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Journal of Ginseng Research

journal homepage: <http://www.ginsengres.org>

Research article

Protective effect of ginsenoside Rh3 against anticancer drug-induced apoptosis in LLC-PK1 kidney cells

Q1 Hye Lim Lee, Ki Sung Kang*

College of Korean Medicine, Gachon University, Seongnam, South Korea

ARTICLE INFO

Article history:

Received 29 September 2016

Received in Revised form

17 January 2017

Accepted 18 January 2017

Available online xxx

Keywords:

cisplatin
ginsenoside Rh3
kidney protection
MAPKs

ABSTRACT

Background: Ginsenosides are active components of *Panax ginseng* that exert various health benefits including kidney protection effect. The medicinal activity of ginsenosides can be enhanced by modulating their stereospecificity by heat processing. Ginsenosides Rk2 and Rh3 represent positional isomers of the double bond at C-20(21) or C-20(22).

Methods: The present study investigated the kidney-protective effects of ginsenosides Rk2 and Rh3 against cisplatin, a platinum based anticancer drug, induced apoptotic damage in renal proximal LLC-PK1 cells.

Results: As a result, ginsenoside Rh3 shows a stronger protective effect than that shown by Rk2. Cisplatin-induced elevated protein levels of phosphorylated c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, and cleaved caspase-3 decreased after cotreatment with ginsenoside Rh3. The increase in the percentage of apoptotic LLC-PK1 cells induced by cisplatin treatment also significantly reduced after cotreatment with ginsenoside Rh3.

Conclusion: These results demonstrate that inhibition of the JNK and ERK mitogen-activated protein kinase signaling cascade plays a critical role in mediating the renoprotective effect of ginsenoside Rh3.

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1. Introduction

Ginsenosides are the main active components in *Panax* species. They show various medicinal activities. Ginsenosides are 30-carbon glycoside derivatives of the triterpenoid dammarane (Fig. 1A). They have a hydrophobic four-ring steroid-like structure with hydrophilic sugar moieties. They have been reported to exhibit antioxidant, anticancer, anti-inflammatory, vasorelaxant, kidney-protective, and lipid profile-improving effects, as well as to influence specific ion channels. Approximately 50 ginsenosides have been identified, and different mechanisms of action have been reported on the basis of the differences in their chemical structures [1,2].

The medicinal activity of ginsenosides can be enhanced by modulating their stereospecificity by heat processing [3]. The heat-processing method has recently been found to increase the biological efficacies including antioxidant and anticancer effects of ginseng. During heat processing, the stereoisomers 20(S)-Rg3 and 20(R)-Rg3 are produced by dissociation of a glycosyl residue located at carbon 20 of the protopanaxadiol-type ginsenoside Rb1

and Rb2. In addition, a dehydration reaction occurs at carbon 20 to produce ginsenosides Rg5 and Rk1 [1,3]. Interestingly, the double bond at carbon-20(22) or the hydroxyl (OH) group at carbon-20 geometrically close to the OH at carbon-12 increase the OH radical-scavenging activity of ginsenosides [1].

Among the various medicinal effects of ginseng, the kidney-protective effect is extensively studied [3]. Cisplatin nephrotoxicity has been recognized as a severe adverse effect for cancer patients [4]. Experimental exposure of kidney cells to cisplatin activates signaling pathways including cell death-promoting mitogen-activated protein kinase (MAPKs), p53, and reactive oxygen species (ROS). In addition, cisplatin induces TNF- α production in tubular cells, which triggers an inflammatory response contributing to tubular cell injury and death [5].

Therefore, the prevention of nephrotoxicity is an important aspect of cisplatin chemotherapy. Recent studies have suggested that nephrotoxicity is dependent on DNA damage related to apoptosis [6]. In addition, dysregulation of MAPK signaling pathways has been demonstrated in acute and chronic kidney disease induced by cisplatin [7]. Compounds isolated from natural sources,

* Corresponding author. College of Korean Medicine, Gachon University, Seongnam 461-701, South Korea.

E-mail address: kkang@gachon.ac.kr (K.S. Kang).

<http://dx.doi.org/10.1016/j.jgr.2017.01.011>

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Please cite this article in press as: Lee HL, Kang KS, Protective effect of ginsenoside Rh3 against anticancer drug-induced apoptosis in LLC-PK1 kidney cells, Journal of Ginseng Research (2017), <http://dx.doi.org/10.1016/j.jgr.2017.01.011>

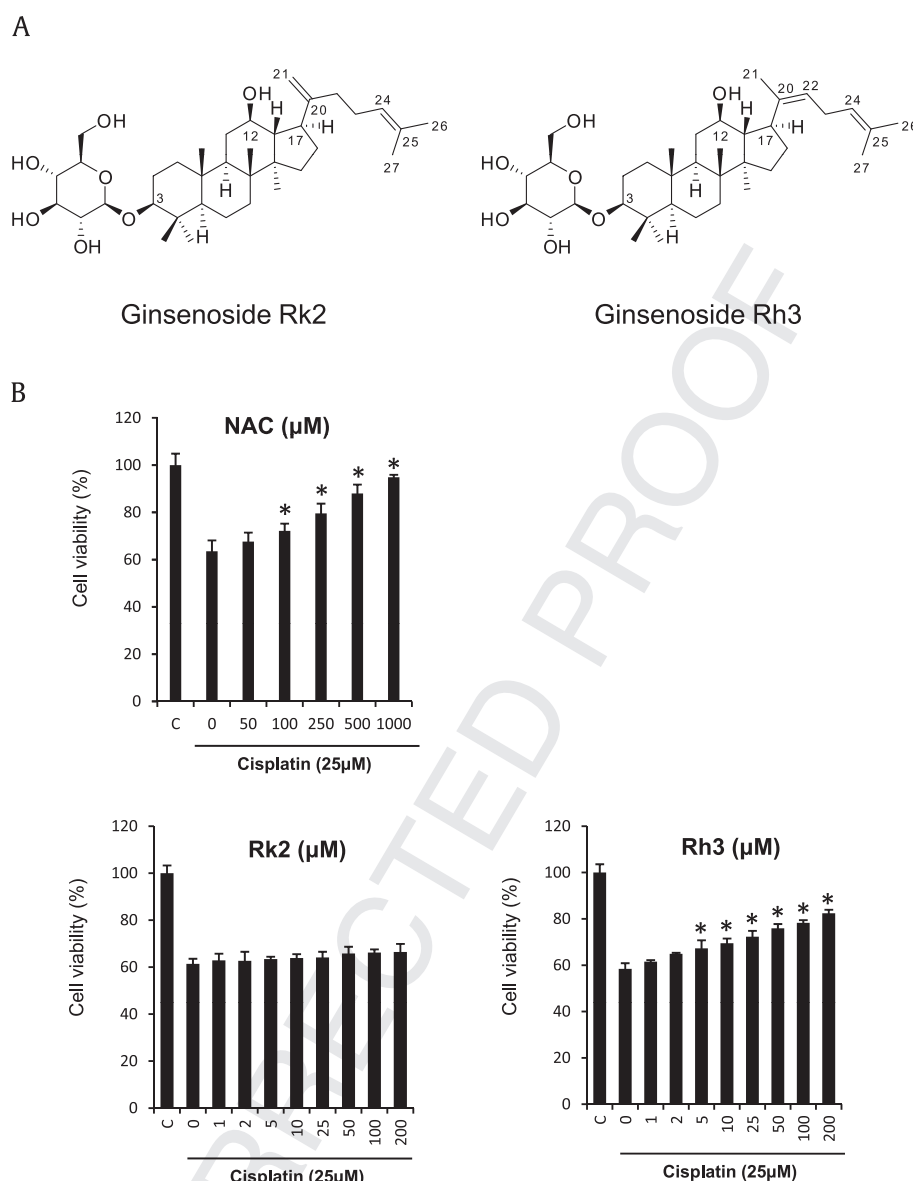


Fig. 1. Comparison of the kidney-protective effects of ginsenosides Rk2 and Rh3 in LLC-PK1 cells. (A) Chemical structures of ginsenosides Rk2 and Rh3. (B) Comparison of protective effects of ginsenosides Rk2 and Rh3 against cisplatin-induced cytotoxicity in LLC-PK1 cells. LLC-PK1 cells were pretreated with various concentrations of ginsenosides Rk2 and Rh3 for 2 h, and then further treated with 25µM of cisplatin for 24 h. Cell viability was assessed using the MTT assay. NAC was used as a positive control compound. * $p < 0.05$ compared with the cisplatin-treated group. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine.

such as *Artemisia asiatica*, pomegranate, and *Chrysanthemum indicum*, have been shown to reduce the nephrotoxicity due to cisplatin [8–12].

In the present study, the kidney-protective effects of the ginsenosides Rk2 and Rh3, produced after heat processing, and their potential roles in MAPK signaling cascade modulation were investigated to identify their mechanisms of action against cisplatin-induced nephrotoxicity. These two ginsenosides represent the positional isomers of the double bond at C-20(21) or C-20(22).

2. Materials and methods

2.1. Chemicals

Cisplatin was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Ginsenosides Rk2 and Rh3 were isolated from *Panax ginseng*

as reported previously [13,14]. Ez-Cytox cell viability assay kit was purchased from DAEIL Lab Service Co. (Seoul, Korea). Tali Apoptosis Kit was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA). Antibodies for p38, p-p38, c-Jun N-terminal kinase (JNK), p-JNK, extracellular signal-regulated kinase (ERK), p-ERK, cleaved caspase-3, and GAPDH were purchased from Cell Signaling (Boston, MA, USA).

2.2. Cell culture and MTT cell viability assay

LLC-PK1 (pig kidney epithelium, CL-101) cells were cultured in DMEM (Cellgro, Manassas, VA, USA), supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen Co.) and 4mM L-glutamine in an atmosphere of 5% CO₂ at 37°C. Viability of cells was

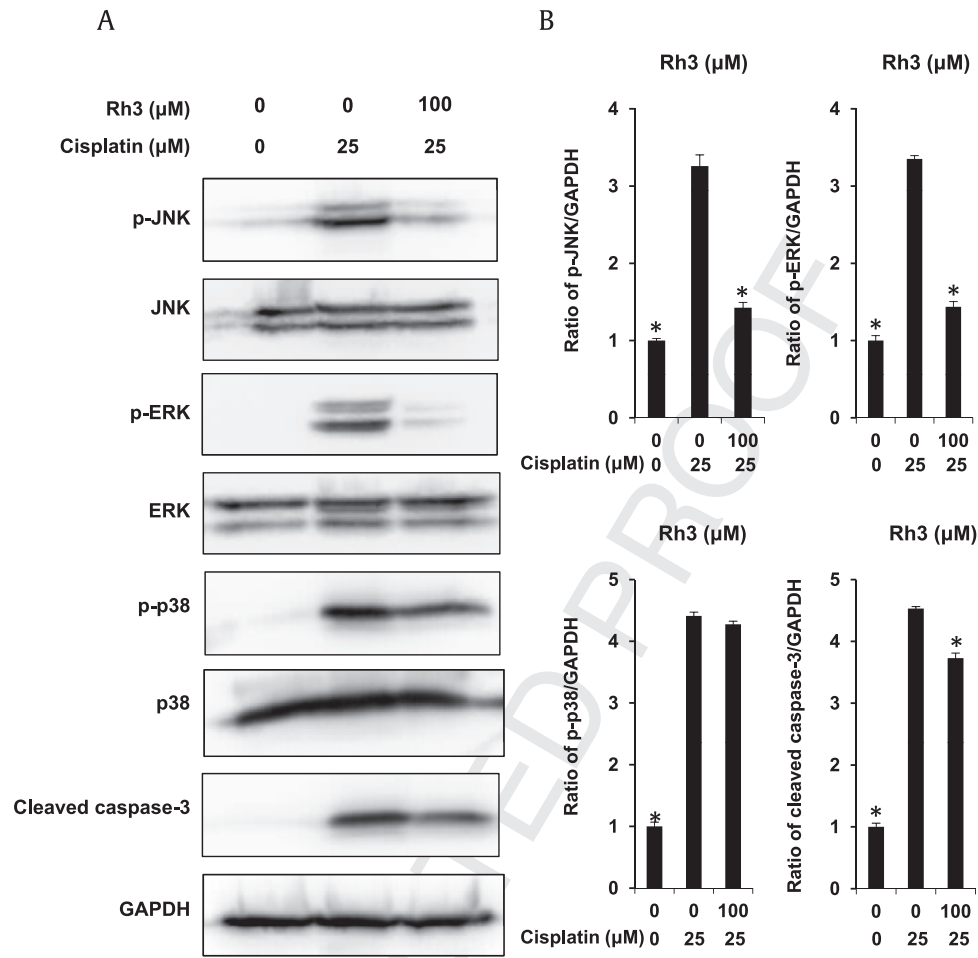


Fig. 2. Western blot analysis of the levels of p-JNK, JNK, p-ERK, ERK, p-p38, p38, and cleaved caspase-3 in LLC-PK1 cells treated with ginsenoside Rh3. Results of the Western blot analysis show the levels of phosphorylated (p)-JNK, JNK, p-ERK, ERK, p-p38, p38, cleaved caspase-3, and GAPDH in LLC-PK1 cells treated with ginsenoside Rh3 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. * $p < 0.05$ compared with the cisplatin-treated group. ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

determined by reported method [15]. In brief, when the cells are ~80% confluent, they were seeded in 96-well culture plates at 1×10^4 cells per well and incubated for 24 h for adhesion. Then cells were treated with indicated concentrations of Rk2/Rh3. After incubation for 2 h, 25 μM cisplatin was added to each well and incubated for 24 h. After incubation, 10 μL of Ez-Cytox reagent was added to each well and incubated for 2 h. Cell viability was measured by absorbance at 450 nm using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA).

2.3. Western blotting analysis

To analyze protein expressions involved in the protection effect of samples, LLC-PK1 cells seeded in 6-well plates were treated with 100 μM Rk2/Rh3. After incubation for 2 h, 25 μM cisplatin was added to each well and incubated for 24 h, then cells were lysed with RIPA buffer immediately before use as described previously [16]. Briefly, equal amounts (20 μg/lane) of protein were separated by electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto polyvinylidene difluoride transfer membranes. Specific proteins were analyzed with epitope-specific primary antibodies and horseradish peroxidase (HRP) conjugated antirabbit antibodies.

2.4. Image-based cytometric assay

To verify whether apoptosis was involved in the cisplatin-induced damage in cells, the number of dead and apoptotic cells was measured using a Tali image-based cytometer (Invitrogen) [17]. LLC-PK1 cells seeded in 6-well plates were pretreated with 100 μM Rk2/Rh3. After incubation for 2 h, 25 μM cisplatin was added to each well and incubated for 24 h, then cells were stained with annexin V Alexa Fluor 488 in darkness for 20 min after suspension in annexin binding buffer and stained with propidium iodide in darkness for 5 min after suspension in annexin binding buffer. Propidium iodide and annexin V Alexa Fluor 488 were used to differentiate dead cells (annexin V-positive/PI positive or annexin V-negative/PI positive) from those that were apoptotic (annexin V-positive/PI negative).

3. Results and discussion

The renoprotective potential of the ginsenosides was assessed using LLC-PK1 cells. As shown in Figure 1B, ginsenoside Rk2 showed no renoprotective or cytotoxic effects, even at high concentrations. However, ginsenoside Rh3 ameliorated cisplatin-induced reduced cell viability in a dose-dependent manner

(Fig. 1B). These two ginsenosides represent the positional isomers of the double bond at C-20(21) or C-20(22). Therefore, stereospecificity exists in kidney protection effects of ginsenosides Rk2 and Rh3. Stereospecific differences in biological activities in ginsenoside isomers have been well defined by us and others [1,3,18,19]. Ginsenoside 20(R)-Rg3, but not 20(S)-Rg3, suppressed lung cancer migration, invasion, and anoikis resistance [18]. However, ginsenoside 20(S)-Rg3, but not 20(R)-Rg3, induced apoptotic cell death in human gastric cancer AGS cells by activating caspases [19,20].

Next, we used Western blotting to measure the expression and phosphorylation of inflammatory molecules, including p-JNK, p-p38, p-ERK, and cleaved caspase-3, in LLC-PK1 cells. Cotreatment with the ginsenoside Rh3 markedly decreased cisplatin-induced phosphorylation of JNK and ERK MAPKs (Fig. 2) that are well defined representative inflammation markers [21,22]. Recently, ginsenoside Rh3 was reported to inhibit the expressions of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines (tumor necrosis factor (TNF)- α and interleukin (IL)-6) in lipopolysaccharide-stimulated microglia [21]. Therefore, beneficial effects of ginsenoside Rh3 against cisplatin-induced damage in LLC-PK1 cells share the same anti-inflammatory mechanism of action.

An image-based cytometric assay was performed to verify the antiapoptotic potential of ginsenoside Rh3 (Fig. 3A). Ginsenoside Rh3 (100 μ M) significantly inhibited the percentage of apoptotic cells (25%) compared with that in the cisplatin-treated group (Fig. 3B). MAPKs play critical roles in cell proliferation, differentiation, and apoptosis [24]. Therefore, these results demonstrate that inhibition of the MAPKs-apoptosis signaling cascade plays a critical role in mediating the renoprotective effect of ginsenoside Rh3.

Cisplatin is one of the most effective chemotherapeutic drugs used for the treatment of various solid tumors. It is potent, demonstrating one of the highest cure rates (e.g., > 90% cure rate in testicular cancers) [25]. Although cisplatin has been a mainstay for cancer therapy, its use is limited mainly by its severe side effects, especially nephrotoxicity. The prevalence of cisplatin nephrotoxicity is high, occurring in approximately one-third of patients undergoing cisplatin treatment [4]. Ginsenoside Rh3 is known to exert moderate cytotoxic effects from concentration range of 5–80 μ M on human cancer HepG2 cells [26]. Therefore, ginsenoside Rh3 may reduce nephrotoxicity without interfering with anticancer activity.

In the present study, the kidney-protective effect of ginsenoside Rh3 was stronger than that of its stereoisomer Rk2, and was mediated via the inhibition of the JNK and ERK MAPKs-caspase-3

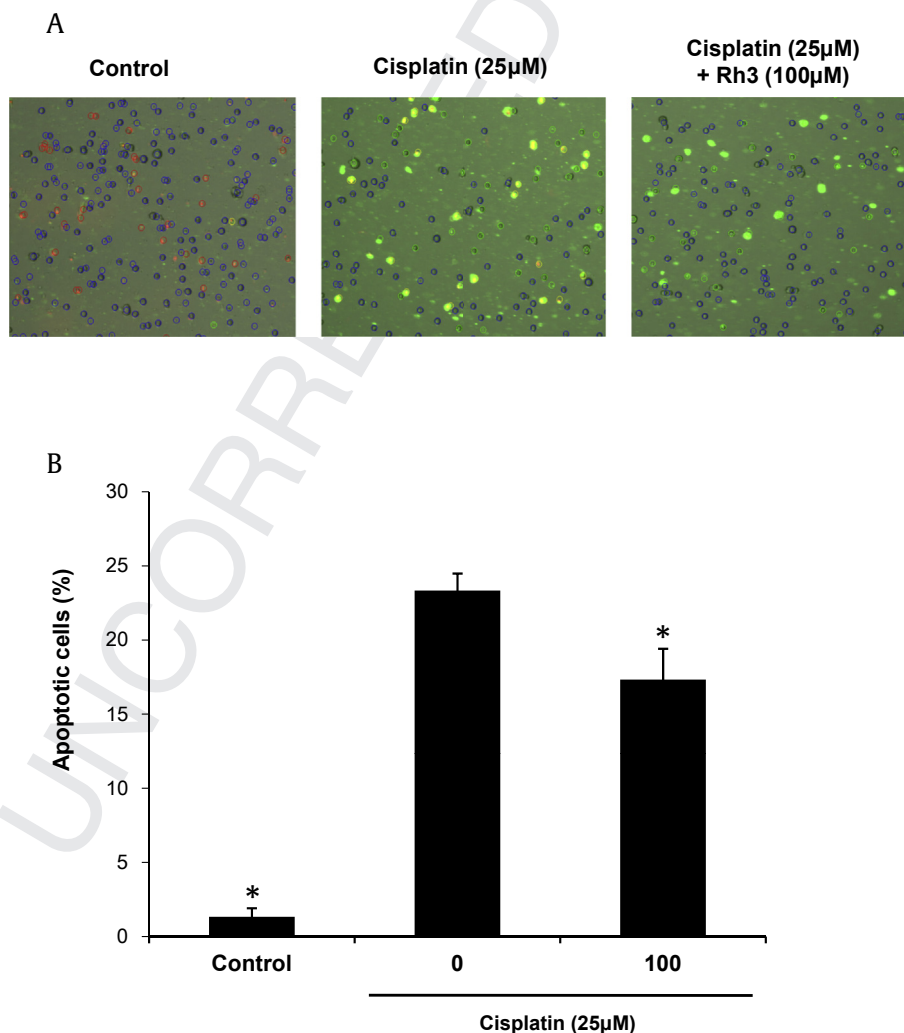


Fig. 3. Effect of ginsenoside Rh3 on apoptosis in LLC-PK1 cells. (A) Representative images of apoptosis detection. (B) Percentage of annexin V-positive-stained apoptotic cells. Dead and apoptotic cells were stained with red and green fluorescence. Apoptosis was determined using a Tali image-based cytometer. * $p < 0.05$ compared with the cisplatin-treated group.

apoptotic cascade. Kang et al [1] compared OH scavenging activities of several ginsenosides. Of these, ginsenoside Rg5 that contains double bond at carbon-20(22) in its structure exerted stronger OH-scavenging IC₅₀ values than those of its isomer Rk1. Ginsenoside Rh3 also contains this double bond at carbon-20(22). Thus, the stronger kidney-protective effects of Rh3 than those of Rk2 were also mediated by the structural characteristics of Rh3.

Conflicts of interest

The authors declare no conflicts of interest.

Uncited reference

Q12 [23].

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2016R1C1B1012787).

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