase (1.5-fold).

Conclusion: Considering the oxidative injury as an early trigger of cisplatin nephrotoxicity, our findings suggest that ginsenosides Rk3 and Rh4 protect the kidney from cisplatin-induced oxidative injury and help to recover renal function by restoring intrinsic antioxidant defenses.

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1. Introduction

cis-Diamminedichloroplatinum (II) (cisplatin) is a chemotherapeutic agent that has been used against cancer of the bladder, cervix, esophagus, head, neck, ovaries, and testes since it gained approval from the United States Food and Drug Administration in 1978 [1]. The main anticancer mechanism of cisplatin

is the inhibition of mitosis through DNA damage in rapidly dividing cells. Upon gaining access into cells, cisplatin is converted to the aquated form through replacement of labile chloride ligands by water molecules [2]. This electrolytic conversion results in a positively charged and highly reactive electrophilic product, thereby leading to deleterious loss of cellular function and subsequent cell death through interaction with cellular

* Corresponding author. College of Pharmacy, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea.
E-mail address: hillpark@snu.ac.kr (J.H. Park).

components such as the DNA, mitochondria, and endoplasmic reticula [3,4].

Despite its chemotherapeutic effectiveness, cisplatin has severe side effects such as vomiting, nausea, digestive-tract disorders, ototoxicity, neurotoxicity, and nephrotoxicity. Cisplatin nephrotoxicity is the main limiting factor restricting the use of cisplatin in the clinics [2,3]. Cisplatin nephrotoxicity is a complex multifactorial process, including preferential accumulation in proximal tubular cells, metabolic activation, oxidative injury, cell death, inflammatory tissue damage, and renal failure [5]. Preferential uptake of cisplatin takes place in renal proximal tubular cells through transporter systems, including high-affinity copper transporter 1 and organic cation transporter 2, which are highly expressed in renal proximal tubular cells [6–9]. Metabolism of cisplatin to nephrotoxin, so called metabolic activation, produces highly reactive cisplatin–thiol conjugates through enzymatic reactions by glutathione transferase, γ -glutamyl transpeptidase, and cysteine-S-conjugate β -lyase [10–12]. Specific accumulation and metabolic activation of cisplatin in renal tubular cells localizes cisplatin toxicity to the renal tissue, thereby resulting in renal cell death, inflammation, fibrogenesis, and tissue remodeling [13]. These processes together culminate in the loss of renal function, triggering nephrotoxicity. Of note, glutathione (GSH) depletion and reactive cisplatin–thiol conjugates found in initial stage of cisplatin exposure lead to an imbalance in the cellular redox status and triggers oxidative stress and cellular loss in renal tissue [14]. Therefore, cisplatin-induced oxidative injury is considered an early event of cellular toxicity and has pivotal roles in the cisplatin-induced nephrotoxicity.

Panax ginseng, commonly known as ginseng, is a medicinal plant that has been used intensively in traditional herbal medicine for more than 2,000 years, and it is now used as a tonic or functional food to improve quality of life [15–18]. Ginseng is one of the best-selling herbal medicines as well as a popular research subject in many fields of life sciences. Bioactive constituents of ginseng have been well-defined including ginsenosides, polysaccharides, phenolics, flavonoids, and polyacetylenes [19]. These compounds are known to be responsible for most of the beneficial effects of ginseng, which has a broad range of protective or therapeutic effects against many diseases [20,21]. In particular, ginsenosides, the unique compounds in *Panax* species, have been studied intensively as the active components of ginseng that have anticancer, antidiabetic, and antioxidant effects [15,22,23]. Steaming of ginseng expands the chemical diversity of the ginsenosides within it. Sun ginseng (SG) is a typical steamed root of *Panax ginseng* that has a more diverse range of ginsenosides not found in raw ginseng [24–26]. SG saponins exhibit various beneficial effects for chronic renal failure, endothelial progenitor cells, and hippocampal neurogenesis [27–29]. They are used as ingredients for many commercial products including functional foods and cosmetics.

Previously, we reported that ginsenosides Rk3 and Rh4 were the principal components in SG responsible for the protective effects reducing cisplatin-induced toxicity in LLC-PK1 cells [30]. Ginsenosides Rk3 and Rh4 are isomers that differ from each other in the location of the double bond ($\Delta 20-21$ and $\Delta 20-22$, respectively). Despite this minor structural difference, ginsenosides Rk3 and Rh4 showed similar efficacy in reducing the cisplatin cytotoxicity [30]. In the present study, we aimed to evaluate the protective effects of ginsenosides Rk3 and Rh4 on kidney function and elucidate the antioxidant effects using *in vitro* and *in vivo* models of cisplatin-induced acute kidney injury (AKI). We demonstrated that ginsenosides Rk3 and Rh4 are potential adjuvants for reducing cisplatin-induced AKI.

2. Materials and methods

2.1. Materials and reagents

Extract of SG was a generous gift from Ginseng Science Inc. (Seoul, Korea). Silica gel (230–400 mesh) was obtained from Merck (Darmstadt, Germany). Solvents for extraction and column chromatography were purchased from Duksan Pure Chemicals (Ansan, Korea). Diaion HP-20 was obtained from Mitsubishi Chemical Industries (Tokyo, Japan). Dulbecco's Modified Eagle Medium supplemented with Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum, and antibiotics (10,000 units/mL of penicillin G and 10,000 μ g/mL streptomycin sulfate) for LLC-PK1 culture were purchased from Invitrogen (Carlsbad, CA, USA). For reactive oxygen species (ROS) measurement, 2',7'-dichlorofluorescein diacetate (DCFH-DA) was obtained from Invitrogen. Diagnostic kits for measurement of blood urea nitrogen (BUN) and creatinine were purchased from Asan Pharmaceuticals (Seoul, Korea). Assay kits for catalase, glutathione reductase, and superoxide dismutase were purchased from Cayman (Ann Arbor, MI, USA). A Bradford Protein Assay kit (Thermo Scientific, Rockford, IL, USA) was used for determination of proteins in cell or kidney homogenates. Cisplatin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), HPLC-grade solvents, and other reagents were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.2. Preparation of an enriched mixture of ginsenosides Rk3 and Rh4

Polar constituents in the extract of SG were removed by passing through the Diaion HP-20 column and repeated washing with water. The ginsenoside fraction bound to Diaion HP-20 resin was recovered by elution with methanol and fractionated using silica column chromatography following the method described by Park et al [26]. An enriched mixture of ginsenosides Rk3 and Rh4 (KG-KH) was prepared by preparative reverse phase-HPLC using C18 column as stationary phase and mixture of MeOH/water as mobile phase.

2.3. Identification and quantitation of ginsenosides Rk3 and Rh4 in KG-KH

Ginsenosides Rk3 and Rh4 in KG-KH were identified and quantitated by reverse phase-HPLC with an evaporative light scattering detector (ELSD) following the method described by Kwon et al [24]. Each in-house reference standard of ginsenosides Rk3 and Rh4 was used for identification and quantitation. Liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) was used to identify ginsenosides Rk3 and Rh4 by measuring molecular ion in negative-ion mode. Sample solutions of KG-KH (0.5 mg/mL) and various concentrations of standards containing ginsenosides Rk3 and Rh4 were prepared by dissolving with methanol. Samples and standards (20 μ L each) were injected, and analyzed using HPLC–ELSD.

2.4. Cell culture, drug treatment, and cell viability assay

LLC-PK1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown with DMEM/F12 medium supplemented with 10% fetal bovine serum and antibiotics (100 units/mL of penicillin G and 100 μ g/mL streptomycin) in a humidified 5% CO₂ incubator at 37°C. LLC-PK1 cells were seeded in appropriate culture plates at 2.0×10^4 cells/cm² and incubated for 1 d before treatment. Cells were treated with 20 μ M of cisplatin with or without KG-KH and incubated further for 24 h. At the end of

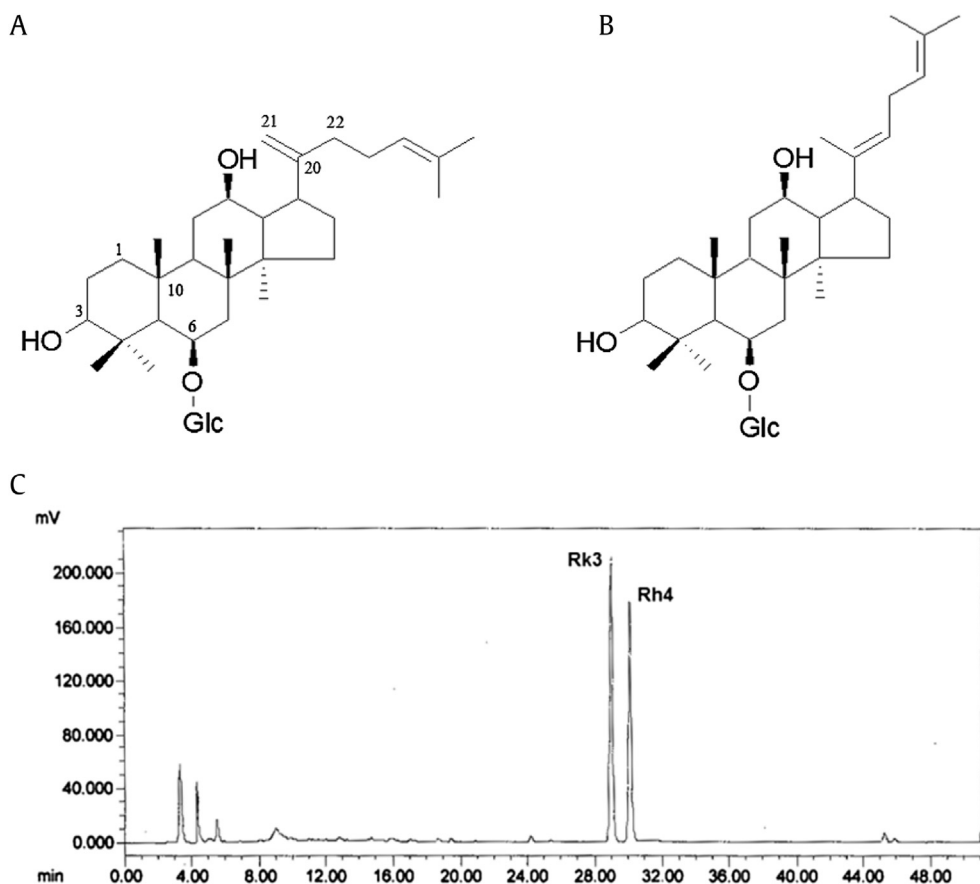


Fig. 1. Structure of ginsenosides and chromatogram of enriched mixture of ginsenosides Rk3 and Rh4 (KG-KH). Structures of (A) ginsenoside Rk3 and (B) ginsenoside Rh4. (C) Chromatogram of KG-KH. Solution of KG-KH (0.5 mg/mL) was analyzed using HPLC coupled with an evaporative light scattering detector.

incubation, cell viability was measured by the MTT assay following manufacturer protocols. Cell viability was expressed as the percentage to that in the normal group (vehicle only).

2.5. ROS measurement

Measurement of intracellular ROS was carried out using an ROS-sensitive fluorescence probe of DCFH-DA [31]. At the end of incubation with cisplatin or KG-KH, cells were incubated with $20\mu\text{M}$ DCFH-DA for 30 min at 37°C and then washed with phosphate-buffered saline (PBS). Cellular fluorescence of DCF, the deacetylated and oxidized product of DCFH-DA, in the presence of cellular ROS was visualized using a fluorescence microscope (Olympus, Tokyo, Japan). For quantitation of ROS generation, flow cytometric analyses were carried out. Labeled cells were detached, washed, and resuspended in PBS. Green fluorescence of DCF in cells was analyzed using a flow cytometer (FACScalibur; BD Bioscience, San Jose, CA, USA).

2.6. In vivo model of cisplatin-induced AKI and drug administration

All experiments were performed in accordance with the National Institutes of Health and Kyung Hee University (Seoul, Korea) guides for Laboratory Animals Care and Use and approved by the Institutional Animal Care and Use Committee in the Kyung Hee Medical Center, Kyung Hee University. Male Sprague–Dawley rats (180–220 g) were purchased from Samtako Biokorea (Seoul, Korea). Animals were housed in a room maintained under 12-h light–dark conditions at $24 \pm 2^\circ\text{C}$ with free access to water and food. Animals were used for experiment after several days of adaptation to their

surroundings. Four groups (6 rats per group) were designated with random clustering to avoid intergroup differences in body weight. AKI was induced by a single intraperitoneal injection of cisplatin (5.0 mg/kg body weight). KG-KH was given via the oral route to cisplatin-treated rats once daily for 5 d from the day of cisplatin injection (2 mg/kg or 6 mg/kg). The normal and control groups were treated with physiological (0.9%) saline or cisplatin injection without KG-KH, respectively. Normal and control rats were given water (*per os*) without KG-KH by following the same treatment schedule. Body weight was measured each day before treatment. Six days after cisplatin injection, blood samples were obtained by cardiac puncture under light ether anesthesia. Rats were sacrificed immediately by cervical dislocation. Serum was recovered immediately by centrifugation (3,000g, 20 min, 4°C). Serum levels of urea nitrogen and creatinine were determined using commercial kits. Simultaneously, kidneys were isolated quickly, washed with cold saline, blotted on filter paper to remove excess saline, and weighed. Thereafter, whole kidneys were dissected into small pieces and homogenized in cold PBS. Aliquots of kidney homogenates were used for the antioxidant enzyme activity assay.

2.7. Antioxidant enzyme activity assay

Cellular or renal levels of the antioxidant enzymes such as catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) were determined using commercial assay kits (Cayman). Cell or tissue homogenates were prepared following manufacturer protocols. GR activity was determined by measuring the oxidation rate of NADPH to NADP^+ . Total SOD activity was

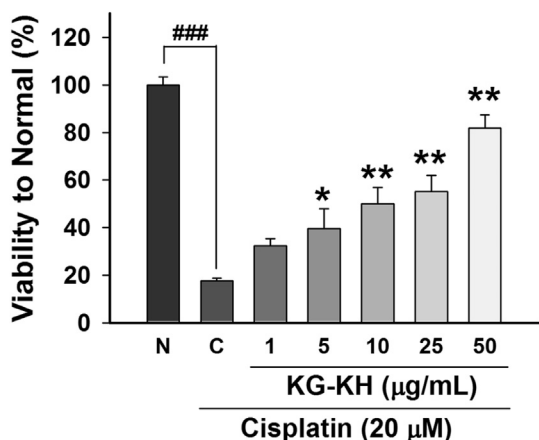


Fig. 2. Ginsenoside Rk3 and Rh4 extract (KG-KH) protects LLC-PK1 cells against cisplatin cytotoxicity. LLC-PK1 cells were treated with cisplatin and/or KG-KH for 24 h. Cell viability was measured using the MTT assay. Bar represents mean \pm standard error of the mean ($n = 3$). Significant differences were determined by the Student t test (### $p < 0.001$ compared with normal cells) or ANOVA (* $p < 0.05$, ** $p < 0.01$ compared with cisplatin control cells). C, cisplatin control cells; N, normal cells.

determined by measuring the dismutation rate of superoxide radicals generated by xanthine oxidase and hypoxanthine. CAT activity was determined by colorimetric detection of formaldehyde produced from methanol by catalase in the presence of hydrogen peroxide. Separately, protein content in each sample was determined using the Bradford Protein Assay kit (Thermo Scientific). Antioxidant enzyme activity was expressed as the activity/mg of protein.

2.8. Histological examinations

Kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 4- μ m sections. Sections were stained with hematoxylin–eosin and analyzed under a light microscope equipped with camera. Histology of kidney was evaluated by the incidences of cell death, exfoliation and shallowing, infiltration, and cast formation. Each pathological marker was scored from 0 to 5 on the basis of the semiquantitative percentage of tissue damage. Tissue injury score was expressed as the sum of all scores [32].

2.9. Statistical analyses

Data are from at least three independent experiments. Results are represented as the mean and standard error of the mean. SAS 9.4 (SAS institute Inc., Cary, NC, USA) was used for statistical analysis. Statistical significance was determined by one-way analysis of variance (ANOVA, Duncan's multiple range tests) or Student t test at indicated p value.

3. Results

3.1. Identification and quantitation of ginsenosides Rk3 and Rh4 in KG-KH

The structure of ginsenosides Rk3 and Rh4 are represented in Fig. 1A and 1B. Identification and quantitation of ginsenosides Rk3 and Rh4 in KG-KH was assessed using HPLC–ELSD and LC–ESI–MS (negative-ion mode) analyses with authentic standards of each ginsenoside. Ginsenosides Rk3 and Rh4 were the major compounds in KG-KH (Fig. 1C). KG-KH was a mixture of 49.3% of Rk3 and 43.1% of Rh4 (w/w %). Separately, the molecular ions of Rk3 and Rh4 [(M-

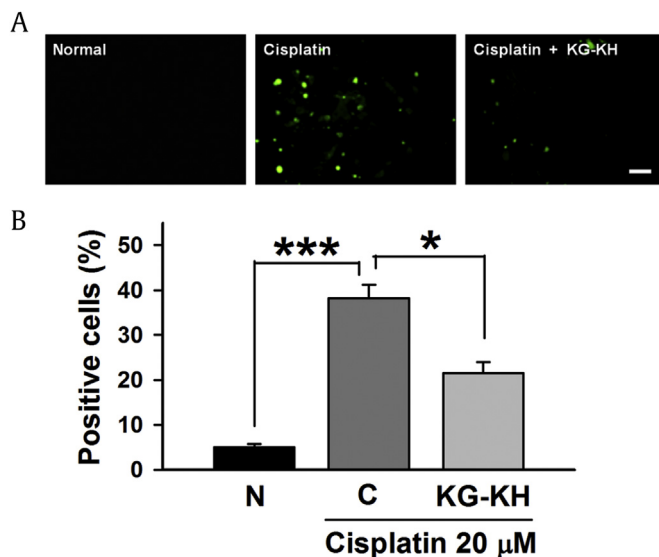


Fig. 3. Ginsenoside Rk3 and Rh4 extract (KG-KH) restores cisplatin-induced reactive oxygen species (ROS) generation in LLC-PK1 cells. LLC-PK1 cells were treated with cisplatin (20 μ M) and/or KG-KH (50 μ g/mL) for 24 h. ROS were determined using a ROS-sensitive fluorescence probe (2',7'-dichlorofluorescein diacetate). (A) Stained cells were visualized using a fluorescence microscope. (B) Scale bar represents 50 μ m. Quantitation was achieved using a flow cytometer. Bar represents mean \pm standard error of the mean ($n = 3$). Significant difference was determined by the Student t test (* $p < 0.05$, *** $p < 0.001$). C, cisplatin control cells; N, normal cells.

H⁻ = 619] were confirmed using LC–ESI–MS analyses (data not shown). Thus, KG-KH was the enriched mixture of ginsenosides Rk3 and Rh4 (total 92.4%, w/w).

3.2. KG-KH reduces cisplatin-induced cytotoxicity in LLC-PK1 cells

To establish a noncytotoxic range, KG-KH was treated alone to LLC-PK1 cells for 24 h and cell viability was measured. KG-KH did not induce noticeable cell death or severe morphologic changes in LLC-PK1 cells at ≤ 50 μ g/mL (data not shown). KG-KH (1–50 μ g/mL) and/or cisplatin (20 μ M) were treated to LLC-PK1 cells for 24 h, and cell viability was assessed using the MTT assay (Fig. 2). Cisplatin induced significant loss of cell viability as compared to the normal cells ($p < 0.01$). Consistent with our

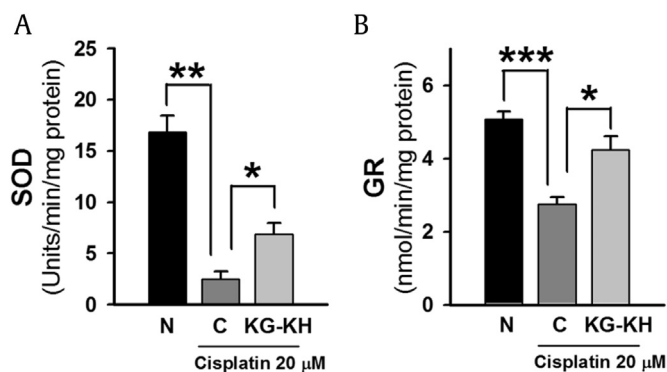


Fig. 4. Ginsenoside Rk3 and Rh4 extract (KG-KH) recovers the antioxidant enzyme activity in cisplatin-treated LLC-PK1 cells. LLC-PK1 cells were treated with cisplatin (20 μ M) and/or KG-KH (50 μ g/mL) for 24 h. At the end of incubation, cells were harvested, and cell homogenates were prepared and used for assays. (A) Superoxide dismutase (SOD) and (B) glutathione reductase (GR) activity was measured using a commercial kit. Bar represents mean \pm standard error of the mean ($n = 3$). Significant differences were determined by the Student t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). C, cisplatin control cells; N, normal cells.

Table 1

Effects of ginsenoside Rk3 and Rh4 (KG-KH) on body weight, kidney weight, kidney index, blood urea nitrogen (BUN), and creatinine in cisplatin-injected rats

	Body weight (g)	Kidney weight (g)	Kidney index ¹⁾	BUN (mg/dL)	Creatinine (mg/dL)
Normal	257.3 ± 9.0	2.00 ± 0.20	0.78 ± 0.08	9.48 ± 3.08	0.78 ± 0.21
CP ²⁾	211.2 ± 7.3 [#]	2.59 ± 0.13 [#]	1.23 ± 0.07 [#]	47.07 ± 6.76 [#]	2.67 ± 0.55 [#]
CP + KG-KH (2 mg/kg)	219.3 ± 13.9	2.39 ± 0.09 ^{**}	1.09 ± 0.04 ^{**}	38.63 ± 4.51 [*]	1.95 ± 0.36 [*]
CP + KG-KH (6 mg/kg)	215.7 ± 17.1	2.29 ± 0.07 ^{**}	1.07 ± 0.1 ^{**}	33.60 ± 4.68 ^{**}	1.80 ± 0.35 ^{**}

Data are presented as mean ± standard deviations (n = 6).

¹⁾ Kidney index represents % ratio of kidney weight to body weight.²⁾ CP indicates cisplatin (5.0 mg/kg, *i.p.*). Statistically significant differences were determined by *t* test ([#] *p* < 0.01 compared with normal group) or ANOVA (^{*} *p* < 0.05; ^{**} *p* < 0.01 compared with CP control group).

previous result, KG-KH significantly reversed cisplatin-induced loss of cell viability in a concentration-dependent manner as compared to the cisplatin control cells (*p* < 0.01 or *p* < 0.05). Protective effect was highest at 50 µg/mL of KG-KH. Next, we tested the effect of KG-KH on cisplatin-induced oxidative injury which is considered as an important factor in the progress of cisplatin nephrotoxicity.

3.3. KG-KH inhibits cisplatin-induced ROS generation in LLC-PK1 cells

ROS generation was assessed by fluorescence microscope and flow cytometer using a ROS-sensitive fluorescent probe (DCFH-DA). Cisplatin induced a predominant increase in the number of green positive cells compared with normal cells, suggesting that cisplatin induced a noticeable increase of ROS generation in cells (Fig. 3A). However, KG-KH reduced the number of green positive cells, suggesting that KG-KH reversed cisplatin-induced ROS generation. Quantitative results using a flow cytometer were also consistent with microscopy data (Fig. 3B). Cisplatin induced a significant increase in the number of green positive cells as compared to normal cells (*p* < 0.001), but KG-KH significantly inhibited the cisplatin-induced increase in the number of green positive cells as compared to cisplatin control cells (*p* < 0.05). These findings implied that KG-KH restored cisplatin-induced oxidative damage in terms of ROS production. Cellular ROS homeostasis is tightly regulated by intracellular antioxidant enzymes, so next we examined the effect of KG-KH on the activity of antioxidant enzymes.

3.4. KG-KH restores cisplatin-induced loss of antioxidant enzyme activity in LLC-PK1 cells (activity of SOD and GR)

We examined the effect of KG-KH on the cellular SOD activity, which detoxifies superoxide anion. To test the function of the cellular GSH recycling system, we also examined the cellular GR activity, which reduces glutathione disulfide to GSH. After incubation with cisplatin (20 µM) or KG-KH (50 µg/mL) in LLC-PK1 cells for 24 h, the cellular activity of SOD and GR was measured. Cisplatin induced a significant decrease of SOD (*p* < 0.01) and GR activity (*p* < 0.001) as compared to normal cells (Figs. 4A and 4B). However, KG-KH significantly recovered the SOD (*p* < 0.05) and GR activity (*p* < 0.05) as compared to cisplatin control cells, suggesting that KG-KH restored the cisplatin-induced alteration of antioxidant enzyme activity.

3.5. KG-KH restores kidney function in cisplatin-induced AKI in rats

Protective effect of KG-KH on the kidney as a whole was assessed using an animal model of cisplatin-induced AKI. AKI was induced by a single intraperitoneal injection of cisplatin (5.0 mg/kg) to male Sprague–Dawley rats. KG-KH or saline (control group) was given to cisplatin-treated rats (2 mg/kg or 6 mg/kg, *per os*) once a day for 5 d starting the day of cisplatin injection. Administered dose of KG-KH did not elicit any noticeable change in the gain of body weight, behavioral disorders, or organ toxicity in the acute toxicity test (data not shown). The effects of KG-KH on body weight, kidney weight, BUN, and creatinine in cisplatin-injected rats are summarized in Table 1. Loss of body weight, a common side effect of chemotherapeutics, was found in all groups treated with cisplatin and/or KG-KH. KG-KH failed to recover the gain of body weight. Cisplatin significantly increased kidney index, BUN, and creatinine as compared to the normal rats (*p* < 0.01). These data suggest that cisplatin induced AKI and nephrotoxicity including kidney edema due to acute inflammation, reduction in interstitial fluid, or impairment of glomerular filtration [33]. KG-KH significantly attenuated cisplatin-induced increase of kidney index, BUN, and creatinine as compared to cisplatin control rats (*p* < 0.01), indicating that KG-KH ameliorated cisplatin-induced AKI and recovered kidney function.

3.6. KG-KH restores the kidney level of antioxidant enzyme activity in cisplatin-induced AKI in rats (SOD and CAT activity)

To examine the effect of KG-KH on oxidative injury in kidney, we determined activity of antioxidant enzymes in the kidney including SOD and CAT. Cisplatin significantly reduced renal SOD (83.8% of normal group, *p* < 0.05) and CAT activity (53.1% of normal group, *p* < 0.01) as compared to normal rats (Fig. 5). As expected, KG-KH significantly recovered the renal SOD (96.4% of normal group at

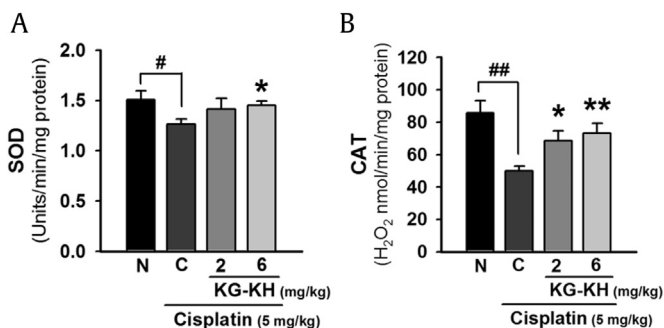


Fig. 5. Ginsenoside Rk3 and Rh4 (KG-KH) recovers the activity of renal antioxidant enzymes in cisplatin-injected rats. Acute kidney injury was induced by single injection of cisplatin (5.0 mg/kg, *i.p.*). Rats were given KG-KH (0 mg/kg, 2 mg/kg, and 6 mg/kg, *per os*) once a day for 5 d from the day of cisplatin injection. Six days after cisplatin injection, kidney levels of (A) superoxide dismutase (SOD) and (B) catalase (CAT) activity were determined in kidney homogenate. Bar represents mean ± standard error of the mean (n = 6). Significant differences were determined by the Student *t* test ([#] *p* < 0.05, ^{##} *p* < 0.01 compared with normal rats) and ANOVA (^{*} *p* < 0.05, ^{**} *p* < 0.01 compared with cisplatin control rats). C, cisplatin control rats; N, normal cells.

6 mg/kg, $p < 0.01$) and CAT activity (85.5% of normal group at 6 mg/kg, $p < 0.01$) in a dose-dependent manner.

3.7. KG-KH improves renal histology in cisplatin-induced AKI in rats

Histopathological study was carried out using hematoxylin–eosin staining of paraffin-embedded kidney sections. Tissue injury was evaluated in the cortex and outer region of the medulla, a region rich in proximal tubules [34]. Representative kidney section images of normal, cisplatin control, and cisplatin with KG-KH groups are shown in Fig. 6A. In normal rats, kidney exhibited normal architecture with clear glomerulus, distal tubules, and proximal tubules in cortex and many ducts in the corticomedullary region (Fig. 6A). Cisplatin increased renal tissue alteration, such as cell death with dilation of tubules and ducts, exfoliation, shallowing, infiltration, and cast formation. Semiquantitative tissue injury score in both regions was significantly higher in cisplatin control than in normal rats ($p < 0.001$; Figs. 6B and 6C). However, KG-KH improved renal histology of both cortex and corticomedullary region in cisplatin-injected rats and significantly ameliorated cisplatin-induced renal tissue damage (Figs. 6B and 6C). Thus, KG-KH reduced renal cell death and tissue alteration, thereby contributing to the restoration of renal function from the cisplatin-induced AKI.

4. Discussion

The major finding of our study was that ginsenosides Rk3 and Rh4 protected renal tubular cells and restored kidney function from cisplatin-induced toxicity in *in vitro* and *in vivo*. We demonstrated that oxidative injury is involved in cisplatin-induced AKI and suggested ginsenosides Rk3 and Rh4 as a potential antioxidant through alleviating cisplatin-induced oxidative injury. Histological results also supported that ginsenosides Rk3 and Rh4 reduced renal cell death and tissue alteration, and protected kidney from cisplatin-induced AKI.

KG-KH protected renal tubular cells and restored kidney function from cisplatin toxicity through reduction of oxidative injury. Cisplatin-induced oxidative injury is considered to be the primary mediator of cisplatin toxicity and orchestrates the subsequent pathologic events leading to tissue damage and failure of the kidney [13,14]. Many antioxidant phytochemicals, including quercetin and narigenin, have been studied for the ability to reduce cisplatin nephrotoxicity through protection against oxidative injury [3,35]. These antioxidants have structural features such as phenolic or polyphenolic groups responsible for the strong radical-scavenging activity. Because of the absence of these functional groups, the antioxidant effect of ginsenosides Rk3 and Rh4 in reducing cisplatin-induced oxidative injury is predicted to be by an

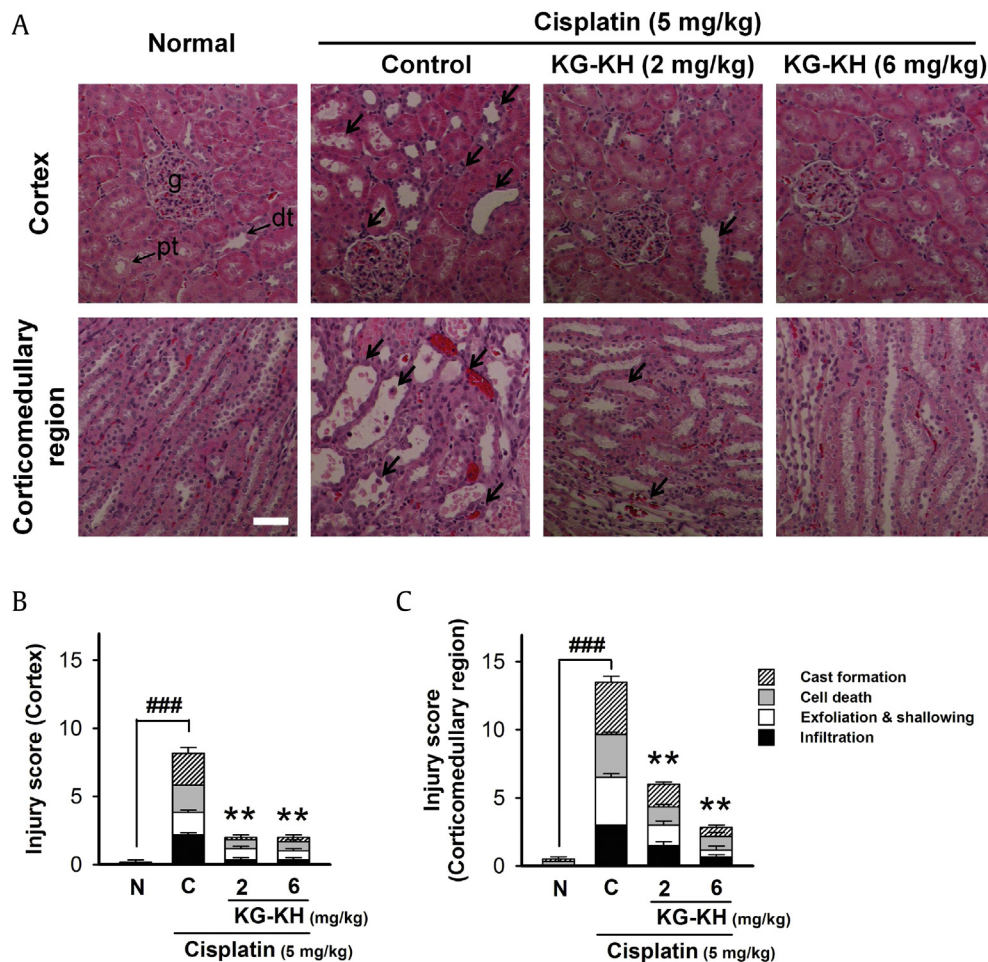


Fig. 6. Ginsenoside Rk3 and Rh4 (KG-KH) improves renal histology in cisplatin-injected rats. (A) Representative images of the renal pathology (hematoxylin–eosin staining) on Day 6 after cisplatin injection with/without KG-KH were presented. Black arrow indicates tissue injury with cell death, infiltration, cast formation, exfoliation and shallowing. Scale bar represents 100 μ m. Tissue injury in the (B) cortex and (C) corticomedullary regions were evaluated by semiquantitative scoring of each pathological marker and expressed as the sum of all scores. Bar represents mean \pm standard error of the mean ($n = 6$). Significant differences were determined by the Student *t* test (### $p < 0.001$ compared with normal rats) or analysis of variance (** $p < 0.01$ compared with cisplatin control rats). C, cisplatin control rats; dt, distal tubule; g, glomerulus; N, normal rats; pt, proximal tubule.

alternative mechanism, i.e., by the restoration of the activity of intrinsic antioxidant enzymes or protection of cellular organelles such as mitochondria rather than by the direct scavenging of radicals. KG-KH improved antioxidant defense enzyme (CAT, GR, SOD) activities inactivated by cisplatin. Because these enzymes play pivotal roles in the metabolism of ROS and recycling of GSH, antioxidant effect of KG-KH might contribute to reduce the cellular burden of cisplatin-mediated oxidative injury.

Several other ginseng saponins have been studied for recovering antioxidant enzyme activity in many models of different types of oxidative stress-mediated diseases. Ginsenosides Rg1 and Re, which are the precursors of Rk3 and Rh4, exhibit protective effects against ischemic brain injury, glutamate-induced lung injury, ROS-induced astrocyte injury, as well as complement-induced podocyte injury through restoration of the antioxidant defense enzyme activities or inhibition of ROS generation [36–39]. Ginsenosides Rg3, Rg5, and Rk1 reduced cisplatin-induced toxicity through inactivation of JNK and p53 [40]. Despite the structural differences in many ginsenosides, ginsenosides exert diverse protective effects on many disease models. Their molecular targets or cellular metabolism should be further studied to understand the bioactivity of individual ginsenoside or ginseng extract.

In conclusion, we have demonstrated that ginsenosides Rk3 and Rh4 alleviate cisplatin cytotoxicity in renal tubular cells and restore kidney function in a rat model of cisplatin-induced AKI. Antioxidant strategies that inhibit ROS generation and recover antioxidant defense enzymes are suggested to be the underlying mechanism of renal protection. Ginsenosides Rk3 and Rh4 could be viable therapeutic agents or adjuvants that reduce nephrotoxicity during cisplatin chemotherapy.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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References

- Lebwohl D, Canetta R. Clinical development of platinum complexes in cancer therapy: an historical perspective and an update. *Eur J Cancer* 1998;34:1522–34.
- Arany I, Safirstein RL. Cisplatin nephrotoxicity. *Semin Nephrol* 2003;23:460–4.
- Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int* 2008;73:994–1007.
- Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. *Nat Rev Drug Discov* 2005;4:307–20.
- Karasawa T, Steyger PS. An integrated view of cisplatin-induced nephrotoxicity and ototoxicity. *Toxicol Lett* 2015;237:219–27.
- Ishida S, Lee J, Thiele DJ, Herskowitz I. Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci USA* 2002;99:14298–302.
- Kuo YM, Gybina AA, Pyatskowitz JW, Gitschier J, Prohaska JR. Copper transport protein (Ctr1) levels in mice are tissue specific and dependent on copper status. *J Nutr* 2006;136:21–6.
- Ludwig T, Riethmuller C, Gekle M, Schwerdt G, Oberleithner H. Nephrotoxicity of platinum complexes is related to basolateral organic cation transport. *Kidney Int* 2004;66:196–202.
- Yonezawa A, Masuda S, Nishihara K, Yano I, Katsura T, Inui K. Association between tubular toxicity of cisplatin and expression of organic cation transporter rOCT2 (Slc22a2) in the rat. *Biochem Pharmacol* 2005;70:1823–31.
- Townsend DM, Deng M, Zhang L, Lapus MG, Hanigan MH. Metabolism of cisplatin to a nephrotoxin in proximal tubule cells. *J Am Soc Nephrol* 2003;14:1–10.
- Townsend DM, Marto JA, Deng M, Macdonald TJ, Hanigan MH. High pressure liquid chromatography and mass spectrometry characterization of the nephrotoxic biotransformation products of cisplatin. *Drug Metab Dispos* 2003;31:705–13.
- Zhang L, Hanigan MH. Role of cysteine S-conjugate beta-lyase in the metabolism of cisplatin. *J Pharmacol Exp Ther* 2003;306:988–94.
- Taguchi T, Nazneen A, Abid MR, Razzaque MS. Cisplatin-associated nephrotoxicity and pathological events. *Contrib Nephrol* 2005;148:107–21.
- Chirino YI, Pedraza-Chaverri J. Role of oxidative and nitrosative stress in cisplatin-induced nephrotoxicity. *Exp Toxicol Pathol* 2009;61:223–42.
- Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C A Meyer. *Acta Pharmacol Sin* 2008;29:1109–18.
- Ernst E. *Panax ginseng*: an overview of the clinical evidence. *J Ginseng Res* 2010;34:259–63.
- Kiefer D, Pantuso T. *Panax ginseng*. *Am Fam Physician* 2003;68:1539–42.
- Park HJ, Kim DH, Park SJ, Kim JM, Ryu JH. Ginseng in traditional herbal prescriptions. *J Ginseng Res* 2012;36:225–41.
- Baek SH, Bae ON, Park JH. Recent methodology in ginseng analysis. *J Ginseng Res* 2012;36:119–34.
- Helms S. Cancer prevention and therapeutics: *Panax ginseng*. *Altern Med Rev* 2004;9:259–74.
- Radad K, Gille G, Liu L, Rausch WD. Use of ginseng in medicine with emphasis on neurodegenerative disorders. *J Pharmacol Sci* 2006;100:175–86.
- Cho IH. Effects of *Panax ginseng* in neurodegenerative diseases. *J Ginseng Res* 2012;36:342–53.
- Qi LW, Wang CZ, Yuan CS. American ginseng: potential structure-function relationship in cancer chemoprevention. *Biochem Pharmacol* 2010;80:947–54.
- Kwon SW, Han SB, Park IH, Kim JM, Park MK, Park JH. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. *J Chromatogr A* 2001;921:335–9.
- Park IH, Han SB, Kim JM, Piao L, Kwon SW, Kim NY, Kang TL, Park MK, Park JH. Four new acetylated ginsenosides from processed ginseng (sun ginseng). *Arch Pharm Res* 2002;25:837–41.
- Park IH, Kim NY, Han SB, Kim JM, Kwon SW, Kim HJ, Park MK, Park JH. Three new dammarane glycosides from heat processed ginseng. *Arch Pharm Res* 2002;25:428–32.
- Im W, Chung JY, Bhan J, Lim J, Lee ST, Chu K, Kim M. Sun ginseng protects endothelial progenitor cells from senescence associated apoptosis. *J Ginseng Res* 2012;36:78–85.
- Kim EJ, Oh HA, Choi HJ, Park JH, Kim DH, Kim NJ. Heat-processed ginseng saponin ameliorates the adenine-induced renal failure in rats. *J Ginseng Res* 2013;37:87–93.
- Lee CH, Kim JM, Kim DH, Park SJ, Liu X, Cai M, Hong JG, Park JH, Ryu JH. Effects of sun ginseng on memory enhancement and hippocampal neurogenesis. *Phytother Res* 2013;27:1293–9.
- Baek SH, Piao XL, Lee UJ, Kim HY, Park JH. Reduction of cisplatin-induced nephrotoxicity by ginsenosides isolated from processed ginseng in cultured renal tubular cells. *Biol Pharm Bull* 2006;29:2051–5.
- Kim YH, Kim YW, Oh YJ, Back NI, Chung SA, Chung HG, Jeong TS, Choi MS, Lee KT. Protective effect of the ethanol extract of the roots of *Brassica rapa* on cisplatin-induced nephrotoxicity in LLC-PK1 cells and rats. *Biol Pharm Bull* 2006;29:2436–41.
- Humanes B, Lazaro A, Camano S, Moreno-Gordaliza E, Lazaro JA, Blanco-Codesido M, Lara JM, Ortiz A, Gomez-Gomez MM, Martin-Vasallo P, et al. Cilastatin protects against cisplatin-induced nephrotoxicity without compromising its anticancer efficiency in rats. *Kidney Int* 2012;82:652–63.
- Bellomo R, Kellum JA, Ronco C. Acute kidney injury. *Lancet* 2012;380:756–66.
- Zsengeller ZK, Ellezian L, Brown D, Horvath B, Mukhopadhyay P, Kalyanaraman B, Parikh SM, Karumanchi SA, Stillman IE, Pacher P. Cisplatin nephrotoxicity involves mitochondrial injury with impaired tubular mitochondrial enzyme activity. *J Histochem Cytochem* 2012;60:521–9.
- Sanchez-Gonzalez PD, Lopez-Hernandez FJ, Perez-Barriocanal F, Morales AI, Lopez-Novoa JM. Quercetin reduces cisplatin nephrotoxicity in rats without compromising its anti-tumour activity. *Nephrol Dial Transplant* 2011;26:3484–95.
- Lopez MV, Cuadrado MP, Ruiz-Poveda OM, Del Fresno AM, Accame ME. Neuroprotective effect of individual ginsenosides on astrocytes primary culture. *Biochim Biophys Acta* 2007;1770:1308–16.
- Shen L, Han JZ, Li C, Yue SJ, Liu Y, Qin XQ, Liu HJ, Luo ZQ. Protective effect of ginsenoside Rg1 on glutamate-induced lung injury. *Acta Pharmacol Sin* 2007;28:392–7.
- Zhang MH, Fan JM, Xie XS, Deng YY, Chen YP, Zhen R, Li J, Cheng Y, Wen J. Ginsenoside-Rg1 protects podocytes from complement mediated injury. *J Ethnopharmacol* 2011;137:99–107.
- Zhou XM, Cao YL, Dou DQ. Protective effect of ginsenoside-Re against cerebral ischemia/reperfusion damage in rats. *Biol Pharm Bull* 2006;29:2502–5.
- Park JY, Choi P, Kim T, Ko H, Kim HK, Kang KS, Ham J. Protective effects of processed ginseng and its active ginsenosides on cisplatin-induced nephrotoxicity: *in vitro* and *in vivo* studies. *J Agric Food Chem* 2015;63:5964–9.