

Protective effect of L-N-acetylcysteine against gentamycin ototoxicity in the organ cultures of the rat cochlea

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Abstract

The *aim* of this study was to test if L-N-acetylcysteine (L-NAC) can protect hair cells against gentamycin-induced damage *in vitro*. Mammalian auditory cells are unable to regenerate when affected by several toxic agents. Aminoglycosides are large-scale antibiotics, extremely useful for the treatment of several Gram-negative bacterial infections, but their use is limited by the extremely severe side effects like ototoxicity and nephrotoxicity. *Materials and Methods*: 1–4-day-old rat cochlea explants were exposed to different doses of gentamycin. Half of the cochleas were pretreated for 24 hours with different doses of L-NAC. The explants were fixed and stained with phalloidin, and the intact hair cells were counted. *Results*: GM treatment resulted in the loss of sensorial cells in the organ of Corti explants in a dose-dependent manner. All doses of L-NAC offered significant protection ($p < 0.001$) when added in culture 24 hours prior to GM. There was no significant difference between the level of protection offered by the different doses of L-NAC, both in the outer and inner hair cells. *Conclusions*: Our results demonstrate that L-NAC can protect cochlear cells against gentamycin toxicity.

Keywords: hair cells, cochlear explants, ototoxicity, otoprotection.

Introduction

Deafness is a major public health issue, 90% of the situations being the result of sensorineural loss, involving damage of sensory cells (hair cells), supporting cells and neurons in the cochlea [1]. They are often damaged by ototoxic drugs, acoustic overstimulation, the lack of essential growth factors, infections, autoimmune conditions or hereditary diseases and processes associated with aging. Neurosensorial hearing loss is irreversible: there is no evidence so far of regeneration in the mammalian cochlea, due to the loss of regenerative capacity of its sensory epithelium [2]. The sensorial epithelium of the cochlea consists of a perfect mosaic of sensorial cells (hair cells – HC) and support cells (SC). In mammals, there are two types of mechanoreceptor cells, the inner hair cells (IHC) disposed on a single row and outer hair cells (OHC), organized on three rows along the cochlear duct. These cells are maintained in a non-proliferative state during the lifetime of the individual, their damage leading to permanent loss, because auditory sensorial cells cannot regenerate in adult mammals, all cells in the organ of Corti becoming terminally mitotic (mitotic quiescence) by embryonic day 15 (E15) [3].

Aminoglycosides are large-scale antibiotics, extremely useful for the treatment of several Gram-negative bacterial infections, but their use is limited by the extremely severe side effects like ototoxicity and nephrotoxicity. Recent studies concluded that aminoglycosides induce apoptosis in the inner ear hair cells [4]. Cell death induced by aminoglycosides implies the

activation of an intrinsic program of cellular “suicide” meaning that it needs the activation of caspase 9. Inhibition of caspase activity with caspase inhibitors reduces ongoing cell death in a dose dependent manner [5, 6]. Overexpression of Bcl-2 increases survival of hair cells after the exposure of organotypic cultures of adult mouse utricles to neomycin, preventing the activation of caspase 9 [7].

Cell death after aminoglycoside exposure is caused by the release of large quantities of reactive oxygen species, or free radicals, into the hair cell cytoplasm [8, 9]. Antioxidants are known to reduce oxidative stress and inflammation; therefore, supplementation with antioxidants appears to be one of the most rational approaches to prevent and improve hearing disorders in combination with standard therapy. Several studies showed that supplementation with antioxidants produced beneficial effects: vitamin E [10], L-N-acetyl-cysteine (L-NAC) [11], acetyl-L-carnitine, coenzyme Q10, vitamin C, etc. [12].

Materials and Methods

Animals

Newborn CD1 rats, 0–6 days *post partum* (P0–P6), (P0 representing the day of birth) from the animal facility of the “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, were used. Every procedure was in accordance with the guidelines and had the approval of the Ethical Committee of the University of Medicine and Pharmacy.

Organ culture

After anesthesia, the rats were killed and decapitated. The organ of Corti was excised and freed from the

surrounding tissues. Dissections were performed under a stereomicroscope, in sterile Dulbecco's phosphate-buffered saline (PBS) (Sigma) (Figure 1).

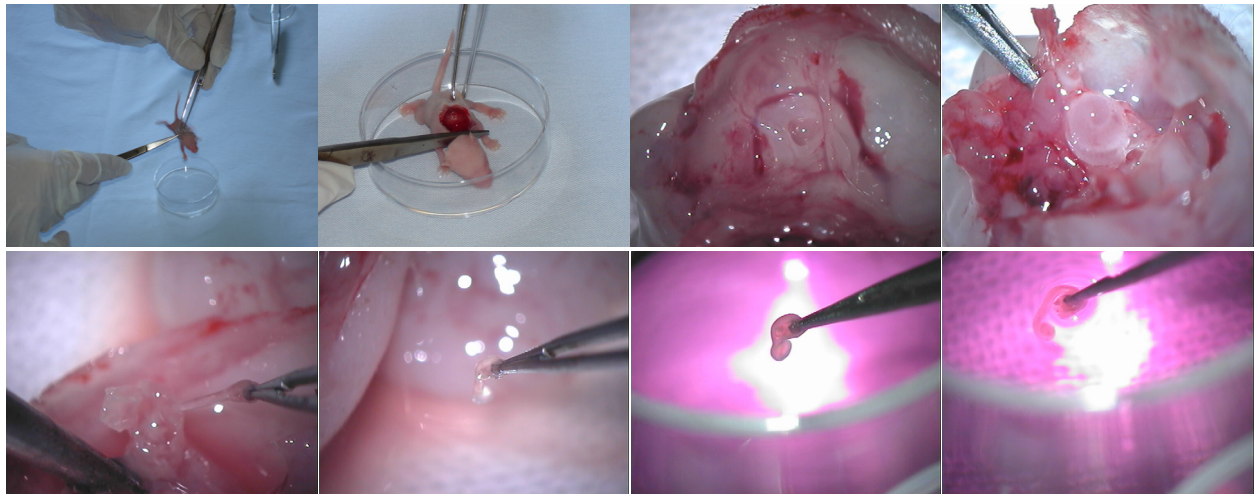


Figure 1 – Dissection of the rat cochlea.

The Corti organs were placed each in one well of a 24-well plate (Nunc) on the surface of Millicell membrane inserts (Millipore), in complete culture media: Dulbecco's modified Eagles' medium (DMEM – Sigma) with 6 g/L glucose, N1 supplement (Sigma). Medium was changed every two days.

Exposure to aminoglycoside antibiotics. Gentamycin (GM) treatment

After one-day culture in complete medium, the explants were exposed to GM, three explants for each concentration, three being considered control explants. The duration of the treatment was 48 hours. The following concentrations of GM were used: 0.1, 0.5, 1 and 3 mM.

Pretreatment with L-NAC

For these experiments, we used two concentrations of GM: 0.5 and 1 mM. Three explants were exposed to different concentrations of L-NAC: 1, 5, and 10 mM. After 24 hours, incubation gentamycin was added. After 48 hours, the explants were fixed and stained with TRITC-phalloidin. Each treatment group consisted of minimum three explants; each experiment was repeated at least three times.

Fluorescent staining of Corti hair cells

We used TRITC (tetramethyl-rhodamine-isothiocyanate) labeled-phalloidin (Sigma), a toxin isolated from *Amanita phalloides* that binds to the F-actin, which is abundant in the stereocilia bundles of cochlear hair cells. This way the stereocilia bundles become visible permitting the easy identification of the intact hair cells as well as of those damaged by different treatments (empty spaces).

The explant was fixed 30 minutes with freshly prepared 4% paraformaldehyde at room temperature then incubated 15 minutes with 1% PBS-Triton X100 for permeabilization. TRITC-conjugated phalloidin was

added (1:60) for 45 minutes at room temperature. After washing with PBS, the explants were mounted on microscope slides with mounting medium. The explants were examined at a Zeiss AxioObserver D1 inverted microscope, equipped with a HBO100 fluorescent lamp and the proper filters for TRITC (excitation 544 nm, emission 572 nm). Images were taken with a monochrome AxioCam MRm camera then processed and analyzed using the AxioVision 4.6 software.

For each explant, the length of the specimen was measured. The first row of OHC is considered the one situated next to the row of IHC. The three rows of OHC and one row of IHC are easily recognized by the presence of the stereocilia bundles, separated by the "pillar" cells. The integrity of the stereocilia bundles was analyzed. The intact hair cells were counted on different segments of the cochlea. The number of the intact cells was expressed as hair cells/length unit. Hair cells were considered missing if the stereocilia bundle is missing entirely. For the quantification of the effects of different ototoxic drugs, OHC and IHC were counted on distances of 0.1 mm chosen from four distinct randomly selected microscopic fields from the basal turn of each explant (using an ocular grid system). For each endpoint, the average of four counts was calculated and the hair cell density/length unit is obtained.

Statistical analysis

Statistical analysis was done and graphs were plotted using GraphPadPrism 5 software.

Results

In vitro culture of cochlear explants

The explants were maintained in culture for four weeks, without infections. The culture medium was changed every 2–3 days (Figure 2). Daily observations of the explants were made on a Nikon Eclipse T100 inverted microscope to examine the outgrowth of the

cells, their morphology, viability and evolution. Explants were also carefully examined for the presence of infection. Images were taken with a Nikon Coolpix 4500 digital camera. When kept in culture dishes without Millicell inserts, the explants adhered to the surface of

the culture dish and after 4–5 days, cells with various morphologies grew out from the explant (Figure 3a). In approximately 20 days, the outgrown cells reached confluence (occupied the entire surface of the dish (Figure 3b).

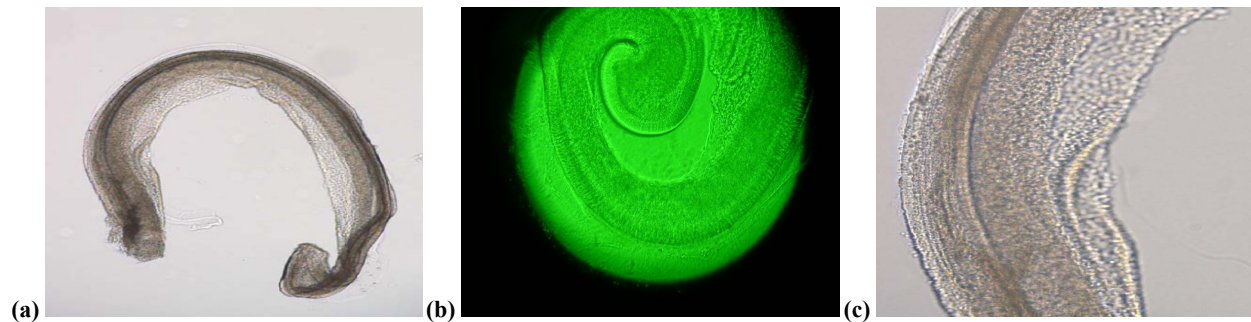


Figure 2 – Microscopic aspect of Corti organ on the first day in culture: (a) and (b) 200× magnification; (c) 400× magnification.

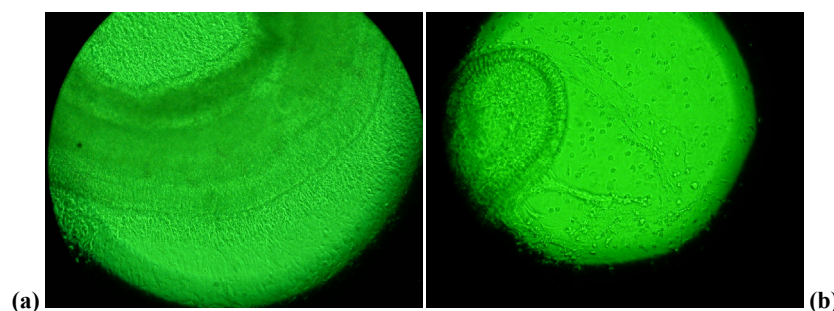


Figure 3 – (a) Organ of Corti explant after 4 DIV (days in vitro) (400×); (b) The explant after 10 DIV (200×).

GM treatment

The Corti organ explants stained with TRITC-phalloidin, showed a normal morphology after two days in culture, with one row of inner hair cells and three rows of outer hair cells, showing a “V”-shape and linear organization (Figure 4a, Figure 8a).

The hair cell count showed that control explants had an average number of 43 OHC/0.1 mm, while IHC were 13.5 cells/0.1 mm. The administration of progressive concentrations of gentamycin resulted in a dose-dependent reduction of the number of intact hair cells. A concentration of 0.1 mM GM in the culture medium led to a decrease of the total number of OHC from 43 (in control explants) to 38, the missing cells (11.3%) being from the third row. At this concentration, IHC remained unaffected.

At 0.5 mM, a massive loss of OHC is present (42.4%), while in IHC the response is still not so impressive (3%). 1 mM GM destroyed the majority of hair cells, both OHC (82%) and IHC (55.6% survival). At 3 mM GM, in the culture medium, almost all hair cells were destroyed (Figure 4b, Figure 8b).

These results lead to the conclusion that GM toxicity on the Corti cells is proportionally increasing with the concentration and that outer hair cells are more susceptible than inner hair cells (Figure 5a). Plotting the survival curve for OHC and IHC, the GM concentration, which reduces cell viability with 50% (IC₅₀) was higher for IHC (0.8986 mM) than for OHC (0.5397 mM), indicating again a higher susceptibility of outer hair cells (Figure 5b).

IC₅₀ for OHC was 0.5397 while for IHC was 0.8986. These data show a greater susceptibility of OHC to GM ototoxicity compared with IHC.

Protective effect of L-N-acetylcysteine (L-NAC)

GM treatment resulted in the loss of sensorial cells in the organ of Corti explants in a dose-dependent manner, compared to control explants, which showed a normal organization of the stereocilia bundles after 72 hours *in vitro*.

The effect of pretreatment with L-NAC, 24 hours before adding GM in the culture medium, was analyzed in the same manner, by counting the intact hair cells in the cochleas stained with TRITC-phalloidin. While both doses of GM used in this experiment, 0.5 and 1 mM affected the cochlear cells, the pretreatment with all doses of L-NAC offered significant protection ($p < 0.001$) (two-way ANOVA, Bonferroni post-tests). At 0.5 mM GM, the number of viable OHC decreased with 42.4% (57.7% survival) and of IHC with 3%. The pretreatment with all concentrations of L-NAC increased cell survival significantly in OHC: 1 mM to 89%, 5 mM and 10 mM to 93 and 94%. When the GM dose was higher, 1 mM, the OHC were killed 82%. L-NAC pretreatment led to a significant increase in cell survival reaching values similar to the control. There was no significant difference between the levels of protection offered by the different doses of L-NAC (Figure 6). This behavior was found both in outer and inner hair cells (Figure 7, Figure 8c).

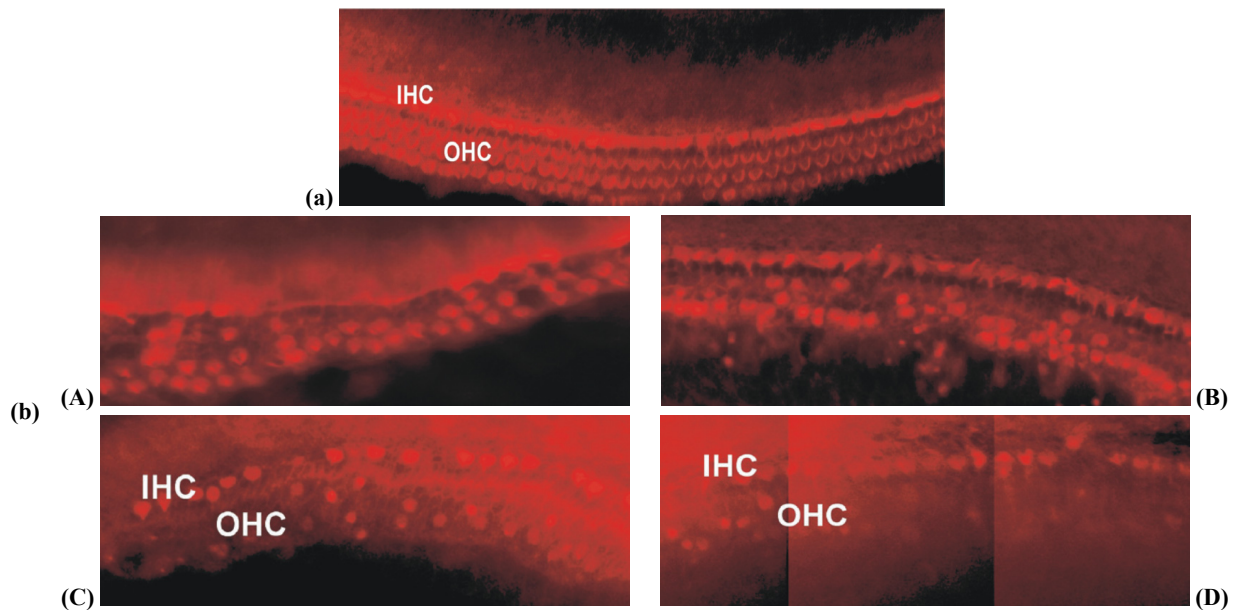


Figure 4 – (a) Normal morphology of inner hair cells (IHC) and three rows of outer hair cells (OHC) (TRITC-phalloidin staining); (b) Representative images with the organ of Corti after GM treatment (48 hours). (A) GM 0.1 mM; (B) 0.5 mM antibiotic; (C) GM 1 mM; (D) Gentamycin 3 mM: loss of stereocilia in approx. 50% OHC. Magnification 1000×.

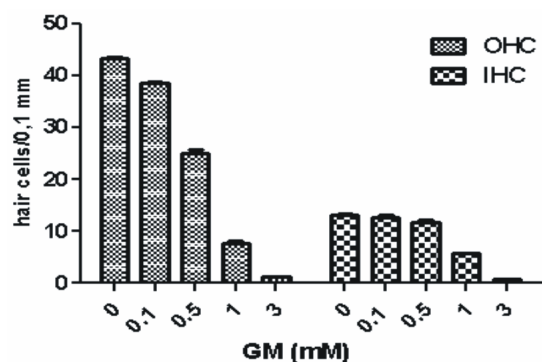


Figure 5 – Comparison of the sensitivity of OHC and IHC to different concentrations of GM. OHC are more susceptible to GM ototoxicity than IHC, the one-way ANOVA analysis of variance showing statistically significant differences ($p < 0.001$).

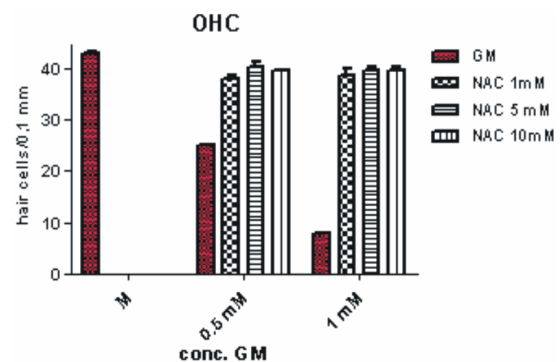


Figure 6 – Significant protection ($p < 0.001$ – two-way ANOVA, Bonferroni post-tests) with all doses of L-NAC on the OHC. No significant differences between the effects of different doses of L-NAC.

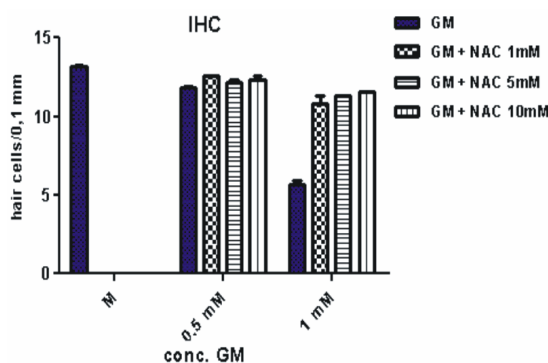


Figure 7 – Significant protection ($p < 0.001$) given by all doses of L-NAC on the IHC. No significant differences between the effects of different doses of L-NAC (two-way ANOVA, Bonferroni post-tests).

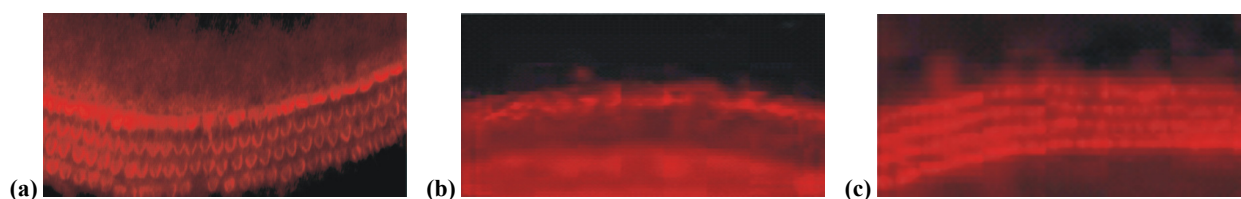


Figure 8 – L-NAC protects hair cells against the toxic action of GM-explants stained with TRITC-phalloidin. (a) Control; (b) Hair cells missing after GM treatment (1 mM); (c) Protection with L-NAC (5 mM). Magnification 1000×.

Discussion

The incidence of gentamycin-induced hearing loss averages 8% for a short course of therapy but may be higher in developing countries, where aminoglycosides are frequently the only affordable antibiotics and are sold over the counter. No therapy presently exists to prevent ototoxicity. Animal models suggest that ototoxicity is caused by reactive oxygen species and is attenuated by antioxidants. Much progress has been made to understand the mechanism of inner ear hair cell degeneration. Intracellular damage caused by noise, aminoglycosides, cisplatin seems to share a common pathway: cytochrome-*c* translocation and caspase activation [13].

The molecular mechanism of aminoglycoside toxicity remains to be elucidated. One possible theory: the aminoglycosides attach to the hair cells' membrane and inhibit the inositol-phosphate cascade resulting in a reduction of intracellular calcium mobilization [14]. Aminoglycoside ototoxicity has a two-step progression: OHC are more susceptible than IHC. When both are affected the damage has an evolution from the base of the cochlea to the apex. This aspect is maintained in the organotypic cultures. The differences are probably due to specific properties of hair cells, for example a low level of glutathione in the basal OHC [15]. When free radical scavengers are added to the culture, a better survival of OHC is obtained. Another essential factor in the hair cell death seems to be the uptake of the aminoglycosides. When fluorescently labeled gentamycin was added to the cultures, it was excluded by the cellular cytoplasm for 6 hours. *In vivo* studies showed that aminoglycosides enter the hair cells through the apical pole after approximately 24 hours after injection by receptor-mediated endocytosis, being sequestered in structures similar to lysosomes [16].

Compelling evidence from animal models suggests that reactive oxygen species are part of the initial mechanisms that triggers apoptotic and necrotic cell death in the inner ear. Consequently, antioxidants protect against aminoglycoside-induced hearing loss in animals and, importantly, they do so without compromising drug serum levels or antibacterial efficacy. The toxicity of aminoglycosides is suppressed by polyanions: poly-L-aspartate and poly-L-glutamate, which interfere with the membrane-attachment by forming aminoglycoside-polyanion complexes [17]. Another possibility is by increasing extracellular calcium concentration, which could compensate the intracellular reduction of calcium, by diffusion through calcium channels [18]. To counteract the formation of free radicals, several research groups have studied and found protective effects *in vitro* of WR-2721 [19], Glutathione or L-NAC. Traditional medicines (natural products) also hold promise as pharmacological protective against aminoglycoside-induced hearing loss [20]. All these information suggest that the clinical use of protective agents could reduce or prevent damage to the inner ear hair cells when ototoxic drugs are used for the treatment of several disorders.

Conclusions

Corti organs from newborn rats can be maintained in culture in proper conditions for up to four weeks. Gentamycin in the culture medium led to a dose-dependent decrease of hair cell number, OHC being more susceptible than IHC, small doses of GM (01 mM) affecting only a few OHC. L-NAC treatment before GM offered significant protection of the Corti hair cells, both IHC and OHC. All doses of L-NAC were efficient, with no significant differences between the protection levels conferred by the different concentrations. Exogen L-NAC could represent a cysteine source for the hair cells leading to the increase of intracellular GSH (glutathione). This way, the protection given by L-NAC might be due to its capacity to increase intracellular cystein, which will serve as substrate for gamma glutamyl cystein synthetase (γ GCS) restoring intracellular levels of GSH. These results enable us to conclude that treatment with L-NAC could be a protection method against aminoglycoside ototoxicity.

Acknowledgements

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References

- [1] Cotanche DA, *Genetic and pharmacological intervention for treatment/prevention of hearing loss*, J Commun Disord, 2008, 41(5):421–443.
- [2] Bermingham-McDonogh O, Rubel EW, *Hair cell regeneration: winging our way towards a sound of future*, Curr Opin Neurobiol, 2003, 13(1):119–126.
- [3] Anniko M, *Cytodifferentiation of cochlear hair cells*, Am J Otolaryngol, 1983, 4(6):375–388.
- [4] Forge A, Li L, *Apoptotic death of hair cells in mammalian vestibular sensory epithelia*, Hear Res, 2000, 139(1–2):97–115.
- [5] Matsui JI, Ogilvie JM, Warchol ME, *Inhibition of caspases prevents ototoxic and ongoing hair cell death*, J Neurosci, 2002, 22(4):1218–1227.
- [6] Tabuchi K, Pak K, Chavez E, Ryan AF, *Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage*, Neuroscience, 2007, 149(1):213–222.
- [7] Staecker H, Liu W, Malgrange B, Lefebvre PP, Van De Water TR, *Vector-mediated delivery of bcl-2 prevents degeneration of auditory hair cells and neurons after injury*, ORL J Otorhinolaryngol Relat Spec, 2007, 69(1):43–50.
- [8] Chen Y, Huang WG, Zha DJ, Qiu JH, Wang JL, Sha SH, Schacht J, *Aspirin attenuates gentamicin ototoxicity: from the laboratory to the clinic*, Hear Res, 2007, 226(1–2):178–182.
- [9] Schacht J, *Aminoglycoside antibiotics*. In: Campbell KCM (ed), *Pharmacology and ototoxicity for audiologists*, Thomson Delmar Learning, Clifton Park, New York, 2007, 163–176.
- [10] Kalkanis JG, Whitworth C, Rybak LP, *Vitamin E reduces cisplatin ototoxicity*, Laryngoscope, 2004, 114(3):538–542.
- [11] Feghali JG, Liu W, Van De Water TR, *L-N-acetyl-cysteine protection against cisplatin-induced auditory neuronal and hair cell toxicity*, Laryngoscope, 2001, 111(7):1147–1155.
- [12] Prasad KN, *Antioxidants in cancer care: when and how to use them as an adjunct to standard and experimental therapies*, Expert Rev Anticancer Ther, 2003, 3(6):903–915.
- [13] Cheng AG, Cunningham LL, Rubel EW, *Mechanisms of hair cell death and protection*, Curr Opin Otolaryngol Head Neck Surg, 2005, 13(6):343–348.
- [14] Malgrange B, Lefebvre PP, Moonen G, *Biologie cellulaire de la prévention et du traitement des surdités neurosensorielles*, MS Médecine Sciences, 1998, 14(5):588–594.

- [15] Sha SH, Taylor R, Forge A, Schacht J, *Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals*, Hear Res, 2001, 155(1–2):1–8.
- [16] Dai CF, Mangiardi D, Cotanche DA, Steyger PS, *Uptake of fluorescent gentamicin by vertebrate sensory cells in vivo*, Hear Res, 2006, 213(1–2):64–78.
- [17] Malgrange B, *Utilisation de la culture in vitro pour l'étude de la neuro- et de l'ototoxicité et l'élaboration de stratégies thérapeutiques neuro- et otoprotectrices*, Mémoire de doctorat en sciences biomédicales expérimentales, Liège, 1993, 1–176.
- [18] Takada A, Schacht J, *Calcium antagonism and reversibility of gentamicin-induced loss of cochlear microphonics in the guinea pig*, Hear Res, 1982, 8(2):179–186.
- [19] Pierson MG, Møller AR, *Prophylaxis of kanamycin-induced ototoxicity by a radioprotectant*, Hear Res, 1981, 4(1):79–87.
- [20] Wang AM, Sha SH, Lesniak W, Schacht J, *Tanshinone (Salviae miltiorrhizae extract) preparations attenuate aminoglycoside-induced free radical formation in vitro and ototoxicity in vivo*, Antimicrob Agents Chemother, 2003, 47(6):1836–1841.

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