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Research article

Protective effect of ginsenosides Rk3 and Rh4 on cisplatin-induced acute kidney injury *in vitro* and *in vivo*

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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 20μM 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.

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

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to deleterious loss of cellular function and subsequent cell death through interaction with cellular 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 (Δ20–21 and Δ20–22, 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 μg/mL streptomycin sulfate) for LLC-PK1 culture were purchased from Invitrogen (Carlsbad, CA, USA). For reactive oxygen species (ROS) measurement, 2′,7′-dichlorofluorescin 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 was 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 μL 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 μg/mL streptomycin) in a humidified 5% CO_2 incubator at 37°C. LLC-PK1 cells were seeded in appropriate culture plates at 2.0×10^4 cells/cm^2 and incubated for 1 d before treatment. Cells were treated with 20μM of cisplatin with or without KG-KH and incubated further for 24 h. At the end of

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