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Corresponding Author: Prof. Ki Sung Kang, Ph.D.

Corresponding Author's Institution: Gachon University

First Author: Myoung-Sik Han

Order of Authors: Myoung-Sik Han; Dahae Lee; Jun Min An; Su-Nam Kim; Myoung-Sook Shin; Noriko Yamabe; Gwi Seo Hwang; Hye Hyun Yoo; Ki Sung Kang, Ph.D.; Hyuk-Jai Jang

Gachon University

Dear Editor

I am sending herewith a manuscript entitled "Protective effects of black ginseng and its ginsenoside 20(S)-Rg3 against cisplatin-induced nephrotoxicity in LLC-PK1 cells" by Myoung-Sik Han, Dahae Lee, Jun Min An, Su-Nam Kim, Myoung-Sook Shin, Noriko Yamabe, Gwi Seo Hwang, Hye Hyun Yoo, Ki Sung Kang and Hyuk-Jai Jang. The paper consists of 16 pages of manuscript (including the title page, abstract, text and figure legends) and 3 figures.

We have been investigating the chemical and biological activity changes of *Panax ginsneg* and its active ginsenosides by heat-processing (Yamabe et al., *Food Chem.* **2013**, 138, 876-883; Jang et al., *J Aric Food Chem.* **2014**, 62, 2830-2836). 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*. We recently discovered that black ginseng and its major ginsenoside 20(S)-Rg3, ameliorated cisplatin-induced nephrotoxicity in LLC-PK1 cells by blocking the MAPKs-p53-caspase-3 signaling cascade.

We would be grateful if the manuscript could be reviewed and considered for publication in *Journal of Ethnopharmacology*. Thank you.

Yours sincerely,

All correspondence concerning this paper should be addressed to:

Prof. Ki Sung Kang

College of Korean Medicine, Gachon University

Tel: +82-31-750-5402

Fax: +82-31-750-5416

E-mail: kkang@gachon.ac.kr

Journal of Ethnopharmacology
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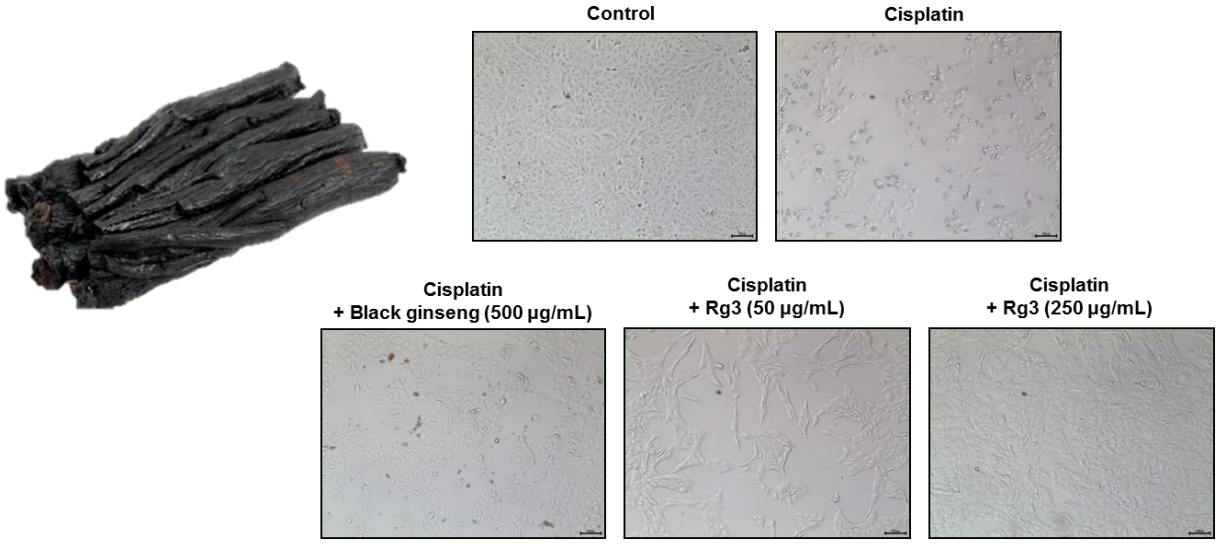
Protective effect of black ginseng and its active component ginsenoside 20(S)-Rg3 against anticancer drug-induced nephrotoxicity in LLC-PK1 cells

Myoung-Sik Han[a,1], Dahae Lee[b,1], Jun Min An[c], Su-Nam Kim[d], Myoung-Sook Shin[d], Noriko Yamabe[b], Gwi Seo Hwang[b], Hye Hyun Yoo[e], Ki Sung Kang[b,*], Hyuk-Jai Jang[a,*]



ABSTRACT

Ethnopharmacological relevance: 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*.

Materials and methods: The present study investigated the kidney-protective effect of black ginseng and its active component ginsenoside 20(S)-Rg3 against cisplatin (chemotherapy drug)-induced damage in LLC-PK1 cells. It focused on assessing the role of mitogen-activated protein kinases (MAPKs) as important mechanistic elements in its kidney protection.

Results: The reduced cell viability induced by cisplatin was significantly recovered with black ginseng 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 co-treatment with black ginseng extract or ginsenoside 20(S)-Rg3. The elevated percentage of apoptotic LLC-PK1 cells induced by cisplatin treatment was significanlty abrogated by co-treatment with black ginseng and the ginsenoside 20(S)-Rg3.

Conclusion: Black ginseng and its major ginsenoside 20(S)-Rg3, ameliorated cisplatin-induced nephrotoxicity in LLC-PK1 cells by blocking the MAPKs-p53-caspase-3 signaling cascade.

Keywords: Panax ginseng, ginsenoside 20(S)-Rg3, nephrotoxicity, mitogen-activated protein kinases, cisplatin

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[a]*Department of Surgery, University of Ulsan College of Medicine, Gangneung 210-711, Korea*

[b]*College of Korean Medicine, Gachon University, Seongnam 461-701, Korea*

[c]*GINSENG BY PHARM. Co., Ltd., Wonju 220-862, Korea*

[d]*Natural Products Research Institute, Korea Institute of Science and Technology, Gangneung 210-340, Korea*

[e]*College of Pharmacy, Hanyang University, Ansan, Gyeonggi-do 426-791, Korea*

[1]These authors contributed equally to the work described in this study.

[*]Corresponding authors:

Tel.: 82-31-750-5402; Fax: 82-31-750-5416; E-mail: kkang@gachon.ac.kr (Kang KS)

Tel.: 82-33-610-3221; Fax: 82-33-641-8120; E-mail: jhj@gnah.co.kr (Jang HJ)

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Keywords: Panax ginseng, ginsenoside 20(S)-Rg3, nephrotoxicity, mitogen-activated protein kinases, cisplatin

1. Introduction

Panax ginseng is one of the best-known herbal medicines and its individual constituents enhance renal function (Lee et al., 2013). *P. ginseng* effectively ameliorated renal dysfunction induced by streptozotocin (STZ) in rats by attenuating oxidative stress (Zakaria et al., 2011). Korean red ginseng not only inhibited the formation of advanced glycation end products (AGEs) and expression of tumor necrosis factor (TNF)-α, but also blocked the mitogen-activa ted protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-mediated inflammatory pathways in STZ-induced diabetic renal damage (Quan et al., 2013).

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 (Kang et al., 2013). Nephrotoxicity is a common side effect of medications and accounts for about 20% of hospital admissions for acute kidney injury (Nasiri-Toosi et al., 2012). Toxic effects on the kidney related to medications are common and may present as a subtle injury or overt renal failure (Choudhury and Ahmed, 2006).

P. ginseng prevented renal impairment induced by gentamicin, an aminoglycoside antibiotic, in rats (Lee et al., 2013). Gentamicin-induced nephrotoxicity is related to oxidative damage. Co-administration with *P. ginseng* decreased the renal damage induced by gentamicin via the inhibition of free radical formation and restoration of the antioxidant systems (Karadeniz et al., 2008; Kalkan et al., 2012). 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 (Qadir et al., 2011).

Cyclosporine, an immunosuppressant drug causes impairment, typical pathologic lesions,

and apoptotic cell death in the kidney (Doh et al., 2013). Ginseng protects against cyclosporine-induced renal injury by decreasing the induction of excessive autophagosomes and protein aggregates (Lim et al., 2014). 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 (iNOS), cytokines, and transforming growth factor (TGF)-β1 in rats (Doh et al., 2013).

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 (NRK) cells. Pretreatment with ginsenosides reduced the increases in serum creatinine, urine protein, blood urea nitrogen, and histological changes in rats. (Xu et al., 2013). 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 (Kim et al., 2013; Le et al., 2014; Lin et al., 2015). 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 (Kang et al., 2013), 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 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 (South Korea). Cisplatin

was purchased from Sigma Aldrich (Seoul, South Korea). Dulbecco's modified Eagle's medium (DMEM) was obtained from Cellgro (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Invitrogen Co. (Grand Island, NY, USA). The antibodies for p38 MAPK, phospho-p38, p44/42 MAPK-extracellular signal-regulated kinases (ERK1/2), phospho-p44/42 (ERK1/2), c-Jun-N-terminal kinase (JNK), phospho-JNK, p53, cleaved caspase-3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the horseradish peroxidase (HRP)-conjugated anti-rabbit 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

The 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 center (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, 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, 24.52, 12.62, 0.63, and 1.32 μg/mL, respectively (Bak et al., 2014).

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 (Yokozawa et al., 2000; Yamabe et al., 2012). The LLC-PK1 (pig kidney epithelium CL-101) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 4 mM 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 per well and allowed to adhere for 2 h. Then, the test sample, radical donor 25 μM 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) 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 (RIPA) buffer (Cell Signaling, MA, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The proteins (whole-cell extracts, 20 μg/lane) were separated using electrophoresis in a precast 4–15% Mini-PROTEAN TGX gel (Bio-Rad, CA, USA) blotted onto polyvinylidene fluoride (PVDF) membranes and analyzed using epitope-specific primary and secondary antibodies. The bound antibodies were visualized using an enhanced chemiluminescence (ECL) advance western blotting detection reagents (GE Healthcare, UK) and a Fusion Solo chemiluminescence system (Peqlab Biotechnologie GmbH, 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, 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. The sample was divided and analyzed independently using both the Tali image and flow cytometers following the manufacturer's recommended protocols. The apoptotic portion of the cell population was determined by staining with annexin V-Alexa Fluor 488 conjugate. Propidium iodide (PI) was used to differentiate the dead cells (annexin V positive/PI positive or annexin V negative/PI positive) from the apoptotic (annexin V positive/PI negative). The percentages of the cell population reported as viable, apoptotic, and dead by the Tali cytometer were comparable with the data from the same samples independently run on the flow cytometer.

2.6. Statistical analysis

The statistical significance was determined using an analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. P values < 0.05 were considered statistically significant. The analysis was performed using the statistics for the social sciences (SPSS) ver. 19.0 (SPSS Inc., Chicago, Il, USA).

3. Results and Discussion

Cisplatin is one of the most effective and widely used anticancer drugs for the treatment of various solid tumors; however, it causes nephrotoxicity, one of the most well-known and clinically important side-effects (Oh et al., 2014). Earlier studies demonstrated that several mechanisms including oxidative stress, DNA damage, and inflammatory responses are closely associated with cisplatin-induced nephrotoxicity (Trujillo et al., 2013; Oh et al., 2014). Cisplatin is known to activate p38, ERK, and JNK/stress-activated protein kinases (SAPK) in the kidney both in vitro and in vivo **(**Ramesh et al., 2005). In addition, genomic DNA is presumably a primary target of cisplatin in tubular cells. The tumor suppressor gene p53 is induced in response to DNA damage, and is implicated in subsequent DNA repair and cell

death by apoptosis (Jiang et al., 2004; Yano et al., 2007). In the present study, we sought to identify the kidney-protective effect of black ginseng extract and its active ginsenoside 20(S)-Rg3, as well as the mechanisms involved, to determine its therapeutic potential.

We carried out a cell-based kidney protection assay to evaluate the protective effects of black ginseng extract and ginsenoside 20(S)-Rg3 on pig kidney epithelial LLC-PK1 cells. The kidney cell protection assay condition was established using the LLC-PK1 cell line, which is commonly used in the evaluation of nephrotoxicity (Lee et al., 2012; Kim et al., 2014). The viability of the LLC-PK1 cells reduced to 60% of the control cell viability after treatment with 25 μM of cisplatin. The reduced cell viability induced by cisplatin was significantly recovered by black ginseng and ginsenoside 20(S)-Rg3 dose-dependently (Figure 1). The black ginseng extract and ginsenoside 20(S)-Rg3 ameliorated the cisplatin-induced nephrotoxicity to control levels at concentrations of 500 and 250 μg/mL, respectively.

Dysregulation of normal MAPK signaling has been implicated in both acute and chronic kidney disease (Cassidy et al., 2012). In the present study, we sought to determine the role of the MAPKs-p53-casapse apoptotic cascade in mediating the protective effects of black ginseng and ginsenoside 20(S)-Rg3 against oxidative cytotoxicity in renal cells. As shown in Figure 2, the phosphorylation of JNK was observed 24 h after cisplatin treatment and decreased following treatment with black ginseng and ginsenoside 20(S)-Rg3.

Cisplatin-induced nephrotoxicity is dependent on DNA damage-induced apoptosis (Pippin et al., 2003). The protein level of p53, which was also markedly increased after treatment with cisplatin, was reduced significantly by a high concentration of black ginseng and ginsenoside 20(S)-Rg3. Similarly, the elevated levels of cleaved caspase-3 also decreased after treatment with black ginseng and ginsenoside 20(S)-Rg3. Figure 3 shows the effects of black ginseng extract and ginsenoside 20(S)-Rg3 on apoptosis in LLC-PK1 cells. As shown in Figure 3A**,** the number of dead and apoptotic cells, which were stained with red and green fluorescence

and increased after cisplatin treatment, decreased following co-treatment with black ginseng and more significantly with ginsenoside 20(S)-Rg3. The elevated percentage of apoptotic LLC-PK1 cells induced by cisplatin treatment was markedly decreased following the co-treatment with black ginseng and ginsenoside 20(S)-Rg3 (Figure 4B).

Cisplatin-induced kidney damage has been correlated with the formation of free radicals and oxidative stress, which can activate MAPKs (Kang et al., 2014; Kim et al., 2014; Song et al., 2015). Treatment with antioxidants and caspase inhibitors can alleviate the effects of cisplatin-related nephrotoxicity. There is considerable evidence showing that the MAPKs, p53, and caspase pathways play important roles in regulation of the apoptosis pathways, as well as the inflammatory process (Francescato et al., 2007; Yano et al., 2007). The present results show that the MAPKs-p53-caspase-3 signaling cascade plays a critical role in mediating the protective effects of black ginseng and ginsenoside 20(S)-Rg3 against oxidative cytotoxicity in cultured LLC-PK1 cells.

Ginseng showed protective effects against cisplatin-induced nephrotoxicity by alleviating the decreased activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and glutathione S-transferase (GST) as well as the levels of glutathione (GHS). This effect consequently reduced the oxidative stress, alterations in biochemical parameters, histological changes, genomic DNA damage, as well as the expression of the TNF-α, interleukin (IL)-6, and tumor suppressor p53 (Yousef et al., 2015). Pretreatment with ginsenoside F11 reduced cisplatin-induced elevation of blood urea nitrogen and creatinine levels, as well as ameliorated the histopathological damage. Further studies showed that F11 suppressed p53 activation, inversed the ratio of Bcl-2 associated X protein (Bax)/B-cell lymphoma 2 (Bcl-2), and the anti-oxidative and free radical levels induced by cisplatin, which in turn inhibited tubular cell apoptosis (Wang et al., 2014). However, the effect and mechanism of action of ginsenoside 20(S)-Rg3 were not identified as

so far.

In conclusion, our results suggest that black ginseng ameliorates nephrotoxicity in LLC-PK1 cells, and ginsenoside 20(*S*)-Rg3 is likely the major component that mediates this effect through blockade of the MAPKs-p53-caspase-3 signaling cascade.

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Conflicts of interest

The authors have declared no conflict of interest.

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Figure legends

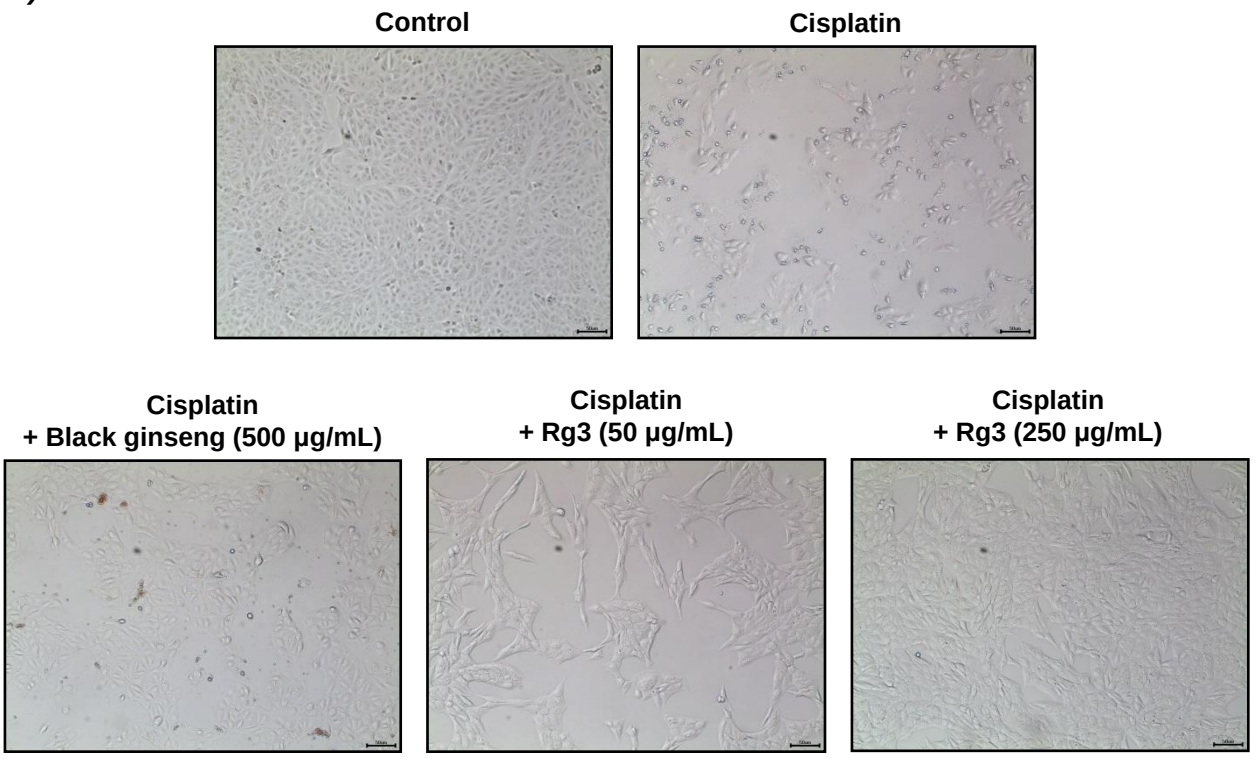
Figure 1. The protective effects of black ginseng and ginsenoside 20(S)-Rg3 on cisplatin-induced renal cell damage. (A) Representative microscopic image of nephroprotective effects of black ginseng and ginsenoside 20(S)-Rg3 following treatment with 25 μM cisplatin. (B) Protection assay of black ginseng and ginsenoside 20(S)-Rg3 in cisplatin-treated (25 μM) LLC-PK1 cells. LLC-PK1 cells were pretreated with various concentrations of black ginseng and ginsenoside 20(S)-Rg3 for 2 h, and then further treated with 25 μM of cisplatin for 24 h. Cell viability was assessed using the MTT assay. $^{*}P < 0.05$ compared with the cisplatin-treated group. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Figure 2. The c-Jun N-terminal kinase (JNK)-p53-caspase-3 signaling pathway mediates the protective effect of black ginseng and ginsenoside 20(S)-Rg3 against cytotoxicity in cultured LLC-PK1 cells. Results of the western blot analysis show the levels of phosphorylated (p)-p38, p38, p-ERK, ERK, p-JNK, JNK, p53, cleaved caspase-3, and GAPDH in LLC-PK1 cells treated with black ginseng and ginsenoside 20(S)-Rg3 with or without cisplatin at different concentrations for 24 h. Whole cell lysates (20 μg) were separated using SDS-PAGE, transferred onto PVDF transfer membranes, and probed with the indicated antibodies. Proteins were visualized using an ECL detection system. ERK, extracellular signal-regulated kinases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence.

Figure 3. Effects of black ginseng and ginsenoside 20(S)-Rg3 on apoptosis in LLC-PK1 cells. (A) Representative images of apoptosis detection. (B) Percentage of annexin V-positive-stained apoptotic cells. Dead and apoptotic cells were stained with red and green fluorescence. Apoptosis was determined using a Tali image-based cytometer.

Figure

(A)



(B)

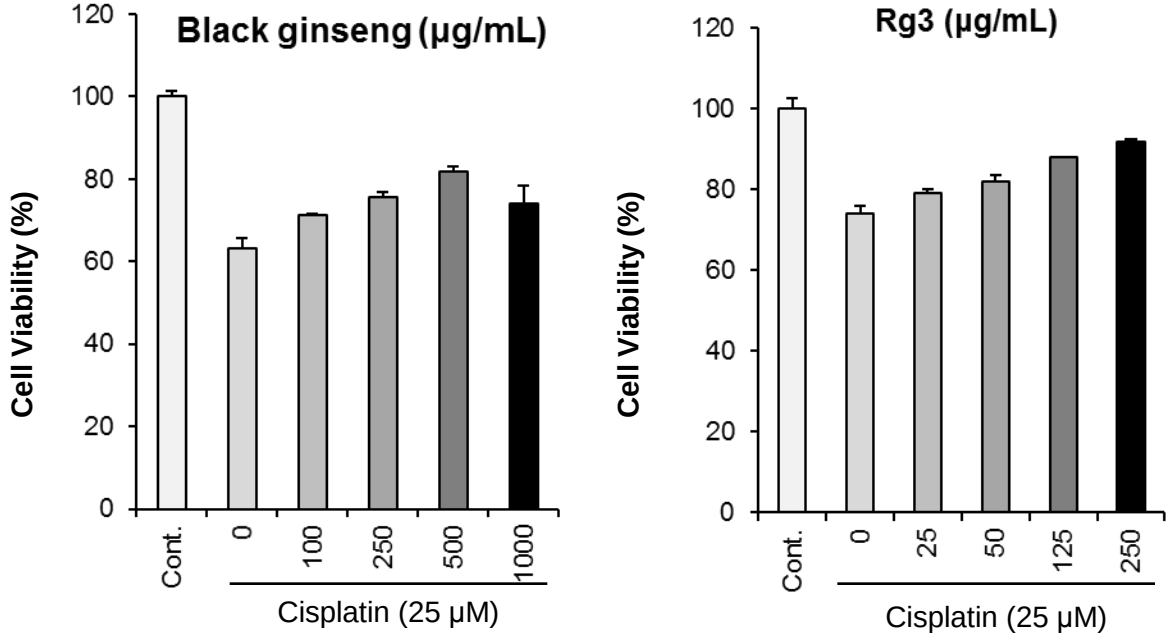


Figure. 1

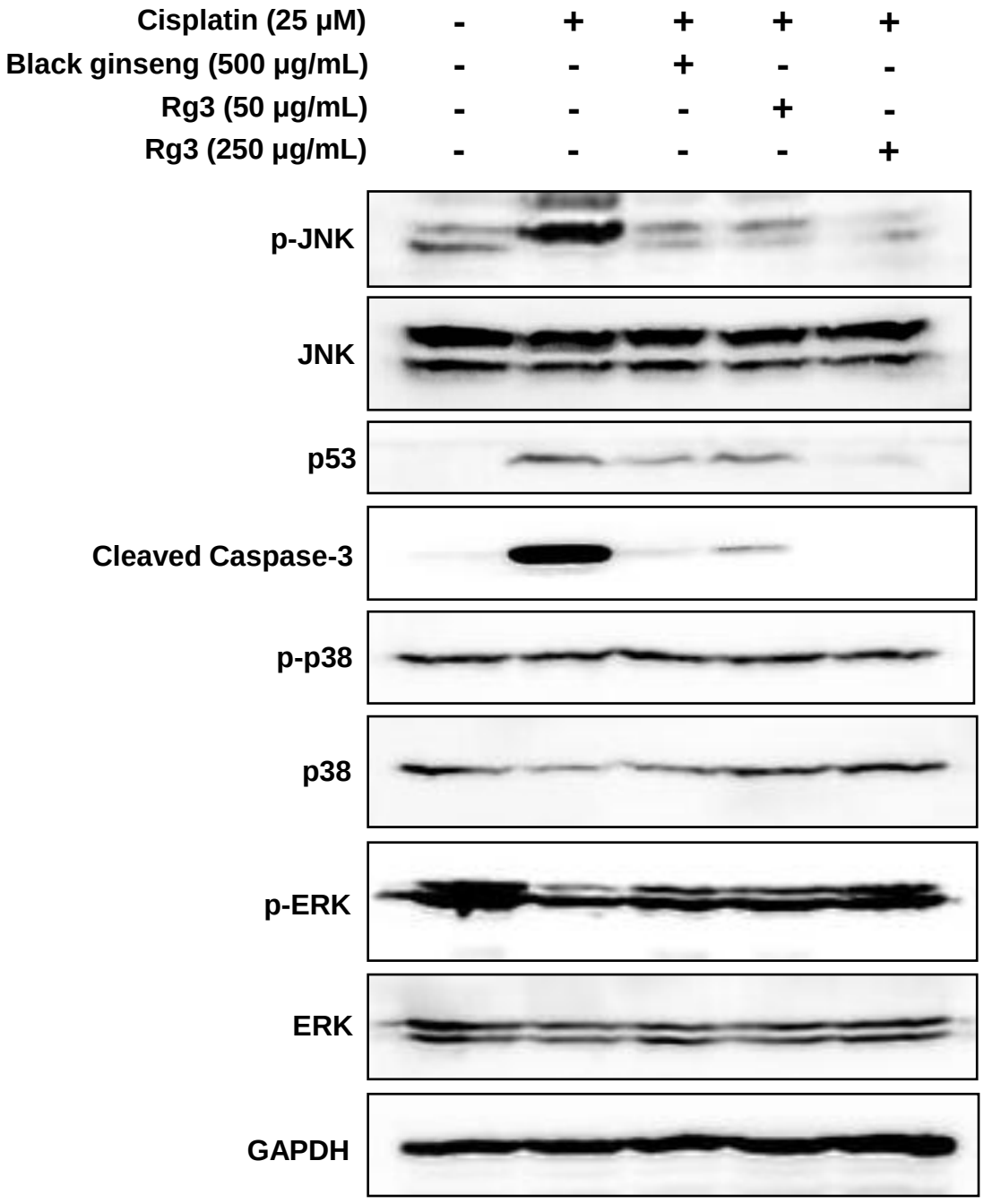
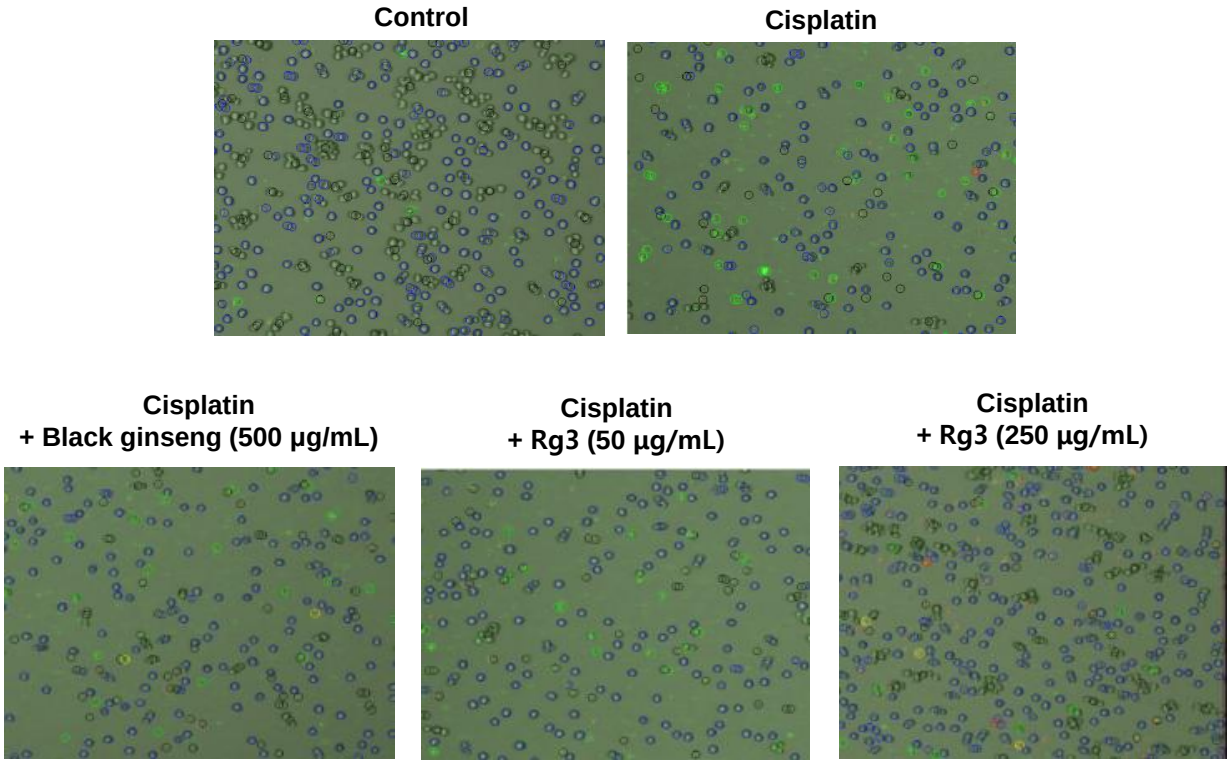

Cisplatin (25 μM) - + + + +
Black ginseng (500 μg/mL) - - + - -
Rg3 (50 μg/mL) - - - + -
Rg3 (250 μg/mL) - - - - +
p-JNK
JNK
p53
Cleaved Caspase-3
p-p38
p38
p-ERK
ERK
GAPDH

Figure. 2

(A)


Control
Cisplatin
Cisplatin
+ Black ginseng (500 μg/mL)
Cisplatin
+ Rg3 (50 μg/mL)
Cisplatin
+ Rg3 (250 μg/mL)

(B)

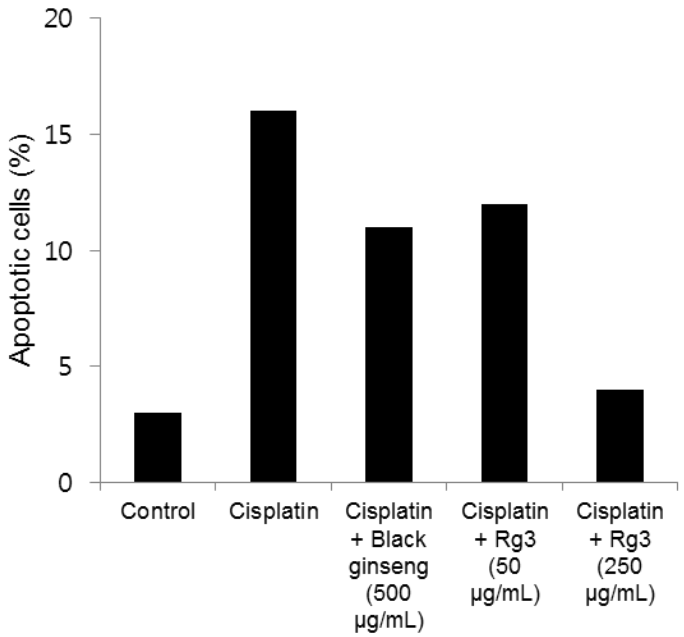

Apoptotic cells (%)
20
15
10
5
0
Control
Cisplatin
Cisplatin + Black ginseng (500 μg/mL)
Cisplatin + Rg3 (50 μg/mL)
Cisplatin + Rg3 (250 μg/mL)

Figure. 3