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Protective role of L-ascorbic acid, *N*-acetylcysteine and apocynin on neomycin-induced hair cell loss in Zebrafish

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ABSTRACT: Hair cells are highly sensitive to environmental insults and other therapeutic drugs. The adverse effects of drugs such as aminoglycosides can cause hair cell death and lead to hearing loss and imbalance. The objective of the present study was to evaluate the protective activity of L-ascorbic acid, *N*-acetylcysteine (NAC) and apocynin on neomycin-induced hair cell damage in zebrafish (*Danio rerio*) larvae at 5 days post fertilization (dpf). Results showed that the loss of hair cells within the neuromasts of the lateral lines after neomycin exposure was evidenced by a significantly lower number of neuromasts labeled with fluorescent dye FM1-43FX observed under a microscope. Co-administration with L-ascorbic acid, NAC and apocynin protected neomycin-induced hair cell loss within the neuromasts. Moreover, these three compounds reduced the production of reactive oxygen species (ROS) in neuromasts exposed to neomycin, indicating that their antioxidant action is involved. In contrast, the neuromasts were labeled with specific fluorescent dye Texas-red conjugated with neomycin to detect neomycin uptake. Interestingly, the uptake of neomycin into hair cells was not influenced by these three antioxidant compounds. These data imply that prevention of hair cell damage against neomycin by L-ascorbic acid, NAC and apocynin might be associated with inhibition of excessive ROS production, but not related to modulating neomycin uptake. Our findings conclude that L-ascorbic acid, NAC and apocynin could be used as therapeutic drugs to protect aminoglycoside-induced listening impairment after further confirmatory studies. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: L-ascorbic acid; N-acetylcysteine; apocynin; hair cell; zebrafish; ototoxicity

Introduction

Neomycin is an important and commonly used aminoglycoside antibiotic around the world. However, the adverse effects of aminoglycoside usage still exist in clinical practice, as it has been documented to cause sensory hair cell damage, which results in permanent hearing loss. While the exact mechanisms in aminoglycoside-induced ototoxicity have not yet been fully clarified, the primarily excessive production of reactive oxygen species (ROS) or free radicals and/or activation of apoptosis markers appear to be involved (Karasawa and Steyger, 2011; Coffin *et al*., 2013). Thus, ROS seems to play a central role in the initiation of hair cell damage via oxidative stress. In order to preserve the sensory function after aminoglycoside exposure, it is necessary to identify the molecules that have the potential to protect hair cell death after ototoxic insults. As ROS may play a key role in hair cell death, neomycin-induced hair cell damage in zebrafish (*Danio rerio*) could be handled by modulation of ROS production and improvement of the antioxidant status.

Although various chemical agents have been identified to inhibit ototoxicity induced by aminoglycosides, none have been approved in clinical use (Selimoglu, 2007; Chang *et al*., 2011). Nowadays, several nutritional supplements are available on the market, and are mostly used to boost the antioxidant system. Apocynin is widely utilized to inhibit ROS production through inhibition of NADPH oxidase, which is responsible for superoxide anion production. Vitamin C, known as ascorbic acid, is an essential nutrient and electron donor, and acts as a potent antioxidant by donating its electron and prevents oxidation of other compounds. It has been reviewed that ascorbate chemical properties allow scavenging of the superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) into a less toxic substance, thereby avoiding oxidative damage of vital molecules (Padayatty *et al*., 2003). *N*-acetylcysteine (NAC), a derivative of cysteine, is commonly used

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as a nutritional supplement to boost the antioxidant system. NAC is essential in the synthesis of glutathione (GSH), which is capable of scavenging free radicals (Dekhuijzen, 2004). According to the documented evidence, aminoglycoside treatment may generate ROS in the inner ear. Then, ROS can induce hair cell death and result in hearing loss and tinnitus effects (Tabuchi *et al*., 2011). Therefore, we proposed that antioxidants, such as L-ascorbic acid, NAC and apocynin, might be good candidates for protecting hair cell toxicity under aminoglycoside exposure.

Zebrafish is an excellent animal model for toxicological and developmental studies, probably because of its large-scale availability, small size and easy husbandry than the rodent models (Lam *et al*., 2011). The zebrafish model is also considered as a non-human model for human diseases and preclinical drug screening studies (Dooley and Zon, 2000; Zon and Peterson, 2005). Furthermore, zebrafish is susceptible to chemical-induced pathology in a similar fashion to mammals (Lam *et al*., 2006). The lateral line in zebrafish is a sensory system that comprises a cluster of neuromasts arrayed on the head and body of the fish (Raible and Kruse, 2000). Neuromasts represent the smallest functional unit of the lateral line, and are composed of 10–20 mechanosensory hair cells, which are compatible with sensory hair cells in the mammalian inner ear and share structural and functional similarities. The responses of zebrafish hair cells to ototoxic drugs are similar to mammalian hair cells (Suli *et al*., 2012; Coffin *et al*., 2013). As hair cells in neuromasts can be directly detected under a microscope *in vivo* by the fluorescent dye FM1-43FX, a reliable indicator to assess hair cell viability, these particular properties make zebrafish a powerful animal model for related studies.

In view of these, prevention of hair cell death is an important target for the protection of hearing loss. It is essential to study neomycin-induced ototoxicity in zebrafish lateral line hair cells, and to find out the novel therapeutic drugs to treat the toxicity. Therefore, in this study we made an attempt to evaluate the beneficial effects of L-ascorbic acid, NAC and apocynin against neomycin-induced hair cell death in zebrafish.

Materials and Methods

Drugs and Chemicals

DAPI, FM1-43FX, YO-PRO-1 and Texas red-X succinimidyl ester were obtained from Molecular Probes, Inc. (Invitrogen, Eugene, OR, USA). Apocynin and neomycin were obtained from Calbiochem (Darmstadt, Germany). NAC, 2′,7′-dichlorofluorescein diacetate (DCF-DA), paraformaldehyde, L-ascorbic acid and other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Embryos of Zebrafish

For this study, zebrafish embryos were produced by group mating of AB wild-type adult fish, and raised at 28.5 °C. Then, the obtained embryos were maintained at a density of about 50 embryos per 100 mm^2 in a Petri dish in E3 embryo medium (NaCl 5 mM, KCl 170 μM, $CaCl_2$ 330 μM, $MgSO_4$ 330 μM and HEPES 10 mM). This procedure was carried out in a zebrafish facility center at Tzu Chi University. All experiments were performed with 5 days post-fertilization (dpf) larvae. The entire study design and experimental procedures were approved by the Tzu Chi University Animal Care and Use Committee, Hualien, Taiwan.

Drug Treatment

The post-fertilized zebrafish larvae were separated into different treatment groups. Six to eight larvae in each treatment group were incubated in a specific E3 embryo medium. Except larvae in the control group, all others were exposed to neomycin at 20 μM with or without treatment with L-ascorbic acid (vitamin C, 2.5–20 μg ml^{-1}; 14.2–113.6 μM), NAC (25–200 μg ml^{-1}; 0.15–1.23 mM) or apocynin (0.1–1 mM) for 30 min at 28.5 °C. Control larvae were incubated in E3 embryo medium. As the aim of this study was to evaluate the beneficial effects of the antioxidant compounds against neomycin-induced ototoxicity, the hair cell survival test was performed by co-administration of different concentrations of L-ascorbic acid, NAC and apocynin with neomycin to zebrafish larvae at 5 dpf.

Hair Cell Labeling and Survival Test

The hair cell survival test in neomycin-exposed zebrafish larvae was performed and observed under a microscope (Nikon eclipse E800). After drug treatment, all the larvae were rinsed with E3 medium and alive hair cells within the neuromast were stained for 30 min with 1 μM fluorescent dye FM1-43FX (Invitrogen), a reliable indicator to assess the hair cell viability. The larvae were then washed twice with E3 embryo medium, euthanized and fixed with 4% paraformaldehyde solution after being washed four times with the E3 embryo medium. The numbers of visible neuromasts, which contain hair cells, were counted on one side of the larvae under a microscope (Nikon eclipse E800). The lateral lines of zebrafish contain hair cells that are compatible with the hair cells of the mammalian inner ear. This assay was performed to detect the effects of L-ascorbic acid, NAC and apocynin on neomycin-induced hair cell loss, which was detected using the fluorescent dye FM1-43FX.

Measurement of Reactive Oxygen Species (ROS)

In order to prove the antioxidant activity of L-ascorbic acid, NAC and apocynin against neomycin-induced hair cell loss, neomycin-induced intracellular ROS production was determined by labeling the membrane permeable green fluorescent dye, DCF-DA. The nuclei of hair cells in Zebrafish larvae were initially counter-stained with DAPI at 0.1 μg ml^{-1} for 5 min and then washed with E3 embryo medium. After washing the larvae were transferred into DCF-DA (10 μM), neomycin (20 μM) and with/without tested drugs for a 5-min incubation. After treatments, larvae were euthanized with 4% paraformaldehyde and neuromasts images were captured immediately for all larvae. A Canon EOS 50D camera was used to take the pictures under the microscope (Nikon eclipse E800). Obtained images were merged by the image processing program, ImageJ, and the images with ROS generation are shown in this study. The above experiments were repeated in triplicate for each tested antioxidant.

Neomycin Uptake Test

Zebrafish larvae were counter-stained with 2 μM YO-PRO-1 for 5 min followed by being washed three times and incubated in E3 embryo medium containing 20 μM Texas red-conjugated neomycin (NRT) with/without testing compounds for 5 min. NRT is prepared for the study of aminoglycoside entry into hair

Journal of AppliedToxicology

cells, whereas YO-PRO-1, the cyanine monomer dye, is used to label hair cell nuclei in neuromasts as counter staining. After treatments, larvae were immediately euthanized with 4% paraformaldehyde and the hair cells were observed under a Leica SP2 AOBS confocal system. NTR was obtained by mixing neomycin with the red fluorescent dye, Texas red-X succinimidyl ester (Molecular Probes; dissolved in DMSO as 5 mg ml^{-1} stock), at a molar ratio of 150:1 and agitating at room temperature overnight. Furthermore, the E3 medium with 0.1 mg ml^{-1} Texas red-X was taken as the control. These experiments were repeated in triplicate for each tested antioxidant.

Statistical Analysis

All values were calculated and presented as the mean ± SEM. Data were evaluated using Student's *t*-test or by one-way analysis of variance with Bonferroni's corrected post-hoc comparisons. A *P*-value of < 0.05 was considered to be statistically significant.

Results

Beneficial Effects of L-ascorbic Acid, NAC, and Apocynin Treatment against Neomycin-induced Hair Cell Death

For quantification of hair cell loss induced by neomycin, the live 5 dpf zebrafish larvae were applied to the fluorescent dye FM1-43FX and the images in the lateral line were observed under a microscope. The obtained fluorescent images of the larval zebrafish lateral line showed convincingly and significantly lower or less number of neuromasts after neomycin exposure for 30 min. Very few visible neuromasts numbers confirmed the hair cell death in the larval lateral line induced by neomycin at the concentration of 20 μM (panel c of Figs. 1A, 2A and 3A). The optimal concentration of neomycin (20 μM) was used in the following studies, as this exposure condition leads to approximately half loss of neuromasts in each zebrafish larvae.

Evidence clearly demonstrated that L-ascorbic acid at concentrations of 2.5, 5, 10 and 20 μg ml^{-1} (14.2, 28.4, 56.8 and 113.6 μM) significantly ($P < 0.001$) attenuated neomycin-induced hair cell death (Fig. 1B). The visible neuromasts number with co-administration of L-ascorbic acid (Fig. 1Ad) was almost similar to the control. It is important to note that L-ascorbic acid alone did not influence hair cell survival at the tested concentrations, but remarkably induced hair cell death at a concentration > 20 μg ml^{-1}.

Furthermore, NAC treatment effectively suppressed neomycin toxicity as it showed a greater number of visible neuromasts (Fig. 2Ad) against neomycin-induced hair cell death. These data indicate that NAC may also play a key role in the protection of zebrafish hair cells. The tested concentrations of NAC at 100 and 200 μg ml^{-1} (0.6 and 1.2 mM) are more effective in the prevention of hair cell damage elicited by neomycin (Fig. 2B), whereas NAC alone did not influence hair cell survival.

Figure 3 shows the effect of apocynin co-administration against neomycin-induced hair cell death. We found that massive hair cell death occurred under 30-min neomycin exposure and was visibly ameliorated by apocynin treatment, evidenced by the reappearance of more neuromasts in the lateral line (Fig. 3Ad). The serial concentrations of apocynin, including 0.1, 0.25, 0.5 and 1 mM, were tested in this study. It was noted that apocynin at the concentration of 0.25 mM is maximally effective in preventing hair cell death induced by neomycin (Fig. 3B), and apocynin itself at the tested concentrations did not alter hair cell survival in zebrafish.

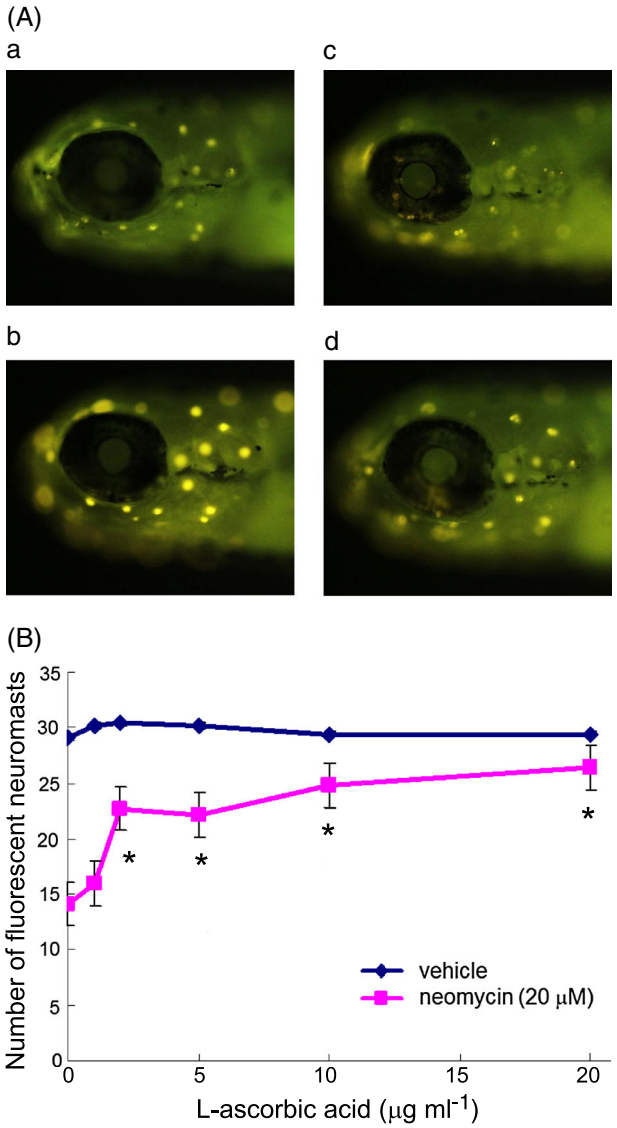

Figure 1. The effects of L-ascorbic acid treatment on neomycin-induced hair cell damage in zebrafish. (A) Neuromasts from zebrafish larvae were labeled with FM1-43FX (a–d images) after 30-min exposure to neomycin with/without L-ascorbic acid. (a) control; (b) 5 μg ml^{-1} L-ascorbic acid; (c) 20 μM neomycin; (d) 5 μg ml^{-1} L-ascorbic acid + 20 μM neomycin. (B) Dose-response curve showing recovered neuromasts stained with FM1-43FX as a function of the increasing L-ascorbic acid concentration under neomycin exposure. Values represent the mean ± SEM (n = 15–20 total fish per group, from triplicate experiments). A significant difference was determined using one-way ANOVA (*$P < 0.05$).

L-ascorbic Acid, NAC and Apocynin Co-administration Attenuates the Neomycin-induced ROS Production in Neuromasts

The neomycin-induced intracellular ROS production was determined by labeling the membrane permeable green fluorescent dye, DCF-DA. Figure 4 illustrates that exposure of neuromasts to neomycin resulted in a significant elevation of ROS levels, as hair cells within neuromasts appeared with green fluorescence

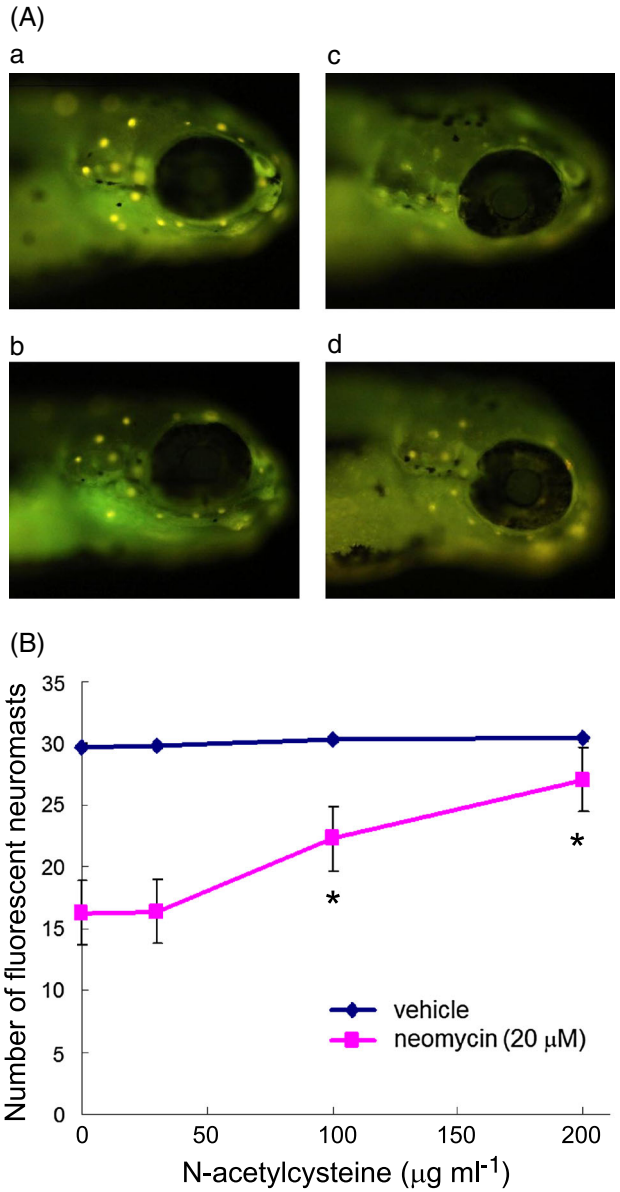

Figure 2. The effects of *N*-acetylcysteine treatment on neomycin-induced hair cell damage in zebrafish. (A) Neuromasts from zebrafish larvae were stained with FM1-43FX (a–d images) after 30-min exposure to neomycin with/without N-acetylcysteine. (a) control; (b) 100 μg ml^{-1} *N*-acetylcysteine; (c) 20 μM neomycin; (d) 100 μg ml^{-1} *N*-acetylcysteine + 20 μM neomycin. (B) Dose response curve showing recovered neuromasts stained with FM1-43FX as a function of increasing *N*-acetylcysteine concentration under neomycin exposure. Values represent the mean ± SEM (n = 15–18 total fish per group, from triplicate experiments). A significant difference was determined using one-way ANOVA (*$P < 0.05$).

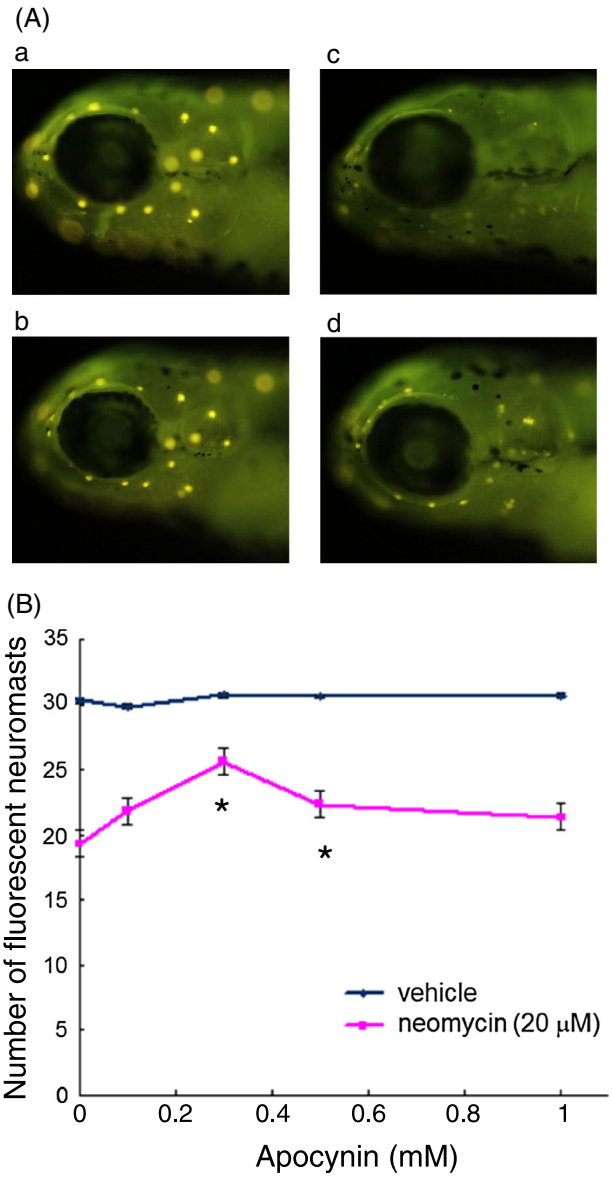

Figure 3. The effects of apocynin treatment on neomycin-induced hair cell damage in zebrafish. (A) Neuromasts from zebrafish larvae were stained with FM1-43FX (a–d images) after 30-min exposure to neomycin with/without apocynin. (a) control; (b) 0.25 mM apocynin; (c) 20 μM neomycin; (d) 0.25 mM apocynin + 20 μM neomycin. (B) Dose response curve showing recovered neuromasts stained with FM1-43FX as a function of increasing apocynin concentration under neomycin exposure. Values represent the mean ± SEM (n = 16–18 total fish per group, from triplicate experiments). Significant difference was determined using one-way ANOVA (*$P < 0.05$).

(Fig. 4B). However, co-administration of L-ascorbic acid, NAC and apocynin with neomycin noticeably attenuated neomycin-induced increases in ROS (Fig. 4C, D and E), compared with neomycin plus vehicle 0.1% DMSO exposure (Fig. 4F).

Texas-red Conjugated Neomycin Uptake Study

We assume that the beneficial effects of tested drugs in this study might be as a result of the reduction in sensitivity of neuromast hair cell to neomycin by interfering with transduction. Thus, we detected whether three tested drugs could block the uptake of neomycin conjugated with a fluorescence dye (Texas red) into neuromasts. Texas-red conjugated neomycin appeared red fluorescence that was more obviously observed in neomycin-exposed larvae (Fig. 5). Control neuromasts exposed to unconjugated Texas red alone did not show any uptake (Fig. 5A). However, Texas-red conjugated neomycin uptake and labeling (Fig. 5B) was not affected in co-administration of neuromasts with L-ascorbic acid, NAC and apocynin (Fig. 5C, D and E, respectively), indicating that these three drugs at the tested concentrations did not impact neomycin uptake. Moreover,

Journal of AppliedToxicology

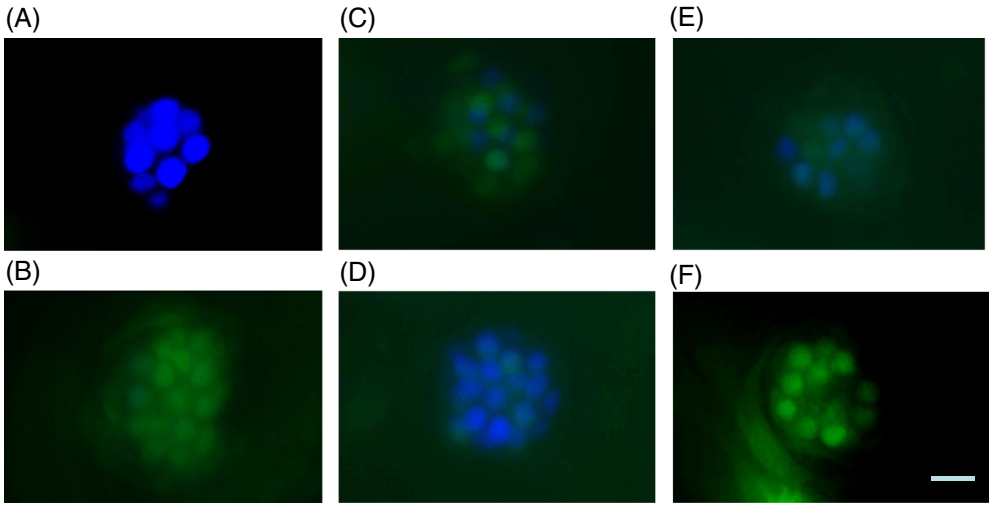

Figure 4. Detection of reactive oxygen species (ROS) by 2′,7′-dichlorofluorescein (DCF) in hair cells of the zebrafish lateral line treated with neomycin and combined compounds. Green fluorescence indicates ROS production after neomycin exposure. Blue dots are nuclei which were counter-stained with DAPI. (A) control; (B) 20 μM neomycin; (C) 20 μM neomycin + 5 μg ml^{-1} L-ascorbic acid; (D) 20 μM neomycin + 100 μg ml^{-1} *N*-acetylcysteine; (E) 20 μM neomycin + 0.25 mM apocynin and (F) 20 μM neomycin + 0.1% DMSO. Scale bar is presented as 10 μm and applied to all panels.

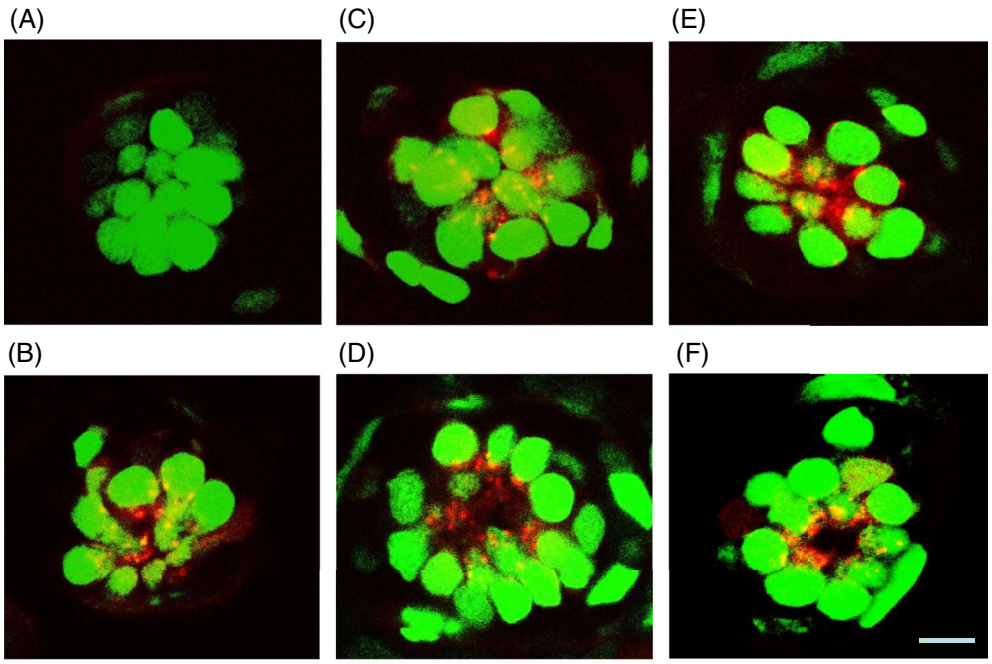

Figure 5. Examples of uptake of Texas-Red conjugated neomycin in neuromast of the zebrafish lateral line. Nuclei of hair cells were counter stained with green fluorescent YO-PRO-1. Normal uptake of Texas-Red conjugated neomycin appears red fluorescence and demonstrates multiple double-labeled hair cells. (A) control; (B) 20 μM neomycin; (C) 20 μM neomycin + 5 μg ml^{-1} L-ascorbic acid; (D) 20 μM neomycin + 100 μg ml^{-1} N-acetylcysteine; (E) 20 μM neomycin + 0.25 mM apocynin and (F) 20 μM neomycin + 0.1% DMSO. Scale bar is presented as 10 μm and applied to all panels.

nuclei of hair cells, counter stained with green fluorescent YO-PRO-1, were visibly similar in the three drug-treated neuromasts compared with the neomycin alone exposed neuromasts.

Discussion

In this study, we have demonstrated that neomycin-induced hair cell death in zebrafish larvae was effectively attenuated by co-administration of antioxidants including L-ascorbic acid, NAC and apocynin with neomycin exposure. Consistently, the experimental evidences indicate that a neomycin-induced increase in ROS production was inhibited by L-ascorbic acid, NAC and apocynin. As ROS plays a significant role in hair cell death leading to ototoxicity (Tabuchi *et al.*, 2011), the decreased ROS production by drug treatment may facilitate to coping the neomycin-induced hearing loss. Thus, the hair cell survival rescued by these drugs might be as a result of their antioxidant properties and could be promising protective agents for future studies. We further tested the therapeutic effects of the drugs against neomycin exposure, and we

observed that neomycin uptake by neuromasts was not ameliorated by L-ascorbic acid, NAC and apocynin co-administered with neomycin. It is assumed that controlling neomycin uptake may not contribute to the protective activity of these antioxidants on neomycin-induced oxidative damage in zebrafish hair cells.

Evidence from numerous laboratory animal and human studies indicates that hearing loss is linked to ageing, ototoxic drugs and noise exposure. The initial cellular basis for most hearing loss is damage and death of the mechanosensory hair cells that reside in the inner ear. Understanding how hair cell death by intrinsic and extrinsic challenges should lead to identification of novel therapeutic targets for protection of hearing loss. Because treatments with aminoglycosides often cause hearing loss, the larval zebrafish lateral line has been successfully used to screen some molecular compounds for the protection of hair cell death induced by aminoglycosides (Ou *et al*., 2009). In the present study, we observed the death of zebrafish lateral line hair cells in response to neomycin exposure at a concentration of 20 μM. The Texas-red fluorescent dye conjugated with neomycin was used further to detect the neomycin uptake. Our data clearly show that Texas-red conjugated neomycin-treated larvae exhibited red fluorescence, which indicates the large amount of neomycin uptake by neuromasts. Thus, the neomycin uptake is relevant to the toxicity in the zebrafish lateral line hair cells. It has been reported that aminoglycoside uptake plays a critical role in hair cell death (Alharazneh *et al*., 2011). Some *in vivo* labeling studies demonstrated that aminoglycosides initially enter through the apical pole of the hair cell by receptor-mediated endocytosis and subsequently sequestered in lysosome-like structure. Under continuous aminoglycosides exposure, lysosomes increase in size until they rapture, and then release their toxic contents into the cytoplasm, which results in cell death (Hashino *et al*., 1997).

Our findings are consistence with several previous studies, which reported the hair cell death induced by neomycin in zebrafish (Harris *et al*., 2003; Owens *et al*., 2009). The exact mechanism behind aminoglycosides-mediated ototoxicity remains unclear, however, initiating of apoptotic cell death pathways and excessive production of reactive free radicals occupied a major role (Forge and Schacht, 2000). The reduced neomycin uptake or accumulation of neomycin in neuromasts possibly decreases the hair cell death. Thus, it is wondered whether the protective activity of antioxidants on neomycin-induced hair cell death is associated with blockade of neomycin uptake. However, our results demonstrated that antioxidants did not affect neomycin uptake, based on Texas-red conjugated neomycin uptake. The current data are consistent with the previous report which showed that the uptake of a chemotherapeutic agent vincristine in human lung cancer PC-9 cells was not influenced by ascorbic acid at 25 μg ml^{-1} (Chiang *et al*., 1994). It is suggested that an ideally protective drug should inhibit intracellular death pathways triggered by neomycin or other aminoglycosides rather than blockade of aminoglycoside uptake. Thus, the tested antioxidants could have more potential applications against various causes of hair cell death, such as oxidative stress.

It has been well documented that excessive ROS production plays a key role in the process of cell death. Sha and Schacht (1999a) demonstrated that aminoglycosides bind to ferric iron (Fe^{III}) and form a redox-active complex (Fe^{II}-aminoglycoside) in the cytosol that can catalyzes ROS formation. Thus excessive production of cytosolic ROS can induce an apoptosis signaling cascade. Furthermore, toxic levels of ROS can damage cells by triggering various cell death mechanisms, such as caspase-dependent and -independent apoptosis and necrosis (Sha and Schacht, 1999a; Genestra, 2007; Karasawa and Steyger, 2011). Recently, it was suggested that the antioxidant drug treatment as an effective therapy to prevent/cure the aminoglycoside-induced hearing loss. Initial clinical evidence demonstrates the preventing effect of antioxidants such as NAC on aminoglycoside-elicited ototoxicity in dialysis patients (Feldman *et al*., 2012). Moreover, caffeic acid phenethyl ester, an important active component of honey bee propolis, has been reported to protect streptomycin-induced ototoxicity in rats, possibly via its antioxidant action (Bakir *et al*., 2013). As ROS might play a key role in killing the zebrafish lateral line hair cells, we proposed that treatment with antioxidant drugs could prevent the neomycin-induced hair cell death. Our current results clearly support this hypothesis that the reduced ROS production after L-ascorbic acid, NAC and apocynin treatment at the respective concentration followed by neomycin exposure could profit regeneration of hair cell number. Over expression of superoxide dismutase (SOD), a primary antioxidant enzyme in quenching the ROS, has been shown to possess more resistance against aminoglycoside-induced ototoxicity (Sha *et al*., 2001), which reveals the toxic role of excessive ROS in aminoglycoside-induced cell death. Previous studies also demonstrated the otoprotective effects of antioxidant compounds, such as alpha-lipoic acid, D-methionine and salicylate against aminoglycoside-induced ototoxicity in animal models (Conlon *et al*., 1999; Sha and Schacht, 1999b, 2000). In a similar fashion, the antioxidant drugs used in this study may play a significant role in ROS elimination and/or inhibit the ROS/free radical production. However, hair cell protection by antioxidants in the zebrafish lateral line could not assure protection in mammalian hair cells. Therefore, it requires further study in mammalian tissue.

The free radical scavenging properties of L-ascorbic acid, NAC and apocynin are well established in various studies against to various stress conditions. Ethanol-induced toxicity was attenuated by ascorbic acid in zebrafish (Reimers *et al*., 2006). The direct and indirect antioxidant properties of NAC and its capability of interacting with ROS has been reported in previous studies (Aruoma *et al*., 1989; Dekhuijzen, 2004). NAC can act as a precursor of glutathione (GSH) (Moldeus *et al*., 1985), hence treatment with NAC may enhance the GSH levels and decrease the neomycin-induced hair cell death. NADPH oxidase is a potential source for the production of ROS after several drugs exposure (Rybak and Whitworth, 2005). Thus apocynin, a specific inhibitor of NADPH oxidase, has been shown to inhibit intracellular ROS generation and prevent tissue damage against various stress conditions (Matsui and Cotanche, 2004; Chan *et al*., 2007; Chirino *et al*., 2008). A recent study by Choi *et al*. (2013) reported that apocynin treatment significantly decreased the ROS production in HEI-OC1 cell, and prevented the loss of neuromasts against cisplatin-induced ototoxicity in transgenic zebrafish. Moreover, it is important to note that apocynin at the concentration of 250 μM maximally prevented cisplatin-induced hair cells loss, and its protection did not increase with a concentration over 250 μM. Consistently, our current findings demonstrate that apocynin at 0.25 mM is maximally effective in preventing hair cell death induced by neomycin. Based on these evidences, we assume that apocynin may inhibit ROS production and protect hair cell death against neomycin exposure. These three

Journal of Applied Toxicology

antioxidants that protect against neomycin-induced hair cell death were identified in present study. Consistent with a previous report, our results demonstrated that there is no significant differential sensitivity to neomycin among neuromasts (Harris *et al*., 2003). With regards to the 'ideal' drug characteristics, antioxidants through these evaluations might be identified as particularly promising drugs for further *in vivo* mammal studies and clinical trials.

Conclusions

Antioxidant compounds are more effective in preventing aminoglycosides-induced toxicity, especially ROS-mediated hair cell death. Our findings showed that all three antioxidant compounds, including ascorbic acid, NAC and apocynin, are effective in controlling ROS production against neomycin exposure. The decreased ROS may help to prevent hair cell death. Our results conclude that ascorbic acid, NAC and apocynin may be developed as potential therapeutic drugs to treat neomycin-induced ototoxicity.

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Conflict of interest

The Authors did not report any conflicts of interest.

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