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Original Article

Protective Effect of Korean Red Ginseng on Cisplatin Ototoxicity: Is It Effective Enough?

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OBJECTIVE: The aim of our study was to investigate the effects Korean Red Ginseng (KRG) on cisplatin (CDDP) ototoxicity in vivo and in vitro.

MATERIALS and METHODS: The first part of the study was conducted on the House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line. Cells were treated with CDDP, KRG, and their combination for 24 h. Cell viability, apoptosis, and the expression of 84 apoptosis-related genes were analyzed. In the second part of the study, 30 Wistar albino rats were divided into five groups. Baseline distortion product otoacoustic emissions (DPOAEs) and auditory brainstem response (ABR) measurements were obtained. In groups I, II, and III, only saline, KRG, and CDDP, respectively, were given. In group IV, 500 mg/kg KRG and in group V, 150 mg/kg of KRG were administered for 10 days. In groups III, IV, and V, 16 mg/kg CDDP injections were administered on day 11. On day 14, final DPOAEs and ABR measurements were completed. The rats were then sacrificed, and their inner ear structures were evaluated by transmission electron microscopy.

RESULTS: In the first part of the study, pretreatment with 1 mg/mL KRG protected cells from CDDP ototoxicity. This protection was mainly due to a decline in apoptotic gene expression and an increase in antiapoptotic gene expression. In the in vivo part of the study, we found that both KRG doses had otoprotective effects. This protection was more prominent at the lower dose, especially on the spiral ganglion and the brainstem.

CONCLUSION: KRG was shown to be an otoprotective agent against CDDP-induced ototoxicity both in vivo and in vitro.

KEYWORDS: Cisplatin, ototoxicity, Korean Red Ginseng

INTRODUCTION

Cisplatin (CDDP) is one of the main agents in majority of chemotherapy protocols. Side effects such as ototoxicity, neurotoxicity, and nephrotoxicity are restricting its usage in both children and adult patients. Ototoxicity could lead to language and speech disabilities in young children [1-2]. The mechanism of CDDP-induced ototoxicity is apoptosis, and outer hair cells, stria vascularis, and spiral ganglion neurons are mainly affected [3-7].

Ginseng is a tonic that has been used in traditional far-east medicine for more than 2000 years. Korean Red Ginseng (KRG) has been extracted from the roots of Panax Ginseng. KRG has beneficial effects on learning and memory impairment. KRG has been found to be effective in various problems that cause hearing loss such as gentamycin ototoxicity, age-related hearing loss, or 3-nitropropionic acid-induced cochlear damage [8-12]. CDDP-induced hearing loss has been studied in one in vitro and in one in vivo study [8, 13]. The aim of this study was to evaluate the effects of different doses of KRG on CDDP ototoxicity both in vitro and in vivo. In the first part of the study, the effects of KRG on CDDP ototoxicity in the HEI-OC1 cell line were studied. In the second part of this study, the effect of KRG was evaluated in a rat model of CDDP ototoxicity.

MATERIALS and METHODS

Part I: *In Vitro* Study

The Local Ethics Committee for non-invasive studies at our University approved this study.

Presented in: This study was presented as an oral presentation at 1st Global Otologic Research Meeting, 13 November 2013, Antalya, Turkey.

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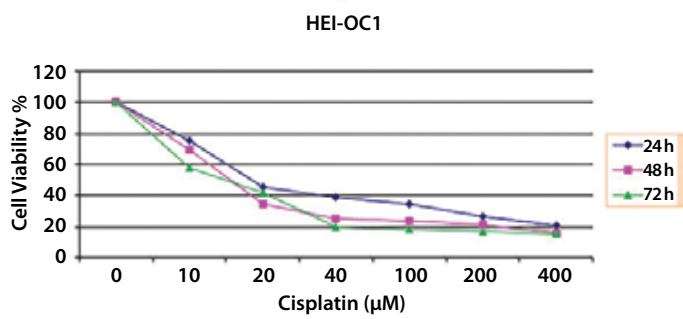


Figure 1. Cells were exposed to 10, 20, 40, 100, 200, and 400 uM doses of cisplatin. Fifty percent of cell growth in HEI-OC1 cells was inhibited at 20 uM cisplatin dose after 24 h incubation

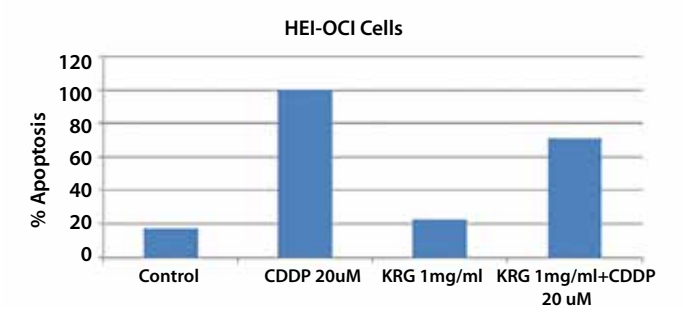


Figure 2. Apoptosis was decreased with a combination of KRG-CDDP. Asterisk indicates significance relative to CDDP (Mann–Whitney U, $p<0.05$). Values represent the mean±SEM of 3 observations.

Cell Culture

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line, an immortalized cell line derived from cochlear cultures of Immortomouse, was obtained from F. Kalinec from the House Ear Institute (Los Angeles, CA, USA). Ginseng extract used in this study was kindly provided from the Korean Red Ginseng Corporation (Taejon, Korea) with the support of Prof. Chong Sun Kim from Seoul National University. Cisplatin vials (Cisplatin-Ebewe® 50 mg/100 mL, Liba; İstanbul, Turkey) and Thiazolyl Blue Tetrazolium Blue MTT (Sigma) were used for the study. All chemicals and solutions were freshly prepared before each experiment. HEI-OC1 cells were exposed to CDDP at 10, 20, 40, 100, 200, and 400 uM doses for growth inhibition experiments.

Korean Red Ginseng extract was used in 0.1, 1, and 10 mg doses. For KRG and CDDP combination experiments, the cells were exposed to KRG for 60 min and then incubated with CDDP for 24 h.

Cell Survival Analysis

Analysis was performed using MTT as published before. All assays were replicated 6 times [14, 15].

Apoptosis Analysis with TUNEL

Apoptosis was analyzed with the TUNEL (terminal gated deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay. The Gen Script TUNEL Apoptosis Detection Kit (Cat. No. L00299, for Adherent cells, FITC-labeled POD) was used according to the manufacturer's instructions [14, 15]. Cells from all groups were smeared on polylysine-coated slides, dried, and fixed with methanol for 5 min. After blocking and proteinase K application, slides were incubated with TdT at 37 °C for 60 min. After application of the secondary antibody, the cells that had TdT attached to double strand DNA breaks were colored with Diaminobensidine. 5000 cells were counted and the apoptosis ratio was scored as %.

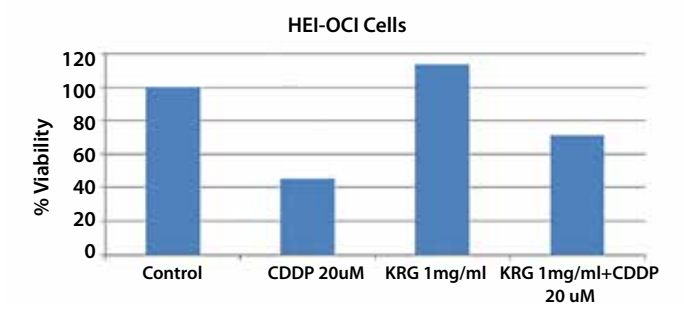


Figure 3. After 60 min pretreatment with 1 mg/mL KRG, cells were protected from 20 μm CDDP-induced toxicity. KRG showed a significant protective effect. Values represent the mean±SEM of 3 observations.

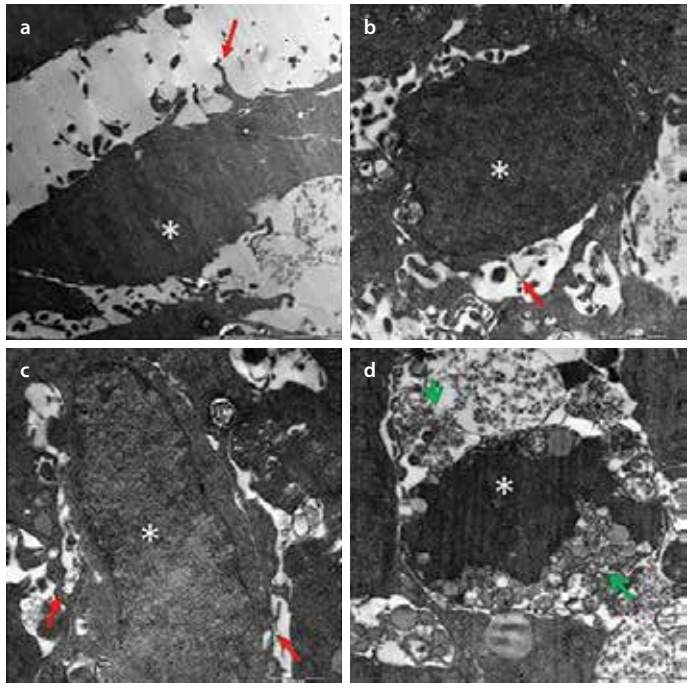


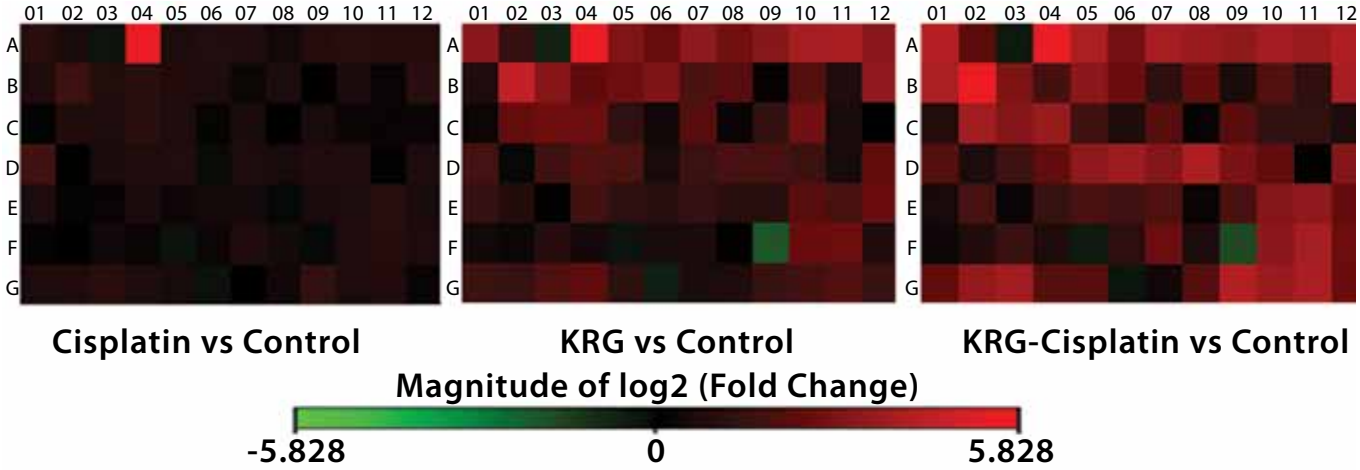
Figure 4. a-d. Ultrastructure of HEI-OC 1 Cells. Control (a), KRG (b), CDDP+KRG (c), and CDDP (d). Hair cells show normal morphology (Red arrows); microvilli-like cytoplasmic prolongations, (a, b) (*): nucleus. Hair cell with normal morphology (*) with protected microvilli-like prolongations (red arrow): Aponecrotic hair cell (*) and an abundance of cytoplasmic debris in the pericellular space (green arrow) (c).

Ultrastructural Findings of HEI-OC1 Cells by Transmission Electron Microcopy

Four groups of HEI-OC1 cells were analyzed with transmission electron microscopy. Groups were HEI-OC1 cells without any treatment, CDDP (20 uM) treated HEI-OC1 cells, KRG (1 mg) treated HEI-OC1 cells, and HEI-OC1 cells treated with a combination of CDDP (20 uM) and KRG (1 mg). The cells were collected by a cell scrapper from the cell culture after incubation with the agents. Cells were fixed with glutaraldehyde and prepared by blocking in resin for routine electron microscopy experiments.

Analysis of Apoptosis-Related Gene Expressions

Ribonucleic acid isolation was performed using an RNA extraction kit. Apoptosis-related gene expression analysis was done with the standard 84 array genes of Mouse apoptosis (SABiosciences; Valencia, USA) by real-time PCR as previously published [16].



A01	Akt1	C01	Bok	E01	Fas	G01	Tnfaf12
A02	Apaf1	C02	Card10	E02	Fasl	G02	Cd401g
A03	Api5	C03	Nod1	E03	Hells	G03	Cd70
A04	Atf5	C04	Card6	E04	Il10	G04	Traf1
A05	Bad	C05	Casp1	E05	Lhx4	G05	Traf2
A06	Bag1	C06	Casp12	E06	Ltbr	G06	Traf3
A07	Bag3	C07	Casp14	E07	Mcl1	G07	Trp53
A08	Bak1	C08	Casp2	E08	Nfkb1	G08	Trp53bp2
A09	Bax	C09	Casp3	E09	Nme5	G09	Trp53inp1
A10	Bci10	C10	Casp4	E10	Nol3	G10	Trp63
A11	Bci2	C11	Casp6	E11	Pak7	G11	Trp73
A12	Bci211	C12	Casp7	E12	Pim2	G12	Zc3hc1
B01	Bci2110	D01	Casp8	F01	Polb	H01	Gusb
B02	Bci212	D02	Casp9	F02	Prdx2	H02	Hprt
B03	Bid	D03	Cflar	F03	Pxcard	H03	Hsp90ab1
B04	Naip1	D04	Cidea	F04	Ripk1	H04	Gapdh
B05	Naip2	D05	Cideb	F05	Rnf7	H05	Actb
B06	Birc2	D06	Cradd	F06	Sphk2	H06	MGDC
B07	Birc3	D07	Dad1	F07	Tnf	H07	RTC
B08	Xiap	D08	Dapk1	F08	Tnfraf10b	H08	RTC
B09	Birc5	D09	Dffa	F09	Tnfraf11b	H09	RTC
B10	Bnip2	D10	Dffb	F10	Tnfraf1a	H10	PPC
B11	Bnip3	D11	Tac22d3	F11	Cd40	H11	PPC
B12	Bnip31	D12	Fadd	F12	Tnfaf10	H12	PPC

Figure 5. The list and heat map presentation of apoptotic gene expressions induced by KRG, CDDP, and the KRG-CDDP combination versus those if the control group. H lines were the array controls (housekeeping genes in H1–5, 1 genomic DNA control in H6, 3 reverse transcriptase controls in H7-9; 3 positive PCR controls in H10-12 lines). The H1-12 lines are not presented in the table. Green color indicated a lower expression of genes, and red color indicated a higher expression of genes.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 software program (Statistical Package for Social Sciences; SPSS Inc.; Chicago, IL, USA). Results were evaluated as mean±SEM. A Mann–Whitney U test was used for comparison of continuous variables. In order to obtain statistically correct data, all experiments were repeated a minimum of 3 times. P<0.05 value was accepted as statistically significant.

RESULTS

Cell Viability and Apoptotic Cell Death

CDDP decreased the viability of HEI-OC1 cells in a dose- and time-dependent manner. Cells were exposed to 10, 20, 40, 100, 200, and 400 uM doses of CDDP. Fifty percent of cell growth was inhibited at 20 uM cisplatin after 24 h incubation in HEI-OC1 cells (Figure 1). This dose was chosen for the rest of the experiment and was used for 24 h. Cells were pretreated with the KRG extract with 0.1 mg/mL, 1 mg/mL, and 10 mg/mL concentrations for 60 min. Protection was dose dependent, and the maximal effect was observed after 60 min of pretreatment with 1 mg/mL KRG. A 1 mg/mL dose of KRG was used for the rest of the experiments. This dose was shown to protect against CDDP (p<0.05) (Figure 2, 3).

Transmission Electron Microscopic Findings

In group I (Control) and group II (KRG) microvilli-like cytoplasmic cellular prolongations were seen as previously observed in cultured epithelial and neuroepithelial cells. These prolongations were prominent in the longitudinal, horizontal, and oblique sections in pericellular spaces. Apoptotic, necrotic, and aponecrotic cell morphologies were scarcely detected. In the CDDP group, large amounts of nuclear fragmentation, as well apoptotic and necrotic cell nuclei, were detected. Plenty of cellular debris was found in the pericellular space. Some of the cells were apoptotic, others had either necrotic or aponecrotic character. The rest of the cells were fusiform and oval shaped as usually seen in cultured cell mediums. Mitochondrial swellings were obvious. In the CDDP and KRG combination group, there were a few necrotic cells but apoptotic and aponecrotic cells were very rare. The rest of the cells were heterochromatic and had normal nuclear morphology. Mitochondrial morphology was also normal (Figure 4).

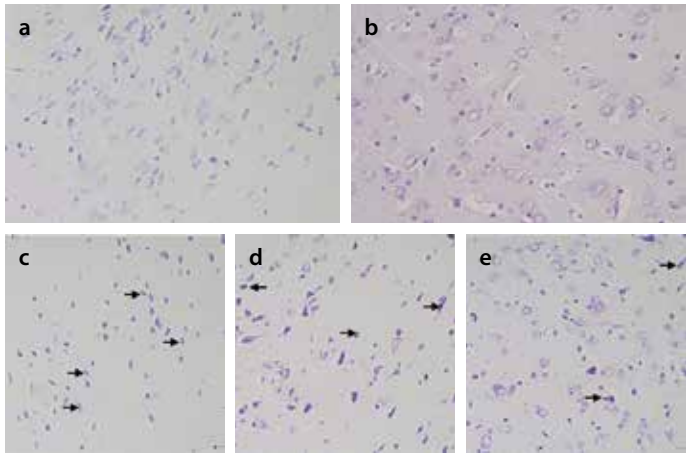


Figure 6. a-e. Cresyl violet staining of the brainstem. Arrows shows pyknotic cells. Control group (a), KRG group (b), CDDP group (c), CDDP+KRG 500 mg group (d), CDDP+KRG150 mg group (e).

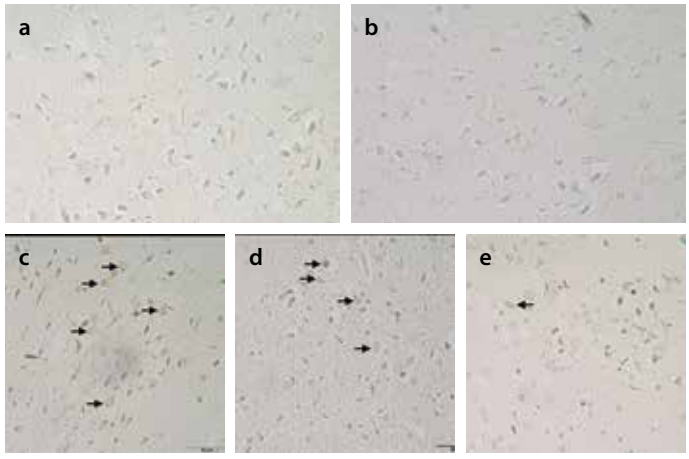


Figure 7. a-e. TUNEL staining of the brainstem. Arrows showing TUNEL-positive cells. Control group (a), KRG group (b), CDDP group (c), CDDP+KRG 500 mg group (d), CDDP+KRG150 mg group (e).

Apoptosis-Related Gene Expression Analysis

CDDP-, KRG-, and KRG-CDDP-induced apoptotic gene expressions were analyzed with the standard 84 array genes for mouse apoptosis (SA, Biosciences; Valencia, USA) (Figure 5). The results of gene analysis showed that CDDP could cause an increase in apoptotic genes such as caspase-8 and genes from the TNF family (Cd40, Cd70). In the CDDP+KRG group, apoptotic gene expression, particularly caspase-8 expression, was reduced. In this group also expression of antiapoptotic genes such as genes from the bcl2 family (bcl2, bcl211, bcl212, and Akt1) were increased.

In the vitro part of this study, KRG was shown to be partially protective against CDDP-related ototoxicity. The molecular mechanism of this protection was mainly due to a decline in apoptotic gene expression and an increase on antiapoptotic gene expression.

Part II: *In Vivo* Study

Materials and Methods

The protocol for this study was approved by the local ethics committee of the University for animal care and use. This study was conducted on 30 adult male Wistar albino rats weighing 200–300 g. Animals with any external or middle ear diseases were excluded from the study.

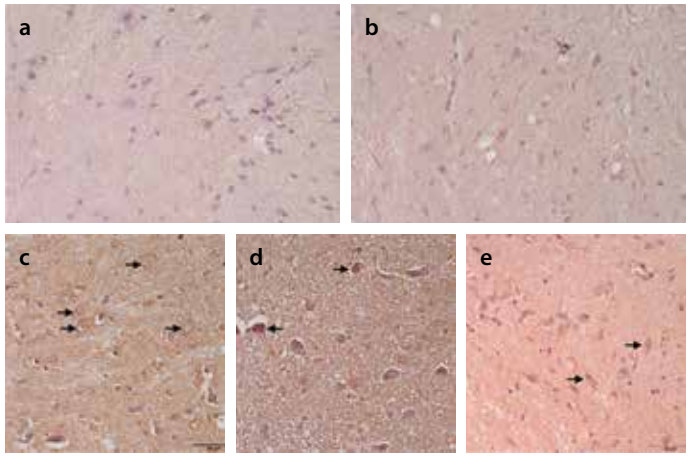


Figure 8. a-e. Brainstem Caspase-3 staining: Arrows showing Caspase-3-positive cells. Control group (a), KRG group (b), CDDP group (c), CDDP+KRG 500 mg group (d), CDDP+KRG 150 mg group (e).

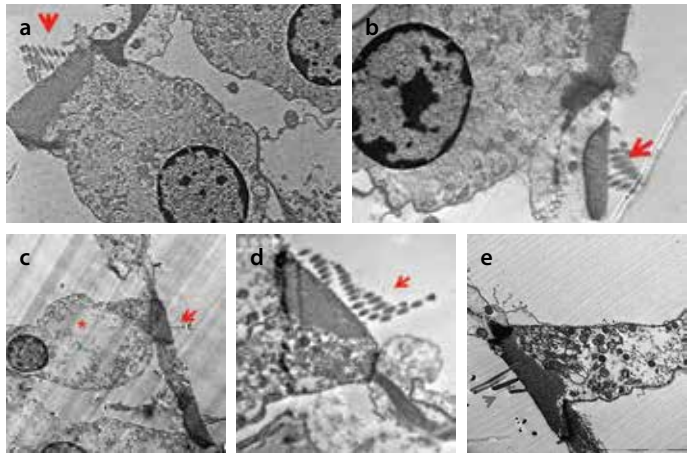


Figure 9. a-e. The ultrastructure of hair cells. A1: Control group, A2: KRG group, A3: CDDP group, A4: CDDP+KRG 500 mg group, A5: CDDP+KRG150 mg group. In the control (a), hair cells with normal ultrastructural properties were seen and cilia (red arrows) were preserved. In the KRG group (b), hair cells with regular nucleus (N) and cilia (red arrows) were seen. In the CDDP group (c), loss of cilia (red arrow) and intracellular degenerative spaces (*) were observed. In the CDDP+KRG 500 mg group (d), cilia were well preserved. In the CDDP+KRG 150 mg group (e), cilia (red arrows) were well preserved (Red arrow: cilia; *: intracellular degenerative areas).

Cisplatin vials (Cisplatin-Ebewe® 50 mg/100 mL, Liba; İstanbul, Turkey) were used for the experiment. Korean Red Ginseng Powder was used for in vivo study. This material was also provided by the Korean Red Ginseng Corporation (Taejon, Korea) with kind support from Professor Chong Sun Kim from Seoul National University. For groups II, IV, and V, 500 mg, 500 mg, and 150 mg of KRG powder was freshly diluted with 0.75 mL saline solution for oral administration.

Experimental Design

Study groups were divided as follows:

Group I (n=5): Saline control

Group II (n=5): 500 mg/kg/day KRG

Group III (n=6): 16 mg/kg CDDP

Group IV (n=7): 500 mg/kg/day KRG+CDDP

Group V (n=7): 150 mg/kg/day KRG+CDDP

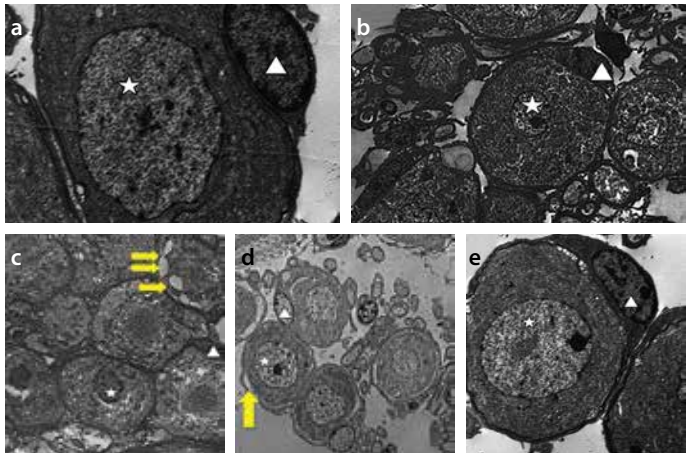


Figure 10. a-e. The ultrastructure of spiral ganglion cells. In the control (a) and KRG group (b), spiral ganglion neurons nucleus (star) and satellite cells (arrowhead) were preserved. In the CDDP group (c), degenerative ganglion cells with irregular cell membranes and intracellular degenerative areas (yellow arrows) were evident. In the CDDP+KRG 500 mg (d) group, satellite cells (arrowhead) were preserved, CDDP+but there were a few intracellular degenerative areas in the cells (yellow arrow). In the CDDP+KRG 150 mg group (e), cell morphology was close to the control group with preserved satellite cells (arrowhead).
Star: nucleus; Arrowhead: satellite cell; Yellow arrow: intracellular degenerative areas.

Groups I and III rats received oral saline (0.75 mL/day) for 10 days. Groups II, IV (500 mg/kg KRG diluted into 0.75 mL saline/day) and V rats (150 mg/kg KRG diluted into 0.75 mL saline/day) were orally given KRG for 10 days. At the 11th day, a single dose of 16 mg/kg CDDP was infused intraperitoneally in groups III, IV, and V. The same volume of saline solution was infused intraperitoneally to groups I and II.

Auditory Assessments

Ketamin hydrochloride (Pfizer; İstanbul, Turkey) (60 mg/kg) and xylazine hydrochloride (Sigma-Aldrich; Lyon, France) (5 mg/kg) was used for anesthesia prior to each auditory investigation. A baseline distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) recording were obtained at the beginning of the study. At 14th day of the study, final audiological investigations were performed.

All recordings were obtained using the same procedures as previously published [16].

Histopathological Examinations

Following the final audiological investigations, the rats were sacrificed under ether anesthesia and the brainstem and temporal bullae were collected. Brainstem samples were investigated under light microscopy by staining with cresyl violet and also evaluated with TUNEL and caspase 3 immunohistochemistry methods as previously published [16].

Inner ear tissue samples were evaluated with transmission electron microscopy according to the previously published method [16].

Statistical Analysis

Statistical analyses were performed using the SPSS 15.0 software package (Statistical Package for Social Sciences; SPSS Inc.; Chicago, IL, USA). Results were given as mean±SEM. All obtained data were analyzed using one-way analysis of variance (ANOVA) and a post hoc Bonferroni test ($p<0.05$ was accepted as statistically significant).

Table 1. Apoptosis ratio in the brainstem

Groups	TUNEL (%)	Caspase-3 (%)
Control	8.67±0.66$^{\#}$	7.0±0.63$^{\#}$
KRG	8.6±1.14$^{\#}$	7.0±0.54$^{\#}$
CDDP	30.1±4.75*	27.1 ±1.92*
CDDP+KRG 500 mg	25.4±1.51*	23.2±0.96*
CDDP+KRG 150 mg	19.8±1.92$^{*\#}$	18.0±0.83$^{*\#}$

KRG: Korean Red Ginseng; CDDP: cisplatin; TUNEL: Tdt-mediated dUTP Nick End Labeling
*Statistically significant compared to the control group.
#Statistically significant compared to the cisplatin group.

Results

Audiological Findings

Baseline ABR and DPOAE values were not statistically different for all groups ($p>0.05$) All test parameters of Group II animals were similar to those of the controls ($p>0.05$). In CDDP group animals, click ABR thresholds were elevated and 8 kHz DPOAE thresholds were lower than in the controls ($p<0.05$). In Group IV and V rats, ABR and DPOAE thresholds were not statistically different from the controls ($p>0.05$).

Histological Examination

Cresyl Violet Staining

All sections were taken from the same area in the brainstem. The cochlear nuclei of the brainstem showed normal morphology in control and KRG only groups. In the CDDP group, the majority of the neurons were shrunken and pyknotic. In both CDDP and KRG combination groups, the number of shrunken neurons were diminished; however, this decrease was more prominent in the 150 mg/kg KRG+CDDP group (Figure 6).

TUNEL Assay

Apoptosis was evident in the CDDP group; TUNEL-positive cells were statistically higher than in the controls ($p<0.05$). Apoptotic cell death diminished in group IV, but this decrease was not significant ($p>0.05$). In group V (150 mg/kg KRG+CDDP), TUNEL-positive cells were significantly decreased in comparison to the CDDP group ($p<0.05$) (Figure 7, Table 1).

Caspase-3 Immunohistochemistry

Immunohistochemical analysis of the rat brainstem revealed caspase-3 reactivity was significantly increased in the CDDP group in comparison with the control and KRG only groups ($p<0.05$). This reactivity was less obvious in groups IV and V; however, it was statistically significant only for group V ($p<0.05$) (Figure 8, Table 1).

Electron Microscopy Findings for the Organ of Corti

In control and KRG only groups, cell membranes, nuclei, and mitochondria from hair cells showed normal properties. In the CDDP group, the ultrastructure of hair cells was disturbed; cellular membranes were irregular, stereocilia were missing, and some intracellular degenerative spaces were seen. In groups IV and V, cellular damage was less evident. Cell membranes and cilia of hair cells were close to normal with very few intracellular degenerative areas (Figure 9).

Electron Microscopy Findings for the Spiral Ganglion

The control and KRG only groups showed normal spiral ganglion neuron properties. In the CDDP group, some irregularities at cell nucleus

and at the cellular membranes were seen. There were cytoplasmic degenerative spaces and losses of satellite cells. In groups IV and V, the ultrastructure of cells were near normal, but in the KRG 500 mg/kg+CDDP group, degenerative areas were still seen. The morphology of the 150 mg/kg KRG+CDDP group's spiral ganglion cells and satellite cells were closer to normal (Figure 10).

The second part of the study showed that KRG had protective effects against cisplatin cochlear damage. This effect was more prominent with lower doses of KRG and at the neuron level.

DISCUSSION

Ototoxicity is an important side effect resulting from anticancer treatment. Hearing loss can cause considerable problems in the social life of both children and adults. Specifically, in small children, it can impair speech and language acquisition. In the elderly population, hearing loss may be associated with social isolation [17]. CDDP leads to the activation of intrinsic and extrinsic apoptotic pathways. While the intrinsic pathway activates with the release of cytochrome-c from the mitochondria, the extrinsic pathway activates by ligand binding to death receptors such as Fas and tumor necrosis factor receptor-1 (TNFR1) [3-7, 9, 10]. Some candidate agents such as sodium thiosulfate, D- or L-methionine, lipoic acid, N-acetyl cysteine, acetyl L-carnitine, alpha tocopherol, and resveratrol were studied both in vitro and in vivo to analyze their protective effects against CDDP-induced ototoxicity [7, 16, 18-21]. However, none of them are currently approved for usage against CDDP-induced ototoxicity.

Ginseng is a herbal medicine taken orally. It is considered a general tonic and is believed to enhance body homeostasis. It is prescribed in oriental medicine predominately for anti-aging, anti-stress effects, and to increase physical health. Its bioavailability is low. Gut microbial flora is responsible for the production of different metabolites such as ginsenosides, polysaccharides, peptides, and gintonins [22, 23]. However, because of poor membrane permeability and low solubility, absorption of these products is limited. More than 100 ginsenosides have been isolated from panax ginseng (KRG).

These ginsenosides, chemically, are steroids. Different ginsenosides with other ginseng saponins have different effects. Some of these effects may differ in different doses. Ginsenosides have been shown to decrease excitability of neuronal cells by stimulating GABA and glycine receptors [24]. Neuroprotective and anxiolytic effects of ginseng are attributed to this action. Cardioprotective and antihypertensive effects of KRG are mostly linked to the relaxation of blood vessels in smooth muscle, inhibition of endothelin production, as well inhibition of platelet aggregation [24].

Some saponins are responsible for anti-inflammatory and immune stimulating properties of KRG by inhibiting leukotrienes, histamine release, and cytokine production and enhancing phagocytosis in B and T cells. However, KRG is consumed after traditional extraction processes and, like many other natural products, cultivation and processing conditions may alter the compositions of ingredients [24]. Because some of the ginsenosides may cause a change in the pharmacodynamics of others, it may not be easy to distinguish which one is responsible for a given effect when they are all taken orally.

Age-related hearing loss, noise-induced hearing loss, and ototoxicity has been shown to occur as a result of an apoptotic process [3-7]. KRG extracts, or its specific saponins, were shown to have antiapoptotic and antioxidant properties in various tissues both in vitro and in vivo [8]. Recently, it was shown that KRG has otoprotective properties against gentamicin [9, 10], 3-nitropropionic acid [12], and CDDP- induced ototoxicity [8,13]. These studies revealed that KRG mainly works by inhibition of some proapoptotic genes such as caspase-1, caspase-3, and the TNF family of genes and by enhancement of antiapoptotic gene expression such as bcl-2 [8-13]. On the other hand, KRG has antiapoptotic effects on neuronal tissues [18]. It was shown that this effect mainly works by up regulation of the p13K/Akt signaling pathway, which is a cell survival pathway. Activation of this pathway inhibits apoptosis by decreasing p53 and caspase-3 expression while also increasing expression of genes in the bcl2 family [8, 11, 18]. KRG has also been shown to be an otoprotective agent following cochlear injury associated with noise-induced hearing loss [25]. Moreover, in a clinical study conducted by Kim [26], the beneficial effects of orally administered KRG on chronic tinnitus symptoms was demonstrated.

This study was conducted in two stages. In the first stage, KRG was tested on HEI-OC1 cells against CDDP ototoxicity. Although in some studies, specific ginsenosides have been used, we preferred to use ginseng extract as it is easily orally taken in daily life. A combination of KRG and CDDP blocked extrinsic pathways by decreasing caspase-8 gene expression. Also it inhibited intrinsic pathways by increasing expressions of bcl-2 family gene. Besides, an increase of Akt1 gene expression has been shown and this expression was thought to have caused activation of another signaling pathway that leads to cell survival by blocking apoptosis.

These findings are in accordance with previous studies that reveal KRG prevents apoptosis by inhibiting extrinsic and intrinsic pathways, as well by activating cell survival pathways [3-8, 11, 18].

As we revealed in the first part of this study, the effect of KRG is time and dose dependent. In the second part of this study, we preferred using two different doses of KRG. Otoprotective effects of KRG were studied in rats in different conditions such as age-related hearing loss and vestibular dysfunction [11], acute 3-nitropropionic-induced cochlear damage, gentamicin-induced hearing loss and balance dysfunction, and CDDP-induced ototoxicity [9, 10, 12]. Audiological evaluation, as well scanning electron microscopy and confocal microscopy findings, revealed that KRG has a protective effect against gentamicin and 3-nitropropionic acid-induced ototoxicity [9, 10, 12].

Recently, Tian et al. [11] used KRG in a two-stage study. In the in vivo part, they found that KRG showed protective effects against CDDP-induced ototoxicity in rats, as shown by ABR results. They also worked on organ of corti explant cultures and found that pretreatment with KRG prevents hair cell impairment [11].

We used DPOAE and ABR to evaluate hearing levels and we found that both 150 mg and 500 mg doses of KRG had otoprotective effects against CDDP-induced ototoxicity.

Sections through the cochlear nuclei of brainstem cells proved that 150 mg/kg/day KRG was more effective against CDDP-induced ototoxicity.

Although with 500 mg/kg/day KRG, the apoptotic cell ratio diminished, this decrement was not significant in comparison to the CDDP group.

Transmission electron microscopy findings showed that treatment with both 150 mg and 500 mg KRG offered protection at the cochlea level. In both treatment groups, spiral ganglion neurons were protected; and this protection was more prominent in the 150 mg/kg/day KRG group. These observations suggest that, although both doses of KRG show otoprotective properties, in lower doses (150 mg/kg) neural tissues such as the spiral ganglion and the brainstem are better protected.

Recently, it was shown that antiapoptotic agents can act differently depending on the used dose. In a recent study, lower doses of KRG was found to be effective in preventing age-related hearing and vestibular dysfunction. But in the same study, it was revealed that a higher dose (500 mg/kg) of KRG aggravated age-related hearing loss and balance dysfunction [11]. Similarly, it was found that while high doses of oral resveratrol increased CDDP-induced ototoxicity and apoptosis, in a lower dose of oral resveratrol, some histopathological improvement was observed. In light of our findings, and the literature, we believe that different doses should be tried in any study concerning this kind of antiapoptotic agents before these studies can be translated to clinical practice.

In conclusion, KRG has a protective effect on HEI-OC1 cells in a dose- and time-dependent manner. This effect mainly worked on increasing antiapoptotic gene expression and decreasing apoptotic gene expression. Moreover, KRG was shown to be effective against CDDP-induced ototoxicity in rats. This effect is more prominent in low doses, particularly on neuronal tissues such as spiral ganglion neurons and the brainstem. Our study can be considered as a frontier for translational studies, and KRG in low doses may be a good nutrient to use in clinical trials against CDDP ototoxicity.

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