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Damage and threshold shift resulting from cochlear exposure to Paraquat-generated superoxide

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Abstract

Superoxide has been implicated as a contributing factor to cochlear pathology from a number of sources, including noise and ototoxic drugs. The effects of NADPH oxidase-dependent superoxide on the cochlea were investigated in the current study using paraquat (PQ). PQ is a toxic herbicide that causes tissue damage by generating superoxide through reduction of molecular oxygen in a reaction catalyzed by NADPH oxidase. The current study examined the effects of round window PQ administration on inferior colliculus (IC) evoked potential thresholds (EVP) and hair cell damage. Using implanted IC electrodes, chinchillas were tested for IC EVP thresholds before and after PQ exposure. Ears were exposed to PQ at one of four concentrations: 10, 5, 3 mM, and vehicle control. Thresholds were increased in a dose-dependent manner, and peaked between one and seven days post-exposure. Thresholds then showed a small amount of recovery before reaching PTS by Day 22. Outer and inner hair cell losses were consistent with PTS. The similarities between PQ ototoxicity and noise-induced hearing loss suggest the possibility of similar biochemical pathways involving superoxide.

Keywords

Paraquat; Superoxide; NADPH oxidase; Cochlea

1. Introduction

Recent studies of cochlear sensory cell damage resulting from noise, aging, and ototoxic chemicals have focused on the molecular phenomena that underlie cell death. Figuring prominently in much of this research has been the involvement of reactive oxygen species (ROS). ROS include oxygen-based molecules that have an unpaired electron in their outer shells (free radicals). ROS can create cell damage by breaking down molecules at different locations in and around cells (Evans and Halliwell, 1999). Overproduction of ROS can lead to protein and DNA damage (Halliwell and Gutteridge, 1999), can cause cell membrane breakdown through lipid peroxidation (Evans and Halliwell, 1999), and can act as a putative trigger for apoptotic cell death (Halliwell and Gutteridge, 1999).

Increases in ROS are associated with drug- and noise-induced cochlear pathologies. Cochlear damage from cisplatin has been linked to changes in the balance of ROS and antioxidants in the ear. Cisplatin-induced hearing loss has been linked to increased ROS formation in cochlear tissue in vitro (Clerici et al., 1996; Dehne et al., 2001); and increases in lipid peroxidation (Rybak et al., 2000) and alterations in antioxidants levels and activity in vivo (Ravi et al., 1995). Pretreatment with antioxidants has also been shown limit cochlear damage and hearing loss from cisplatin exposure (Campbell et al., 1996; Kopke et al., 1997). Similar observations

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have been made with carboplatin, regarding increases in ROS after administration (Husain et al., 2001) and protection with antioxidant pretreatment (Lockwood et al., 2000). Additionally, Clerici and Yang (1996) were the first to show that generators of superoxide, hydrogen peroxide, and the hydroxyl radical induced significant shifts in the compound action potential threshold when introduced into the perilymph (Clerici and Yang, 1996).

The ROS/antioxidant pathways have also been implicated as significant contributor to aminoglycoside-induced cochlear damage. Gentamicin (Priuska and Schacht, 1995; Hirose et al., 1997; Clerici et al., 1996) and kanamycin (Clerici et al., 1996) stimulate increases in ROS in cochlear cell cultures. Antioxidant treatments have been used to attenuate cochlear damage and hearing loss from amikacin (Conlon et al., 1999) and gentamicin (Sha and Schacht, 2000). Treatment with iron chelators to interrupt ROS generation through Fenton chemistry has been shown to attenuate aminoglycoside-induced damage. Salicylate, which acts as both an iron chelator and antioxidant, has also been used to protect against gentamicin in vivo (Sha and Schacht, 1999). Superoxide dismutase (SOD) has also been shown to protect against aminoglycoside ototoxicity through in vivo supplementation of SOD prior to gentamicin exposure (Takumida et al., 1999). Additionally, overexpression of SOD1 in transgenic mice rendered the mice more resistant to kanamycin ototoxicity (Sha et al., 2001a).

Significant increases in the ROS superoxide ($O_2^{\bullet-}$) (Yamane et al., 1995) and the hydroxyl radical ($OH^{\bullet}$) (Ohlemiller et al., 1999) have been detected in the cochlea following a noise exposure. Additionally, pretreatment with antioxidants or antioxidant enzymes, which scavenge ROS or catalyze reactions that convert them into other molecules, renders the ears less susceptible to noise damage (Seidman et al., 1993; Quirk et al., 1994; Hu et al., 1997; Yamasoba et al., 1999; Kopke et al., 2000, 2002; Hight et al., 2003).

While the exact sources of ROS in the cochlea are not completely understood, the electron transport chain in the mitochondria is thought to be a major source of intracellular superoxide (Evans and Halliwell, 1999). The hair cells are known to be highly demanding of energy (Thalmann et al., 1975) and very oxygen consumptive. Superoxide can accumulate at the mitochondria as an inefficient product of respiration (Halliwell and Gutteridge, 1999). With high level noise exposure, the demand for energy increases in the hair cells, taxing the mitochondria further. Consequently, more and more superoxide is generated in higher quantities. In addition, high level noise exposures can influence cochlear blood supply, leading to ischemia/reperfusion (Yamane et al., 1995), which may also contribute to the ROS that appear in the cochlea during and after toxic insults. The increased superoxide levels may lead to direct damage to hair cells and/or increase the generation of more toxic ROS, including: hydrogen peroxide (through a reaction catalyzed by superoxide dismutase), the hydroxyl radical (through Fenton chemistry), and peroxynitrite (through a reaction of superoxide with nitric oxide) (Halliwell and Gutteridge, 1999).

Superoxide can also be generated through enzymatic reactions, including reactions catalyzed by xanthine–xanthine oxidase (Clerici and Yang, 1996) or NADPH oxidase. NADPH oxidase has been studied extensively in neutrophils and phagocytic cells (Heyworth et al., 1992). In these cells, NADPH oxidase is activated in the presence of bacteria or foreign invaders. When activated, NADPH oxidase catalyzes a reaction in which NADPH donates an electron to molecular oxygen to form superoxide. The generated superoxide molecules can work against foreign microbes directly, or can be converted into other ROS (hydrogen peroxide, the hydroxyl radical, etc.) which act as antimicrobial agents (Heyworth et al., 1992). Recently, increases in the activity of NADPH oxidase have been detected in the cochlea following a noise exposure (Ramkumar et al., 2004), introducing the possibility that NADPH oxidase could be involved in the superoxide accumulation that has been detected in the cochlea after noise.

Paraquat (PQ) provides a tool that allows examination of the effects of intracellular NADPH oxidase-dependent superoxide generation in the cochlea. PQ is a toxic herbicide known for its toxicity in lung tissue (Bus and Gibson, 1984;Suntres, 2002). PQ in solution exists as a dication (PQ^{++}) which is readily taken up into cells. In a reaction catalyzed by NADPH oxidase, intracellular NADPH donates an electron to PQ^{++} to form the paraquat cation (PQ^+). PQ^+ then donates its electron to molecular oxygen, creating superoxide, and returning PQ to its dication state (Bus and Gibson, 1984).

The introduction of PQ into the cochlea provides the conditions for examination of cochlear cell death and hearing loss patterns resulting directly from intracellular NADPH oxidase-dependent superoxide. Specifically, in the current study, the appearance of any temporary threshold shift (TTS) from PQ was assessed, along with measures of permanent threshold shift (PTS) and loss of outer (OHC) and inner hair cells (IHC). PQ provides the opportunity to evaluate the potential for NADPH oxidase-dependent superoxide to cause cochlear damage and hearing loss independent of the mechanical stress and other damage processes associated with traumatic noise exposure.

2. Methods

Twelve adult chinchillas (24 ears) weighing between 400 and 700 g were divided into four different experimental groups ($n = 6$ ears per group). Prior to surgical procedures and between test times, the animals were housed in a quiet colony. All procedures involving use and care of the animals were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

2.1. Inferior colliculus electrode implantation

To test hearing sensitivity, all animals were implanted with inferior colliculus (IC) electrodes. The animals were intramuscularly injected with ketamine (60 mg/kg) and acepromazine (0.5 mg/kg) to achieve a deep plane of anesthesia. The animals were then placed in a stereotaxic apparatus. The electrodes were inserted bilaterally into the region of the IC. A ground electrode was placed in the rostral cranium (Henderson et al., 1973). The animals were allowed to recover for two weeks prior to evoked potential testing.

2.2. Evoked potential testing

During evoked potential threshold testing, the chinchillas were awake and secured in a restraint tube (Snyder and Salvi, 1994). Test stimuli consisted of alternating phase tone bursts at frequencies of 0.5, 1, 2, 4, and 8 kHz. Signals were generated using Tucker Davis Technologies (TDT) SigGen software. Each tone burst had a 2 ms rise/fall time, a 1 ms plateau with a cosine ramp, and were presented at a 21/s rate. Signals were generated digitally with a TDT D/A converter at a 50 kHz sampling rate, and were 20 kHz low-pass filtered. Signals were routed to an earphone (Etymotic ER-1) that was inserted into the canal of the test ear. Acoustic stimuli were calibrated prior to each testing session, by inserting the earphone into a 2 cc coupler and recording output with a probe tube microphone (Etymotic ER-7C). The evoked responses were amplified by 20,000 times by a TDT Head-stage-4 bioamplifier, and bandpass filtered at a range of 100–3000 Hz. One hundred samples were taken at each stimulus level. Averaged responses were displayed on a PC monitor using TDT BioSig software. The level of the signal was decreased in 5-dB steps from 90 to a point at which no response was detectable. Threshold was determined as the midpoint between the level at which a clear response was elicited and the next lowest level.

2.3. Paraquat solution preparation and application surgery

Paraquat dichloride hydrate (Sigma chemicals M2254) was first dissolved in distilled water to create a 0.1735 M stock solution. The stock solution was then diluted in 0.2 M phosphate-buffered saline (PBS) to create the 10, 5 and 3 mM PQ solutions. The animals were again intramuscularly injected with ketamine (60 mg/kg) and acepromazine (0.5 mg/kg) to achieve deep anesthesia. An incision was made to expose the auditory bulla on the right side. A hole was then formed in the bone using a scalpel. A portion of the basal turn of the cochlea, and a portion of the round window were identified under an operating microscope. Using a 10- μ L microsyringe (World Precision Instruments Inc) 10 μ L of one of four PQ concentrations was applied to the round window: 10 mM PQ, 5 mM PQ, 3 mM PQ, or 0.2 M PBS. Ears were randomly assigned one of the four concentrations, but no animal received the same PQ concentration on both ears. After application of the PQ onto the round window niche, the hole in the auditory bulla was covered with dental cement, which was given ample time to harden prior to suture of the incision (silk 3/0 suture). The process was then repeated for the left ear of the animal. For each ear, the bulla was open for twenty-thirty minutes. All of the 10-L drops were allowed to diffuse across the round window membrane. Upon cochlear removal at the conclusion of the study, all middle ear spaces were examined for any signs of infection.

2.4. Assessment of threshold shift

To assess PQ-induced threshold shift in the animals, IC evoked potential testing was performed at seven time points following PQ application: 24 h post-application (Day 1), two days post (Day 2), three days post (Day 3), seven days post (Day 7), 15 days post (Day 15), and 22 days post (Day 22). Pre-exposure thresholds were subtracted from the seven threshold measurements to calculate threshold shift at each time point. The 22-day measurement provided the data for calculation of PTS.

2.5. Histology

After the final physiological measurements were obtained, the animals were rapidly sacrificed using inhalation of CO₂. Both auditory bullae were removed from each animal. The stapes were removed from each cochlea, and the cochleae were then perfused with 10% buffered formalin. The organs of Corti were then dissected out from the cochleae, and each was double stained with propidium iodide and FITC-phalloidin. The propidium iodide stained the nuclei of the hair cells and the FITC-phalloidin permitted examination of the stereocilia and cuticular plate. The stained specimens were mounted on slides and examined under light microscopy. Missing OHC and IHC were counted along the entire length of the cochlea to allow assembly of a cochleogram for each ear.

2.6. Statistical analysis

A three-factor ANOVA (concentration $\times$ frequency $\times$ time) was used to analyze differences between the means of the four experimental groups (10, 5, 3 mM, and control PBS) across the five different test frequencies at the seven different time points post-PQ application. Concentration and Frequency were analyzed as between-subjects variables, and Day post-PQ application was analyzed as a repeated measure. If a significant main effect occurred for PQ concentration or frequency, post-hoc testing with Tukey A tests was performed to delineate the nature of the differences. If a significant main effect of Day occurred, the different days were compared with paired subjects *t*-tests. For comparing PTS differences between concentration groups, two-way ANOVAs (concentration $\times$ frequency) were used, with Tukey A post-hoc testing as needed. For comparing differences between concentration groups with respect to OHC and IHC loss, two-way ANOVAs (concentration $\times$ distance along cochlea from the apex) were used, with post-hoc Tukey A tests to delineate any main effect of concentration.

3. Results

3.1. Pre-exposure thresholds

One ear was deleted from the study due to middle ear pathology, leaving groups of $n = 6$ for the 10 mM PQ, the 5 mM PQ, and the vehicle control groups, and $n = 5$ for the 3 mM PQ group. Statistical analysis (two-way ANOVA with Tukey post-hoc test) revealed a significant main effect of frequency, but no main effect of concentration or concentration $\times$ frequency interaction. The absence of differences between groups prior to PQ exposure gave confidence that the differences observed in threshold shift between groups were a result of the differing concentrations of PQ solution that each group received.

3.2. Threshold shift induced by paraquat

Recovery curves for the five frequencies tested over the 22-day testing period for 10 mM PQ, 5 mM PQ, and 3 mM PQ are shown in Figs. 1-3, respectively. By the 24 h time point, mean thresholds for the three concentrations of PQ are significantly greater than pre-exposure thresholds ($p < 0.01$). Thus, each concentration of PQ induced threshold shifts of varying degree. During the period between the 24 h test and the Day 22 test, all three groups' mean threshold shifts showed recovery. The control group receiving only the PBS vehicle solution showed no significant shifts relative to pre-exposure baselines at any frequencies or time points post-exposure ($p > 0.05$).

The means of the 10 mM group peaked at Day 1 and began to recover between Days 3 and 7 ($p < 0.01$). The means of the 5 mM group peaked at Day 7, and recovered between Days 7 and 15 ($p < 0.001$). The means of the 3 mM group were flat from Day 1 through Day 7 ($p > 0.05$) and then recovered from Day 7 through Day 15 ($p = 0.001$) and Day 22 ($p < 0.05$). The PQ treatment led to a change in hearing threshold across the whole tested frequency range, implying that the PQ, and resulting superoxide molecules, affected cells from base to apex.

3.3. Dose response of paraquat on PTS

Fig. 4 shows the mean of each concentration group (including the vehicle control group) across frequency. Dose response distributed as expected, with 10 mM inducing the greatest PTS ($p < 0.001$) with mean shifts on the order of 30–45 dB. Five millimolar induced means of 5–25 dB PTS. Three millimolar induced means of 0–10 dB shifts. Five millimolar and 3 mM were not statistically different from one another, due in large part to high variability.

3.4. OHC and IHC loss

Figs. 5 and 6 show OHC and IHC loss for the three PQ groups and the PBS control group. Patterns of loss are qualitatively consistent with the PTS patterns (Figs. 5 and 6 compared to Fig. 4). Ten millimolar induced the greatest OHC and IHC loss ($p < 0.001$), with more damage occurring at the basal end than at the middle or apex. Five millimolar induced significantly less OHC loss than 10 mM, and only sporadic IHC loss. Three millimolar induced no OHC loss until the point roughly 60% from the apex, at which a small basal lesion was detected. Similarly, a small basal IHC lesion was created by the 3 mM PQ. Overall, 3 mM was not statistically different from the vehicle controls, which induced no OHC or IHC loss.

4. Discussion

4.1. NADPH-oxidase dependent superoxide

The current project was undertaken to examine the cochlear damage and threshold shift patterns induced by PQ delivered to the cochlea via the round window. The damage is presumed to occur through a mechanism involving superoxide radicals generated from PQ and NADPH.

PQ is known to activate NADPH oxidase, which catalyzes a reaction that converts molecular oxygen to superoxide (Bus and Gibson, 1984), which can cause damage through a variety of mechanisms (Halliwell and Gutteridge, 1999). PQ is readily taken up by cells and can be used as a model for intracellular super-oxide generation (Suntres, 2002). The round window administration of PQ allowed direct analysis of hearing sensitivity changes and hair cell loss induced by cochlear exposure to PQ. The cochlear damage and threshold shift induced by PQ confirms the presence of NADPH oxidase in the hair cells and provides evidence that NADPH oxidase-dependent superoxide by itself is a potent mechanism for induction of hair cell death. Exposure of cochlear cell cultures to PQ and a superoxide dismutase mimetic provided evidence that superoxide is able to induce cell death without needing to be converted to more toxic free radicals (Nicotera et al., 2004). Noise is likely to be damaging cells through a complex series of ROS, reactive nitrogen species, and a variety of cell death pathways. Superoxide is a likely component in this complex cell death sequence. The extent to which, if at all, NADPH oxidase is involved in the generation of superoxide in conditions of noise or ototoxic drugs is currently unknown, and future investigation of the role of NADPH oxidase in noise- and drug-induced hair cell death may be warranted.

4.2. Appearance of TTS

PQ at 10, 5, and 3 mM induced a maximal threshold shift of 20–60 dB, and then partially recovered to a lower PTS. Peak threshold shift occurred between one and seven days, and most of the recovery occurred by Day 15. The observed recovery was small, but statistically significant, in each of the three exposure conditions. Previous studies have examined the effects of PQ on the hair cells in vitro finding a toxic effect of PQ on the hair cells (Nicotera et al., 2004) as well as evidence of both apoptotic and necrotic cell death (Nicotera et al., 2002). Results from the current study reiterate the finding of toxic effects of PQ on hair cells, and add the novel finding of TTS from PQ-generated superoxide.

Since PQ is damaging the cochlea through ROS generation, the results suggest that ROS activity leads to both TTS and PTS changes in the cochlea. A number of pathologies have been implicated as sources of TTS following noise exposure, including damage to stereo-cilia (Pickles et al., 1987; Saunders and Flock, 1986), excitotoxic damage to the IHC-afferent auditory nerve fiber junctions (Spoendlin, 1971; Puel et al., 1996), and damage to supporting cells (Nordmann et al., 2000; Wang et al., 2002). Superoxide might induce any of these three types of pathology. A study in which PQ was applied to cochlear cell cultures found that damage to stereocilia was evident in a number of cells that had not died at the time of examination (Nicotera et al., 2004).

4.3. Relationship between hearing loss and cochleogram data

Both OHC and IHC cochleograms showed the same dose response patterns as the hearing loss data. The 10 mM group showed the highest OHC loss, IHC loss, and PTS. Five millimolar showed the next greatest OHC loss, IHC loss, and PTS. The 3 mM group showed minimal losses all three categories, with just a small amount of OHC loss (~20%) in the basal region, a small peak of IHC loss in the base, and a small amount of high frequency PTS. The control group showed no significant changes in PTS, OHC loss, or IHC loss.

Consistent with other forms of cochlear pathology (noise, ototoxicity, aging), the OHC appear more vulnerable to PQ-induced damage than the IHC. In animals that showed significant IHC damage, there were also large OHC lesions. The reverse, significant IHC damage in the absence of OHC damage, was not true of any of the animals. Overall, a substantial portion of the hearing sensitivity changes observed after PQ exposure can be attributed to loss of OHC and IHC, but permanent PQ-induced damage to other structures in the cochlea (supporting cells, stria vascularis, stereocilia, eighth nerve fibers) cannot be ruled out. As with the threshold shift data,

variability in the cochleogram data was substantial, which is likely a reflection of individual animals' inherent differences in susceptibility to superoxide and ROS damage and to the variability in actual number of PQ molecules to which each cochlea was exposed.

4.4. Threshold shift across frequencies

One of the hallmarks of aminoglycoside (Forge and Schacht, 2000) or cisplatin ototoxicity (Nakai et al., 1982; Böheim and Bichler, 1985) is progressive hearing loss starting with the high frequencies. This progressive high frequency hearing loss is the result of progressive loss of hair cells in the basal portion of the cochlea. The vulnerability of basal hair cells has been linked to a lower level of antioxidant defenses in the basal hair cells, relative to the apical hair cells (Sha et al., 2001b). Additionally, drug tracer studies have found that drugs delivered to the cochlea through the round window will be present in greater quantities in the base than in the apex (Salt and Ma, 2001; Laurell et al., 2002). Yet in the current study, though mean threshold shifts for each of the three concentrations of PQ were higher in the high frequencies and mean hair cell losses were greater in the basal portion of the cochlea, differences were not statistically significant. The damage to cells in the apex does provide evidence that the PQ, when applied to the round window, can influence hair cells across the length of the cochlea. But the lack of differential vulnerability to PQ between the base and the apex is surprising. The result could be due to the magnitude of the damage induced by PQ at the higher concentrations. In many ears, the OHC were nearly completely lost across the entire length of the cochlea, suggesting the possibility that the magnitude of the cochlear insult from PQ may have overwhelmed any disparate cochlear defenses. Additionally, variability between animals in terms of both threshold shift and hair cell loss was high, and may have obscured a difference in vulnerability between the base and apex. Finally, Sha et al. (2001b) found that basal hair cells had lower levels of glutathione than apical cells, a finding that possibly accounts for the difference in vulnerability between these groups of cells. In the case of PQ in cell cultures, there was evidence that the PQ-induced superoxide radicals were able to induce cell death without being converted into the more hazardous hydroxyl radicals or peroxynitrite (Nicotera et al., 2004). Since glutathione affects those more toxic free radicals, it cannot be ruled out that differences in glutathione would not have an effect on susceptibility to PQ toxicity, since some portion of that toxicity is the result of the action of superoxide itself.

5. Conclusion

As the knowledge base of the molecular processes underlying cell death in the cochlea grows, the involvement of ROS in pathologies resulting from noise, ototoxic drugs, and aging becomes clearer. Whether through increases in ROS production or depletion of antioxidant defenses, an imbalance in the ROS/antioxidant ratio can lead to cell death due to damage to proteins, DNA, and cell membranes. Additionally, ROS can act as a putative trigger of apoptosis. Apoptotic cell death has been implicated as major cell death process in the expansion of the hair cell lesion after noise exposure (Hu et al., 2002).

Endogenous levels of antioxidants that can counteract the production of ROS may be an important factor in determining an individual's inherent susceptibility to cochlear damage from noise (Jacono et al., 1998), ototoxic drugs, or aging. Altering antioxidant levels through exogenous antioxidant delivery has consistently been shown to lower animals' susceptibility to all three of these forms of pathology. Additionally, greater understanding of the downstream events that are set in motion in a cell after exposure to ROS will provide even more understanding of cell death from ROS-related pathology and will provide more potential avenues for intervention to prevent cell death in the cochlea. The use of PQ as a model of intracellular superoxide generation provides a unique look at the damage that can be induced by this type of ROS, and represents another tool to examine ROS-related cell death patterns in the cochlea and the events that occur downstream of ROS exposure that lead to cell death. The

finding of NADPH oxidase-dependent superoxide as a potent ototoxic agent provides cause to investigate the role of NADPH oxidase in ROS-related damage in toxic cochlear insults.

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Abbreviations

PQ, Paraquat; IC, inferior colliculus; EVP, evoked potential; ROS, reactive oxygen species; SOD, superoxide dismutase; TTS, temporary threshold shift; PTS, permanent threshold shift; OHC, outer hair cells; IHC, inner hair cells; PBS, phosphate buffered saline; TDT, Tucker Davis technologies.

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Fig 1. Recovery patterns of the 10 mM concentration group for the five frequencies tested, plotted as threshold shift in dB against the Day of testing post-exposure. Error bars are +1 standard deviation.

Fig 2. Recovery patterns of the 5 mM concentration group for the five frequencies tested, plotted as threshold shift in dB against the Day of testing post-exposure. Error bars are +1 standard deviation.

Fig 3. Recovery patterns of the 3 mM concentration group for the five frequencies tested, plotted as threshold shift in dB against the Day of testing post-exposure. Error bars are +1 standard deviation.

Fig 4. PTS for the four experimental groups plotted as dB PTS across frequency. Error bars are +1 standard deviation.

Fig 5.
Mean OHC loss cochleograms for the four concentration groups, plotted as % OHC lost against % distance from the apex of the cochlea.

Fig 6.
Mean IHC loss cochleograms for the four concentration groups, plotted as % IHC lost against % distance from the apex of the cochlea.