Medical Science Research Institute and Department of Otorhinolaryngology - Head and Neck Surgery[1], Dongguk University Ilsan Hospital, Gyeonggi; Graduate School of Oriental Medicine[2], Dongguk University, Seoul; and Department of Oriental Opthalmotolaryngology & Dermatology[3], Dongguk University Ilsan Hospital, Gyeonggi, Korea

Ethanol extract of *Piper longum* L. attenuates gentamicin-induced hair cell loss in neonatal cochlea cultures

Xiao Fei Du[1], Jae-Jun Song[1], Seungug Hong[2,3], Jihye Kim[2]

Received September 5, 2011, accepted October 7, 2011

Dr. Jae-Jun Song, Department of Otorhinolaryngology - Head and Neck Surgery, Dongguk University Ilsan Hospital, 814 Siksa-dong, Goyang, Gyeonggi, 410-773, Korea.
jjsong23@gmail.com

Pharmazie 67: 559–563 (2012) *doi: 10.1691/ph.2012.1681*

Piper longum L. (PL), also as known as long pepper, a well-known spice and traditional medicine in Asia and Pacific islands, has been reported to exhibit wide spectrum activity including antioxidant activity. However, little information is available on its protective effect on gentamicin (GM) induced ototoxicity which is commonly regarded as being mediated by reactive oxygen species and reactive nitrogen species. This study was undertaken to investigate the protective effect of PL ethanol extract on gentamicin-induced hair cell loss in neonatal cochlea cultures. Cochlea cultures from postnatal day 2–3 mice were used for analysis of the protective effects of PL against gentamicin-induced hair cell loss by phalloidin staining. *E. coli* cultures were used to determine whether PL interferes with the antibiotic activity of GM. Nitric oxide (NO)-scavenging activity of PL was also measured *in vitro*. GM induced significant dose-dependent hair cell loss in cochlea cultures. However, without interfering with the antibiotic activity of GM, PL showed a significant and concentration-dependent protective effect against GM-induced hair cell loss, and hair cells retained their stereocilia well. In addition, PL expressed direct scavenging activity toward NO radical liberated within solution of sodium nitroprusside. These findings demonstrate the protective effect of PL on GM-induced hair cell loss in neonatal cochlea cultures, and suggest that it might be of therapeutic benefit for treatment of GM-induced ototoxicity.

1. Introduction

Gentamicin (GM) is a widely used aminoglycoside antibiotic. However the clinical usefulness of aminoglycosides is limited by their ototoxicity and nephrotoxicity (Forge and Schacht 2000). GM can induce hearing loss due to destruction of inner ear hair cells (Theopold 1977). Hair cells are auditory receptor cells that convert sound signals into electrical impulses (Hudspeth 1989). Many studies have reported that GM-induced ototoxicity is commonly regarded as being mediated by reactive oxygen species (ROS) and reactive nitrogen species (Heinrich et al. 2008; Lopez-Gonzalez et al. 1998; Wu et al. 2002). Overproduction of ROS triggers the signaling pathways of cellular apoptosis, resulting in inner ear damage. In addition, GM also enhances formation of nitric oxide (NO) in the inner ear through regulation of inducible NO synthase (iNOS) expression and later forms destructive peroxynitrite with ROS (Takumida and Anniko 2001). Several agents that scavenge ROS or block their formation (McFadden et al. 2003; Sha and Schacht 2000; Wang et al. 2003a) or reduce apoptosis *via* inhibition of various stages of the cell death pathway have been proposed for protection of the inner ear (Bodmer et al. 2002; Wang et al. 2003b; Ylikoski et al. 2002). *Piper longum* L. (PL), also as known as long pepper, is used as a spice by people all over the world. For centuries now, people in the Mediterranean region, India, Africa and Indonesia have been using this spice, not just because of its taste, but also for the various benefits as a traditional medicine particularly in Indian medicine and Chinese traditional medicine (Shoba et al. 1998). The benefits of PL fruits have been advocated in treatment of diseases and ailments such as gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic intestinal pain, and certain forms of arthritis (Choi and Hwang 2003; Ghoshal et al. 1996; Mata et al. 2004). Other beneficial effects of PL include analgesic and diuretic effects, relaxation of muscle tension, and alleviation of anxiety (Das et al. 1996; Vedhanayaki et al. 2003). The chemical constituents present in the ethanol extract include piperine, pipalartene, piperlongumine, volatile oils, starch, resins, gum, fatty oils and inorganic matter (Jalapure 2003). Previous studies have shown that the ethanol extract of PL showed significant protection against oxidative stress by virtue of its antioxidant activity (Thomas et al. 2009; Wakade et al. 2008). Hence, PL shows promise in amelioration of GM-induced ototoxicity. To our knowledge, a protective effect of PL against GM-induced hair cell loss has never been reported. Therefore, we investigated its preventive effect and possible mechanism in GM-treated neonatal cochlea cultures.

2. Investigations and results

2.1. GM induces hair cell damage

Organotypic cultures of cochlea isolated from postnatal mice were incubated in the medium with addition of 0.1–1 mM GM

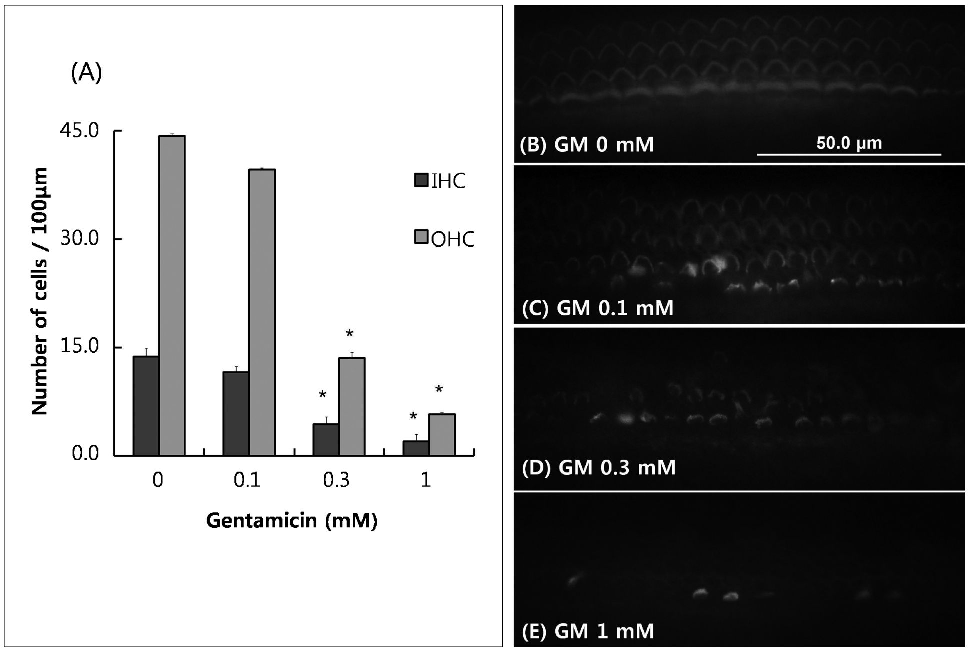

Fig. 1: Dose dependent cytotoxicity of GM on hair cells of postnatal mice cochlear cultures. Cochleae were cultured for 24 h in various concentrations of GM. Hair cells labeled with Alexa Fluor 350 phalloidin were counted under a fluorescence microscope. (A) Mean number (± SE) of IHCs, OHCs and total hair cells per 100 µm length of the basal and middle turn of the cochlea ($^{*}p < 0.05$ as compared with the untreated group). Representative photographs of each group are demonstrated as follows: (B) control explant without GM; (C) explants exposed to 0.1 mM GM; (D) explants exposed to 0.3 mM GM; (E) explants exposed to 1 mM GM. GM: gentamicin

for 24 h. In the control group, three rows of outer hair cells and one row were preserved (Fig. 1B). In the GM-treated group, viable hair cells showed a decrease in number (Fig. 1A), and they also lost normal contour and alignment (Fig. 1C∼E). The average hair cell count showed a significant decline in the middle and basal turns. Because significant hair cell loss of the mouse cochlea occurred in the 0.3 mM GM group, this concentration was used to evaluate the effect of PL on GM-induced ototoxicity.

2.2. *Ethanol extract of PL protects against GM-induced hair cell loss*

Cochlear cultures of postnatal mice were incubated in the medium containing ethanol extract of PL at concentrations of 1.25, 5 and 10 mg/ml with 0.3 mM GM. Viable phalloidin-labeled hair cells were counted after 24 h. Compared with untreated controls (Fig. 2B), GM induced severe distortion of the anatomy of the organ of Corti in the GM-only group (Fig. 2C). Ethanol extract of PL was not toxic to the cochlea (Fig. 3) and

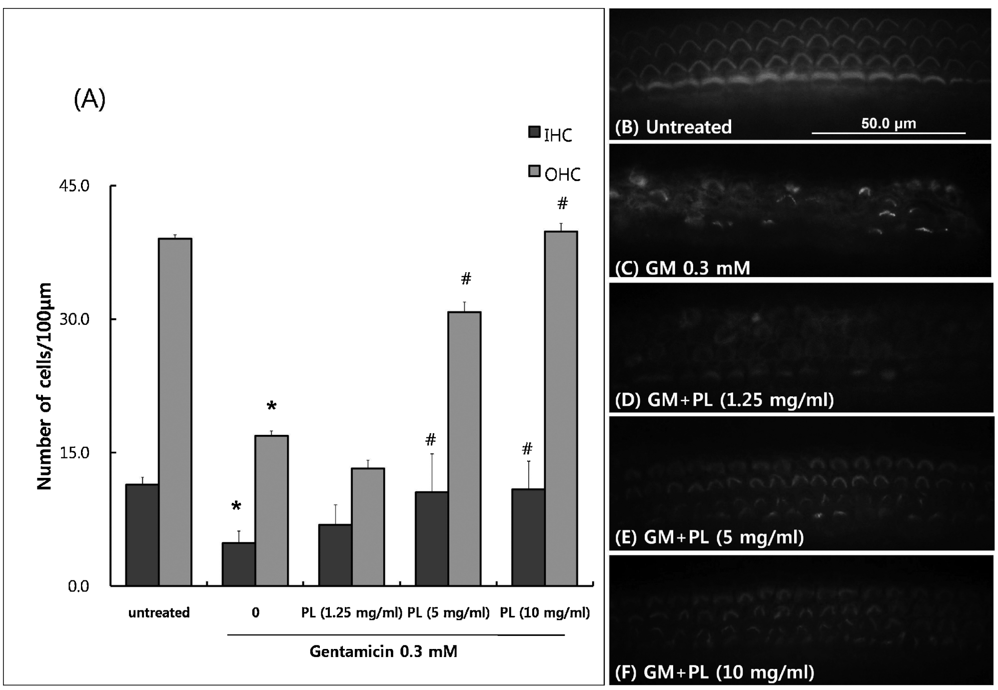

Fig. 2: Protective effect of PL against GM-induced hair cell loss in organotypic cochlear cultures of postnatal mice. (A) Quantitative analysis of the protective effect of ethanol extract of *Piper longum* L. (PL) on hair cells against GM (0.3 mM) at different concentrations in cultures maintained for 24 h. Hair cells labeled with Alexa Fluor 350 phalloidin were counted under a confocal microscope and are presented as the mean number (± SE) of IHCs, OHCs and total hair cells per 100 µm length of the basal and middle turn of the cochlea ($^{*}p < 0.05$ as compared with the untreated group; $^{\#}p < 0.05$ as compared with the GM-only group). Representative photographs of each group are presented as follows: (B) control explant without any treatment; (C) explants exposed to 0.3 mM GM; (D) explants exposed to 0.3 mM GM and PL (1.25 mg/ml); (E) explants exposed to 0.3 mM GM and PL (5 mg/ml); (F) explants exposed to 0.3 mM GM and PL (10 mg/ml). GM: gentamicin; PL: ethanol extract of *Piper longum* L

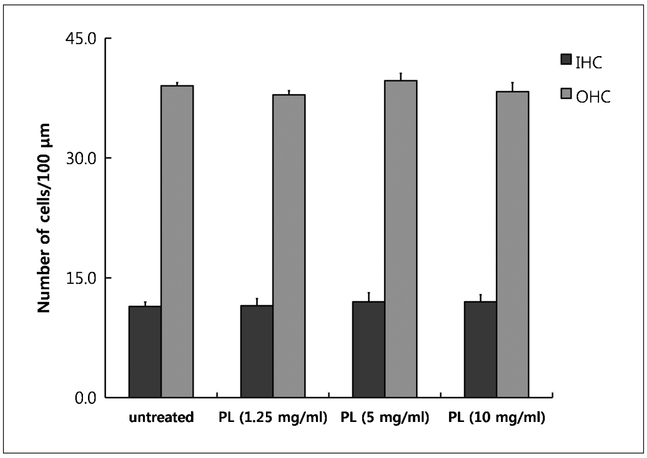

Fig. 3: PL alone had no adverse effects on hair cell survival in organotypic cochlear cultures of postnatal mice. Cochleae were cultured for 24 h in various concentrations of PL (1.25, 5, and 10 mg/ml). Hair cells labeled with Alexa Fluor 350 phalloidin were counted under a fluorescence microscope. Mean number (± SE) of IHCs, OHCs and total hair cells per 100 µm length of the basal and middle turn of the cochlea

reduced GM-induced ototoxicity (Fig. 2E∼F); the alignment of cells in the organ of Corti was somewhat irregular; however, hair cells retained their shape and stereocilia (Fig. 2E∼F). The preventive effect was dose-dependent (1.25∼5 mg/ml) (Fig. 2A). Five miligram per mililiter of PL provided effective protection against GM (Fig. 2E).

2.3. Ethanol extract of PL doses not interfere with the antibiotic activity of GM

To determine whether PL interferes with the antibiotic activity of GM, we performed experiments with *E. coli* cultures. Because the number of *E. coli* cells is proportional to the OD of the cultures, measurement of OD (at 600 nm) of *E. coli* cultures with a spectrometer is a good way to reflect the growth of bacteria and the antibiotic activity of GM. When *E. coli* cultures were maintained at 37 °C overnight, the growth of *E. coli* was evident. When GM concentrations were above 30 µM, all bacteria were killed (Fig. 4). If GM concentrations were below 0.3 µM, bacterial growth was not inhibited. Co-treatment wih PL and 0.3 mM GM (the same concentrations as those used in the cochlear explant cultures) also killed all bacteria. As shown

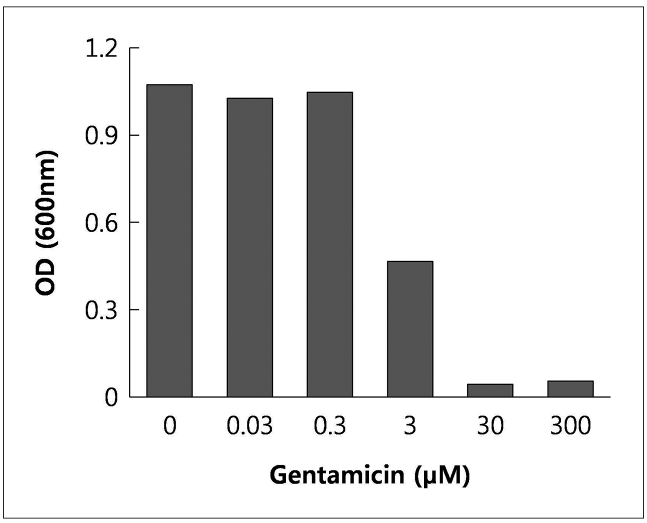

Fig. 4: OD (600 nm) readings of *E.coli* cultures after exposure to various concentrations of GM for 24 h. Triplicate cultures were performed for each experimental group, and the mean OD readings for each group are plotted.

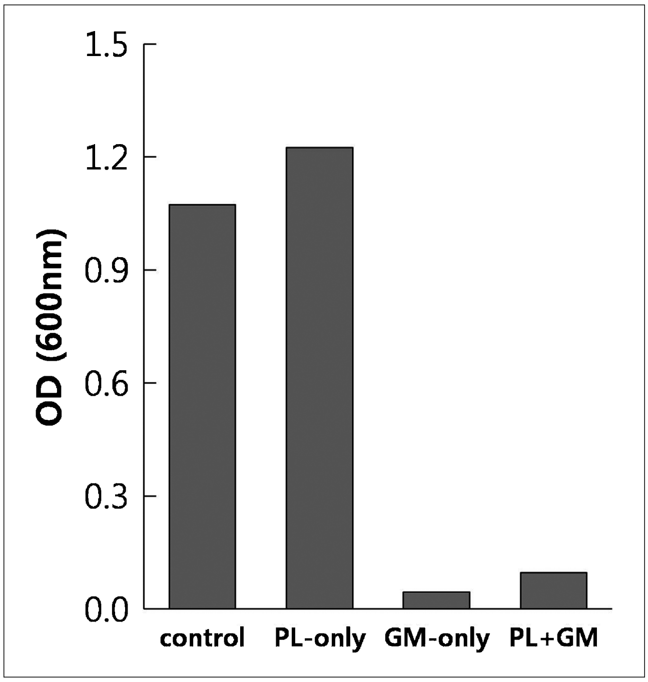

Fig. 5: OD (600 nm) readings of E.coli cultures after exposure to 5 mg/ml ethanol extract of *Piper longum* L. (PL), 0.3 mM gentamicin (GM), or both for 24 h. Triplicate cultures were performed for each experimental group, and the mean OD reading for each group is plotted.

in Fig. 5, OD readings of the cultures co-treated with PL and GM were essentially the same as those of cultures treated with GM alone. Thus, these results demonstrate that PL does not interfere with the antibiotic activity of GM.

2.4. NO-Scavenging activity of ethanol extract of PL

A release of NO from SNP and held on light and room temperature was time- and dose-dependent (Fig. 6). Co-incubation of SNP with PL resulted in a dose-dependent decrease of the level of nitrite, in comparison with nitrite levels obtained when SNP was dissolved alone. For the three lines, EC_{50} values were approximately 10 mg/ml.

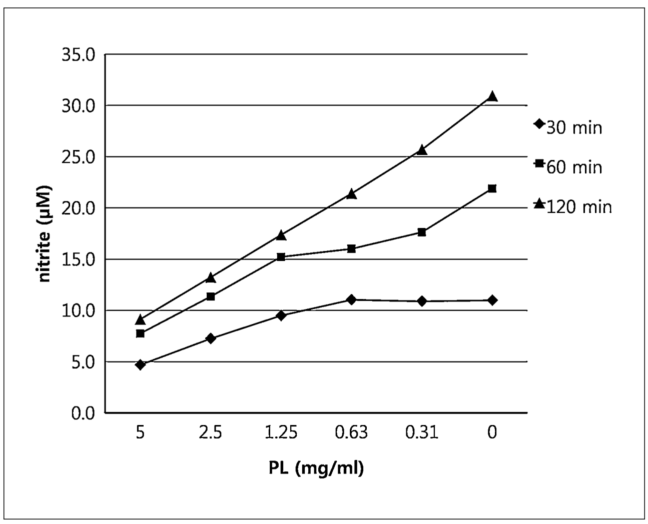

Fig. 6: Effect of PL on nitrite levels in SNP solution after incubation periods of 30, 60, and 120 min. SNP solution (5 mM) was incubated alone or in combination with different concentrations of PL (0.31∼5 mg/ml) on light at room temperature and nitrite levels were estimated after 30, 60, and 120 min using Greiss reagent. Points represent means from four replicates. Standard error mean was 1–5%. PL: ethanol extract of *Piper longum* L

3. Discussion

PL, a well-known traditional medicine in Asia and the Pacific Islands, has been reported to exhibit wide spectrum activity. We hypothesized that PL might be useful by virtue of its antioxidant activity if applied to patients with ototoxicity induced by GM. In the present study, an ethanol extract of PL alone had no adverse effects on hair cell survival. Combination of PL with GM resulted in attenuated GM-induced hair cell loss. Addition of 5 and 10 mg/ml PL resulted in enhanced OHC survival by 82% and 136% in cochlea cultured with 0.3 mM GM, respectively. Meanwhile, PL appears not to interfere with the antibiotic activity of GM and showed NO-scavenging activity *in vitro*.

Aminoglycoside antibiotics, including GM, are widely used clinically in treatment of bacterial infections. Unfortunately, their side effects on the kidney and inner ear remain a problem. The finding that PL can protect hair cells from GM insult but does not interfere with the antibiotic activity of GM suggests that PL may be of therapeutic value in prevention of aminoglycoside-induced hearing loss.

Aminoglycoside-induced ototoxicity progresses from high to low frequencies with increases in dose or duration of treatment (Tablan et al. 1984). Hair cell loss begins in the basal, high-frequency region of the cochlea and progresses towards the apex, consistent with the pattern of hearing loss. Therefore, we counted the number of hair cells from the basilar membrane for the basal and middle turn segments of each organ of Corti explants. While the protective effect of PL against GM-induced hair cell loss appears to be quite potent *in vitro* in P3 cochlear cultures, it remains to be seen whether it is efficacious *in vivo* in adult mammals.

Aminoglycosides such as GM can react with iron to generate ROS within the inner ear, with permanent damage to hair cells and neurons (Choung et al. 2009; Rybak and Ramkumar 2007). ROS are believed to promote apoptotic and necrotic cell death. One signaling pathway activated by aminoglycosides *via* ROS is the c-Jun N-terminal kinase (JNK) pathway, which contributes to cell apoptosis (Davis 2000). One of the downstream targets of the JNK is the transcription factor, activating protein-1, of which the main component was found to be the c-Fos protein (Albinger-Hegyi et al. 2006).

Overproduction of ROS was suggested as an initial step in triggering apoptotic pathways, resulting in cell death due to aminoglycoside-induced ototoxicity. Methods of blocking ROS in the cochlea under *in vitro* or *in vivo* aminoglycoside exposure have been demonstrated in many studies (Rizzi and Hirose 2007; Rybak and Ramkumar 2007). NO is also involved in GM ototoxicity. Peroxynitrite, formed by NO together with superoxide, is more reactive and toxic and plays an important role in GM-induced ototoxicity (Takumida et al. 1999). Recent *in vivo* studies have shown that GM is capable of inducing up-regulation of iNOS (Liu et al. 2008) and endothelial NO synthase (Heinrich et al. 2006) expression in the cochlea of guinea pigs. Heinrich et al. (2008) discovered the correlation between hearing threshold shift and NO production in the cochlea and concluded that increased NO contributed to GM-induced hearing loss. In this work, we found that PL in SNP solution induced a decrease in levels of nitrite, a stable oxidation product of NO liberated from SNP. Therefore, these findings suggest that PL possesses the ability to antagonize GM-induced ototoxicity, possibly *via* inhibition of ROS and NO formation.

The present report demonstrates a novel property of the extract from *Piper longum* L., indicating an otoprotective activity. PL appears not to interfere with the antibiotic activity of GM and showed NO-scavenging activity *in vitro*. On these bases, we conclude that the PL extract can represent a valuable tool for use in prevention of GM ototoxic side effects, and can be proposed for further investigations, for identification of specific components involved in otoprotection and development of a suitable intervention scheme against GM side effects.

4. Experimental

4.1. Ethanol extract of PL

Authenticated *Piper longum* L. was obtained from the Department of Pharmacy, Dongguk University Ilsan Hospital. Dried fruit powder was extracted twice in 10 volumes of 30% ethanol for 3 h at 85∼90 °C, and filtered with 40 μm filter. Ethanol was removed in a vacuum at 60 °C and the yield obtained was 4.7%. The extract was resuspended in PBS (pH 7.2).

4.2. Antibiotic activity test in Escherichia coli (E. coli) cultures

To examine the antibiotic activity of GM, 2 μl of *E. coli* stock was incubated in culture tubes with 2 ml of LB broth culture medium (Sigma, S. Louis, MO, USA). GM (Sigma, S. Louis, MO, USA) was added to some of the *E. coli* cultures either alone or together with PL at the beginning of culture. Cultures were incubated at 37 °C overnight and the optical density (OD) of the cultures was measured with a spectrometer at 600 nm. Data were collected from duplicate cultures and the mean OD readings from each group were plotted.

4.3. Cochlea organotypic culture

Cochlea organotypic culture was performed as described previously (Corbacella et al. 2004). All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of Dongguk University Ilsan Hospital and NIH guidelines. Every effort was made to minimize the number of animals required to provide explants for this study and to minimize any suffering. ICR mouse (Koatech laboratory animal company, Gyeonggi, Korea) pups were decapitated on postnatal day 2–3 (P2–3). The cochlea was carefully dissected as a flat-surface preparation of the organ of Corti. A drop of rat tail collagen (Type 1, BD Biosciences, USA, 3.86 mg/ml in 0.02 N acetic acid, 10x basal medium Eagle (Sigma, S. Louis, MO, USA), 2% sodium carbonate; 9:1:1 ration) was placed in a 35 mm culture dish (Nunc, Thermo, Waltham, MA, USA) and allowed to gel. Afterwards, 1 ml of serum-free Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Korea) supplemented with N-1 supplement (1%, Sigma, S. Louis, MO, USA) and penicillin G (50 U/mL, Sigma, S. Louis, MO, USA) were added to the culture dish. The organ of Corti was gently pressed on to the surface of the collagen gel with forceps and held in place by the surface tension of the culture medium. Cochlea cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 overnight and then exposed to a medium containing specific concentrations of GM (0.1 0.3, and 1 mM, Sigma, S. Louis, MO, USA), ethanol extract of PL (1.25, 5 and 10 mg/ml), or a combination of the two for 24 h.

4.4. Hair cell counts

At the end of the experiment, the cultures were fixed, permeabilized, and stained with Alexa Fluor 350 phalloidin (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The explants were then mounted on a slide with Gel Mount Biomedia (Fisher Scientific, Waltham, MA, USA), coverslipped, and examined with a fluorescence microscope (Olympus DP70, Tokyo, Japan). Each labeled hair cell was counted in the three OHC rows and one IHC row. Cells were considered missing, if there was a gap in the normal geometric array and no stereocilia or cuticular plates were observed. The number of cells was counted over a distance of 100 μm from five randomly selected fields of basilar membrane for the basal and middle turn segments of each organ of Corti explant. The average of hair cell counts from five fields was considered as a single sample, and typically 5–11 specimens were evaluated for each condition. The means and standard errors of the mean (SE) were calculated for all parameters measured. One way-ANOVA followed by a LSD post hoc test was used for comparison of hair cell damage between the experimental groups by SPSS 11.5.

4.5. NO-Scavenging

NO-scavenging activity was measured spectrophotometrically (Mirkov et al. 2004). Sodium nitroprusside (SNP, 5 mM) in phosphate buffer saline was mixed with different concentrations of ethanol extract of PL (1.25, 5 and 10 mg/ml). SNP is an inorganic complex where NO is found as NO^+ and light irradiation is necessary for release of NO. Therefore, incubation mixtures were incubated on light, at room temperature, and nitrite levels were determined exactly after 30, 60, and 120 min. For nitrite measurements, 130 μl of the incubated solution were removed and diluted with 10 μl of Griess reagent (1% sulfanilamide, 2% phosphoric acid, and 0.1% naphthyl ethelene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethelene diamine was measured at 548 nm along with a control. Sodium nitrite was used as a standard.

Acknowledgements: The authors thank Dr. Mukesh Kumar Yadav for his great help with *E.coli* experiments. This study was supported by a grant of the Korea Healthcare Technology R&D Project (A090084), Ministry for Health, Welfare & Family Affairs, Republic of Korea.

References

Albinger-Hegyi A, Hegyi I, Nagy I, Bodmer M, Schmid S, Bodmer D (2006) Alteration of activator protein 1 DNA binding activity in gentamicin-induced hair cell degeneration. Neuroscience 137: 971–980.

Bodmer D, Brors D, Pak K, Gloddek B, Ryan A (2002) Rescue of auditory hair cells from aminoglycoside toxicity by Clostridium difficile toxin B, an inhibitor of the small GTPases Rho/Rac/Cdc42. Hear Res 172: 81–86.

Choi EM, Hwang JK (2003) Investigations of anti-inflammatory and antinociceptive activities of Piper cubeba, Physalis angulata and Rosa hybrida. J Ethnopharmacol 89: 171–175.

Choung YH, Taura A, Pak K, Choi SJ, Masuda M, Ryan AF (2009) Generation of highly-reactive oxygen species is closely related to hair cell damage in rat organ of Corti treated with gentamicin. Neuroscience 161: 214–226.

Corbacella E, Lanzoni I, Ding D, Previati M, Salvi R (2004) Minocycline attenuates gentamicin induced hair cell loss in neonatal cochlear cultures. Hear Res 197: 11–18.

Das B, Kashinatham A, Srinivas KV (1996) Alkamides and other constituents of Piper longum. Planta Med 62: 582.

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103: 239–252.

Forge A, Schacht J (2000) Aminoglycoside antibiotics. Audiol Neurootol 5: 3–22.

Ghoshal S, Prasad BN, Lakshmi V (1996) Antiamoebic activity of Piper longum fruits against Entamoeba histolytica *in vitro* and *in vivo*. J Ethnopharmacol 50: 167–170.

Heinrich UR, Helling K, Sifferath M, Brieger J, Li H, Schmidtmann I, Mann WJ (2008) Gentamicin increases nitric oxide production and induces hearing loss in guinea pigs. Laryngoscope 118: 1438–1442.

Heinrich UR, Selivanova O, Brieger J, Mann WJ (2006) Endothelial nitric oxide synthase upregulation in the cochlea of the guinea pig after intratympanic gentamicin injection. Eur Arch Otorhinolaryngol 263: 62–68.

Hudspeth AJ (1989) How the ear's works work. Nature 341: 397–404.

Jalapure SS PM, Prakash NS, Hemalatha K, Manvi FV (2003) Hepatoprotective activity of the fruits of Piper longum Linn. Indian J Pharm Sci 65: 636.

Liu HY, Chi FL, Gao WY (2008) Taurine attenuates aminoglycoside ototoxicity by inhibiting inducible nitric oxide synthase expression in the cochlea. Neuroreport 19: 117–120.

Lopez-Gonzalez MA, Lucas M, Delgado F, Diaz P (1998) The production of free oxygen radicals and nitric oxide in the rat cochlea. Neurochem Int 33: 55–59.

Mata R, Morales I, Perez O, Rivero-Cruz I, Acevedo L, Enriquez-Mendoza I, Bye R, Franzblau S, Timmermann B (2004) Antimycobacterial compounds from Piper sanctum. J Nat Prod 67: 1961–1968.

McFadden SL, Ding D, Salvemini D, Salvi RJ (2003) M40403, a superoxide dismutase mimetic, protects cochlear hair cells from gentamicin, but not cisplatin toxicity. Toxicol Appl Pharmacol 186: 46–54.

Mirkov SM, Djordjevic AN, Andric NL, Andric SA, Kostic TS, Bogdanovic GM, Vojinovic-Miloradov MB, Kovacevic RZ (2004) Nitric oxide-scavenging activity of polyhydroxylated fullerenol, C60(OH)24. Nitric Oxide 11: 201–207.

Rizzi MD, Hirose K (2007) Aminoglycoside ototoxicity. Curr Opin Otolaryngol Head Neck Surg 15: 352–357.

Rybak LP, Ramkumar V (2007) Ototoxicity. Kidney Int 72: 931–935.

Sha SH, Schacht J (2000) Antioxidants attenuate gentamicin-induced free radical formation *in vitro* and ototoxicity *in vivo*: D-methionine is a potential protectant. Hear Res 142: 34–40.

Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 64: 353–356.

Tablan OC, Reyes MP, Rintelmann WF, Lerner AM (1984) Renal and auditory toxicity of high-dose, prolonged therapy with gentamicin and tobramycin in pseudomonas endocarditis. J Infect Dis 149: 257–263.

Takumida M, Anniko M (2001) Nitric oxide in guinea pig vestibular sensory cells following gentamicin exposure *in vitro*. Acta Otolaryngol 121: 346–350.

Takumida M, Popa R, Anniko M (1999) Free radicals in the guinea pig inner ear following gentamicin exposure. ORL J Otorhinolaryngol Relat Spec 61: 63–70.

Theopold HM (1977) Comparative surface studies of ototoxic effects of various aminoglycoside antibiotics on the organ of Corti in the guinea pig. A scanning electron microscopic study. Acta Otolaryngol 841: 57–64.

Thomas M, Sujatha KS, George S (2009) Protective effect of Piper longum Linn. on monosodium glutamate induced oxidative stress in rats. Indian J Exp Biol 47: 186–192.

Vedhanayaki G, Shastri GV, Kuruvilla A (2003) Analgesic activity of Piper longum Linn. root. Indian J Exp Biol 41: 649–651.

Wakade AS, Shah AS, Kulkarni MP, Juvekar AR (2008) Protective effect of Piper longum L. on oxidative stress induced injury and cellular abnormality in adriamycin induced cardiotoxicity in rats. Indian J Exp Biol 46: 528–533.

Wang AM, Sha SH, Lesniak W, Schacht J (2003a) Tanshinone (Salviae miltiorrhizae extract) preparations attenuate aminoglycoside-induced free radical formation *in vitro* and ototoxicity *in vivo*. Antimicrob Agents Chemother 47: 1836–1841.

Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A (2003b) A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. J Neurosci 23: 8596–8607.

Wu WJ, Sha SH, Schacht J (2002) Recent advances in understanding aminoglycoside ototoxicity and its prevention. Audiol Neurootol 7: 171–174.

Ylikoski J, Xing-Qun L, Virkkala J, Pirvola U (2002) Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. Hear Res 166: 33–43.