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Research article

Beneficial effects of fermented black ginseng and its ginsenoside 20(S)-Rg3 against cisplatin-induced nephrotoxicity in LLC-PK1 cells

Myoung-Sik Han [1,☆], Im-Ho Han [2,3,☆], Dahae Lee [4], Jun Min An [5], Su-Nam Kim [3], Myoung-Sook Shin [3], Noriko Yamabe [4], Gwi Seo Hwang [4], Hye Hyun Yoo [6], Suk-Jung Choi [2], Ki Sung Kang [4,*], Hyuk-Jai Jang [1,**]

[1] Department of Surgery, University of Ulsan College of Medicine, Gangneung, Korea
[2] Department of Chemistry, Gangneung-Wonju National University, Gangneung, Korea
[3] Natural Products Research Institute, Korea Institute of Science and Technology, Gangneung, Korea
[4] College of Korean Medicine, Gachon University, Seongnam, Korea
[5] GINSENG BY PHARM Co., Ltd., Wonju, Korea
[6] College of Pharmacy, Hanyang University, Ansan, Korea

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A B S T R A C T

Background: Nephrotoxicity is a common side effect of medications. *Panax ginseng* is one of the best-known herbal medicines, and its individual constituents enhance renal function. Identification of its efficacy and mechanisms of action against drug-induced nephrotoxicity, as well as the specific constituents mediating this effect, have recently emerged as an interesting research area focusing on the kidney protective efficacy of *P. ginseng*.
Methods: The present study investigated the kidney protective effect of fermented black ginseng (FBG) and its active component ginsenoside 20(S)-Rg3 against cisplatin (chemotherapy drug)-induced damage in pig kidney (LLC-PK1) cells. It focused on assessing the role of mitogen-activated protein kinases as important mechanistic elements in kidney protection.
Results: The reduced cell viability induced by cisplatin was significantly recovered with FBG extract and ginsenoside 20(S)-Rg3 dose-dependently. The cisplatin-induced elevated protein levels of phosphorylated c-Jun N-terminal kinase (JNK), p53, and cleaved caspase-3 were decreased after cotreatment with FBG extract or ginsenoside 20(S)-Rg3. The elevated percentage of apoptotic LLC-PK1 cells induced by cisplatin treatment was significantly abrogated by cotreatment with FBG and the ginsenoside 20(S)-Rg3.
Conclusion: FBG and its major ginsenoside 20(S)-Rg3, ameliorated cisplatin-induced nephrotoxicity in LLC-PK1 cells by blocking the JNK–p53–caspase-3 signaling cascade.
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1. Introduction

Panax ginseng is one of the best-known herbal medicines and its individual constituents enhance renal function [1]. *P. ginseng* effectively ameliorated renal dysfunction induced by streptozotocin in rats by attenuating oxidative stress [2]. Korean Red Ginseng not only inhibited the formation of advanced glycation end products and expression of tumor necrosis factor (TNF)-α, but also blocked the mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cell-mediated inflammatory pathways in streptozotocin-induced diabetic renal damage [3].

Nephrotoxicity is a common side effect of medications and accounts for approximately 20% of hospital admissions for acute

* Corresponding author. College of Korean Medicine, Gachon University, 1342 SeongnamDaero, Sujeong-Gu, Seongnam 461-701, Korea.
** Corresponding author. Department of Surgery, University of Ulsan College of Medicine, 38 Bangdong gil, Sachean Myeon, Gangneung 210-711, Korea.
E-mail addresses: kkang@gachon.ac.kr (K.S. Kang), jhj@gnah.co.kr (H.-J. Jang).
☆ These authors contributed equally to the work described in this study.

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kidney injury [4]. Toxic effects on the kidney related to medications are common and may present as a subtle injury or overt renal failure [5]. Therefore, evaluation of the efficacy of *P. ginseng* in drug-induced nephrotoxicity, as well as the mechanisms and active constituents involved in this action, have recently emerged as an interesting area of ginseng research focused on its potential efficacy for kidney protection [6].

P. ginseng prevented renal impairment induced by gentamicin, an aminoglycoside antibiotic, in rats [1]. Gentamicin-induced nephrotoxicity is related to oxidative damage. Coadministration with *P. ginseng* decreased the renal damage induced by gentamicin via the inhibition of free radical formation and restoration of the antioxidant systems [7,8]. Among the several constituents of ginseng, phenolic acids and flavonoids, which are responsible for the increase in renal blood flow and elimination of free radicals, exhibited protective effects against gentamicin-induced oxidative nephrotoxicity [9].

Cyclosporine, an immunosuppressant drug causes impairment, typical pathologic lesions, and apoptotic cell death in the kidneys [10]. Ginseng protects against cyclosporine-induced renal injury by decreasing the induction of excessive autophagosomes and protein aggregates [11]. Korean Red Ginseng also exhibited protective effects in cyclosporine-induced renal injury via the reduction of renal dysfunction, oxidative stress, and proinflammatory molecules such as induced nitric oxide synthase, cytokines, and transforming growth factor-β1 in rats [10].

Ginsenosides, which are 30-carbon glycosides derived from the triterpenoid dammarane, are major active constituents of *P. ginseng*. Ginsenosides inhibited the cantharidin-induced cytotoxicity in normal rat kidney cells. Pretreatment with ginsenosides reduced the increases in serum creatinine, urine protein, blood urea nitrogen, and histological changes in rats [12]. These findings may reflect the improvements of renal dysfunction by certain ginsenosides in drug-induced nephrotoxicity.

Research has been conducted to develop methods for increasing the pharmacological efficacy of ginseng by converting the dammarane-based saponins using thermal processing [13]. In line with this notion, black ginseng has been prepared by heat processing and fermentation of raw ginseng. Although some studies of ginseng have focused on its renoprotective effects in diabetes [3], little is known about the effects of black ginseng against nephrotoxicity induced by medications.

The present study sought to investigate the renoprotective effect of black ginseng and its active component ginsenoside 20(S)-Rg3, against cisplatin (a chemotherapy drug)-induced damage in pig kidney (LLC-PK1) cells. In addition, we focused on assessing the role of MAPKs as important mechanistic elements in the kidney protective effects of black ginseng.

2. Materials and methods

2.1. Chemicals and reagents

Ginsenoside 20(S)-Rg3 was purchased from the Ambo Institute (Dajeon, Korea). Cisplatin was purchased from Sigma Aldrich (Seoul, Korea). Dulbecco's modified Eagle's medium was obtained from Cellgro (Manassas, VA, USA). Fetal bovine serum was purchased from Invitrogen Co. (Grand Island, NY, USA). The antibodies for p38 MAPK, phospho-p38, p44/42 MAPK-extracellular signal-regulated kinases, phospho-p44/42, c-Jun-N-terminal kinase (JNK), phospho-JNK, p53, cleaved caspase-3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the horseradish peroxidase-conjugated antirabbit antibodies were procured from Cell Signaling (Boston, MA, USA). Other chemicals and reagents were of high quality and obtained from commercial sources.

2.2. Preparation of ginseng extracts

Dried powder extract of black ginseng was supplied by the Ginseng By Pharm Co., Ltd., (Wonju, Korea). Four-year-old white ginseng was purchased from a local ginseng market (Geumsan, Korea). The authenticity of the ginseng was determined based on the ingredient profile. The black ginseng was prepared by nine cycles of repeated steaming of the white ginseng at 85°C for 8 h and drying at 50°C for 48 h. To prepare the ginseng extracts, black ginseng was crushed into a powder and extracted once with 10 volumes of distilled water at 80°C for 72 h, and then filtered and chilled. The ginseng extract was fermented with *Saccharomyces cerevisiae* (Lallemand, Grenaa, Denmark) at 34°C for 25 h. Following fermentation, the black ginseng extract was sterilized at 85°C for 22 h, and then lyophilized. The ginsenosides present in the black ginseng extracts used in this study were Rg2, Rg3, Rh1, Rh2, and Rf at a content of 2.86 μg/mL, 24.52 μg/mL, 12.62 μg/mL, 0.63 μg/mL, and 1.32 μg/mL, respectively [14].

2.3. Renoprotective effect against cisplatin-induced damage in kidney cells

The protective effect against oxidative renal cell damage was evaluated using LLC-PK1 cells [15]. The LLC-PK1 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 4mM L-glutamine at 37°C in an atmosphere of 5% CO_2. The cells were seeded in 96-well culture plates at 1×10^4 cells/well and allowed to adhere for 2 h. Then, the test sample, radical donor 25μM cisplatin, or both were added to the culture medium. Following a 24-h incubation, the medium containing the test sample, radical donor, or both was removed. The cells were incubated in serum-free medium (90 μL/well) and Ez-Cytox reagent (10 μL/well, Itsbio, Seoul, Korea) at 37°C for 2 h. The cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (PowerWave XS; BioTek Instruments, Winooski, VT, USA).

2.4. Western blot analysis

Whole-cell extracts were prepared according to the manufacturer's instructions using a radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, USA) supplemented with 1mM phenylmethylsulfonyl fluoride. The proteins (whole-cell extracts, 20 μg/lane) were separated using electrophoresis in a precast 4–15% Mini-PROTEAN TGX gel (Bio-Rad, Hercules, CA, USA) blotted onto polyvinylidene fluoride (PVDF) membranes and analyzed using epitope-specific primary and secondary antibodies [16]. The bound antibodies were visualized using an enhanced chemiluminescence advance western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and a Fusion Solo chemiluminescence system (Peqlab Biotechnologie GmbH, Erlangen, Germany).

2.5. Image-based cytometric assay

LLC-PK1 cells were used for an image-based apoptosis assay system. All assays were conducted in accordance with the guidelines for operating the Tali image-based cytometer (Invitrogen, Carlsbad, CA, USA). The cells were treated with samples for 24 h at 37°C under 5 % CO_2. The cells were harvested by trypsin treatment using the TrypLE reagent and stained using the Tali apoptosis kit (Invitrogen). The sample was divided and analyzed independently using both the Tali image and flow cytometers following the

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