저작자표시 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.
- 이 저작물을 영리 목적으로 이용할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer: 

Collection
Effects of Korean Red Ginseng against Ototoxic Drugs and Age-related Cochleo-vestibular Dysfunction

by

Chunjie Tian

Major in Medicine
Department of Medical Sciences
The Graduate School, Ajou University
Effects of Korean Red Ginseng against Ototoxic Drugs and Age-related Cochleo-vestibular Dysfunction

by

Chunjie Tian

A Dissertation Submitted to The Graduate School of Ajou University in Partial Fulfillment of the Requirements for the Degree of Ph. D. in Medicine

Supervised by

Yun-Hoon Choung, M.D., D.D.S., Ph.D.

Major in Medicine
Department of Medical Sciences
The Graduate School, Ajou University
February, 2014
This certifies that the dissertation
of Chunjie Tian is approved.

SUPERVISORY COMMITTEE

Keehyun Park
Yun-Hoon Choong
Chul-Ho Kim
Seon-Yong Jeong
Shi-Nae Park

The Graduate School, Ajou University
November, 4th, 2014
- ABSTRACT -

Effects of Korean Red Ginseng against Ototoxic Drugs and Age-related Cochleo-vestibular Dysfunction

Gentamicin (GM) is one of the most widely used aminoglycoside antibiotics. However, it can damage inner ear hair cells and cause both hearing loss and vestibular impairment. 3-Nitropropionic acid (3-NP), a mitochondrial toxin, has been reported to induce an acute cochlear damage. There are no therapeutic interventions have been developed for age related inner ear disorder which is one of the most common sensory deficits in the elderly. Korean red ginseng (KRG) with its ginsenosides has neuron beneficial effects and anti-ageing effects. In the present study, the authors evaluated (1) the protective effects of KRG against gentamicin (GM)-induced unilateral vestibular and hearing dysfunction and investigated its effective mechanism using in vitro cell cultures; (2) the protective effects of KRG in an ototoxic animal model BALB/c mice using 3-NP; (3) the effects of KRG on age-related hearing loss (AHL) and balance disturbance in C57BL/6 mouse.

In the first study, Sprague-Dawley rats were classified into GM group (n = 12) and KRG+GM group (n = 10). Head tilt, tail hanging, and swimming tests for balance function and auditory brainstem response test (ABR) for hearing function were performed for hearing and vestibular function evaluation. Cochleae and utricles/saccules were harvested for scanning electron microscope (SEM). The ventral otocyst-epithelial clone 36 (VOT-E36) cell was used to explore protective mechanism of Ginsenoside Rb1. In the second study, Dose-dependent toxic effects of 3-NP were investigated for an appropriate toxicity level of 3-NP, and then 23 mice were grouped into 3-NP (n = 12) and KRG + 3-NP (n = 11) groups for observation of the protective effects of KRG. ABR and cochlear morphological evaluations were performed before and after drug administration. In the third study, 22 mice were randomly distributed into control (n = 8), KRG (150 mg/kg, n = 7), and KRG (500 mg/kg, n = 7) groups. Hearing/vestibular function and related morphology were evaluation during ageing process.

In the first study, Vestibular function was comprehensively evaluated by a scoring system
that ranged from 0 (normal) to 3 (worst) points, using head tilt, tail hanging, and swimming tests. The GM group showed significantly more deteriorated vestibular function (0 point – 5 rats, 1 point – 1 rat, 2 points – 3 rats, and 3 points – 3 rats) than the KRG + GM group (0 point – 9 rats and 1 point – 1 rat) \( (p < 0.01) \). The hearing thresholds were better in the KRG + GM group than in the GM group \( (p < 0.05) \). Quantitative analysis of hair cell damage in the scanning electron microscopy was closely related with vestibular and hearing functional results. In vitro study showed that ginsenoside Rb1 \( (\text{gRb}1) \) attenuated reactive oxygen species production, suppressed JNK activation, up-regulated Bcl-xL and down-regulated Bax, cytochrome \( c \), caspase 3, and cleaved poly (ADP-ribose) polymerase in GM-treated VOT-E36 cells. In the second study, The ABR thresholds in the 800–5000 mM groups exceeded the maximum recording limit at 16 and 32 kHz 1 day after 3-NP administration. The ABR threshold in the 500 mM 3-NP + KRG group was significantly lower than that in the 500 mM 3-NP group from post 1 week to 1 month. The mean type II fibrocyte counts significantly differed between the control and 3-NP groups and between the 3-NP and 3-NP + KRG groups. Spiral ganglion cell degeneration in the 3-NP group was more severe than that in the 3-NP + KRG group. In the third study, No mice in the control and KRG1 groups died at the age of 12 month, however, 4 in 7 mice died in the KRG (500 mg/kg) group. ABR recording demonstrated high frequency hearing loss at 32 kHz \( (38.8 \pm 5.6 \text{ dB}) \) at 6 month of age, and 16 kHz \( (41.9 \pm 6.0 \text{ dB}) \) at 9 month of age, elevated to 52.2 \( \pm 8.6 \text{ dB} \) at 16 kHz and 55.6 \( \pm 8.3 \text{ dB} \) at 32 kHz at 12 month in the control group. However, the thresholds shift was delayed significantly \( (P < 0.05) \) in the KRG (150 mg/kg) group (from the age of 6 month \( (32.1 \pm 5.7 \text{ dB}) \) to 12 month \( (50.0 \pm 16.6 \text{ dB}) \) at 32 kHz, and 9 month \( (34.6 \pm 8.2 \text{ dB}) \) to 12 month \( (47.9 \pm 18.1 \text{ dB}) \) at 16 kHz). The hearing thresholds increased to 61.7 \( \pm 20.7 \text{ dB} \) at 16 kHz and 64.2 \( \pm 16.9 \text{ dB} \) at 32 kHz at 12 month in the 500 mg/kg fed mice. The age-associated vestibular dysfunction was observed with tail hanging and swimming tests, the severity score in the tail hanging test and swimming time in swimming test were significantly different between the KRG (150 mg/kg) group and the control group at 12 month \( (P < 0.05) \). Histological observation supported the hearing and vestibular function findings. The number of the Bcl-xL immunopositive SGCs and type II fibrocytes was markedly more in KRG (150 mg/kg) group than that in the control group \( (P < 0.05) \)
These findings suggest that KRG including gRb1 component protects against vestibular/hearing dysfunction by inhibiting apoptotic pathways when ototoxicity is induced by unilateral intratympanic injection with GM in rats. Mice model of 3-NP-induced hearing loss exhibited a dose-dependent hearing loss with histological changes. KRG administration ameliorated the deterioration of hearing by 3-NP. C57BL/6J mouse showed early onset of hearing and vestibular dysfunction with ageing, which were delayed by KRG treatment in dose of 150 mg/kg by inhibiting mitochondrial apoptotic pathway; however, long-term treatment of 500 mg/kg KRG may induce aggressive behavior and aggravate age-related hearing and balance dysfunction.

In conclusion, Korean red ginseng might be an effective treatment for protecting hearing loss caused by various etiologies.

**Key words:** Korean red ginseng, Gentamicin, Ginsenoside Rb1, Hair cell, Fibrocyte, Spiral ligament, Spiral ganglion cell, 3-Nitropropionic acid, Age-related hearing loss
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................................................. i
TABLE OF CONTENTS ...................................................................................................................................................... iv
LIST OF FIGURES ........................................................................................................................................................... vii
LIST OF TABLES ........................................................................................................................................................... ix

PART 1 Korean red ginseng protects against gentamicin-induced balance dysfunction and hearing loss in rats through antiapoptotic functions of ginsenoside Rb1 ................................................................. 1

I. INTRODUCTION .......................................................................................................................................................... 1

II. MATERIALS AND METHODS ....................................................................................................................................... 3

A. Preparation of animal study groups ............................................................................................................................. 3
B. Evaluation of vestibular function ................................................................................................................................... 5
C. Evaluation of hearing function ........................................................................................................................................ 5
E. Cell culture and viability assays ..................................................................................................................................... 6
F. Intracellular levels of reactive oxygen species (ROS) ................................................................................................. 6
G. Western blot assay ........................................................................................................................................................ 7
H. Statistical analysis .......................................................................................................................................................... 8

III. RESULTS ........................................................................................................................................................................ 9

A. Evaluation of vestibular/balance function ..................................................................................................................... 9
B. Otolith organ (saccule and utricle) morphology ........................................................................................................ 13
C. Evaluation of hearing function ....................................................................................................................................... 15
D. Cochlear morphology ....................................................................................................................................................... 15
E. gRb1 exerts a protective effect in vitro ....................................................................................................................... 17

IV. DISCUSSION ................................................................................................................................................................ 23
PART 2  Korean red ginseng ameliorates acute 3-nitropropionic acid-induced cochlear damage in mice

I.  INTRODUCTION

II.  MATERIALS AND METHODS

A. Animals
B. Korean red ginseng
C. Study design
D. Intratympanic administration of 3-NP
E. Auditory brainstem response (ABR) measurement
F. Morphological evaluation
G. Statistical analysis

III.  RESULTS

A. 3-NP dose-dependent hearing threshold shift
B. Histological analysis after 3-NP administration
C. Protective effects of KRG on 3-NP-induced hearing threshold shift
D. Histological analysis after KRG treatment

IV.  DISCUSSION

V.  CONCLUSION

PART 3  Korean red ginseng delays age-related hearing loss and vestibular dysfunction in C57BL/6 mouse

I.  INTRODUCTION
II. MATERIALS AND METHODS........................................................................................................... 58

A. Study design ................................................................................................................................ 58

B. Auditory and vestibular function assessment. ............................................................................. 58

C. Morphological evaluation of hair cells....................................................................................... 59

D. Immunohistochemistry .................................................................................................................. 59

E. Statistical analysis .......................................................................................................................... 59

III. RESULTS...................................................................................................................................... 60

A. Mortality ....................................................................................................................................... 60

B. KRG’s effect on age related hearing loss in C57BL/6J mouse ...................................................... 62

C. KRG’s effect on age related balance dysfunction ......................................................................... 66

D. Detection of Bcl-xL and cytochrome c expression ...................................................................... 70

IV. DISCUSSION.................................................................................................................................. 72

V. CONCLUSION .................................................................................................................................. 75
# LIST OF FIGURES

## Part 1

<table>
<thead>
<tr>
<th>FIG. 1. EVALUATION OF VESTIBULAR FUNCTION</th>
<th>1 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIG. 2. SCANNING ELECTRON MICROGRAPHS OF SACCULAR MACULAE AND</td>
<td></td>
</tr>
<tr>
<td>UTRICULAR MACULAE</td>
<td>1 4</td>
</tr>
<tr>
<td>FIG. 3. FUNCTIONAL AND MORPHOLOGICAL MANIFESTATIONS OF THE INDUCED</td>
<td></td>
</tr>
<tr>
<td>HEARING LOSS</td>
<td>1 6</td>
</tr>
<tr>
<td>FIG. 4. CELL VIABILITY</td>
<td>1 8</td>
</tr>
<tr>
<td>FIG. 5. MEASUREMENT OF INTRACELLULAR ROS PRODUCTION</td>
<td>2 0</td>
</tr>
<tr>
<td>FIG. 6. THE EFFECT OF GRB1 ON GM-INDUCED APOPTOSIS</td>
<td>2 2</td>
</tr>
</tbody>
</table>

## Part 2

<table>
<thead>
<tr>
<th>FIG. 1. AUDITORY BRAINSTEM RESPONSE</th>
<th>3 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIG. 2. CONFOCAL MICROSCOPIC IMAGES</td>
<td>3 7</td>
</tr>
<tr>
<td>FIG. 3. LIGHT MICROSCOPIC IMAGES OF LATERAL WALLS</td>
<td>3 8</td>
</tr>
<tr>
<td>FIG. 4. LIGHT MICROSCOPIC IMAGES OF SPIRAL GANGLION CELLS</td>
<td>3 9</td>
</tr>
<tr>
<td>FIG. 5. CHANGES IN MEAN AUDITORY BRAINSTEM RESPONSE</td>
<td>4 1</td>
</tr>
<tr>
<td>FIG. 6. CONFOCAL MICROSCOPIC IMAGES OF THE ORGAN OF CORTI</td>
<td>4 3</td>
</tr>
<tr>
<td>FIG. 7. CELLULAR CHANGES IN THE COCHLEAR LATERAL WALLS</td>
<td>4 5</td>
</tr>
<tr>
<td>FIG. 8. CELLULAR CHANGES IN THE SPIRAL GANGLION</td>
<td>4 7</td>
</tr>
</tbody>
</table>

## Part 3

<table>
<thead>
<tr>
<th>FIG. 1. MORTALITY OF 12 MONTHS OLD MICE</th>
<th>6 1</th>
</tr>
</thead>
</table>
LIST OF TABLES

TABLE 1. GINSENOSIDE COMPONENTS OF THE KOREAN RED GINSENG.......................... 4

TABLE 2. COMPREHENSIVE EVALUATION OF BALANCE IMPAIRMENT......................... 1 2
PART 1
Korean red ginseng protects against gentamicin-induced balance dysfunction and hearing loss in rats through antiapoptotic functions of ginsenoside Rb1

I. INTRODUCTION

Aminoglycoside antibiotics were first introduced into clinical practice in the 1940s and have proved highly effective in the treatment of gram-negative infections such as tuberculosis (Warchol, 2010). Gentamicin (GM) is renowned for its effect against a wide variety of pathogens, including gram-negative organisms and methicillin-resistant staphylococci. Because of its specificity, availability, and low cost, GM is the aminoglycoside antibiotic used most commonly to treat gram-negative infections (Black et al., 2004). The ototoxic effects of aminoglycosides became evident shortly after their introduction into practice. The drug induced permanent hearing loss and/or vestibular deficits in numerous individuals. The reported incidence of hearing loss ranges from a few percent up to 33%. Vestibular toxicity occurs in about 15% of patients treated with aminoglycosides (Chen et al., 2007). Subsequent histological studies revealed that the administration of aminoglycosides can destroy sensory hair cells (Warchol, 2010). Aminoglycoside-induced hearing loss begins at high frequencies and is related to the progressive and irreversible damage of outer hair cells (OHCs) in the cochlea (Choung et al., 2009). A level of vestibulotoxicity that is sufficient to damage the vestibular sensory neuroepitheliums induces a whirling sensation and/or disequilibrium in affected patients (Rybak et al., 2006). This loss of vestibular hair cells represents a common cause of balance dysfunction (Staecker et al., 2007). Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), has been used in herbal medicines for 2000 years. Ginseng root is widely prescribed in Asia for the treatment of numerous ailments. Recent accumulating evidence has shown that treatment with ginseng saponins, which are active ingredients isolated from Panax ginseng, inhibit the increase of intracellular Ca$^{2+}$ (Rhim et al., 2002). The protective
effects of ginseng saponins against 3-nitropropionic acid (3-NP)-induced neurotoxicity in vivo manifests at the molecular level as the inhibition of 3-NP-induced increases of intracellular Ca\textsuperscript{2+}, which reduces the compound’s cytotoxicity in striatal neurons. This maintenance of homeostatic intracellular Ca\textsuperscript{2+} levels could be the basis for protection against excitatory amino acid- or neurotoxin-induced neuronal cell damage (Kim et al., 2005). Ginsenoside Rb1 (gRb1) deregulates Bcl-xL expression, which is known to suppress procaspase-9 activation by forming a complex with Apaf-1. This, in turn, prevents the release of cytochrome \(c\) from mitochondria, thereby maintaining cell viability and cell survival (Zhang et al., 2006). Post ischemic administration of gRb1 may suppress apoptotic cell death in spiral ganglion cells by activating the Bcl-xL signaling pathway, and minimize injury resulting from cochlear ischemic. Therefore, gRb1 may be effective for treating sensorineural hearing loss that eventually follows transient ischemia of the cochlea (Fujita et al., 2007). Choung et al. (2011) demonstrated that Korean red ginseng (KRG) was effective in protecting against GM-induced hearing loss in rats (Choung et al., 2011). The effect of KRG treatment on vestibular dysfunction was not investigated because the animal models with intraperitoneal injection of GM did not exhibit any signs of vestibular dysfunction. We therefore induced unilateral vestibular dysfunction with intratympanic injection (ITI) of GM and evaluated the protective effect of KRG in rats. The active component of KRG and its mechanism was identified through \textit{in vitro} experiments.
II. MATERIALS AND METHODS

The Ajou University School of Medicine – Institutional Animal Care and Use Committee approved the surgical procedures in accordance with the guidelines regarding the care and use of animals for experimental procedures (AUSM-IACUC). All efforts were made to minimize animal suffering as well as the number of animals. Twenty-two female Sprague-Dawley rats (8 weeks, 200–250 g) were housed in a temperature-controlled (23 ± 2 ℃) room with a 12 h light/dark cycle. The rats were provided with free access to standard laboratory food and tap water. They were allowed to acclimatize to their cages for at least 72 h after shipment.

A. Preparation of animal study groups

Twenty-two rats were divided into 2 groups, a GM only-treated group (GM) (12 rats) and a KRG plus GM-treated group (KRG + GM) (10 rats). Rats were intraperitoneally anesthetized with Zoletil 50 (Virbac Laboratoires, Carros, France) 0.1 cc/100 g and Rompun 2% (Bayer Korea, Ansan, Korea) 0.02 cc/100 g. KRG containing ginsenosides (Table 1) was provided from the Korea Ginseng Center (KGC®, Korea) and administrated orally (dissolved in tap water; 500 mg/kg; 1.5 ml/rat; once per day for 3 weeks including 1 week for pre-treatment) in the KRG + GM group. Both groups received ITI of GM in the left ear every other day for 2 weeks. The contralateral ears were used as controls. Under an operating microscope, GM (500 mg/ml) was slowly administered through the anterosuperior quadrant of the left tympanic membrane to fill the middle ear cavity (approximately 20 μl/rat). The rats were maintained in the same position with the left ear up for >30 min. Before, during, and after drug administration, the rats were carefully observed for any behavioral abnormalities. A complete battery of tests to assess hearing loss and vestibular toxicity was undertaken two days before and on days 1, 8, 15, 22, and 30 after drug administration. After day 30, all rats were sacrificed, and the cochlea, utricles, and saccules were harvested for morphological evaluation.
Table 1. Ginsenoside components of the Korean Red Ginseng

This 100% powderized ginsenosides comprise the roots (70%) and hair roots (30%) of ginseng at 6 years of plant age.

<table>
<thead>
<tr>
<th>Ginsenoside components</th>
<th>Rb1</th>
<th>Rg1</th>
<th>Rc</th>
<th>Re</th>
<th>Rb2</th>
<th>Rf</th>
<th>Rh1</th>
<th>Rg2(s)</th>
<th>Rg2(r)</th>
<th>Rd</th>
<th>Rg3(s)</th>
<th>Rg3(r)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content (mg/g)</td>
<td>5.16</td>
<td>2.89</td>
<td>2.22</td>
<td>2.16</td>
<td>1.82</td>
<td>0.93</td>
<td>0.13</td>
<td>0.37</td>
<td>0.21</td>
<td>0.47</td>
<td>0.14</td>
<td>0.08</td>
<td>16.58</td>
</tr>
<tr>
<td>Component ratio (%)</td>
<td>(31.1)</td>
<td>(17.4)</td>
<td>(13.4)</td>
<td>(13.0)</td>
<td>(11.0)</td>
<td>(5.6)</td>
<td>(0.8)</td>
<td>(2.2)</td>
<td>(1.3)</td>
<td>(2.8)</td>
<td>(0.8)</td>
<td>(0.5)</td>
<td>(100)</td>
</tr>
</tbody>
</table>
B. Evaluation of vestibular function

Head tilt test: Rats were placed in a black plastic channel with its roof open. Pictures were taken when the rat’s head and body were aligned. The tilt angle from the vertical plane to the sagittal plane of the head was quantified with a protractor. The head tilt was performed six times; one time before and five times after drug administration on days 1, 8, 15, 22, and 30. Tail hanging test: The rat was lifted by the tail and kept hanging at a height of 30 cm. The number of head rotations was counted at 10 s intervals. This test was performed just after the head tilt test. Swimming test: Each rat was tested just after the tail hanging test. A stainless steel pool (length, 28 cm; width, 45 cm; depth, 25 cm) was filled with body-temperature water at a depth of 19 cm. A rat was lifted by its tail and dropped into the center of the pool from a height of 20 cm. The time in seconds between the rat’s contact with the water and climbing up to the top of the platform was noted by an independent observer.

C. Evaluation of hearing function

The auditory brainstem response (ABR) was tested with the Biosig 32 system (Tucker-Davis Technologies, Gainesville, FL, USA) as previously described (Choung et al., 2011). The threshold was defined as the lowest intensity level at which a clear waveform was visible in the evoked trace and was determined by visual inspection of the responses.

D. Morphological evaluation

Rats were decapitated quickly after being anesthetized, and the temporal bones were dissected out. The perilymphatic space was perfused with 4% gluteraldehyde. Each specimen was then placed in the same gluteraldehyde solution overnight. After rinsing 3 times with phosphate-buffered saline (PBS), the specimen was perfused with 1% osmium tetroxide and placed on a tissue rotator for 15 min. The bony capsule of the cochlea was then carefully removed after rinsing with PBS; the lateral wall was cut away to reveal the organ of Corti. The otolith was carefully removed to reveal hair cells on the saccule/utricle macula. Samples were serially dehydrated in 50%, 70%, 90%, 95%, and 100% acetone. Each specimen was exposed to hexamethyldisilazane and allowed to air dry, then placed on a stub for gold-sputter coating. Photographs were taken with a JSM-5410 LV SEM camera (Jeol, Tokyo,
Japan). The number of hair cells in the organ of Corti was analyzed in 2 different representative areas of the middle and basal turns corresponding to 16 and 32 kHz, respectively (Choung et al., 2011).

**E. Cell culture and viability assays**

The ventral otocyst-epithelial clone 36 (VOT-E36) cell line represents epithelial progenitors with potential to differentiate into sensory and non-sensory epithelial cells and express a number of specific molecular markers for hair cells (Liu et al., 2006). The cells were maintained in Dulbecco’s Modified Eagle’s Medium with 10% fetal bovine serum at 33°C in a CO2 incubator. The cells (3000 cells/well in a 96-well plate) were incubated with 2 mM GM for 0, 24, 48, and 72 h. The timedependent effects were measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega, Madison, WI, USA). GRb1 (Ambo Institute, Seoul, Korea) was dissolved in methanol (Merck KGaA, Darmstadt, Germany) to yield a 50 mM stock solution. To test the effects of gRb1 on cell viability and GM-mediated cytotoxicity, cells were treated with different concentrations of gRb1 (2.5, 5.0, 10, 20 μM) for 48 h. The cells were pretreated with gRb1 for 2 h and exposed to GM (2 mM) for 48 h, respectively. A 20 μl aliquot of MTS solution was added to 100 μl of medium, after which the cells were incubated for 4 h at 33 °C in 10% CO2. The absorbance of formazan at 490 nm was measured directly from the 96-well assay plates using a microplate reader (Model 680, Bio-Rad, Tokyo, Japan). All experiments were repeated 3 times.

**F. Intracellular levels of reactive oxygen species (ROS)**

Intracellular ROS levels were measured using a Total ROS Detection kit (Enzo Life Sciences, Plymouth Meeting PA, USA). After cell culture and treatment with GM in the presence or absence of gRb1 in 12-well plates on cover slips, the cells were loaded with ROS Detection solution and incubated for 1 h. After washing with 1× wash buffer, the samples were immediately observed under a confocal microscope (LSM710, Carl Zeiss, Jena, Germany). The cells were cultured and treated with GM in the presence or absence of gRb1 in 6-well plates for the quantitative flow cytometry analysis. Media were removed, and the cells were
washed twice with 1× wash buffer. The cells were detached with trypsin and centrifuged for 5 min at 400 g at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 500 μl of ROS detection solution. The cells were stained for 30 min at 33 °C in the dark and analyzed by flow cytometry using a FACSARia™III device (BD Biosciences). The experiments were repeated 3 times.

G. Western blot assay

Western blots were performed to evaluate the involvement of the cell apoptotic pathway. The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): α-tubulin, poly-ADP-ribose polymerase (PARP), Bcl-xL, BAX, cytochrome c, caspase 3, and JNK. The treated cells were washed twice with PBS and then lysed in cold RIPA lysis buffer (Biosesang) by adding Xpert Protease Inhibitor Cocktail Solution (100) (GenDEPOT, Seoul, Korea). The reaction was allowed to proceed for 30 min on ice. The homogenates were centrifuged at 13,000 rpm for 30 min at 4 °C, and the supernatant fraction was collected for further analysis. Protein concentrations were measured using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Samples containing protein (45 μg, 80 μg for caspase 3) were immediately heated for 10 min at 100 °C, and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk in PBS containing 0.05% Tween (PBS-T) and subsequently incubated with primary polyclonal antibodies at a final dilution of 1:1000 at 4 °C overnight. After three washes in PBS-T, the membranes were incubated with peroxidase-conjugated secondary antibody (final dilution, 1:2000) in PBS-T at room temperature for 1 h. After washing the membranes, the protein bands were visualized using enhanced chemiluminescence on X-ray film according to the manufacturer’s instructions (West-Q Chemiluminescent Substrate kit, GenDEPOT). Band intensity was measured using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). The experiments were repeated 3 times for statistical analysis.
H. Statistical analysis

All the data were presented as mean ± standard deviation (SD). The statistical analysis was carried out using the SPSS 12.0 statistical software package for Windows (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed with Mann-Whitney test or Fisher’s Exact Test to determine the significance. A $p \leq 0.05$ was considered as statistically significant.
III. RESULTS

A. Evaluation of vestibular/balance function

In the head tilt test, the onset of head tilt (Fig. 1A) was observed around days 1 and 2 just after the seventh ITI of GM only in the GM group (7 of 12), not in the KRG + GM group. Four rats showed severe head tilt > 40°, and three rats showed tilts of <40°. The difference between groups was statistically significant ($p < 0.05$, Fig. 1C). The average degree of head tilt with time is shown in Fig. 1B. The differences between the groups were statistically significant ($p < 0.05$ at post-treatment days (P) 1, 8, 15, 22 and 30, respectively). In the tail hanging test, 4 of 12 rats in the GM group showed uncontrollable head turning, but no head turning was observed in the KRG + GM group. The difference between groups was not statistically significant ($p > 0.05$, Fig. 1F). The number of head-turns was significantly different at P8 and P15 ($p < 0.05$, Fig. 1E).

In the swimming test, the average swimming time for all 22 animals was $7.0 \pm 5.0$ s before drug administration. Rats with an average swimming time >12.0 s (mean + 1 SD) were considered as abnormal. Each rat, which could not get to the platform because of a severe balance deficit, was observed for 5 min. Swimming time was prolonged for 5 rats (41.7%) in the GM group but only one rat (10%) in the KRG + GM group (Fig. 1I). Among the five abnormal rats in the GM group, two exhibited swimming disabilities. The GM alone-treated animals had average swimming times of $61 \pm 113.4$ s, $57 \pm 113.6$ s, $57.5 \pm 113.7$ s, $32.2 \pm 81.0$ s, and $29.3 \pm 85.3$ s at post-treatment days 1, 8, 15, 22, and 30, respectively. In contrast, the KRG + GM group had times of $8.1 \pm 6.5$ s, $7.3 \pm 8.9$ s, $8.3 \pm 6.4$ s, $7.3 \pm 6.5$ s, and $6.1 \pm 3.5$ s (Fig. 1H). No significant difference in average swimming time was observed between the groups.

We performed a comprehensive evaluation of balance function by awarding one point each for failure on the head tilt test, tail hanging test and swimming test (Table 2). In the GM group, 5 rats earned zero points, 1 rat earned 1 point, 3 rats earned 2 points, and 3 rats earned 3 points. In the KRG + GM group, 9 rats earned 0 points and 1 rat earned 1 point. The
difference was statistically significant ($p < 0.01$). Since rat rated $\geq 1$ point indicate the occurrence of balance impairment, the incidence of GM-induced balance impairment in the GM group was 7/12, and 1/10 in the KRG + GM group. The difference was statistically significant ($p < 0.05$).
Fig. 1. Evaluation of vestibular function

The head tilt (A) was observed in the head tilt test. The differences of the average degrees of the head tilt with time between GM and KRG + GM groups were statistically significant (B), and the difference of the incidences between two groups was significant (C). Head turning was observed in the tail hanging test (D). The number of head-turns and incidence were shown in E and F, respectively. The abnormal swimming was detected in the swimming test (G). The time and incidence of the abnormal swimming were shown in H and I, respectively. *p < 0.05.
Table 2. Comprehensive evaluation of balance impairment

Each rat’s behavioral change in head tilt, tail hanging and swimming test was given 1 point. Scores show the total points of the individual rat.

<table>
<thead>
<tr>
<th>Vestibular Function Tests</th>
<th>GM</th>
<th>KRG + GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>R2</td>
<td>R3</td>
</tr>
<tr>
<td>Head Tilt</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tail Hanging</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Swimming</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Scores</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>


B. Otolith organ (saccule and utricle) morphology

The saccules and utricles in the GM group had an extremely reduced number of hair cells, which exhibited an uneven distribution and abnormal appearance (Fig. 2B and F) compared to those in the control ears (Fig. 2A and E). Hair cells in the KRG + GM group were well preserved in a high density of stereocilia with normal morphology (Fig. 2C and G). Five unit areas were chosen in a saccule macula and utricle macula of the control ear, GM treated ear, and KRG + GM treated ear, respectively. Hair cells were counted in each unit area. Quantitative analysis showed that the number of intact hair cells in the saccules was 34 ± 7 in the control group, 13 ± 3 in the GM group, and 30 ± 6 in the KRG + GM group (Fig. 2D). With respect to the utricles, the number of intact hair cells in the control group, GM group, and the KRG + GM group was 38 ± 5, 27 ± 5, and 35 ± 4, respectively (Fig. 2H). The hair cells in the saccule and utricle of the KRG + GM group were well preserved with their hair cell number significant more than that in the GM group ($p < 0.01$ and $p < 0.05$, respectively).
Fig. 2. Scanning electron micrographs of saccular maculae and utricular maculae.
The number of stereocilia in hair cells in the GM group (B and F) was extremely reduced in comparison to the numbers observed in normal controls (A and E). However, the KRG + GM groups (C and G) had dense, well-preserved stereocilia. The differences in hair cell number are depicted in panels D and H. *p < 0.05; **p < 0.01. Scale bars = 100 µm and 10 µm.
C. Evaluation of hearing function

There was no change in hearing thresholds in the control side (right) ears in both the GM and KRG + GM groups (data not shown). The average hearing threshold at 16 kHz/32 kHz in pre-treatment ears in the GM and the KRG + GM groups was 17.1 ± 4.5 dB/ 16.7 ± 4.9 dB and 15.5 ± 5.0 dB/16.0 ± 5.2 dB, respectively. On the first day after drug treatment, marked hearing loss was noted in GM-treated ears at 16 kHz and 32 kHz in the GM and KRG + GM treated ears (Fig. 3). The ABR threshold at 16 and 32 kHz was 58.3 ± 5.8 dB/58.3 ± 9.8 dB in the GM group and 50.5 ± 10.1 dB/ 50.0 ± 8.8 dB in the KRG + GM group (p < 0.05). As shown in Fig. 3, from days 1–30, the hearing thresholds at 16 kHz and 32 kHz recovered significantly in both groups. On day 30, the ABR threshold at 16 and 32 kHz was 43.3 ± 8.3 dB/47.9 ± 7.5 dB in the GM group and 35.5 ± 9.0 dB/39.0 ± 9.7 dB in the KRG + GM group. There were significant differences between the groups (p < 0.05).

D. Cochlear morphology

SEM was used to determine that all hair bundles in the middle turns (16 kHz) and basal turns (32 kHz) of the control ears remained structurally unaffected (Fig. 3B and G). Each hair bundle was comprised of well-integrated stereocilia. In the GM group (Fig. 3C and H), the number of hair cell bundles and stereocilia within a bundle was extremely reduced (particularly for OHCs in basal turns), but relatively intact IHCs were evident. In contrast, in the KRG + GM group (Fig. 3D and I), the outer and inner hair cells in middle turns had stereocilia that were nearly intact and of a density similar to that observed in normal cells. Only a few stereocilia were lost in OHCs of basal turns, and the hair bundles were well shaped. The difference between the GM and KRG + GM groups in the number of hair cells was statistically significant at both 16 kHz (p < 0.05) and at 32 kHz (p < 0.01). The hair cell damage in the basal turn was more severe than that observed in the middle turn.
Fig. 3. Functional and morphological manifestations of the induced hearing loss.

Hearing thresholds (dB) were measured at 16 kHz (A) and 32 kHz (F) before and after (P1, P8, P15, P22 and P30) drug administration. Scanning electron micrographs of the organ of Corti obtained at 16 kHz (B–D) and 32 kHz (G–I). The number of the outer hair cells hair bundles and stereocilia within a bundle was extremely reduced in the GM group (C and H) and almost intact in the KRG + GM group (D and I). A statistical difference in the number of intact outer hair cells (OHC) was observed upon comparison of the GM and KRG + GM groups (E and J). *\( p < 0.05 \); **\( p < 0.01 \). Scale bars = 10 µm.
**E. gRb1 exerts a protective effect in vitro**

Cell viability test: GM decreased cell viability in a time-dependent manner as shown in Fig. 4A. 2 mM GM administered for 48 h was chosen for the following studies. The cells were exposed to 2 mM GM in the absence or presence of gRb1 for 48 h. Viability increased slightly in gRb1 (10 µM)-treated cells (111.2%) (Fig. 4B) and decreased in the GM-treated cells (78.9%). However, when the cells were exposed to GM (2 mM) with gRb1 (10 µM) including gRb1 pretreatment for 2 h, cell viability was 94.3%, which was significantly different from the level observed in GM-treated cells ($p < 0.05$) (Fig. 4C). Cell viability was similar in control cells incubated in medium containing the gRb1 vehicle, 2 µM methanol (data not shown).
Fig. 4. Cell viability.
(A) GM-induced VOT-E36 cell toxicity. (B) Dose-dependent effect of gRb1. (C) Dose-dependent effect of gRb1 on GM-induced toxicity. *p < 0.05.
ROS generation: GM induced an increase in ROS production but not when cells were exposed to GM after pretreatment with gRb1 (Fig. 5A). Flow cytometry analysis was performed to quantify the level of intracellular ROS generation (Fig. 5B). The difference between the normal control and the GM only treated-group was significant ($p < 0.05$). However, pretreatment with gRb1 significantly attenuated GM-induced ROS production in gRb1 + GM treated cells than the GM-only treated cells ($p < 0.05$).
Fig. 5. Measurement of intracellular ROS production.

(A) A higher density of green fluorescence was observed in the GM group as compared to the gRb1-treated cells. (B) Flow cytometry revealed significant difference between the gRb1 + GM and gRb1 groups. *p < 0.05. Scale bars = 10 µm.
Western blot: Western blot analysis was performed to elucidate the mechanism underlying the protective effects of gRb1 in VOT-E36 cells. A representative western blot is shown in Fig. 6. GM-induced JNK activation was significantly suppressed in gRb1-treated cells ($p < 0.05$); gRb1 deregulated the expression of Bcl-xL and down-regulated BAX expression ($p < 0.05$). This, in turn, prevented the release of cytochrome $c$ from mitochondria and therefore reduced caspase 3 activation ($p < 0.05$). This chain of events maintained cell viability, as represented by reduced levels of cleaved PARP ($p < 0.05$).
Fig. 6. The effect of gRb1 on GM-induced apoptosis.
Representative western blots are shown on the right. Each ratio indicates the level of phosphor-protein (after normalization to a-tubulin levels) in comparison to the control. *$p < 0.05$. 
IV. DISCUSSION

Vestibular hair cell function is necessary to convert head acceleration into a neural discharge. The loss of these hair cells results in changes in the horizontal vestibulo-ocular reflex (VOR) response (Rauch, 2001). Central compensation after a unilateral fixed vestibular loss can result in the partial recovery of VOR gain. Bilateral vestibular loss results in permanent chronic balance dysfunction and oscillopsia. Fifteen percent of patients taking GM show vestibular toxicity (Chen et al., 2007). However, the rate of occurrence may be even higher due to spontaneous compensation. To date, there are no effective prosthetic devices such as hearing aids or cochlear implants to treat patients who have vestibular dysfunction (Wall et al., 2002). Therefore, precautions against vestibular hair cell loss are an important issue.

In a previous study, we found that KRG protects against GM-induced hearing loss and hair cell death (Choung et al., 2011). However, no significant difference in walking ability was observed in treated animals. The systemic administration of low-dose GM might not induce detectable vestibular function impairments. Alternately, rats with bilateral vestibular dysfunction may nonetheless be able to walk relatively well on the rotarod treadmill used for testing. To solve these problems, ITI (500 mg/ml) of GM was performed to induce unilateral vestibular dysfunction and to explore the protective effects of KRG. In the present study, symptoms of vestibular dysfunction including head tilt, head rotation, and swimming dysfunction were observed in the GM group. Head tilt is the most sensitive of the four tests for the evaluation of vestibular function, because it directly reflects the ocular compensation to vestibular dysfunction. Head tilt was the most sensitive sign with the highest incidence rate. Head tilts were observed 1–2 days after seventh GM dose. A tilt angle of < 20° can be recovered slightly or completely within 1 month, but only one in four severe tilts (> 40°) recovered by 5° within 1 month. Rats in the KRG + GM group showed normal performance in the vestibular functional tests with only one rats had prolonged swimming time in the swimming test. Consistently, these behavioral findings were supported by the morphological results. KRG may be a promising agent that has significant potential for protection against
GM-induced toxicity in vestibular structures, and to our knowledge, the current study is the first time to explore KRG’s effects on GM- induce vestibular dysfunction. Aminoglycosides enter into the OHCs through the mechanoelectrical transducer channels and form an aminoglycoside-iron complex that reacts with electron donors, such as arachidonic acid to form ROS such as superoxide, hydroxyl radical, and hydrogen peroxide. ROS then activate c-Jun-NH2-terminal kinase, which translocates to the nucleus to activate genes in the cell death pathway. These genes then translocate to the mitochondria, causing the release of cytochrome c, which triggers apoptosis via caspases. Cell death may also result from caspase-independent mechanisms (Rybak and Ramkumar, 2007). The demonstration that hair cell death occurs via an active, multistep cellular process raised the possibility of blocking the progression of this pathway, thus rescuing hair cells from ototoxic injury. Iron-chelators lead to a dramatic reduction in aminoglycoside ototoxicity without interfering with aminoglycoside therapeutic efficacy (Rauch, 2001). Chen et al. (2007) showed that aspirin attenuates GM ototoxicity in double-blind trials (Chen et al., 2007). Antioxidants and antioxidant gene therapy also protect against aminoglycoside ototoxicity in experimental animals (Kawamoto et al., 2004; Lesniak et al., 2005). Delivery of the math1 gene results in the generation of vestibular hair cells in vitro after aminoglycoside-mediated hair cell loss (Staecker et al., 2007). CEP-11004 prevented hair cells death by inhibiting the neomycin-induced phosphorylation of JNK through the direct inhibition of JNK signaling (Sugahara et al., 2006). In the current study, KRG protected against GM-induced hearing loss. Ginseng root has two major classes of ingredients: saponin and nonsaponin components. The saponins are classified into three major groups according to their chemical structures: protopanaxadiol, protopanaxatriol, and oleanolic acid. Ginsenoside Rb1, ginsenoside Rg1, and ginsenoside Ro are representative substances (Shibata et al., 1994). GRb1 has been reported to have numerous biological effects. In H2O2-exposed cardiomyocytes, pretreatment with gRb1 for 2 h and concurrent treatment with H2O2 for 2 h resulted in a dose-dependent reduction in cell death. This cardioprotective effect of gRb1 was associated with attenuated intracellular ROS generation and therefore the preservation of mitochondrial membrane potential. Furthermore, the increased p-JNK levels in H2O2-exposed cells were suppressed by pretreatment with gRb1 (Li et al., 2012). GRb1 and compound K significantly
inhibited JNK activation in a mouse model of 2,4,6-trinitrobenzene sulfuric acid (TNBS)-induced colitis (Joh et al., 2011). The neuroprotective effect of gRb1 on cerebral ischemia–reperfusion injury is related to the inhibition of neuronal apoptosis increased the expression of Bcl-2, and reduced the expression of Bax (Yang et al., 2008). In the previous study, Rb1 and gRb2 showed definite protective effects against GM-induced apoptosis, more prominent than gRg1 and gRe (Choung et al., 2011). Since gRb1 has been reported to have numerous biological effects, the current in vitro study sought to explore the roles of gRb1 in signaling pathways regulating GM-induced apoptosis in VOT-E36 cells. Consistent with these published data, gRb1 attenuated ROS production, reduced JNK activation, up-regulated the expression of Bcl-xL, and down-regulated Bax levels. This in turn resulted in reduced levels of cytochrome c, cleaved caspase 3, and cleaved PARP. gRb1 may be an active component that protects against GM-induced vestibular and hearing dysfunction via anti-apoptotic effects. However KRG contains various ginsenoside components as shown in table 1. Ginsenoside Rg3 is one of ginsenosides that are the well-known bioactive principles of Panax ginseng. It is reported that 20(S)-Rg3, as one of the major stereoisomeric form of ginsenoside Rg3, is capable of suppressing the nitric oxide (NO), reactive oxygen species (ROS) and prostaglandin E2 (PGE2) productions induced by lipopolysaccharide (LPS) in RAW264.7 macrophage cells in a concentration-dependent manner (Shin et al., 2013). Rd has a significant neuroprotective effect on rats insulted by Ab1–40 by anti-inflammation, anti-oxidation and anti-apoptotic (Liu et al., 2012). In the central nervous system, ginsenoside has been reported to be effective in decreasing ROS formation in cultured astrocytes (Lopez et al., 2007). Consistently, a study has demonstrated that ginsenoside Rd can protect PC12 cells from H2O2-induced oxidative stress (Ye et al., 2008). Other than apoptosis, a study about of gRb1 suggested that inhibition of autophagy could be responsible for neuroprotective effects in glutamate-induced injury (Chen et al., 2010). According to these reports, KRG’s preventive effects against GM-induced vestibular and hearing dysfunction in rats may involve in direct actions on other targets, such as inflammation or autophagy. The therapeutic effects are probably based on a multi-target mechanism and synergistic effects of different ginsenosides. More effective components of KRG and their mechanism should be further explored.
V. CONCLUSION

In conclusion, the present study indicates that KRG protects against vestibular dysfunction as well as hearing loss when ototoxicity is induced by unilateral ITI with GM in rats. Ginsenoside gRb1 may mediate this effect on cochleo-vestibular function by inhibiting apoptotic pathway.
Korean red ginseng ameliorates acute 3-nitropropionic acid-induced cochlear damage in mice

1. INTRODUCTION

Mitochondria play an important role in the life and death of cells. They are the intracellular organelles mainly responsible for cellular adenosine triphosphate (ATP) production by oxidative phosphorylation, and also have a series of specific functions, including apoptosis and oxidative stress control (Kokotas et al., 2007). Mitochondrial dysfunctions are critically involved in cellular processes underlying necrotic and apoptotic cell deaths, which are thought to have a major role in the pathogenesis of neurodegenerative diseases (Beal et al., 2000). Apoptosis and formation of reactive oxygen species (ROS) have been reported to be involved in pathophysiological mechanisms of cochlear damage by ischemia, ototoxins, and noise (Huang et al., 2000; Yamane et al., 1995). Mitochondrial dysfunction in the cochlea is thought to be an important cause of sensorineural hearing loss (SNHL). The frequency of mitochondrial hearing loss is unknown. It has been estimated that 1% of prelingual deafness cases result from constitutional mutations in mtDNA (Marazita et al., 1993). As it is for postlingual hearing loss, it has been estimated that 20% may be caused by mutations in the mitochondrial genome (Estivill et al., 1998). 3-Nitropropionic acid (3-NP) is an irreversible inhibitor of succinate dehydrogenase (Ludolph et al., 1991a) and complex II respiratory enzyme required for mitochondrial energy production (Nony et al., 1999). It is a compound found in crops contaminated with a naturally occurring neurotoxin produced by legumes of the genus Astragalus and Arthrium fungi (Ming, 1995) that causes neurotoxicity in animals and humans (Ludolph et al., 1991b), inducing ATP exhaustion by mitochondrial dysfunction (Pang and Geddes, 1997). Mitochondrial Ca\(^{2+}\) homeostasis is impaired along with these serial cascades and results in elevation of intracellular Ca\(^{2+}\) levels and impaired buffering capacity of intracellular Ca\(^{2+}\) in astrocytes and neurons (Calabresi et al., 2001; Nasr et al.,
An animal model of acute cochlear energy failure induced by administering 3-NP, the mitochondrial toxin, into the round window niche of the rat cochlea was established (Hoya et al., 2004). In one study using the permanent auditory threshold shift model induced by 3-NP, a remarkable degeneration was detected in type II fibrocytes in the spiral ligament and marginal/intermediate cells in the stria vascularis after 3-NP administration (Mizutari et al., 2008). These results indicate that a permanent auditory threshold shift caused by acute cochlear mitochondrial dysfunction is primarily mediated by cellular degeneration in the cochlear lateral wall and suggest that therapy for hearing loss due to acute energy failure may be achieved through protection or regeneration of the cochlear lateral wall. Ginseng refers to the root of some species of the genus Panax (C.A. Meyer Araliaceae). Among these species, Panax ginseng is mainly cultivated in Korea and China and is the most widely used ginseng. It has a cultural and medical history of more than 5000 years. The active ingredients in ginseng are called ginsenosides or ginseng saponins (Liu and Xiao, 1992; Back et al., 1996). According to their structural differences, they were classified into 3 major groups: the panaxadiol (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1), panaxatriol (Re, Rf, Rg1, Rg2, and Rh1), and oleanolic acid groups (Ro) (Wen et al., 1996; Tackikawa et al., 1999). To date, more than 40 ginsenosides, the major components of ginseng extract, have been isolated from several species of ginseng (Lee et al., 2008). Red ginseng refers to the type that is dried after being steamed, which may have healthful elements not found in fresh ginseng. Korean red ginseng (KRG) has various health-promoting effects on the human body, such as reducing the incidence of cancers, protective effects on gastric ulcer, anti-inflammatory and antioxidative effects, enhancing the mind, stimulating libido, and positive effects on patients with Alzheimer disease (Calabresi et al., 2001; Nasr et al., 2003; Yun et al., 2010; Oyagi et al., 2010; Hong and Lyu, 2011; Im et al., 2010a). In addition, KRG containing 42 natural minerals offers a wide variety of beneficial nutrients to the body. Accumulative studies showed that KRG had protective effects on hearing loss induced by cisplatin, gentamicin, and noise through the protection of outer hair cells; pretreatment with ginseng extract significantly attenuated the cisplatin-induced increase in ROS and also inhibited the
expression of caspase-3 and poly-adenosine diphosphate-ribose polymerase related to cisplatin-induced apoptosis in HEI-OC1 auditory cells (Lee et al., 2008; Choung et al., 2011a; Kang and Chung, 2010). Given the results of the previous studies, some forms of hearing loss may be prevented through preservation of sensory hair cells, spiral ganglion cells, and nonsensory cells like fibrocytes in the cochlear lateral wall. However, there is no current report on the protective role of red ginseng against 3-NP-induced cochlear damage. We speculated that KRG could protect from hearing loss due to 3-NP-induced acute cochlear damage. In the present study, the animal model of acute cochlear damage using intratympanic injection of 3-NP was established and the preventive effect of KRG against cochlear damage induced by 3-NP was investigated.
II. MATERIALS AND METHODS

A. Animals
Male BALB/c mice (6 weeks, 25–30g) involved in this study were housed in the temperature-controlled (23 ± 28 °C) room with 12-h light/dark cycles and provided with free access to standard laboratory food and tap water. Institutional Animal Care and Use Committee approved the surgical procedures in accordance with the guidelines regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals and their suffering.

B. Korean red ginseng
KRG was provided from Korea Ginsengs Center (KGC®, Korea). This 100% powderized ginsenosides comprise the roots (70%) and hair roots (30%) of ginseng at 6 years of plant age. The whole ginsenoside components of KRG was melt with tap water, and given to animals at a dose of 300 mg/kg for 7 days before and after 3-NP administration.

C. Study design
This study was performed in 2 phases as follows:

Phase I: investigation of 3-NP dose-dependent toxic effects on auditory function. The study was performed with 15 BALB/c mice, which were randomly assigned to 5 groups. For each animal, the intratympanic injection of 3-NP (7 ml, Sigma, St. Louis, MO, USA) in concentration of 300, 500, 800, 1000, and 5000 mM was performed on left ear and the right ear was used as control. Auditory brainstem response (ABR) test for the assessment of hearing loss was undertaken before and on post 1 day, 1 week, and 1 month after 300, 500, 800, 1000, and 5000 mM of 3-NP administration. Cochlear organs were harvested for morphological evaluation 1 month after 500, 800, and 1000 mM of 3-NP administration.

Phase II: determining the protective effect of KRG on 3-NP-induced hearing loss with animal model established in phase I. Twenty-three mice were classified into 3-NP group (n = 12) and KRG-treated 3-NP group (n = 11). For 7 days before 3-NP administration, mice in KRG-treated 3-NP group were pretreated with KRG (300 mg/kg; 0.8 ml/each animal;
once a day; per os). All mice in both groups were administrated with 3-NP (500 mM; 7 ml; intratympanic injection) and the contralateral ears remained as controls. After 3-NP administration, additional KRG treatment was performed for 7 days in the KRG-treated 3-NP group. Auditory function was evaluated 2 days before 3-NP treatment, then 1 day, 1 week, and 1 month after 3-NP treatment. Cochleae in each group were harvested 1 month 3-NP treatment for morphological evaluation.

D. Intratympanic administration of 3-NP
Animals were anesthetized by intraperitoneal injection of a mixture of xylazine 10 mg/kg (Rompun1, Bayer-Korea, Korea) and Zolazepam–Tiletamine 30 mg/kg (Zoletile1, Virvac, France). Under an operating microscope, 3-NP solution (7 ml/each animal) was given slowly through the anterosuperior quadrant of the left tympanic membrane with a 0.45-gauge dental needle to fill the middle ear cavity. The mouse was then kept in the same position with the left ear facing up for more than 30 min.

E. Auditory brainstem response (ABR) measurement
The animals were anaesthetized with above agents. Their body temperature was maintained with a warm blanket. All experiments were performed in an acoustically isolated booth. Subcutaneous platinum needle electrodes were placed on vertex (positive), ipsilateral (negative), and contralateral (ground) ear. The stimulus consisted of a 15-ms two tone-burst sounds, with a rise-fall time of 1 ms at frequencies of 16 and 32 kHz. Two tone-burst sounds (16 and 32 kHz) programmed by SigGen software were delivered to the left external ear of the subjects using BioSig amplifier and transducer (RP2.1 and PA5; Tucker–Davis Technologies, Alachua, FL, USA), as previously described (Kim et al., 2010). The cosine window was applied to all tone-burst stimuli. The number of iterations was fixed 1024 during all experiments. The gain of the biological amplifier was 100,000 and band-pass filter (from 300 Hz to 3 kHz) was applied to obtain ABR data. Before acquiring an EEG signal, electrodes were adjusted, if necessary, so that the impedance was less than 2 kOhm. Responses for 1024 sweeps were averaged at each intensity level and the stimuli typically progressed from 75 dB SPL to 10 dB SPL with 5 dB steps. Hearing threshold was defined as
the lowest stimulus intensity level that showed a reliable waveform in the ABR trace by visual inspection. The interpretation of ABR data was done by one audiology specialist. ABR threshold beyond the maximum recording limit was defined as 100 dB SPL.

F. Morphological evaluation

Phalloidin staining in the surface preparation: Rhodamine phalloidin staining was taken to detect hair cell changes in the organ of Corti. Dissected cochleae were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) and kept in the fixative overnight at 4 °C. For preparation of PBS used in this study, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ were dissolved in 800 ml distilled water and adjusted to pH 7.4, and then made to 1 l with additional distilled water. They were washed with PBS three times, and then decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA) for 7 days. The buffer for Calci-Clear Rapid was changed daily. Following decalcification, otic capsule was removed and followed by removal of the lateral wall, Reissner’s membrane, and tectorial membrane under a microscope. The remaining organ of Corti was stained with Texas Red1-X phalloidin (Invitrogen, Eugene, Oregon, USA) for an hour. Thereafter, the whole mount was rinsed in PBS and mounted on a glass slide. Hair cells in the organ of Corti were visualized under a confocal microscope (LSM710, Carl Zeiss, Jena, Germany). The number of hair cells in the organ of Corti was counted in two different representative areas of middle and basal turns corresponding to 16 and 32 kHz, respectively based on previous study (Viberg and Canlon, 2004). Hematoxylin and eosin staining: Cochleae were fixed with 4% paraformaldehyde in PBS. After immersion in the fixative overnight at 4 °C, the bones were rinsed in PBS containing 10% sucrose for 1 h. After decalcification with Calci-Clear Rapid (National diagnostics, Atlanta, GA) for 6 h at room temperature and then PBS washing, they were rinsed in PBS containing 10% sucrose, 20% sucrose, and 30% sucrose for 1 h at room temperature, in regular order. Then, the tissues were embedded with OCT compound and frozen blocks were cut into 10 mm thick sections in the horizontal plane parallel to the modiolus, following staining with hematoxylin and eosin for histopathology. Fibrocytes in lateral wall and spiral ganglion cells were examined under light microscopy.
G. Statistical analysis

All the data were presented as mean standard deviation (SD). The statistical analysis was performed using the SPSS 12.0 statistical software package for Windows (SPSS, Inc., Chicago, IL, USA). The comparison of the hearing thresholds between the 2 groups was analyzed by Mann–Whitney test. The hair cell damage was analyzed by counting outer hair cells. Quantitative analysis was performed by evaluating 30 outer hair cells in a microscope field as follows: 3 microscope fields were randomly selected for evaluating outer hair cell loss in middle and basal turns per sample, using 5 samples per group, and the mean cell counts were determined. The fibrocyte cell counts in the spiral ligament and the ganglion cells in the spiral ganglion area were determined as follows: the unit area was determined as 30,000 mm² in the spiral ligament area and 50,000 mm² in the spiral ganglion area. The unit area (type II area) in the spiral ligament was made below the midline of the spiral prominence, and 2 unit areas were made in the spiral ligament, using 5 samples per group. One unit area was made in the spiral ganglion, using 5 samples per group. The cell count was performed using the Olympus BX51 microscope and DP2-BSW2 program. Statistical analysis was performed with a Mann–Whitney test to determine the significance of the main and interactive effects. A $p \leq 0.05$ was considered as statistically significant.
III. RESULTS

A. 3-NP dose-dependent hearing threshold shift

The ABR thresholds for both frequencies (16 and 32 kHz) were elevated 1 day after administration of 300, 500, 800, 1000, and 5000 mM 3-NP, whereas a threshold shift was not detected in contralateral ears (Fig. 1). The ABR thresholds in the 800–5000 mM groups exceeded the maximum recording limit for 16 and 32 kHz in post 1 day. The decrease in auditory threshold was observed in various extents over time in the 300–800 mM groups, whereas 1000 and 5000 mM groups beyond maximum recording limit showed no auditory threshold shift 1 month after 3-NP administration.
Fig. 1. Auditory brainstem response (ABR)

The ABR thresholds at both frequencies were elevated 1 day after administration of 300, 500, 800, 1000, and 5000 mM 3-NP compared with those of the control animals. The ABR thresholds in the 800–5000 mM groups exceeded the maximum recording limit for 16 and 32 kHz in post 1 day. The decrease in auditory threshold was observed in various extents over time in the 300–800 mM groups, whereas the 1000 and 5000 mM groups beyond maximum recording limit showed no auditory threshold shift 1 month after 3-NP administration. The mean ± SD values are presented (n = 3 in each group).
B. Histological analysis after 3-NP administration

Histological comparisons for the organ of Corti, lateral wall, and spiral ganglion area between both groups are shown in Figs. 2–4, respectively. In surface preparation using phalloidin staining, hair cells in the organ of Corti of the control ears showed normal features; however, the number of missing outer hair cells increased as the concentration of 3-NP increased from 300 to 1000 mM (Fig. 2). Light microscopy revealed that the 3-NP administration induced the loss of fibrocytes in cochlear lateral walls (Fig. 3). In contrast to the normal finding from the lateral walls of the control group, the fibrocytes in the 300, 500, and 800 mM groups were reduced remarkably and most fibrocytes in the ears treated with 1000 mM 3-NP were degenerated with the shrinkage of the spiral ligament and atrophy of the stria vascularis. Spiral ganglion cells were degenerated dose dependently in the 3-NP-treated ears, similar to the findings in the lateral walls (Fig. 4).
Fig. 2. Confocal microscopic images

Confocal microscopic images of phalloidin-stained organ of Corti in middle and basal turns of cochleae in control and 3-NP groups (300, 500, 800, and 1000 mM 3-NP) 1 month after 3-NP administration. In surface preparation, hair cells in the organ of Corti in the control group showed normal features in both turns, whereas the number of missing outer hair cells increased as the 3-NP concentration increased from 300 to 1000 mM. Scale bar = 50 mm.
Fig. 3. Light microscopic images of lateral walls

Light microscopic images of hematoxylin–eosin-stained lateral walls in the middle and basal turns of cochleae 1 month after 3-NP administration. In contrast to the normal findings in the lateral walls of the control group, the fibrocyte counts in the 300, 500, and 800 mM 3-NP groups were reduced remarkably and most fibrocytes in the ears treated with 1000 mM 3-NP were degenerated with the shrinkage of spiral ligament and atrophy of stria vascularis.
Fig. 4. Light microscopic images of spiral ganglion cells
Light microscopic images of hematoxylin–eosin-stained spiral ganglion cells in the middle turns of cochleae 1 month after 3-NP administration. The spiral ganglion cells were degenerated dose dependently in the 3-NP-treated ears, similar to the findings in the lateral walls.
C. Protective effects of KRG on 3-NP-induced hearing threshold shift

Based on the above-mentioned results, 500 mM 3-NP was selected in the study for the evaluation of the protective effect of KRG on a 3-NP-induced cochlear damage model. The ABR thresholds of the 3-NP and KRG-treated 3-NP groups are shown in Fig. 5. One day after 3-NP administration, 3-NP and KRG-treated 3-NP groups demonstrated a remarkable auditory threshold shift at 16 (45 and 40 dB, respectively) and 32 kHz (45 and 39 dB, respectively); however, thresholds at 16 and 32 kHz of the KRG treated 3-NP group were significantly lower than those of the 3-NP group post 1 week and post 1 month (p < 0.001, Mann–Whitney test). The threshold shifts for hearing recovery from 1 day after 3-NP administration in 3-NP group were 2.9 dB (1 week) and 5.8 dB (1 month) at 16 kHz, and 6.2 dB (1 week) and 7.5 dB (1 month) at 32 kHz. For the KRG-treated 3-NP group, threshold shifts of 8.2 dB (1 week) and 15.5 dB (1 month) at 16 kHz, and 10.4 dB (1 week) and 14 dB (1 month) at 32 kHz were observed. No change in threshold shift was seen in the contralateral ears of either group.
Fig. 5. Changes in mean auditory brainstem response (ABR)

Changes in mean auditory brainstem response (ABR) thresholds in the 3-NP and KRG-treated 3-NP groups before (Pre) and after drug administration (P1D, P1W, and P1M) at 16 kHz (A) and 32 kHz (B). Marked auditory threshold shifts at 16 and 32 kHz in both groups were noted 1 day after 3-NP administration; however, the auditory thresholds at both frequencies in the KRG-treated 3-NP group were significantly lower than those in the 3-NP group post 1 week and post 1 month (*p < 0.001, Mann–Whitney test). No change in threshold shift was seen in the contralateral ears of either group.
D. Histological analysis after KRG treatment

The hair cells in organ of Corti were observed in basal and middle turns of cochleae in the control, 3-NP, and KRG-treated 3-NP groups (Fig. 6). The loss of some outer hair cells was observed in the 3-NP group (Fig. 6B and E), whereas those in the control group showed normal appearance (Fig. 6A and D) and KRG-treated 3-NP group demonstrated minimal loss (Fig. 6C and F). The mean outer hair cell counts in organ of Corti at middle and basal turns were 30 ± 0.0 at both turns in the control group, 28.8 ± 0.8 and 28.6 ± 1.5 in the 3-NP group, 29.6 ± 0.5 and 29.4 ± 0.9 in the KRG-treated 3-NP group. The difference of mean counts of outer hair cells among the 3 groups and between basal and middle turns was not statistically significant.
Fig. 6. Confocal microscopic images of the organ of Corti

Confocal microscopic images of the phalloidin-stained organ of Corti in the middle and basal turns in the control, 3-NP, and KRG-treated 3-NP groups. The loss of some outer hair cells was observed in the 3-NP group (B, E), whereas those in the control group showed normal features (A, D) and those in the KRG-treated 3-NP group demonstrated a minimal loss (C, F). The difference of mean counts of outer hair cells among the 3 groups and between basal and middle turns was not statistically significant. Scale bar = 50 mm.
The cellular change of the cochlear lateral wall was observed under light microscopy (Fig. 7). Compared with the findings from the control group that showed normal morphological features of the type II fibrocytes (Fig. 7A and E), diffuse degeneration of fibrocytes and atrophy of stria vascularis were seen in the 3-NP group (Fig. 7B and F) and similar findings with that of the control group were obtained from the KRG-treated 3-NP group (Fig. 7C and G). Comparison of the mean cell counts in the type II area of the cochlear lateral wall revealed a statistically significant difference between the control and 3-NP groups (p < 0.001 at basal and middle turns, 15.6 ± 2.8 cells/30,000 mm², 17.2 ± 2.4 cells/30,000 mm², respectively in control group, 6.8 ± 1.5 cells/30,000 mm², 6.4 ± 1.1 cells/30,000 mm², respectively in 3-NP group, Mann–Whitney test) and between the 3-NP and KRG-treated 3-NP groups (p = 0.002 at basal turn and p = 0.027 at middle turn, 13.2 ± 2.8 cells/30,000 mm², 12.8 ± 4.3 cells/30,000 mm², respectively in KRG-treated 3-NP group, Mann–Whitney test), however not between the control and KRG-treated 3-NP groups (Fig. 7D and H).
Fig. 7. Cellular changes in the cochlear lateral walls

Cellular changes in the cochlear lateral walls under light microscopy in the control, 3-NP, and KRG-treated 3-NP groups. The control group showed normal morphological features of type II fibrocytes (A, E), and the KRG-treated 3-NP group displayed similar findings with that of the control group (C, G). In contrast, the diffuse degeneration of fibrocytes and atrophy of stria vascularis were seen in the 3-NP group (B, F). The comparison of the mean cell counts in the type II area of the cochlear lateral wall demonstrates the statistically significant difference between the 3-NP and KRG-treated 3-NP groups (*$p < 0.027$ at middle turn, †$p < 0.002$ at basal turn, Mann–Whitney test) and between the control and 3-NP groups ($^{‡}p < 0.001$, at both basal and middle turns, Mann–Whitney test), however not between the control and KRG-treated 3-NP groups (D, H).
The cellular change in the spiral ganglion was observed under light microscopy (Fig. 8). Compared with the findings from the control group, which shows normal spiral ganglion cell population (Fig. 8A), severe spiral ganglion cell degeneration was seen in the 3-NP group (Fig. 8B) and mild degeneration was observed in the KRG-treated 3-NP group (Fig. 8C). The mean cell counts in the spiral ganglion area in the control, 3-NP, and KRG-treated 3-NP groups were 37 ± 6.1 cells/50,000 mm², 15.2 ± 3.4 cells/50,000 mm², and 32.8 ± 2.8 cells/50,000 mm², respectively. The mean cell counts in the spiral ganglion area did not show a statistically significant difference between the control and KRG-treated 3-NP groups; however, there were statistically significant differences between the control and 3-NP groups and between the 3-NP and KRG-treated 3-NP groups (Fig. 8D; $p < 0.001$, Mann–Whitney test).
Fig. 8. Cellular changes in the spiral ganglion

Cellular changes in the spiral ganglion under light microscopy in the control, 3-NP, and KRG-treated 3-NP groups. The control and KRG-treated 3-NP groups showed normal spiral ganglion cell population (A) and mild degeneration (C), respectively, whereas the 3-NP group showed severe spiral ganglion cell degeneration (B). The mean cell counts in the spiral ganglion area do not show a statistically significant difference between the control and KRG-treated 3-NP groups; however, there are statistical significant differences between the control and 3-NP groups and between the 3-NP and KRG-treated 3-NP groups (D; *$p < 0.001$, Mann–Whitney test).
IV. DISCUSSION

Given that it was reported that 3-NP, an irreversible mitochondrial complex II inhibitor, could induce the selective striatal pathology similar to that seen in Huntington disease, 3-NP has been widely used as an agent to establish a good model recapitulating Huntington disease phenotypes in rodents (Bossi et al., 1993). Moreover, various protective agents against the 3-NP brain toxicity have been introduced to date (Wu et al., 2009; Kumar et al., 2009, 2010; Al Mutairy et al., 2010; Sandhir et al., 2010; Napolitano et al., 2011; Rosenstock et al., 2011); that is, studies investigated the neuroprotective effects of these agents against 3-NP brain toxicity using neurotrophic factors such as brain-derived neurotrophic factors (Wu et al., 2009); antioxidants such as sesamol, trolox, and lycopene (Kumar et al., 2009; Al Mutairy et al., 2010; Sandhir et al., 2010); cyclosporine A, a nitric oxide modulator (Kumar et al., 2010); peroxisome proliferator-activated receptor γ activator such as pioglitazone (Napolitano et al., 2011); and calcineurin inhibitor such as FK506 (Rosenstock et al., 2011). Taken together, the results of the previous studies suggest that such agents might at least partly protect cells or neurons from 3-NP-induced mitochondrial dysfunction. Among these studies, some reported that systemic administration of ginseng saponin, a major component of ginseng extract, produced significant protective effects against systemic 3-NP- and intrastriatal malonate-induced lesions in rat striata in a dose-dependent manner (Kim et al., 2005; Lian et al., 2005). Some authors reported that ginseng saponin significantly improved 3-NP-induced behavioral impairment and extended survival and that these effects might be achieved via in vitro inhibition of 3-NP-induced elevation of intracellular Ca\(^{2+}\) levels and cytotoxicity of striatal neurons (Kim et al., 2005).

Recently, 3-NP has been used as an effective pharmacological agent for an animal model of hearing loss by acute cochlear energy failure. It is known to induce a severe degeneration of type II fibrocytes in the spiral ligament and marginal/intermediate cells in the stria vascularis by mitochondrial dysfunction through mitochondrial ATP depletion and cochlear energy failure, leading to temporary or permanent auditory threshold shift. In 2004, the first study on cochlear damage by 3-NP was conducted and histological changes in the cochlea by 3-NP
were first described (Hoya et al., 2004). Whereas ototoxic agents like gentamicin and cisplatin mainly target hair cells in the organ of Corti, saccule, and utricle, the degeneration of inner hair cells and that of spiral ganglion cells are the secondary events after the loss of outer hair cells as retrograde damage (Rybak et al., 2006). On the contrary, in an early-stage 3-NP-induced hearing loss animal model, the degeneration of fibrocytes in the spiral ligament and spiral ganglion cells happened earlier than that of the hair cells in the organ of Corti (Hoya et al., 2004). It has been reported that fibrocytes in the cochlear lateral wall and sensory hair cells or spiral ganglion cells have important roles in the maintenance of hearing (Fujinami et al., 2010). Loss of fibrocytes in the spiral ligament was identified as the first morphological sign of cochlear degeneration in a mouse model of aging (Hequembourg and Liberman, 2001). Moreover, the extreme vulnerability of fibrocytes to noise insult was also observed in the mouse spiral ligament and spiral limbus (Wang et al., 2002). These close relationships between fibrocytes and hearing function seem to be highly related with the role of fibrocytes. It was reported that fibrocytes in the spiral ligament were critical for maintaining the ion concentration of the endolymph in the cochlea by K⁺ recycling from the perilymph in the cochlea (Spicer and Schulte, 1998), and the disturbance of the endolymphatic ion concentration leads to immediate hearing loss. The endolymph with a high K⁺ level and positive potential of approximately +80 mV, known as the endocochlear potential, is essential for a normal auditory function. Furthermore, it is maintained by K⁺ circulation from the perilymph to the endolymph through the cochlear lateral wall. That is, fibrocytes in the spiral ligament supply K⁺ to the stria vascularis via gap junctions by recirculation mechanism of ions and stria vascularis maintains the homeostasis of K⁺ gradient between the endolymph and perilymph. In particular, marginal cells within the stria vascularis maintain the ionic balance in the endolymph and the endocochlear potential via ion channels using Na–K-ATPase. In addition, types II and IV fibrocytes contain numerous mitochondria, and an extensive endoplasmic reticulum that play significant roles in the maintenance of the endolymph, expresses various types of ion transporters, channels, and pumps (Fujinami et al., 2010). Therefore, lethal damage of fibrocytes in the spiral ligament may induce the interruption of ion changes and disturbance of the endocochlear potential, leading to disorder of the cochlear auditory function and permanent hearing loss in a short
time. In contrast, as seen in Fig. 2, missing outer hair cell’s number increases slightly as 3-NP concentration increases, however its degree seems to be less severe compared to results of the spiral ligament and spiral ganglion as demonstrated in Figs. 3 and 4. Mitochondrial ATP depletion in the cochlea induced by 3-NP and the resultant cochlear energy failure may be a mechanism of various SNHL. This type of hearing loss may explain a portion of the pathogenesis of idiopathic sudden SNHL, age-related SNHL, or progressive SNHL.

The KRG used in the present study was a kind of biological drug made from ginseng provided from a Korean company developing ginseng products and is known to be nontoxic. It comprised the roots (70%) and hair roots (30%) of ginseng at 6 years of plant age. And then, the plant parts were powderized after steaming for 3 h and artificial and sun drying. Its main components were Rb1 (31.1%), Rg1 (17.4%), Re (13.4%), Rg2 (11.0%), Rb2 (11.0%), and Rf (5.6%). As summarized in Table 1, this KRG contains more than 50% of the panaxadiol group (i.e., Rb1, Rb2, and Re). The previous 2 studies suggested that panaxadiol saponins might enhance superoxide dismutase activity, reduce the levels of free radicals and lipid peroxides, and stimulate proliferation of human bone marrowhemopoietic progenitor cells (Wang et al., 1995; Niu et al., 2004). When considering the results of the present study, panaxadiol saponins, the main components of KRG, may have roles as antioxidants against free radicals responsible for the mitochondrial dysfunction caused by 3-NP. Moreover, ginsenosides, including Rg1, Rh1, and Rb1, are known to have steroid-like activities and to be abundant in KRG (Yue et al., 2007). They may bind with the intracellular steroid hormone receptors, triggering cellular responses. However, because ginseng has various ingredients and multi-effects to the human body, the protective effects and action mechanisms of KRG remain to be fully elucidated. The protective effect of ginseng against the ototoxicity has been reported in some studies (Im et al., 2010b; Hong and Lyu, 2011; Hong et al., 2011; Choung et al., 2011b). The previous studies investigated the protective effect of ginseng against cisplatin-, noise-, and gentamicin-induced ototoxicity, respectively, and their results supported the present findings that KRG had ameliorating effects on 3-NP-induced cochlear damage. In addition, some authors reported that ginsenoside Rb1 prevented hearing loss from damage to spiral ganglion cells by cochlear ischemic injury (Fujita et al., 2007). One of
limitations in this study may be the fact that KRG was given prior to 3-NP administration. This is hardly a useful protocol when considering unexpected exposure to stimulants such as sudden intense noise. Sometimes, in some experiments evaluating drugs’ efficacy, the pretreatment protocol may be necessary to enhance or maintain the effective concentration of the drug. In aspects of the clinical application, this kind of pretreatment protocol is not appropriate to expect its effect on the treatment of sudden hearing loss. However, the pretreatment on noise-induced hearing loss, drug-induced hearing loss, and age-related hearing loss can be considered from the experience. For example, this KRG can be started before exposure in cases of use of ototoxic drugs or working in the intense noise environment.

Some studies on ginseng root toxicity were performed in rats and dogs (Popov and Goldwag, 1973; Hess et al., 1983). They found no toxic effects after ingestion of ginseng extract at daily doses in rats and at various concentrations in dogs. When considering the body weights of the animals used in this study, the total content of KRG administered to the animals may be 7.5–9 mg and the content of Rb1 and Rg1 may be approximately 4 mg, half of the total KRG content because this KRG contains approximately 48.5% of Rb1 and Rg1. The amount of Rb1 and Rg1 delivered to the mice is very similar to the recommended daily allowance for humans (Rb1 + Rg1 4.0 mg per day). Other studies with human subjects showed that the most common adverse effects with inappropriate use of ginseng extract were reported to be hypertension (probably by a steroid-like action mechanism via the adrenal cortex or pituitary gland), diarrhea, sleeplessness, mastalgia, eruptions, and vaginal bleeding (Bahrke and Morgan, 1994; Coon and Ernst, 2002). Although some adverse effects may not be serious to humans, the clinical application of ginseng for protection of hearing remains to be evaluated more. Drug interactions with P. ginseng are expected with warfarin, phenylzine, and alcohol (Coon and Ernst, 2002). The method of 3-NP administration used in the present study was quite different from that used by Hoya et al. (2004), who were the first to report on 3-NP-induced cochlear damage. They used an intracochlear microinjection through a round window membrane in rats, whereas we used a slow diffusion method through the round window membrane using intratympanic injection of 3-NP in the mice. Although the former
can have an advantage such as administration of accurate dose in the method, the slow diffusion method used in the present study also showed the model appropriate to perform the interventional animal study. The intratympanic injection method used in this study has some advantages such as its easy approach, avoidance of unnecessary cochlear damage, and avoidance of surgery. With the same 3-NP concentration (500 mM), a permanent auditory threshold shift and severe histological changes were induced in the previous study, whereas a temporary threshold shift was induced in the present study, indicating that intratympanic injection of 3-NP is an appropriate method for the evaluation of the protective effectiveness of some agents. The present study revealed stria vascularis atrophy, hair cell loss in the organ of Corti, and spiral ganglion cell and fibrocytes losses, whereas Hoya et al. (2004) found fibrocyte loss in the spiral ligament, prominent swelling of the supralimbal epithelium, and preservation of hair cells, supporting cells, and nerve fibers in the organ of Corti. One of the reasons for these differences may be the time of histological investigation. Histological observation in the present study was performed 1 month after administration of 3-NP; however, their findings might appear less severe because the observation was made 14 days after treatment, although a more direct and invasive injection technique of 3-NP was used. In these 2 studies, the loss of fibrocytes in the spiral ligament was observed in the early period after 3-NP administration and hair cell loss in the organ of Corti and spiral ganglion cell degeneration may be followed in the late period. However, this speculation needs to be verified in further studies. Our previous study has shown that acute cochlear damage by 3-NP was ameliorated by geranylgeranylacetone, a heat shock protein inducer (Kim et al., 2010). Because 3-NP may induce the damage at the cochlear cellular level in the acute stage, interventional therapeutic studies using various agents may be useful. Similar to the previous studies (Hoya et al., 2004; Mizutari et al., 2008; Kim et al., 2010), the present study showed fibrocyte degeneration in the spiral ligament, especially in the type II and IV areas, which might be the primary cause of 3-NP-induced hearing loss. Moreover, the significant hearing recovery in the KRG-treated 3-NP group might have resulted from the functional restoration of the fibrocytes by the administration of KRG. Therefore, fibrocytes in the spiral ligament may have a substantially important role in the maintenance of the cochlear function as reported in hair cells in the organ of Corti and spiral ganglion cells. In addition, the
protection and regeneration of fibrocytes in the spiral ligament might be an important rescue method against hearing loss by mitochondrial dysfunction.
V. CONCLUSION

In conclusion, the present animal model of acute cochlear dysfunction induced by intratympanic administration of 3-NP demonstrated that the hearing impairment was dose dependent and that functional changes were well matched with the histological changes. KRG may ameliorate the cochlear damage induced by 3-NP in the acute stage through the suppressing degeneration of fibrocytes in lateral walls and spiral ganglion cells.
PART 3

Korean red ginseng delays age-related hearing loss and vestibular dysfunction in C57BL/6 mouse

I. INTRODUCTION

Age-related hearing loss (AHL) or presbycusis affects more than 40% of people older than 65 years of age in the United States (US) and is projected to afflict more than 28 million Americans by 2030 (Yamasoba, et al., 2007). AHL is the most common sensory deficit in the elderly and has become a severe social and health problem (Yamasoba, et al., 2007). Therefore, AHL presents as an urgent research and public health concern. Patients and animals with AHL typically show degeneration and death of multiple cell types (Kidd Iii and Bao, 2012). As a result of impaired function of the cochlea, loss of auditory neurons such as spiral ganglion neurons (SGNs) is a major cause of AHL (Schuknecht and Gacek, 1993). Recent studies suggest the involvement of the reactive oxygen species (ROS) pathway in the development of AHL. Both hair cells and SGNs are protected against ROS by an interacting network of enzyme systems and antioxidants (Bared, et al., 2010). Current view holds that most of cell death during aging occurs via apoptosis, whether in the brain (Mattson, 2007) or the cochlea (Nevado, et al., 2006). Consistent with these points, it is reported that oral supplementation of antioxidants could significantly delay AHL in rodents (Someya, et al., 2009). Caloric restriction suppresses apoptotic cell death in the mouse cochlea and prevents late onset of AHL (Someya, et al., 2007).

From 2001 through 2004, 35.4% of US adults aged 40 years and older (69 million Americans) had vestibular dysfunction, which significantly increases the likelihood of falls, and are in the most morbid and costly health conditions affecting older individuals. These data suggest the importance of diagnosing, treating, and potentially screening for vestibular deficits to reduce the burden of fall-related injuries and deaths (Agrawal, et al., 2009). However, to our
knowledge, there are very rare studies about age-associated vestibular dysfunction. Therefore, age-related vestibular dysfunction presents as an urgent research request.

The C57BL/6 strain of inbred mice has been suggested as a useful animal model of AHL, seen in human populations (Li and Borg, 1991). This strain begins to lose hearing by 6 months of age and progresses to complete hearing loss by the time the animal is 1 year-old (Staecker, et al., 2001). The AHL in C57BL/6 mice begins at the highest test frequencies spreads to low frequencies. Progressive loss of hair cells, degeneration of afferent neurons, the stria vascularis, and the spiral ligament have all been observed in the C57BL/6 mouse strain, which overlaps with each of the major types of AHL (Schuknecht and Gacek, 1993). However, there is only one study about age-related vestibular dysfunction in C57BL/6 mouse (Shiga, et al., 2005), and no study about its protection. Therefore, little is known about the mechanisms involved in age-related changes in vestibular function.

Ginseng refers to the root of some species of the genus Panax (C.A. Meyer Araliaceae). Among these species, Panax ginseng is mainly cultivated in Korea and China and is the most widely used ginseng. It has a cultural and medical history of more than 5000 years (Tian, et al., 2013). Many recent studies reported that KRG with its ginsenosides has neuronal beneficial effects and anti-ageing effects (Liao, et al., 2002). Ginsenoside may mediate beneficial effects on memory loss in aged C57BL/6J mice via increasing the activities of antioxidant enzymes such as total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px), decreasing the level of lipid peroxidation such as thiobarbituric acid reactive substances (TBARS) and protein carbonyl to block oxidative pathways, and preventing mitochondrial decay and the lipofuscin accumulation (Zhao, et al., 2011). These results raise the possibility of the preventive effect of ginseng against hearing loss induced by ototoxic chemicals, noise, and ageing. Choung et al. reported that KRG administration prevents gentamicin-induced hearing loss and vestibular dysfunction via inhibiting mitochondrial apoptotic pathway (Tian, et al., 2013). Ginsenoside Rb1 protects against damage to the spiral ganglion cells after cochlear ischemia in Mongolian gerbils by activating the Bcl-xL signaling pathway (Fujita, et al., 2007). These findings suggest that long-term therapy of
KRG may retard the onset of AHL. However, until now there are no studies about it. The purpose of the present study was to explore the protective effects of KRG on age-related hearing loss and balance disturbance in C57BL/6 mice.
II. MATERIALS AND METHODS

The Ajou University School of Medicine Institutional Animal Care and Use Committee approved the surgical procedures in accordance with the guidelines regarding the care and use of animals for experimental procedures. All efforts were made to minimize the number of animals used and their suffering. C57BL/6 mice (male, 5 weeks, 18-22 g) were purchased from Orient Bio Inc. (Seoul, Korea). KRG was provided by Korea Ginsengs Center (KGC®, Korea). This 100% powderized ginsenosides comprise the roots (70%) and hair roots (30%) of ginseng at 6 years of plant age. The whole ginsenoside components of KRG are summarized in Table 1.

A. Study design
Mice were allowed 1 week to adapt to their environment before being used for experiments. A total 22 mice were randomly distributed into control group (n = 8), KRG (150 mg/kg, n = 7), and KRG (500mg/kg, n = 7) group. Mice aged 3 months were chronically treated with KRG in their drinking water until 12 months (every Monday to Friday). Water containing KRG was freshly prepared every day, and water left in the bottle was measured to determine amounts consumed (to ensure that a little bitter taste KRG water have no influence on experimental mice’s water intake). All mice performed the whole battery of tests for assessment of hearing and balance function including auditory brainstem response (ABR) test, tail hanging test, and swimming test.

B. Auditory and vestibular function assessment.
The ABR was tested with the Biosig 32 system (Tucker-Davis Technologies, Gainesville, FL, USA) as described previously (Choung, et al., 2011). The tail hanging test was used to evaluate vestibular function; the mouse was lifted by the tail and kept hanging at a height of 30 cm, the response was carefully observed and rated (Al Deeb, et al., 2000): 0 = straight body posture with extension of forelimbs towards the earth, 1 = slightly bending the body ventrally, 2 = persistently bending the body, sometimes crawling up towards its tail, and 3 = bending the body with head rotation. For the swimming test, a stainless steel pool (length, 40
cm; width, 30 cm; depth, 10 cm) was filled with body-temperature water. Mouse was lifted by tail and dropped into the center of the pool. The time in seconds between contact with the water and separation from the water to the platform was noted by an independent observer.

C. Morphological evaluation of hair cells.
Scanning Electrical Microscope was used for the hair cell morphological evaluation in the cochlea and sacculles /utricles as described previously (Choung, et al., 2011). Hematoxylin and eosin (HE) staining was performed. The tissues were embedded with optimal cutting temperature compound (USA, CA, Sakura Finetek USA, Inc.) and frozen blocks were cut into 10 μm thick sections in the horizontal plane parallel to the modiolus, following staining with hematoxylin and eosin for histopathology. Fibrocytes in lateral wall and spiral ganglion cells were examined under light microscopy.

D. Immunohistochemistry
Frozen sections were dried at 37 °C for 1 hour. After permeabilized with 0.2% TritonX-100, blocked with 1% BSA (Bovine serum albumin) for 30 min, and washed with 0.5% BSA in PBS, the sections were subsequently incubated with anti-Bcl-xL antibody (1:100 dilution; CST, MA, USA) and anti-cytochrome c antibody (1:100 dilution; CST, MA, USA) respectively at 4°C for overnight. Following PBS washing, the sections were flooded with FITC-conjugated secondary antibodies (1:400 dilution; Jackson immunoresearch lab, PA, USA) for 1 h. After washing with 0.5% BSA in PBS, the sections were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and then visualized with confocal microscope (LSM710, Carl Zeiss, Jena, Germany).

E. Statistical analysis
All the data were presented as mean ± standard deviation (SD). The statistical analysis was performed using the SPSS 12.0 statistical software package for Windows (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed with Mann-Whitney test and t-tests to determine the significance. A p ≤ 0.05 was considered as statistically significant.
III. RESULTS

A. Mortality
No mice died at the age of 12 months both in the control and 150 mg/kg KRG groups (Fig. 1), however, in the 500 mg/kg KRG group, after 3 months high dose KRG intake, aggressive behavior was frequently observed among mice from the age of 6 month. Most mice hurt from fighting with their back skin bite off (Fig. 1 black arrow), resulted in 4 of 7 mice died at the age of 12 months, significantly higher than that in the control group ($p < 0.05$).
Fig. 1. Mortality of 12 months old mice.

No mice died at the age of 12 months both in the control and 150 mg/kg KRG groups, however, the mice feed with 500 mg/kg KRG had 4 in 7 died, significantly higher than that in the control group (* $p < 0.05$). The living mice in the 500 mg/kg KRG group were injured (black arrow) from aggressive fighting behavior.
B. KRG’s effect on age related hearing loss in C57BL/6J mouse

Mice at the age of 3 month had well hearing thresholds both at 16 kHz (28.5 ± 6.9 dB) and 32 kHz (29.7 ± 3.9 dB). As time progressed, in the control group, the AHL began at 32 kHz (38.8 ± 5.6 dB) at the age of 6 month, and increased to 45.0 ± 6.6 dB at 9 month and then 55.6 ± 8.3 dB at 12 month (Fig. 2B). The AHL gradually spread to 16 kHz (41.9 ± 6.0 dB) at the age of 9 month, and increased to 52.2 ± 8.6 dB at 12 month (Fig. 2A). Compared to the control group, the delayed hearing thresholds shift was detected in the 150 mg/kg KRG treated mice. At 32 kHz, the average thresholds were 32.1 ± 5.7 dB at 6 month ($p < 0.05$), 37.5 ± 7.8 dB at 9 month ($p < 0.05$) and 50.0 ± 16.6 dB at 12 month ($p < 0.05$). At 16 kHz, the average thresholds were 29.6 ± 5.0 dB at 6 month ($P > 0.05$), 34.6 ± 8.2 dB at 9 month ($p < 0.05$) and 47.9 ± 18.1 dB at 12 month (Fig. 2, $p < 0.05$). However, the 500 mg/kg KRG treated mice showed elevated hearing thresholds progressed with age, even higher than that in the control group at 12 month old with average thresholds 61.7 ± 20.7 dB at 16 kHz and 64.2 ± 16.9 dB at 32 kHz (Fig. 2, $p > 0.05$). The histopathological changes in the cochlea were studied in the 12 month old mice (Fig. 3). The hair cells loss was found in the basal (for 32 kHz) and middle (for 16 kHz) turns in all three groups (Fig. 3A). In the control group, the typical age-related stereocilia loss in the remained outer hair cells was observed. However, the stereocilia in the 150 mg/kg treated mice were well shaped, and the number of the remained outer hair cells was significant more than that in the control group in the middle turn ($p < 0.05$) and basal turn ($p < 0.05$). The hair cells in the 500 mg/kg treated mice were extremely degenerated. Cellular changes in the spiral ganglion region were evaluated under light microscopy (Fig. 3B). Compared to the degeneration of the spiral ganglion neurons in the control group, the prevention by the 150 mg/kg KRG treatment was observed, and the SGC density (cells/10.000 μm$^2$) was markedly higher than that in the control group ($p < 0.05$), however, the 500 mg/kg KRG treated mice showed no significant difference from the control group. The diagram of the lateral wall shows the different types of fibrocyte. The age-related degeneration of type II fibrocytes was detected in three groups (Fig. 3C). The 150 mg/kg KRG treated mice displayed significantly more type II fibrocytes than that in the control group ($p < 0.05$). However, the 500 mg/kg KRG treated mice showed no prevention of type II fibrocytes loss.
Fig. 2. Assessment of age-related auditory dysfunction.
Hearing thresholds elevated from the age of 6 month to 12 months both at 16 kHz (A) and 32 kHz (B) in the control group, however, delay of hearing threshold shift was detected in the 150 mg/kg KRG group. The hearing thresholds increased in the 500 mg/kg KRG group. * $p < 0.05$. 

* Indicates significant difference compared to the control group.
Fig. 3. Morphological changes in cochlear.

(A), The typical stereocilia loss were found in the control group, however, the well shaped stereocilia were observed in the KRG (150 mg/mg) fed mice, the number of which was significantly different from that in the control group both at 16 kHz and 32 kHz. Scale bar = 10 μm. (B), The SGCs loss was found in the control group, which was prevented in the KRG (150 mg/kg) group significantly. Scale bar = 50 μm. (C). The diffuse loss of type II fibrocytes was found in the control group, which was prevented significantly in the KRG (150 mg/kg) group both in the middle and basal turns. * p < 0.05, Scale bar = 50 μm.
C. KRG’s effect on age related balance dysfunction

As mice aged from 3 month to 12 month, there were balance behavioral changes detected by tail hanging and swimming tests. The severity scores in the tail hanging test were gradually increased in three groups (Fig. 4), however, the severity score in the 150 mg/kg treated mice was markedly lower ($p < 0.05$) than that in the control group at the age of 12 month (Fig. 4A), and 500 mg/kg KRG treated mice showed no different compared to the control group (table 2). In the swimming test (Fig. 4B), swimming time was counted, prolonged swimming time was found at the age of 12 months, however, the averaged swimming time was shorter in the KRG (150mg/kg) group than that in the other two groups ($p < 0.05$) (table 2). The utricle/saccule was evaluated with SEM (Fig. 5). The uneven distribution and abnormal appearance (black arrow) of stereocilia was observed at the age of 12 months old mice in all three groups. The HCs with normal stereocilia (white arrow) were counted. The utricle and saccule in the KRG (150 mg/kg) group have more HCs with well shaped stereocilia ($p < 0.05$) than the other two groups. Age-related loss of vestibular sensory hair cells in the crista ampullaris was detected in three groups (Fig. 6); however, relative preserved hair cells were found in 150 mg/kg KRG fed mice.
Fig. 4. Balance function evaluation.

(A). In the tail hanging test, the balance responses were scored, the severity scores were increased in three groups at the age of 12 months, however, the 150 mg/kg KRG fed mice were lower than that in other two groups. (B). swimming time duration in the swimming test were counted, prolonged swimming time was found at the age of 12 months, however, the averaged swimming time was shorter in the KRG (150 mg/kg) group than that in the other groups. (* $p < 0.05$).
Fig. 5. Evaluation for HCs in utricle and saccule.
The uneven distribution and abnormal appearance (black arrow) of stereocilia were observed at the age of 12 months old mice in all three groups. The HCs with normal stereocilia (white arrow) were counted. The utricle and saccule in the KRG (150 mg/kg) group have more HCs with well shaped stereocilia. (* $p < 0.05$). Scale bar = 10 μm.
Fig. 6. Age-related hair cells loss in the crista ampullaris
Age-related loss of vestibular sensory hair cells in the crista ampullaris was detected in three groups, however, relative preserved hair cells were found in 150 mg/kg KRG fed mice. Scale bar = 10 µm.
D. Detection of Bcl-xL and cytochrome c expression

To determine whether KRG’s protection against age-related inner ear dysfunction was through mitochondrial apoptotic pathway, the cochlear tissues were examined by immunohistochemistry using antibody specific to Bcl-xL and cytochrome c (Fig. 7). The Bcl-xL immunopositive (arrow) cells of spiral ganglion (Fig. 7A) and fibrocytes in the lateral wall (Fig. 7B) were detected in the control group; however, strongly stained cells were present in the cochleae from 150 mg/kg treated mice, from which the number of the immunopositive cells were significantly more than that in the control group both in the spiral ganglion region \( p < 0.05 \) and type II fibrocytes in lateral wall \( (p < 0.05) \). Correspondingly, the low expressed Bcl-xL in the control group presented strongly cytochrome c expressed cells throughout the spiral ganglion region and lateral wall; however, weak cytochrome c stained cells were detected in the KRG treated mice both in the spiral ganglion region (Fig. 7C) and lateral wall (Fig. 7D), which was consistent with the high expressed Bcl-xL, interestingly, the relatively higher expressed Bcl-xL in the type I fibrocytes region of lateral wall of the KRG treated group showed very weak cytochrome c expression (Fig. 7D arrow).
Fig. 7. Expression of Bcl-xL and cytochrome c

Expression of Bcl-xL (green) and cytochrome c (green) in the 12 months old mice inner ears, the nuclear dye DAPI (blue). Up-regulated Bcl-xL expression was observed in 150 mg/kg KRG treated group both in the spiral ganglion neurons (A) and lateral walls (C), * \( p < 0.05 \). Correspondingly, low Bcl-xL expression in the control group showed relatively higher expression of cytochrome c in spiral ganglion cells (B) and lateral (D) than that in the KRG (150 mg/kg) group. Scale bar = 50 \( \mu \text{m} \).
IV. DISCUSSION

AHL is thought to be the result of ageing, oxidative damage, mitochondrial impairment, and environmental factors (Kokotas, et al., 2007). Experimental evidence suggests that mitochondrial dysfunction associated with reactive oxygen species (ROS) plays a central role in the aging process of cochlear cells (Someya and Prolla, 2010). Chronic generation of ROS in the regions of the organ of Corti: stria vascularis, spiral ligament and spiral ganglion cells is a common mechanism of different insulting factors in ageing, and this ROS over load lead to apoptotic cell death (Mukherjea, et al., 2011). AHL in C57BL/6J mice is mediated by mitochondrial apoptotic pathway (Someya, et al., 2009). It is proposed that treatment with antioxidants or dietary restriction can attenuate age-related hearing loss. It is proved that, in response to dietary restriction, SIRT3 activates IDH2, thereby increasing NADPH levels in mitochondria, This in turn leads to an increased ratio of GSH:GSSG and decreased levels of ROS, thereby resulting in protection from oxidative stress and prevention of AHL (Someya, et al., 2010). It is reported that a combination antioxidant therapy prevents AHL in C57BL/6J (Heman-Ackah, et al., 2010). In the present study, ABR test data showed that, across the ages of 3, 6, 9, and 12 months of age, there was a linear increase in hearing threshold for both 16 and 32 kHz. Consistently, the related histopathological analysis depicts an evident loss of outer hair cells, spiral ganglion neurons, and type II fibrocytes of the lateral wall. Treatment with KRG (150 mg/kg) significantly retarded hearing threshold shift and protected cells in the organ of Corti, spiral ganglion, and lateral wall. In our previously published data, we demonstrated that Ginsenoside Rb1 is one of the effective compound protected auditory cell lines (HEI-OC1 cell and VOT-E36 cell) against gentamicin-induced cytotoxicity through mitochondria apoptotic pathway (Tian et al., 2013). It is reported that Ginsenoside Rb1 protected GM-treated VOT-E36 cell by attenuating ROS production, suppressing JNK activation, up-regulating Bcl-xL and down-regulating Bax, followed decreased cytochrome c, caspase 3 and cleaved poly (ADP-ribose) polymerase. Since the insulting factors such as gentamicin, ageing, and noise share a general apoptotic mechanism (Mukherjea, et al., 2011), we speculated that KRG prevented AHL via mitochondrial apoptotic pathway. Agreed with this speculation, in the present study, the significantly increased Bcl-xL and decreased
cytochrome c indicated that KRG prevented AHL via mitochondrial apoptotic pathway. In the present study, vestibular dysfunction was detected in the tail hanging and swimming tests at the age of 12 months, and hair cells loss in the vestibular epithelia was observed. Such a delay is consistent with the findings of Atsushi Shiga et al. They reported that age-related changes in the vestibular function of C57BL/6 mice followed a different time course when compared the changes in auditory function, which is identical to previous findings in a longitudinal study in humans (Enrietto, et al., 1999). To our knowledge, this is the first study about the exploration of the protection against age-related vestibular disorder. Animals treated with KRG (150 mg/kg) revealed markedly better vestibular function than that in control group at the age of 12 months, which is supported by hair cells morphological assessment in saccule/utricle.

KRG powder contained all kinds of active ginsenocides was used in dose of 150 mg/kg and 500 mg/kg in the present study. Since different doses showed totally opposite effects, dose and duration of treatment are very important issues for the expected outcome. For human, the recommended daily dose is 0.5 to 2 g per day of dried root or 100 to 300 mg per day of a standardized extract containing 1.5 to 7% ginsenosides (Mahady, et al., 2000), and followed by a rest period of two weeks. Results from numerous clinical trials have reported very few side effects and demonstrate that KRG is safe for human use when administered at the recommended dosage. Extensive testing of KRG in five different animal models (mice, rats, cats, guinea pigs, beagle dogs) using conventional toxicologic methods failed to show any acute or chronic toxicity (Mahady, et al., 1999). Since previous studies demonstrated that 7-day pretreatment of KRG in dose of 500 mg/kg had significant effects to prevent gentamicin-induced hearing loss in rats (Choung, et al., 2011). In the present study 500 mg/kg was given to reveal its effects on AHL. However, the irritability was observed after 3-month treatment. Most back skin was bitten off due to aggressive behavior, and resulted in high mortality, which was consistent with the reports about high dose long-term treatment KRG related adverse reactions, which were associated with ingestion of excessive doses of KRG (Mahady, et al., 2000) such as hypertension, nervousness, irritability, and so on, collectively called “Ginseng Abuse Syndrome (GAS)” (Siegel, 1979). Since ginseng is one of the most popular herbs in the world, the treatment in terms of dose and course is such an important issue.
Together with our previous studies, we find that short-term high dose KRG treatment may be effective to acute insulting factors such as gentamicin or noise induced hearing loss (Choung, et al., 2011), long-term low dose KRG with a rest period should be recommended for chronic insulting factors such as age associated inner ear dysfunction, and long-term high-dose use of KRG product without of a break should be avoided. The mechanism for the KRG-induced aggressive behavior is unclear; however, aggression and competitive behaviors are facilitated directly by raised testosterone levels (Archer, 2006). Interestingly, it is reported that use of Panax Ginseng extract showed an increase in plasma total and free testosterone in patients (Salvati, et al., 1996), and adult male rats were treated with 5% Panax Ginseng in their diet for 60 days experienced a significant increase in blood testosterone level (Fahim, et al., 1982). So the relation between KRG and testosterone level should be further studied to reveal the mechanism of the long-term high dose KRG treatment induced irritability.
V. CONCLUSION

In the present study, C57BL/6J mouse showed early onset of hearing and vestibular dysfunction with ageing, which were delayed by KRG in dose of 150 mg/kg by inhibiting mitochondrial apoptotic pathway; however, long-term treatment of 500 mg/kg KRG may induce aggressive behavior and aggravate age-related hearing and balance dysfunction. KRG treatment may provide a promising method for preventing AHL in the growing elderly population.
REFERENCES


20. Fujinami Y, Mutai H, Kamiya K, Mizutari K, Fujii M, Matsunaga T: Enhanced expression of C/EBP homologous protein (CHOP) precedes degeneration of fibrocytes in the lateral wall after acute cochlear mitochondrial dysfunction induced by 3-


31. Kang WS, Chung JW: Ingestion of Korean red ginseng after noise exposure can


42. Li J, Shao ZH, Xie JT, Wang CZ, Ramachandran S, Yin JJ, Aung H, Li CQ, Qin G,


75. Siegel RK: Ginseng abuse syndrome. Problems with the panacea. *JAMA: the journal*


85. Tackikawa E, Kudo K, Harada K, Kashimoto T, Miyate M, Kakizaki A: Effects of ginseng saponins on responses induced by various receptor stimuli. *Eur J Pharmacol*


102. Zhao HF, Li Q, Li Y: Long-term ginsenoside administration prevents memory loss in aged female C57BL/6J mice by modulating the redox status and up-regulating the plasticity-related proteins in hippocampus. *Neuroscience* 183: 189-202, 2011.
이독성 및 노화 관련 청각소실 및 전정기능 이상에 관한 고려홍삼의 효과

이재혁

아주대학교 대학원 의학교육과

Chunjie Tian

(지도교수: 정연훈)

게타마이신은 가장 널리 사용되는 아미노글리코시드계 항생제이다. 그러나 이는 내이 청세포에 손상을 일으킬 수 있고 청력소실과 전정기능 장애를 유발할 수 있다. 미토콘드리아 독소인 3-Nitropropionic acid (3-NP)는 급성 와우 손상을 일으킨다고 보고되고 있다. 노인들에게 가장 흔한 감각 소실 중 하나인 노화성 내이질환을 위해 개발된 치료 방법은 없다. 진세노이드를 가진 고려홍삼은 신경세포에 유익한 효과와 항노화 작용을 가진다. 본 연구에서 저자는 (1) 겐타마이신에 의한 한쪽 평형장애 및 난청에 대한 한국홍삼의 보호 작용, (2) 3-NP을 이용한 이독성 동물 모델인 BALB/c 마우스에서 한국홍삼의 보호 작용, (3) C57BL/6 마우스에서 노화성 난청 및 평형장애에 대한 고려홍삼의 효과를 알아보고자 하였다.

첫 실험에서, Sprague-Dawley 쥐는 겐타마이신 처리군 (12 마리)과 홍삼섭취 및 겐타마이신 처리군 (10 마리)으로 나뉘어진다. 평정기능 평가를 위한 head tilt, tail hanging test, swimming test와 청력 평가를 위한 청성뇌간반응검사 (auditory brainstem response test, ABR)를 시행하였다. 와우와 구형낭/난형낭은 SEM (scanning electron microscope)을 위해 적출되었다. VOT-E36 (Ventral otocyst-epithelial clone 36) 세포는 Ginsenoside Rb1의 보호기전을 연구하기 위해 사용되었다. 두번째 실험에서, 3-NP의 복용량에 따른 독성효과는 3-NP의 적정 독성 농도 측정을 위해 조사하였고, 그 다음 23 마리의 마우스는 3-NP 처리군 (12 마리)와 홍삼섭취 및 3-NP 처리군 (11 마리)으로 홍삼의 보호작용 관찰을 위해 나뉘어졌다. ABR와 와우의 형태학적 관찰을 약물 주입 전후에 시행하였다. 세번째 실험에서는, 22 마리의 마우스가 무작위로 대조군 (8 마리), 홍삼섭취군 1 (150 mg/kg, 7 마리), 홍삼섭취군 2 (500 mg/kg, 7 마리)로 나누었다. 청력 및 전정기능 검사, 관련된 형태학적 관찰이 노화과정 중에 시행되었다.

첫 실험에서, 전정기능검사는 head tilt, tail hanging, and swimming test를 이용해서 0 (정상)점에서 3 (가장 나쁨)점까지 범위에서 점수체로 평가되었다.
겐타마이신 처리군 (0 점 – 5 마리, 1 점 – 1 마리, 2 점 – 3 마리, 그리고 3 점 – 3 마리)은 홍삼섭취 및 겐타마이신 처리군 (0 점 – 9 마리 및 1 점 – 1 마리) 보다 통계적으로 더 심한 전정기능장애를 보였다 (p < 0.01). 청력 역치는 홍삼섭취 및 겐타마이신 처리군에서 겐타마이신 처리군보다 더 좋은 결과를 보였다 (p < 0.05). SEM 에서 청신경 세포의 손상을 양적으로 분석하였을 때 청력 기능과 전정기능 검사 결과와 밀접하게 연관되었다. In vitro 연구는 Ginsenoside Rb1 이 반응 산소중 생성을 감소, JNK 활성을 억제, Bcl-xL 을 활전시키고 Bax, cytochrome c, caspase 3 을 억제시키며 GM-treated VOT-E36 세포에서 poly (ADP-ribose) polymerase 를 붕괴시킨다고 보여주고 있다. 두번째 실험에서는 ABR 역치가 3-NP 처리 후 1 일째 16 과 32 kHz 에서 최대 측정한계를 넘었다. 500 mM 3-NP 처리 및 홍삼섭취군에서 ABR 역치는 500 mM 3-NP 처리군보다 향상 1 주에서 1 달까지 유의하게 낮았다. 평균 type II fibrocyte 숫자는 대조군과 3-NP 처리군 사이에, 그리고 3-NP 처리군과 3-NP 처리 및 홍삼섭취군사이에 유의한 차이를 보였다. 세번째 실험에서는 대조군과 홍삼섭취군 (150 mg/kg) 에서 모두 12 개월의 연령에 죽은 마우스는 없었지만, 홍삼섭취군 (500 mg/kg) 에서는 7 마리중 4 마리가 죽었다. ABR 측정에서는 6 개월령에 32 kHz (38.8 ± 5.6 dB) 에서 고주파 청력감소를 보였고, 9 개월령에서는 16 kHz (41.9 ± 6.0dB) 에서 고주파와 청력감소를 보였으며, 대조군에서 12 개월령에서 16 kHz 에서 52.2 ± 8.6 dB 까지 역치가 증가했고, 32 kHz 에서 55.6 ± 8.3 dB 까지 역치가 증가했다. 그러나, 역치 변화는 홍삼섭취군 (150 mg/kg) 에서 유의하게 증가되었다 (p < 0.05) (32 kHz 에서 6 개월령 (32.1 ± 5.7 dB) 에서 12 개월령 (50.0 ± 16.6 dB) 까지, 16 kHz 에서 9 개월령 (34.6 ± 8.2 dB) 에서 12 개월령 (47.9 ± 18.1 dB) 까지). 청력 역치는 500 mg/kg 를 선택한 마우스에서 12 개월에 16 kHz 에서 61.7 ± 20.7 dB 까지, 32 kHz 에서 64.2 ± 16.9 dB 까지 증가하였다. 노화성 전정장애에는 tail hanging 과 swimming test 에서 관찰되었고, tail hanging test 에서 심각성의 정도 및 swimming test 에서 수영시간은 12 개월령에, 홍삼섭취군 (150 mg/kg) 과 대조군 간에 유의한 차이를 보였다 (P < 0.05). 조직학적 관찰은 청력 및 전정기능 결과를 뒷받침해 주고 있다. Bcl-xL immunopositive SGCs 와 type II fibrocytes 의 숫자는 대조군보다 홍삼섭취군 (150 mg/kg) 에서 현격하게 많았다 (P < 0.05).

이러한 결과는 쥐에서 한쪽 고막내 겐타마이신 주입으로 청각세포를 주 타겟으로 하는 이독성을 유발했을 때, gRb1 을 포함하는 홍삼섭취군이 전정장애 및 난청에 대한 보호작용을 한다는 것을 보여주고 있다. 3-NP 처리로 유도된 난청의 마우스 모델은 외부 외측벽과 청신경절 손상을 주로 보이는 조직학적 변화와 함께 복용량에 따른 난청을 보여준다. 홍삼섭취군은 3-NP 로 인한 청력감소를 개선시켰다. C57BL/6J 마우스는 노화작용과 함께 이른 나이에 난청 및 평형장애를 보여주었고, 이는 미토콘드리아의 세포사멸과정을 억제함으로써
150 mg/kg 용량의 홍삼섭취군에서 지연되었다. 그러나 장기간의 500 mg/kg의 홍삼투여는 부작용을 유발해서 노화성 난청 및 평형장애를 오히려 악화시켰다.

결과적으로 고려홍삼추출물은 다양한 원인의 난청을 효과적으로 예방하는 치료제로 사용될 수 있을 것 같다.