

# Protective mechanism of Korean Red Ginseng in cisplatin-induced ototoxicity through attenuation of nuclear factor- $\kappa$ B and caspase-1 activation

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**Abstract.** Cisplatin is an effective anti-cancer drug; however, one of its side effects is irreversible sensorineural hearing damage. Korean Red Ginseng (KRG) has been used clinically for the treatment of various diseases; however, the underlying mechanism of KRG treatment of ototoxicity has not been studied extensively. The present study aimed to further investigate the mechanism of KRG on cisplatin-induced toxicity in auditory HEI-OC1 cells *in vitro*, as well as *in vivo*. The pharmacological effects of KRG on cisplatin-induced changes in the hearing threshold of mice were determined, as well as the effect on the impairment of hair cell arrays. In addition, in order to elucidate the protective mechanisms of KRG, the regulatory effects of KRG on cisplatin-induced apoptosis-associated gene levels and nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation were investigated in auditory cells. The results revealed that KRG prevented cisplatin-induced alterations in the hearing threshold of mice as well as the destruction of hair cell arrays in rat organ of Corti primary explants. In addition, KRG inhibited cisplatin-mediated cell toxicity, reactive oxygen species generation, interleukin-6 production, cytochrome *c* release and activation of caspases-3

in the HEI-OC1 auditory cell line. Furthermore, the results demonstrated that KRG inhibited the activation of NF- $\kappa$ B and caspase-1. In conclusion, these results provided a model for the pharmacological mechanism of KRG and provided evidence for potential therapies against ototoxicity.

## Introduction

Cisplatin is a chemotherapeutic agent, which is commonly used in the treatment of cancer (1,2). However, cisplatin has been shown to induce a number of severe side effects, including peripheral neuropathy and nephrotoxicity. One of the most serious adverse effects of cisplatin treatment is ototoxicity, which is manifested as irreversible sensorineural hearing loss (3,4). Ototoxicity occurs due to cisplatin-induced auditory and vestibular sensory cell apoptosis (5).

Accumulating evidence has indicated that pro-inflammatory cytokines may be involved in cochlear damage (6-10). These cytokines were shown to induce secondary inflammatory responses, including leukocyte infiltration and scar formation. In addition, one study demonstrated that cisplatin induced ototoxicity via the apoptosis of outer hair cells (OHCs) and supporting cells in the organ of Corti, which occurred in parallel with the production and expression of pro-inflammatory cytokines (11). Nuclear factor (NF)- $\kappa$ B was reported to be involved in the regulation of cell proliferation, survival, angiogenesis, apoptosis and differentiation (12,13). NF- $\kappa$ B was shown to activate genes inside the nucleus, which are involved in the regulation of apoptosis, the inflammatory response and oxidative stress (14,15). Numerous studies have indicated that NF- $\kappa$ B may have a significant role in the mediation of hearing loss. Ototoxic stimulants, including noise exposure and ototoxic drugs, were found to induce the activation of NF- $\kappa$ B (16,17), which results in damage to the cochlear lateral wall via the production of large amounts of reactive oxygen species (ROS) (18,19). Caspase-1, a member of the caspase family characterized by large pro-domains (20), has been reported to be involved in apoptosis and inflammation following activation (21). Caspase-1 activation results in

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inflammation through the generation of pro-inflammatory cytokines (22). In addition, caspase-1 has a significant role in the cisplatin-induced apoptosis of cochlear hair cells and spiral ganglion neurons.

Korean red ginseng (KRG) has previously been used as a supplementary herbal medicine in the treatment of numerous diseases, including inflammation, diabetes and atherosclerosis (23,24). However, the underlying protective mechanisms of KRG in the context of cisplatin have remained to be elucidated. The present study aimed to further investigate the mechanism of KRG in cisplatin-induced toxicity in auditory HEI-OC1 cells. Furthermore, the protective effects of KRG on cisplatin-induced hair cell damage were investigated in the organ of Corti in a rat model.

## Materials and methods

**Reagents.** Fetal bovine serum (FBS) and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco-BRL (Grand Island, NY, USA). cis-Diamineplatinum(II) dichloride (cisplatin; cat. no. 479306), paraformaldehyde, Tween<sup>®</sup> 20, avidinperoxidase, Triton X-100 and liquid nitrogen were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10X phosphate-buffered saline was purchased from bioWORLD (Dublin, OH, USA). ABTS was purchased from BD Bioscience (San Diego, CA, USA). Rabbit polyclonal NF- $\kappa$ B (cat. no. sc-7151), mouse monoclonal cytochrome (cyt) *c* (cat. no. sc-13156), mouse monoclonal  $\alpha$ -tubulin (cat. no. sc-8035), rabbit polyclonal histone H1 (cat. no. sc-10806) and rabbit polyclonal caspase-3 (cat. no. sc-7148) antibodies (Abs) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Caspase-1, caspase-3 and caspase-9 colorimetric kits were supplied by R&D systems, Inc. (Minneapolis, MN, USA). Purified anti-mouse interleukin (IL)-6 (cat. no. 554400), biotinylated IL-6 (cat. no. 554402) and recombinant IL-6 (cat. no. 554401) Abs were obtained from BD-Pharmingen (San Diego, CA, USA). Peroxidase-conjugated AffiniPure goat anti-rabbit immunoglobulin (Ig)G (cat. no. 111-035-003) and peroxidase-conjugated AffiniPure goat anti-mouse IgG (cat. no. 115-035-062) Abs were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Nonidet P (NP)-40 was purchased from Abcam (Cambridge, UK).

**Preparation of KRG.** KRG was manufactured by the Korea Ginseng Corporation (Seoul, Korea). Extract was prepared by decocting the KRG with boiling distilled water (100 g/l). The extraction was decocted for ~3 h, and was subsequently filtered with LabSystem 2 (EMD Millipore, Billerica, MA, USA), lyophilized with a freeze dryer (Ilshin Bio Base Co., Ltd., Yangju, Korea) and stored at 4°C. Dilutions were made using saline and filtered through a 0.22- $\mu$ m Millex-GS Syringe Filter unit (EMD Millipore). The KRG extracts contained the following seven glycosides, known as ginsenosides (mg/g): Rg<sub>1</sub> (2.481), Rb<sub>1</sub> (5.481), Rg<sub>3</sub>(S) (0.197), Re (2.975), Rc (2.248), Rb<sub>2</sub> (2.175) and Rb (0.566) (25).

**Cell culture.** The HEI-OC1 cell line was provided by Dr Federico Kalinec (House Ear Institute, Los Angeles, CA, USA), which was produced as previously described (26).

The passage number of the cells used was five, and to avoid mycoplasmic infection, the provided cell stocks were reconstituted with Mycoplasma Removal Agent (cat. no. 9030500) from MP Biochemicals (Santa Ana, CA, USA). HEI-OC1 cells express multiple molecular markers that are characteristic of the organ of Corti sensory cells, including  $\alpha$ -thyroid hormone, brain-derived neurotrophic factor, calbindin, calmodulin, Connexin 26, Math 1, Myosin 7a, organ of Corti protein 2, tyrosine kinase receptors B and C, platelet-derived growth factor receptor and prestin; in addition, HEI-OC1 cells are particularly sensitive to ototoxic drugs (26). These cells were maintained in DMEM supplemented with 10% FBS at 33°C with 5% CO<sub>2</sub>. Cells were treated with various concentrations of KRG (0.5, 1 and 2 mg/ml) for 1 h, and subsequently treated with cisplatin (20  $\mu$ M) for 48 h at 33°C.

**Ethics statement.** All animal procedures and experiments were approved by the Animal Care and Use Committee of Wonkwang University (approval no. WKU14-05; Iskan, Korea). All animals were treated humanely and with regard for alleviation of suffering.

**Organ of Corti explant culture.** The organ culturing procedure was similar to that described previously (27). In brief, two-day-old Sprague Dawley rats (n=5/group; weighing 6.5 $\pm$ 0.5 g) were anesthetized with CO<sub>2</sub> and sacrificed by decapitation. Subsequently, the cochlea was carefully removed by dissection. The cochlear explants were treated with KRG (2 mg/ml) for 1 h, and subsequently treated with cisplatin (30  $\mu$ M) for 24 h at 33°C. When the experimental treatment was completed, the culture was prepared for histological analysis. The organ of Corti explants were fixed for 15 min in 2% paraformaldehyde in PBS. The specimens were rinsed in PBS, incubated in 0.25% Triton X-100 for 2 min and immersed in tetramethylrhodamine (TRITC)-labeled phalloidin (1:100; Sigma-Aldrich) in PBS for 20 min. Following rinsing with PBS, the specimens were examined using fluorescence microscopy (IX71, Olympus Corporation, Tokyo, Japan) with the appropriate filters for TRITC.

**Animal experiment.** All experiments were performed using male Balb/c mice (four-weeks-old; weighing 16-18 g), which were housed in stainless steel cages in a temperature controlled (25°C) room equipped to maintain a 12-h light/dark cycle. The animals were distributed at random into three groups (n=5 per group) and fed a regular diet. Group 1, regarded as the control group, received intraperitoneal (i.p.) injection of 200  $\mu$ l PBS; group 2 was administered cisplatin (4 mg/kg) via i.p. injection for four consecutive days; and group 3 was orally administered KRG (200 mg/kg) once a day for four days; and group 4 was orally administered KRG (200 mg/kg) 1 h prior to i.p. injection of cisplatin (4 mg/kg). An auditory brainstem response (ABR) test was subsequently performed to investigate the effect of KRG on hearing function. Following the ABR test, all of the mice were sacrificed by cervical dislocation.

**Assessment of hearing function.** ABR tests were conducted under ketamine/xylazine (equivalent to 172.4 mg/kg ketamine and 5.5 mg/kg xylazine) sedation. The ABR was used to determine the auditory threshold of mice. While under sedation,

ABR testing was performed on the animals in response to 4, 8 and 32 kHz tone bursts. A computer-based signal-averaging system (ABR workstation with SigGen software) from Tucker Davis Technologies (Gainesville, FL, USA) was used to compile ABR data. The ABR was recorded using three platinum-iridium needle electrodes (Ambu® Neuroline Disposable Subdermal Needle Electrodes; Cadwell, Kennewick, WA, USA), positioned subdermally over the vertex (positive), mastoid (negative) and dorsum (reference/ground) areas of the animal. Sound was generated through an Etymotic ER-2 earphone (Etymotic Research, Elk Grove Village, IL, USA), which was placed directly in the ear canal. The ABR threshold began at 90 dB and decreased in 10 dB steps, and each response was repeated in triplicate.

**Cytokine assays.** Expression levels of IL-6 in HEI-OCI cells were examined using a modified ELISA. In brief, the ELISA was set up by coating 96-well plates with mouse monoclonal Ab against IL-6. The coated plates were then washed with PBS containing 0.05% Tween 20. All reagents used in this assay were incubated for 2 h at 37°C. Recombinant IL-6 was diluted and used as a standard. Standards were diluted 1:1 with diluents (PBS with 10% FBS) from 10 ng/ml to 0.34125 ng/ml and were used to construct the standard curve. Following 2 h incubation of standards and cell supernatants ( $3 \times 10^5$  cells/well) at room temperature, the assay plates were sequentially exposed to biotinylated mouse IL-6, avidin peroxidase and [2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt] substrate solution containing 30%  $H_2O_2$ . The plates were read at a wavelength of 405 nm using a VersaMax™ microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Spectrofluorimetric measurement of intracellular ROS production.** Intracellular ROS levels in HEI-OCI cells were measured using fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA). In the presence of an oxidant, DCFH is converted into a highly fluorescent molecule, 2',7'-dichlorofluorescein (DCF). Cells ( $3 \times 10^5$  cells/well) were treated with KRG (0.5, 1 and 2 mg/ml) for 1 h, cultured in the presence or absence of cisplatin (20  $\mu$ M) for 48 h and subsequently incubated with 5  $\mu$ M DCFH-DA for 30 min. The fluorescence intensity was measured using a spectrofluorometer (RF-5301PC; Shimadzu Corp., Kyoto, Japan) at excitation and emission wavelengths of 485 and 538 nm, respectively.

**Preparation of cytoplasmic and nuclear extracts.** Nuclear and cytoplasmic extracts were prepared as described previously (28). In brief, following activation of the HEI-COI cells ( $7 \times 10^6$  cells/well) with cisplatin, they were washed with ice-cold PBS. These cells were resuspended in 60  $\mu$ l buffer A [10 mM Hepes/KOH, 2 mM  $MgCl_2$ , 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF); pH 7.9]. The cells were allowed to swell on ice for 15 min, lysed gently with 2.5  $\mu$ l of 10% NP-40 and centrifuged at 2,000 x g for 10 min at 4°C. The supernatant was collected and used as the cytoplasmic extract. The nuclei pellet was resuspended in 40  $\mu$ l of buffer B (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and 0.5 mM PMSF; pH 7.9), left on ice for 20 min,

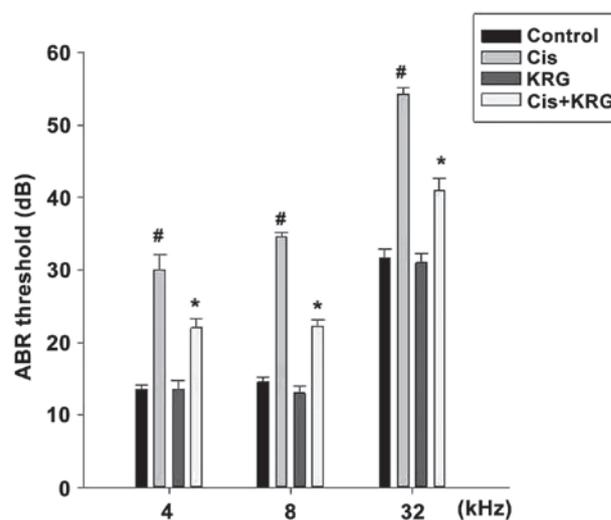


Figure 1. Effect of KRG on cisplatin-induced ABR threshold shift in mice. Mice (n=5) were orally administered KRG (200 mg/kg) prior to injection of cisplatin (4 mg/kg). ABR was used to determine the auditory threshold in response to 4, 8 and 32 kHz tone bursts. Values are presented as the mean  $\pm$  standard error of the mean of triplicate results from three separate experiments. <sup>#</sup>P<0.05 vs. control and <sup>\*</sup>P<0.05 vs. cisplatin alone. KRG, Korean Red Ginseng; ABR, auditory brainstem response; Cis, cisplatin.

inverted and centrifuged at 15,000 x g for 15 min to remove nuclear debris. The supernatant (nuclear extract) was collected, frozen in liquid nitrogen and stored at -70°C until required for further analysis.

**Western blot analysis.** For analysis of the expression levels of *cyt c*, the cells ( $5 \times 10^6$  cells/well) were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (1% Triton, 1% NP-40, 0.1% SDS and 1% deoxycholate in PBS). Samples were centrifuged at 15,000 x g for 5 min at 4°C and the supernatants were mixed with an equal volume of 2X SDS sample buffer (ELIPIS-Biotech, Inc., Dajeon, Korea), boiled for 5 min and separated through a 10% denaturing protein gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Uppsala, Sweden) by electrophoretic transfer. The membranes were blocked in 5% Difco skim milk (BD Diagnostics, Sparks Glencoe, MD, USA) for 2 h, rinsed and incubated overnight at 4°C with primary antibodies (1:500). Following three washes (30 min per wash) in PBS/0.5% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (secondary antibodies). Following three washes (30 min per wash) in PBS/0.1% Tween 20, the protein bands were visualized using an enhanced chemiluminescence assay (Amersham Pharmacia, Piscataway, NJ, USA) according to the manufacturer's instructions and subsequently exposed to X-ray film (Agfa-Gevaert N.V, Mortsel, Belgium). The relative protein expression levels were evaluated using Image J software, version 1.48.

**Luciferase assay.** Cells were transiently transfected with NF- $\kappa$ B luciferase reporter plasmid (Stratagene, Cedar Creek, TX, USA) using the transfection reagent, Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Following 36-h incubation, the cells were treated with various concentrations of

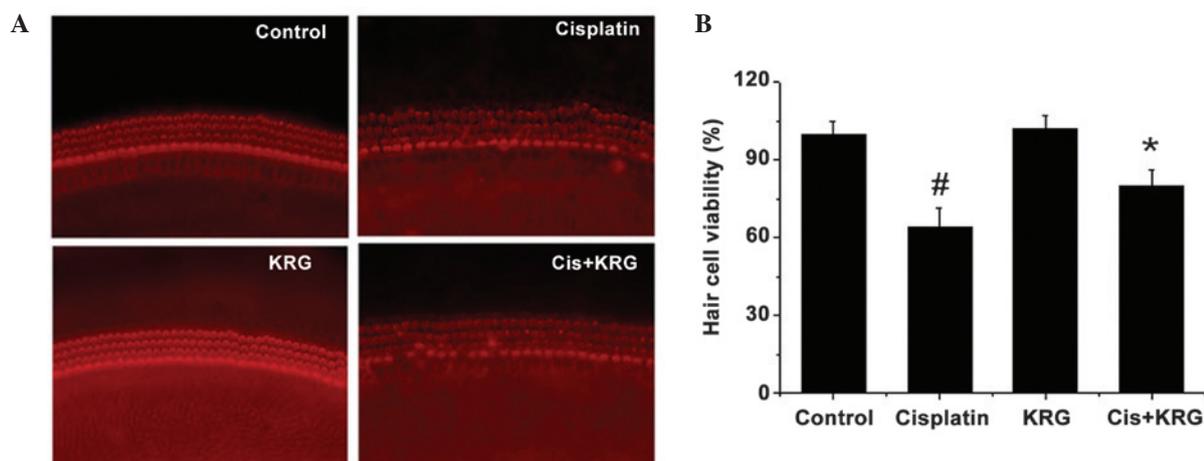


Figure 2. Effect of KRG on cisplatin-induced effects in the organ of Corti in rat primary explants. (A) Explants were pretreated with KRG (2 mg/ml) for 1 h, and then treated with cisplatin. The organ of Corti explants were fixed with paraformaldehyde, stained with tetramethylrhodamine-conjugated phalloidin and observed under a fluorescent microscope (magnification, x200). (B) Relative hair cell viability of each treatment group. Values are presented as the mean  $\pm$  standard error of the mean of triplicate determinations from three separate experiments. # $P$ <0.05 vs. control and \* $P$ <0.05 vs. cisplatin alone. KRG, Korean Red Ginseng; Cis, cisplatin.

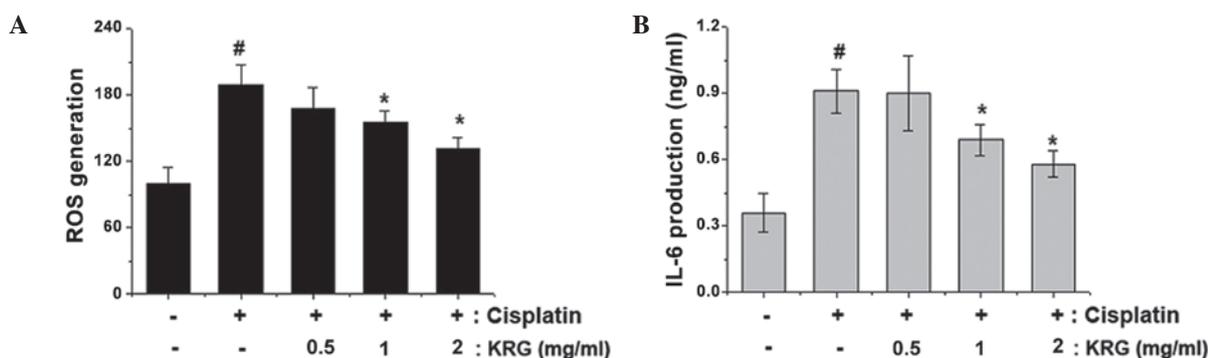


Figure 3. Effect of KRG on cisplatin-induced ROS generation and IL-6 production in HEI-OC1 cells. Cells ( $3 \times 10^5$  cells/well) were treated with various concentrations of KRG (0.5, 1 and 2 mg/ml) for 1 h and then treated with cisplatin (20  $\mu$ M) for 48 h. (A) Intracellular ROS levels were determined using a spectrofluorometer and the peroxide-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate. (B) IL-6 levels were measured in the cell supernatant using ELISA. Values are presented as the mean  $\pm$  standard error of the mean of triplicate determinations from three separate experiments. # $P$ <0.05 vs. control and \* $P$ <0.05 vs. cisplatin alone. KRG, Korean Red Ginseng; ROS, reactive oxygen species; IL-6, interleukin 6.

KRG (1 and 2 mg/ml) for 1 h and then treated with cisplatin (20  $\mu$ M) for 24 h. The cells were then washed twice with PBS buffer and subsequently lysed in reporter lysis buffer (Promega Corporation, Madison, WI, USA). A 20- $\mu$ l aliquot of the lysate was then mixed with 100  $\mu$ l luciferase assay reagent, after which the emitted light intensity was measured using an AutoLumat LB 953 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Finally, the luciferase activity was measured in triplicate, averaged, and then normalized against the  $\beta$ -galactosidase activity using the galactosidase assay system (Galacto-Light; Tropix, Inc., Bedford, MA, USA) according to the manufacturer's instructions.

**Assay of caspase-1, -3 and -9 activity.** The enzymatic activity of caspase-1, -3 and -9 was evaluated using a caspase colorimetric assay kit (R&D Systems, Inc.) according to the manufacturer's instructions. Cells ( $5 \times 10^6$  cells/well) were treated with various concentrations of KRG (0.5, 1 and 2 mg/ml) for 1 h and then treated with cisplatin (20  $\mu$ M) for 48 h. In brief, the cytoplasmic protein supernatant was incubated with 50  $\mu$ l reaction buffer

and 5  $\mu$ l caspase substrate at 37°C for 2 h. The absorbance was measured using a plate reader (VersaMax™) at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid (BCA) protein quantification kit (Sigma-Aldrich) according to the manufacturer's instructions. Inhibition rate was calculated as follows: Inhibition rate = [(KRG OD) - (control OD)] / [(cisplatin-only OD) - (control OD)]  $\times$  100%; where OD stands for optical density.

**Statistical analysis.** Values are presented as the mean  $\pm$  standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance with Tukey's post hoc test (PASW Statistics 18.0.0.; SPSS Inc., Chicago, IL, USA)  $P$ <0.05 was considered to indicate a statistically significant difference between values.

## Results

**KRG attenuates cisplatin-induced changes in the hearing threshold of mice.** In order to investigate the effect of KRG

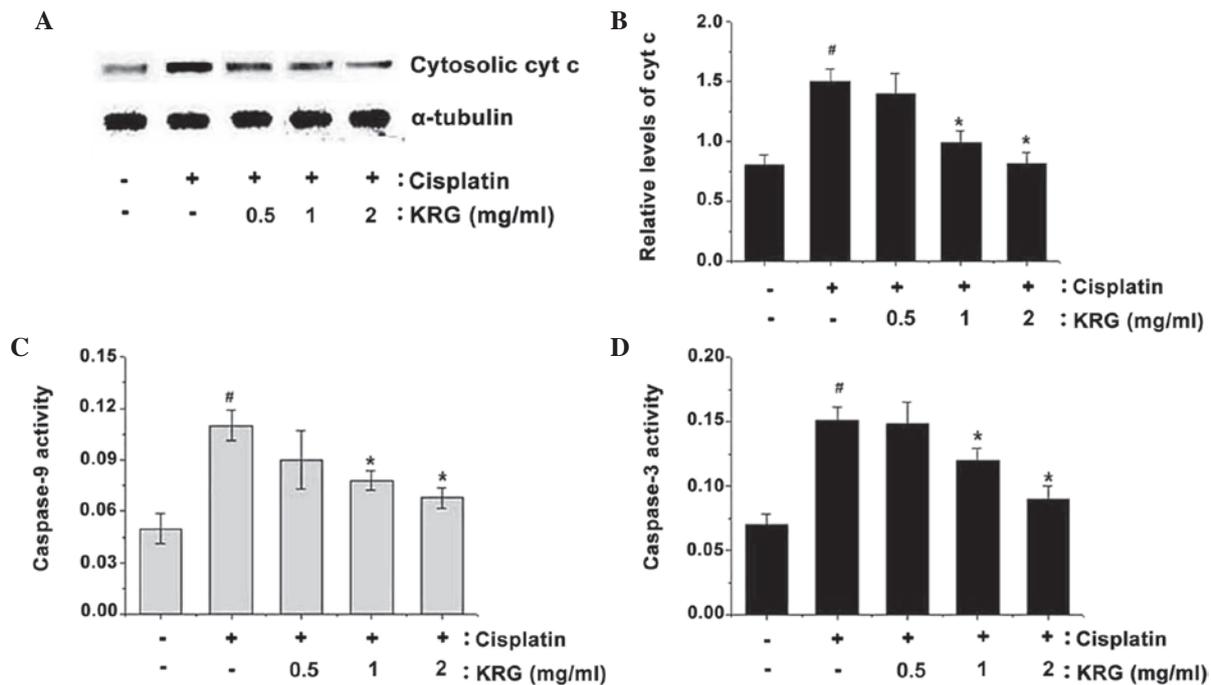


Figure 4. Effect of KRG on cisplatin-induced cyt *c* release and caspase activation in HEI-OC1 cells. Cells ( $5 \times 10^6$  cells/well) were treated with various concentrations of KRG (0.5, 1 and 2 mg/ml) for 1 h and then treated with cisplatin ( $20 \mu\text{M}$ ) for 48 h. (A) Following isolation of the cytoplasmic fraction, cyt *c* was measured in the protein extracts using western blot analysis.  $\alpha$ -tubulin was used as the loading control. (B) Relative expression level of cyt *c* was measured using an image analyzer. Activity of (C) caspase-9 and (D) caspase-3 was determined using a colorimetric kit. Values are presented as the mean  $\pm$  standard error of the mean of triplicate determinations from three separate experiments. Relative protein levels were determined using the amount of  $\alpha$ -tubulin reference protein in each condition, and band intensities were measured using the Image J 1.48 software. <sup>#</sup> $P < 0.05$  vs. control and <sup>\*</sup> $P < 0.05$  vs. cisplatin alone. KRG, Korean Red Ginseng; cyt *c*, cytochrome *c*.

on hearing function, ABR was recorded at 4, 8 and 32 kHz. The results indicated that the cisplatin-only group had a significantly increased ABR threshold shift at 4, 8 and 32 kHz compared with that of the control group ( $P < 0.05$ ). However, KRG (200 mg/kg) was shown to significantly attenuate the cisplatin-induced hearing threshold shift (Fig. 1).

**KRG attenuates cisplatin-induced toxicity in organ of Corti explants.** The organ of Corti was isolated from rats on post-natal day two and treated with cisplatin ( $30 \mu\text{M}$ ) in presence of KRG (2 mg/ml). As indicated in Fig. 2A, cisplatin treatment impaired the orderly arrangements of the three rows of OHCs and a single row of inner hair cells (IHCs) in the organ of Corti. However, treatment with KRG in combination with cisplatin significantly attenuated this effect ( $P < 0.05$ ). The relative hair cell viability is presented in Fig. 2B, and indicates a significant decrease in hair cell viability in the cisplatin-treated group, compared with that of the control group ( $P < 0.05$ ) that is attenuated following KRG pretreatment ( $P < 0.05$ ).

**KRG suppresses the cisplatin-induced increase in IL-6 levels and ROS generation in HEI-OC1 cells.** ROS generated in the mitochondria may induce damage to the mitochondrial membrane, resulting in apoptosis (29). In order to determine the regulatory effects of KRG on ROS production, cells were treated with DCFH-DA, which is converted to a fluorescent substance following intracellular oxidation. The results revealed that KRG effectively suppressed the significant increase in ROS levels induced by cisplatin treatment ( $P < 0.05$ ; Fig. 3A).

Previous studies have reported that the expression of inflammatory cytokines is significantly enhanced in the inner ear under various damaging conditions (30-33). In order to determine whether KRG was able to regulate the enhanced IL-6 levels induced by cisplatin, ELISAs were performed. As shown in Fig. 3B, IL-6 levels were significantly increased following treatment with cisplatin compared with those of the control group ( $P < 0.05$ ); however, this effect was markedly reduced following treatment with KRG ( $P < 0.05$ ).

**KRG inhibits the increased apoptosis-associated gene levels induced by cisplatin in HEI-OC1 cells.** Pro-apoptotic stimuli induce the release of cyt *c* into the cytosol, which leads to the activation of pro-apoptotic factors as well as the maturation of caspase-3 (34,35). As shown in Fig. 4A and B, the effect of KRG on cisplatin-induced cyt *c* release was examined. The results revealed that cisplatin significantly increased the quantity of cyt *c* released into the cytosol compared with that of the control group ( $P < 0.05$ ); however, KRG significantly inhibited cisplatin-induced cyt *c* release ( $P < 0.05$ ).

Caspase-3 is known to have a significant role in the initiation and implementation of apoptosis (36,37). In the present study, the inhibitory effect of KRG on caspase-9 and caspase-3 activity was examined. Cisplatin significantly induced caspase-9 and caspase-3 activation compared with that of the control group ( $P < 0.05$ ), while KRG effectively inhibited this induction ( $P < 0.05$ ) (Fig. 4C and 4D).

**KRG inhibits cisplatin-induced NF- $\kappa$ B activation in HEI-OC1 cells.** Reduced NF- $\kappa$ B activation has previously been associated

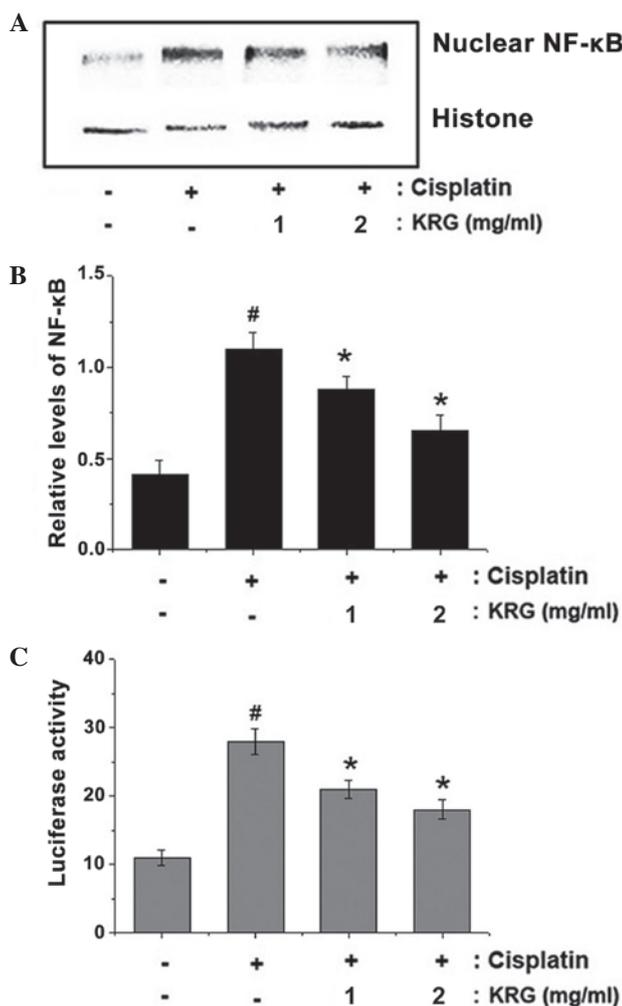


Figure 5. Effect of KRG on cisplatin-induced NF- $\kappa$ B activation in HEI-COI cells. Cells ( $7 \times 10^6$  cells/well) were treated with various concentrations of KRG (1 and 2 mg/ml) for 1 h and then treated with cisplatin ( $20 \mu\text{M}$ ) for 24 h. (A) Following isolation of the nuclear fraction, the NF- $\kappa$ B protein levels were determined using western blot analysis. (B) Relative protein expression of NF- $\kappa$ B. (C) NF- $\kappa$ B activity was examined using a luciferase assay. Values are presented as the mean  $\pm$  standard error of the mean of triplicate determinations from three separate experiments. Relative protein levels were determined using the amount of Histone reference protein in each condition, and band intensities were measured using the Image J 1.48 software. # $P < 0.05$  vs. control and \* $P < 0.05$  vs. cisplatin alone. KRG, Korean Red Ginseng; NF- $\kappa$ B, nuclear factor  $\kappa$ B.

with hearing loss (38,39); therefore, it was hypothesized that the effects of KRG may be mediated, at least in part, by the suppression of NF- $\kappa$ B activation. In addition, NF- $\kappa$ B activation requires the nuclear translocation of the RelA/p65 subunit of NF- $\kappa$ B (40); therefore, the present study aimed to evaluate the effects of KRG on the nuclear pool of RelA/p65 protein using western blot analysis. In cisplatin-treated cells, the levels of Rel/p65 were markedly increased compared with those of the untreated group; however, KRG reduced these enhanced nuclear levels of Rel/p65 (Fig. 5A). The relative nuclear levels of NF- $\kappa$ B are exhibited in Fig. 5B. The cisplatin-induced increase in NF- $\kappa$ B expression was significantly attenuated following KRG pretreatment in a dose-dependent manner.

Furthermore, a luciferase assay was performed in order to investigate the effects of KRG on NF- $\kappa$ B promoter activity. As shown in Fig. 5C, cisplatin treatment enhanced NF- $\kappa$ B promoter

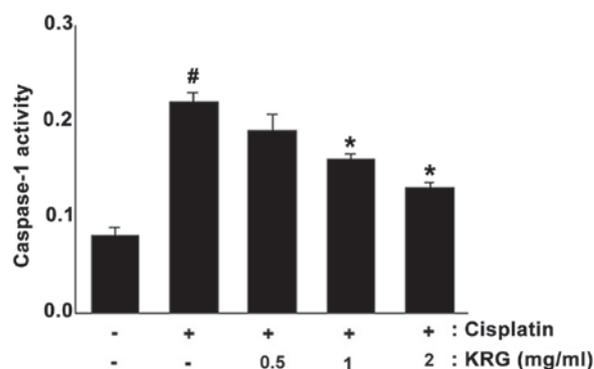


Figure 6. Effect of KRG on cisplatin-induced caspase-1 activation in HEI-COI cells. Cells ( $5 \times 10^6$  cells/well) were treated with various concentrations of KRG (0.5, 1 and 2 mg/ml) for 1 h and then treated with cisplatin ( $20 \mu\text{M}$ ) for 48 h. The activity of caspase-1 was determined using a colorimetric kit. Values are presented as the mean  $\pm$  standard error of the mean of triplicate determinations from three separate experiments. # $P < 0.05$  vs. control and \* $P < 0.05$  vs. cisplatin alone. KRG, Korean Red Ginseng.

activity compared with that of the control group ( $P < 0.05$ ), while KRG treatment inhibited this cisplatin-induced increase in NF- $\kappa$ B promoter activity ( $P < 0.05$ ).

*KRG attenuates cisplatin-induced caspase-1 activation in HEI-OCI cells.* The activation of caspase-1 induces the activation of NF- $\kappa$ B and production of inflammatory cytokines (41). In order to elucidate the mechanism underlying the protective effects of KRG on ototoxicity, the effects of KRG on caspase-1 activation were evaluated. The results demonstrated that the enhanced caspase-1 activity induced by cisplatin was significantly reduced by KRG in a dose-dependent manner ( $P < 0.05$ ) (Fig. 6). The maximal inhibition rate of KRG (2 mg/ml) was  $42.21 \pm 3.1\%$  compared with that of the cisplatin-only group.

## Discussion

KRG has been reported to have numerous beneficial therapeutic effects including anti-cancer, anti-inflammatory and anti-oxidant activities (42). However, the underlying protective mechanisms of KRG in the auditory system have remained to be fully elucidated. The results of the present study demonstrated that KRG attenuated the adverse effects of cisplatin in the organ of Corti as well as apoptosis in auditory cells.

Cisplatin is an effective, commonly used anticancer agent; however, its major side effects include hearing damage. A previous study investigated the mechanisms underlying cisplatin-induced ototoxicity (43). The results of the present study demonstrated that KRG pretreatment effectively prevented cisplatin-induced impairment of hair cell arrays in the rat organ of Corti primary explants. Cisplatin has been confirmed to induce the apoptosis of auditory sensory cells (44). The mitochondrial apoptotic pathway has been demonstrated to be activated by the release of proteins from the mitochondrial intermembrane space into the cytosol, which subsequently initiate the activation of apoptotic pathways (44). Cyt *c* is a mitochondrial protein, which induces caspase-dependent cell apoptosis; cyt *c* initiates caspase-9 activation, which in turn activates caspase-3 (45). It was

reported that cisplatin-mediated ototoxicity was associated with caspase-3 activity, cyt *c* release and ROS generation in auditory cells (46,47). These previous studies therefore indicated that mitochondrial signaling pathways may represent potential therapeutic targets for use in the prevention of cisplatin-mediated ototoxic damage. In the present study, KRG was demonstrated to inhibit the cyt *c* release, ROS generation and caspase-3 activation induced by cisplatin. These results therefore suggested that the protective effect of KRG in auditory cells occurred via the regulation of caspase-mediated apoptosis. Although KRG attenuated the caspase-mediated pathway, the present study did not investigate the effects of KRG on non-mitochondrial signaling pathways. Therefore, further studies are required in order to evaluate the potential role of KRG on non-mitochondrial signaling pathways in the auditory system.

Numerous previous studies have reported that inflammatory cytokines are associated with hearing loss (7-10,32). These cytokines were reported to induce secondary inflammatory responses, including leukocyte infiltration and scar formation. In addition, it was reported that cisplatin-induced cochlear injury occurred via pro-inflammatory cytokine expression (11). Flunarizine, a T-type Ca<sup>2+</sup> channel antagonist, was demonstrated to have cyto-protective effects against cisplatin cytotoxicity, the mechanism of which was shown to proceed via the inhibition of pro-inflammatory cytokine expression in HEI-OC1 cells (46). It was therefore hypothesized that the protective effect of KRG may occur via the regulation of pro-inflammatory cytokines. In the present study, it was demonstrated that KRG inhibited IL-6 expression levels, which indicated that the protective effect of KRG against cisplatin cytotoxicity occurred due to the suppression of inflammatory cytokine levels in auditory cells. Although it was demonstrated that KRG attenuated IL-6 expression levels, the effects of KRG on other cytokine pathways, or any pathways that may be involved, were not evaluated. Therefore, further studies are required in order to clarify the role of KRG in the cytokine-associated pathways in the auditory system.

The association between NF-κB activation and hearing loss has been investigated in numerous previous studies (38,39,48). Certain studies have reported that NF-κB family proteins identified in the inner ear were essential for the regular function of hair cells (49), while other studies have demonstrated that signal-transduction pathways respond rapidly to ototoxic stimulants, including noise exposure (38) and ototoxic drugs (16,50,51). NF-κB activation was reported to induce damage to the cochlear lateral wall through the generation of large quantities of ROS (52). In addition, acoustic overstimulation enhances inflammatory factor expression levels via NF-κB activation in the inner ear (39). The results of these studies suggested that NF-κB activation may be the primary target for the prevention of cisplatin-induced ototoxic damage. Therefore, in the present study, whether the protective mechanism of KRG proceeded via NF-κB pathway regulation was investigated. The results revealed that KRG attenuated the cisplatin-induced activation of NF-κB in auditory cells.

The activation of caspase-1 is known to be involved in the mediation of apoptosis and inflammation (21). Activated caspase-1 induces inhibitor of κB (IκB) kinase (IKK) phosphorylation by RIP2 dependent pathway. Caspase-1 interacts with RIP2 through the caspase recruitment domain, then RIP2

recruits the IKK complex via interaction with IKK-γ. This is independent of the enzymatic activity of caspase-1, and results in IκB-α degradation and NF-κB activation (41). Therefore, released NF-κB is translocated to the nucleus, where it is involved in the induction of gene transcription (53). The addition of pro-inflammatory cytokines, including IL-1β, has previously been shown to increase the nuclear translocation of NF-κB in HEI-OC1 auditory cells (11). Caspase-1 may contribute to NF-κB activation via the autocrine action of IL-1β. Therefore, it was hypothesized that the NF-κB pathway may interact with caspase-1 in auditory cells. Zhang *et al* (54) reported that caspase-1 initiated cyt *c* release and caspase-3 activation in ischemia/hypoxia-mediated neuronal cell death. In addition, it was demonstrated that cisplatin induced caspase-1 activation in cochlear hair cells and spiral ganglion neurons (55). In the present study, KRG was found to inhibit cisplatin-induced caspase-1 activation. These results suggested that the protective mechanism of KRG may be mediated via the modulation of signaling molecules involved cisplatin-induced apoptosis.

In conclusion, KRG was demonstrated to attenuate ototoxicity through prevention of the impairment of the hair cell arrays in organ of Corti rat primary explants. In addition, KRG inhibited cisplatin-induced ROS production, enhanced IL-6 levels, cyt *c* release, caspase-3 activation and NF-κB translocation to the nucleus in auditory cells. Therefore, these results have contributed to the elucidation of the pharmacological mechanism underlying KRG function and have provided evidence for potential therapeutic approaches for the treatment of ototoxicity.

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