

Effects of delayed and extended antioxidant treatment on acute acoustic trauma

CHUL-HEE CHOI¹, KEJIAN CHEN², XIAOPING DU³, ROBERT A. FLOYD^{4,5},
& RICHARD D. KOPKE^{3,4,6}

¹Audiology & Speech-Language Pathology & Research Institute of Biomimetic Sensory Control, College of Medical Sciences, Catholic University of Daegu, Kyungsansi, South Korea, ²Department of Defense Spatial Orientation Center, Naval Medical Center, San Diego, California, USA, ³Hough Ear Institute, Oklahoma City, Oklahoma, USA, ⁴Experimental Therapeutics Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA, ⁵Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA, ⁶Department of Otorhinolaryngology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

(Received 3 April 2011; Accepted 26 June 2011)

Abstract

Objective: Hair cell death caused by acute acoustic trauma (AAT) reaches a secondary maximum at 7–10 days after noise exposure due to a second oxidative stress. Therefore, this study tested the effects of a combination of hydroxylated alpha-phenyl-tert-butyl nitron (4-OHPBN), N-acetyl-L-cysteine (NAC) and acetyl-L-carnitine (ALCAR) on AAT when the duration of treatment was extended over the period of 7–10 days after noise exposure as well as when the initial treatment was delayed 24 to 48 h after noise exposure. **Methods:** Thirty chinchilla were exposed to a 105 dB octave-band noise centred at 4 kHz for 6 h and received the following treatments: (1) noise + saline (2–5) 4-OHPBN (20 mg/kg) + NAC (50 mg/kg) + ALCAR (20 mg/kg) intraperitoneally injected beginning 24 or 48 h after noise exposure twice daily for the next 2, 8 or 9 days. Auditory brainstem response (ABR) threshold shifts, outer hair cell (OHC) counts and organ of Corti immunohistochemistry were analyzed. **Results:** The combination administration decreased ABR threshold shifts, inhibited OHC loss and reduced 4-hydroxynonenal (4-HNE) immunostaining. Significant decreases in the threshold shifts and reduction in OHC loss were observed with a shorter delay before starting treatment (24 h) and longer duration (9 days) treatment. **Conclusions:** These results demonstrate that the administration of antioxidant drugs extended up to 10 days after noise exposure can effectively treat AAT in a chinchilla model. This may provide significant and potentially clinically important information about the effective therapeutic window for AAT treatment.

Key Words: Hearing loss, antioxidant drugs, hydroxylated alpha-tert-butyl nitron (4-OHPBN), N-acetyl-L-cysteine (NAC), acetyl-L-carnitine (ALCAR), auditory brainstem response (ABR), outer hair cell loss, 4-hydroxy-2-nonenal (4-HNE), nitrotyrosine (NT)

Introduction

Acute acoustic trauma (AAT) results in oxidative stress exceeding the capacity of the antioxidant defence mechanisms in the cochlea through the excessive production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipid peroxidation. Oxidative stress leads to hair cell death by apoptosis or to a lesser extent by necrosis, causing hair cell damage and sensorineural hearing loss. Pharmacological approaches for the prevention

or treatment of AAT-induced hearing loss have been developed using antioxidant drugs, which increase antioxidant defences in the cochlea. The antioxidant drugs in this study include N-acetyl-L-cysteine (NAC), a glutathione (GSH) prodrug, and acetyl-L-carnitine (ALCAR), a mitochondrial biogenesis agent, as well as 4-hydroxy phenyl N-tert-butyl nitron (4-OHPBN), a nitron-based free radical trap and an inhibitor of inducible nitric oxide synthase [1–6].

Correspondence: Chul-Hee Choi, PhD, The Catholic University of Daegu, Kyungsanbukdo Kyungsansi Hayangup Gumgokri, South Korea, 712-702. Tel: +82-53-850-3185. Fax: +82-53-850-3383. E-mail: cchoi@cu.ac.kr

NAC has been shown to be effective in protecting hair cells from AAT-induced damage presumably by primarily increasing levels of GSH [3–5], while ALCAR inhibits age-related and AAT-induced hearing loss caused by mitochondrial injury [2,7–9]. Recently, 4-OHPBN was shown to be effective in treating AAT [6]. 4-OHPBN reduced AAT in a dose-dependent manner (from 10 to 75 mg/kg). In addition, administration of 4-OHPBN in combination with other antioxidant drugs [4-OHPBN (50 mg/kg) plus NAC (100 mg/kg) and 4-OHPBN (20 mg/kg) plus NAC (50 mg/kg) plus ALCAR (20 mg/kg)] has greater efficacy, compared with each antioxidant drug alone and at lower dosages based partly on historical data. The three-drug combination of 4-OHPBN plus NAC plus ALCAR showed the strongest synergistic effect in treating AAT, since each agent can target a distinctly different mechanism of AAT injuring.

The main objective of our laboratory is to develop realistic pharmacological interventions for AAT that can be administered to combatants exposed to explosions, blasts or loud noises in the military environment and to workers exposed to hazardous noise levels in the civilian environment. Our previous study suggested that the three-drug combination initially given 4 h after noise exposure can be utilized as rescue agents for treating hearing loss in animals with AAT [6]. However, although early treatment of AAT is essential in many clinical situations (especially in the military environment), the treatment for AAT might be delayed for 1–2 days after noise exposure. It is clinically important to understand the therapeutic window for the three-drug combination treatment. Therapeutic effectiveness of these antioxidant drugs may depend on the timing and duration of treatment. The effective therapeutic window for antioxidant drugs was reported to be 1–4 h after noise exposure [5,6]. However, when the single administration of NAC or ALCAR was initiated 12 h after noise exposure, therapeutic effects were not shown [10]. Due to the synergistic effect of the three-drug combination shown in our previous study, the three-drug combination may expand the therapeutic time window for AAT. In addition, the duration of treatment should be associated with the time course of ROS/RNS formation in the cochlea. It has been shown that there is an increase in free radicals in the cochlea during noise exposure, immediately after noise exposure, and for a few hours or several days after noise exposure [11–13]. Thus, in previous studies, the duration of treatment was up to 3 days after noise exposure [5,14]. Recently, it has been shown that an increase in free radical formation also occurs 7–10 days after noise exposure. This is referred to as a secondary oxidative burst and results in delayed cell death [14,15]. A pharmacological intervention extending up to 10 days could perhaps counter this secondary oxidative burst.

Therefore, the present study was performed to test the hypotheses that the combination of antioxidant drugs (4-OHPBN plus NAC plus ALCAR) would be more effective in treating AAT if the duration of treatment was extended over the period of 7–10 days after noise exposure. We tested whether delaying the initial treatment up to 1–2 days after noise exposure altered the outcomes. We tested these hypotheses by assessing ABR threshold shifts, the number of missing outer hair cells (OHCs), and 4-hydroxynonenal (4-HNE) and nitrotyrosine (NT) as markers of ROS and RNS formation.

Methods

Animals

Animal preparation and experimental methods were similar to those previously described, except for the duration and timing of treatment and immunohistochemical assays [6]. The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the Office of Naval Research and the Oklahoma Medical Research Foundation (OMRF).

Thirty 3–5-year-old female *chinchilla laniger* (Moulton Chinchilla Ranch, Rochester, MN) weighing 500–850 g were used in this study. The animals were housed in plastic cages in the OMRF animal facility with free access to a standard chinchilla diet (Mazuri Chinchilla Diet, 5MO1, PM1 Nutrition International Inc., Brentwood, MO) and tap water. Ambient noise levels measured in our animal facility (68–72 dB SPL at 70–80 Hz) were within permissible normal noise levels recommended by the National Institute for Occupational Safety and Health. Five groups of six randomly assigned animals each were as follows: (1) a control group, which received only saline after noise exposure; (2) an experimental group treated for 3 days with injections starting 48 h after noise exposure; (3) an experimental group treated for 8 days with injections starting 48 h after noise exposure; (4) an experimental group treated for 3 days with injections starting 24 h after noise exposure; and (5) an experimental group treated for 9 days with injections starting 24 h after noise exposure. Table I shows the relationship between the specific groups and the treatments that they received (Table I). 4-OHPBN was synthesized at OMRF using a previously described method [6]. For administration, this chemical was dissolved in dimethyl sulfoxide (DMSO, 0.8 ml/100 mg, Sigma-Aldrich Inc., St. Louis, MO) at 37 °C, then polyethylene glycol (PEG) 400 (0.8 ml/100 mg, Sigma-Aldrich Inc., St. Louis, MO) was added. Sterile saline (0.4 ml/100 mg) was added just before injection. A combination of 20 mg/kg of 4-OHPBN, 50 mg/kg of NAC (Hospira Inc., Lake Forest, IL) and 20 mg/kg of ALCAR (Sigma-Aldrich

Table I. Different groups with different treatment delays and durations.

Groups	Treatment
Group 1	Noise + saline (24 h delay with 9-day treatment)
Group 2	Noise + three-drug combination (48 h delay with 3-day treatment)
Group 3	Noise + three-drug combination (48 h delay with 8-day treatment)
Group 4	Noise + three-drug combination (24 h delay with 3-day treatment)
Group 5	Noise + three-drug combination (24 h delay with 9-day treatment)

Inc., St. Louis, MO) was intraperitoneally injected into the experimental animals. Experimental animals received the initial injection at 24 or 48 h after noise exposure and additional injections twice daily for the next 2, 8 or 9 days. In experimental groups 3 and 5, the treatments continued for 10 days after noise exposure with 48 or 24 h of delay before treatment initiation and either 8 or 9 days of actual treatment. Equal volumes of carrier solution (DMSO, PEG 400, and sterile saline, 2:2:1 ratio) were injected into group 1 using the same method as in the experimental animals. The carrier solution (saline) for group 1 was paired with the three-drug combination experimental group treated for 9 days with injections starting 24 h after noise exposure.

Noise Exposure

During noise exposure, two animals at a time were placed in two small wire restraint cages on a wooden plate. These animals each wore a breeding collar and were exposed to a 105 dB SPL octave-band noise centred at 4 kHz for 6 h in a sound isolation booth [Industrial Acoustics Company (IAC), New York, NY]. The noise was digitally generated by a Tucker Davis Technologies (TDT, Alachua, FL) device and passed through a real-time attenuator (TDT, RP2), filtered, amplified with a preamplifier (QSC audio power, Costa Mesa, CA) and transduced with a high frequency acoustic driver and an acoustic speaker (JBL 2350, Northridge, CA) suspended from the ceiling of the sound booth and positioned directly above the wire cages. During noise exposure, the noise level was continuously monitored by a PULSE software system [B&K Sound & Vibration Measurement (version 10.0), Norcross, GA] with a condenser microphone (B&K 2804, Norcross, GA) placed between the two wire cages at the level of the animals' heads and coupled to the preamplifier.

Auditory Brainstem Responses

Auditory brainstem response (ABR) for both ears of each animal was measured before initial noise

exposure (baseline threshold), immediately after and then 21 days after noise exposure for each frequency. Permanent ABR threshold shift (PTS) was obtained as the difference between the baseline threshold and the final ABR threshold measured at 21 days after noise exposure. The ABR measurement after noise exposure was completed within 1.5 h after completion of noise exposure. ABR was recorded under light ketamine (20 mg/kg) and xylazine (1 mg/kg) anaesthesia and small supplemental doses (1/3 of initial dose) if needed. For ABR recordings, an active needle electrode and a reference electrode were subcutaneously placed proximal to the test ear and the non-test ear, respectively, while a ground electrode was placed at the vertex. Auditory stimuli consisting of tone pips (5 ms duration and 1 ms Blackman rise and fall) at frequencies of 0.5, 1, 2, 4, 6 and 8 kHz were generated by a computer-aided system (Intelligent Hearing Systems, Miami, FL) coupled to high frequency transducers and transduced through the computer-controlled attenuator to a 3A insert earphone [Etymotic Research (ER)-3A, Etymotic Research Inc., Elk Grove Village, IL] placed about 5 mm from the tympanic membrane. The insert earphone was calibrated with a coupler mounted to a sound level meter. The electrical responses obtained from the electrodes were amplified ($\times 100,000$), filtered (100–3000 Hz), digitized through an A/D converter on a signal processing board and averaged at a sample rate of 1024 for each level. Hearing threshold was determined with 10 dB descending steps and then 5 dB ascending steps until near the threshold, the mid-point between the lowest level of a clear response and the next level where no response was observed. The ABR hearing thresholds were confirmed by two investigators who were blinded as to the identity of the animal groups.

Histological Examination

After the final ABR tests were measured at 21 days after noise exposure, the animals were humanely euthanized by transcardial injection of sodium pentobarbital under anaesthesia with a mixture of ketamine (20 mg/kg) and xylazine (1 mg/kg). One ear of each animal was used for hair cell counting, whereas the other was used for immunohistochemical staining. After immediately removing the temporal bones from the skull, the cochleae were perfused from the round and oval windows through a hole made at the apex with a solution of 4% paraformaldehyde in phosphate buffer and were submerged in the same fixative for 24 h at 4° C. The cochlear tissues were dissected and washed with PBS, permeabilized with 0.3% Triton X-100 for 10 min and stained with actin with 1% rhodamine phalloidin for 30 min. After staining, the cochleae were mounted on a glass slide as surface preparations and examined for hair cell counts under a light microscope (Olympus BH-2; Olympus Optical Co. Ltd.).

Percentages of missing OHCs were obtained by dividing the OHC count for the experimental animals by control value hair cell counts for normative values developed for each cochlear section from 100 animals. Finally, as a cytochrome, the percentage of the missing OHCs was plotted as a function of percent distance from the apex by entering inputs into a worksheet to construct continual data points along the entire cochlear length [6,16]. The average at each 0.5 mm segment was plotted to create the cytochrome. An equation of cochlear frequency-place map ($F = 125e^{0.051d}$, where F is the frequency in Hz and d is percent distance from the apex) was used to evaluate OHC losses at specific cochlear regions corresponding to specific stimulus frequencies of 2, 4, 6 and 8 kHz [17,18]. Counting of OHC losses was also confirmed by two investigators who were blinded as to the identity of the animal groups.

Immunohistochemistry

Biomarkers 4-HNE and NT have been used to assess oxidative stress, monitor ROS and RNS activity in the inner ear and evaluate the effects of antioxidant treatment after noise exposure [14,15,19,20]. Since 4-HNE reacts extensively with DNA and proteins, depletes intracellular GSH and alters many cell signalling cascades, it has been used as an indicator of oxidative damage [21]. NT has been used as a marker for endogenous nitric oxide production and nitration of a tyrosine residue in proteins [22].

Immunohistochemistry for 4-HNE and NT was carried out on the left ear of each of the animals in each group. After overnight fixation, the tissues were washed in dH₂O three times at room temperature and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for about 2 weeks with 3–4 changes of solution. After decalcification, the tissues were cryoprotected in 30% sucrose in PBS at 4°C overnight and then placed in a Tissue-Tek mold (Sakura Finetek USA, Inc., Torrance, CA). The frozen sections (18–20 µm) were cut with a Cryotome (Thermo Fisher Scientific, Inc. Waltham, MA) parallel to the modiolus. The serial sections were collected and mounted onto slides pre-coated with gelatin. The sections were washed three times with PBS for 30 min, incubated in blocking solution consisting of 1% bovine serum albumin and 1% normal goat serum and permeabilized in 0.2% triton X-100 in PBS (PBS/T) for 30 min. The sections were incubated with a 1:100 dilution of rabbit anti-4-hydroxy-2-nonenal Michael Adducts IgG (EMD Chemicals, Inc. Gibbstown, NJ) or a 1:200 dilution of mouse anti-nitrotyrosine antibody IgG (Upstate, Lake Placid, NY) at room temperature for 2 h. After washing with PBS/T, a 1:1000 dilution of Alexa Fluor 594 donkey anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA) for 4-HNE or a 1:1000 of Alexa Fluor 594 donkey anti-mouse IgG secondary antibody for NT was used

for 1 h at room temperature to visualize staining. After rinsing with PBS/T, a 1:40,000 dilution of 4'-6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. A negative control was obtained by omitting the primary antibody during processing of tissues randomly selected across experimental groups. Visual images were obtained from the basal and middle turns of the cochlea by an observer blinded to the identity of the animal groups using a confocal microscope (Leica SP2 Confocal Microscope, Leica Microsystems, Heidelberg, Germany) with a 60× objective.

Immunostaining semi-quantitation and statistical analysis

All the data are reported as mean ± S.E.M. Significant differences in PTS among the different groups at each frequency were evaluated using one-way ANOVA (SPSS 14.0 for Windows). We used the ABRs from both ears of each animal. Before we used the ABRs from both ears, we compared the ABRs from one ear with those from the other ear. With a paired samples *t*-test at each frequency, there was no significant difference between them. This result was not different from that of a one-way ANOVA. Therefore, we treated the ABRs from both ears of each animal as independent observations [2]. The ABR results from six animals of each group resulted in a sample size of 12.

Measurement of 4-HNE relative fluorescence intensity and a semi-quantitative procedure to count an NT immunostaining index were similar to previously described procedures [23]. Relative fluorescence intensity of 4-HNE in the organ of Corti was evaluated with LAS AF Lite software (Leica Microsystems CMS GmbH, Heidelberg, Germany). Two to three images from mid-modiolar sections of each cochlea by fluorescence microscopy were collected using the same camera settings (40×) so that a similar shape and size of the organ of Corti could be measured in all ears. The distance between two sections was 200–400 µm to ensure non-duplicate measurement. Measurement of the relative fluorescence intensity was conducted with the software by drawing a line around the border of the organ of Corti, from which the mean pixel intensity of the labelling was derived. A modified semi-quantitative procedure was used to count an NT immunostaining index in the spiral ligament of the middle turn [23]. Images were taken from the spiral ligament by fluorescence microscopy (40×) for every other section. The distance between two sections was about 400 µm to ensure non-duplicate counting. After five to six images were collected from each middle turn, an immunostaining index using the ImageJ software was calculated by dividing the number of NT positive cells by total number of cells (number of nuclei stained by DAPI) within the image.

Statistical differences in relative fluorescence intensity of 4-HNE and NT immunostaining index

among the different groups were also evaluated using one-way ANOVA. In addition, differences in percentage of missing OHCs among the different groups were also tested with one-way ANOVA. The Fisher's least squares difference (LSD) post hoc test was then used to evaluate the statistical differences of specific pairs of values. A p -value less than 0.05 was considered to indicate a statistically significant difference.

Results

ABR threshold shifts

There were no significant differences in temporary threshold shifts measured immediately after noise exposure among any of the groups (data not shown). However, PTS varied with animal group ($p < 0.001$) and frequency ($p < 0.001$). As shown in Figure 1, mean PTS in group 1 ranged from about 16 dB at low frequencies to 42 dB at high frequencies, while the threshold shifts of the three-drug combination treatment groups were reduced across frequencies (Figure 1). No significant differences were found across frequencies in PTS between group 1 and group 2. However, significant differences in PTS between group 1 and group 3 were shown at high frequencies of 4–8 kHz. For group 4, the threshold shifts were significantly reduced at 0.5, 4, 6 and 8 kHz, compared to group 1. For group 5, the PTS were significantly

reduced at all frequencies except at 1 kHz, compared to group 1. There were no significant differences in threshold shifts among experimental groups.

Figure 2 compares PTS averaged at higher frequencies (2–8 kHz) among groups 1–5 (Figure 2). Reduction in PTS became greater as the duration of treatment increased and time delay before starting the treatment was shortened. The mean threshold shifts for groups 3, 4 and 5 were significantly decreased compared to those of group 1. Significant differences between groups 2 and 5 were found. With these results, the PTS could be averaged at higher frequencies (2–8 kHz) for the experimental groups based on the delay in starting the treatment (24 and 48 h) and different durations (3 and 8/9 days) of treatment. The mean threshold shifts for the experimental groups with treatment delay of 24 h after noise exposure were significantly decreased compared to those of group 1. The amount of reduction was 17 dB. Although there was no significant difference, the mean threshold shifts of the experimental group with treatment delay of 48 h were reduced, compared to group 1. The amount of reduction was 9 dB. The amount of reduction for the 3-day and 8/9 day duration groups was 11 and 14 dB, respectively.

Hair cell counts

The OHC loss varied with animal group ($p < 0.05$) and frequency ($p < 0.01$). Figure 3 shows a cytochrome c oxidase (cytochrome c oxidase) relating mean percentage of missing

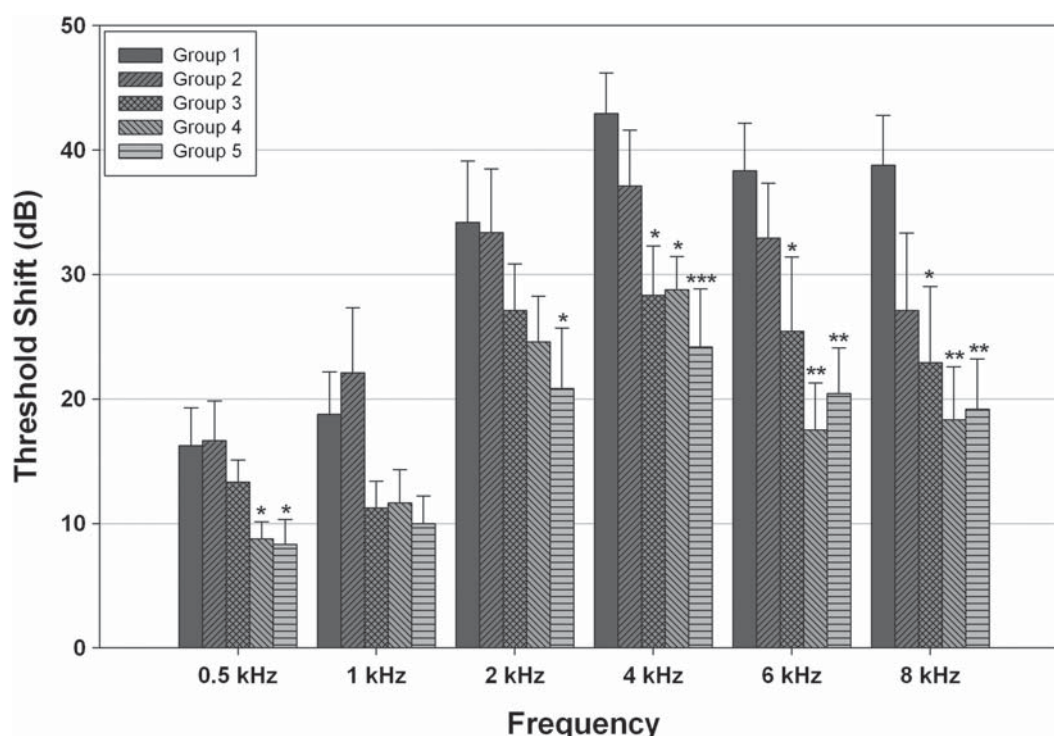


Figure 1. Permanent ABR threshold shifts of different groups. The ABR threshold shifts of the groups 3, 4 and 5 were significantly reduced, compared to group 1, at high frequencies (2–8 kHz). There were no significant differences among experimental groups (2, 3, 4, 5). At lower frequencies (0.5–2 kHz), compared to group 1, significant reductions were found in groups 4 and 5 at 0.5 kHz and in group 5 at 2 kHz. Asterisks: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

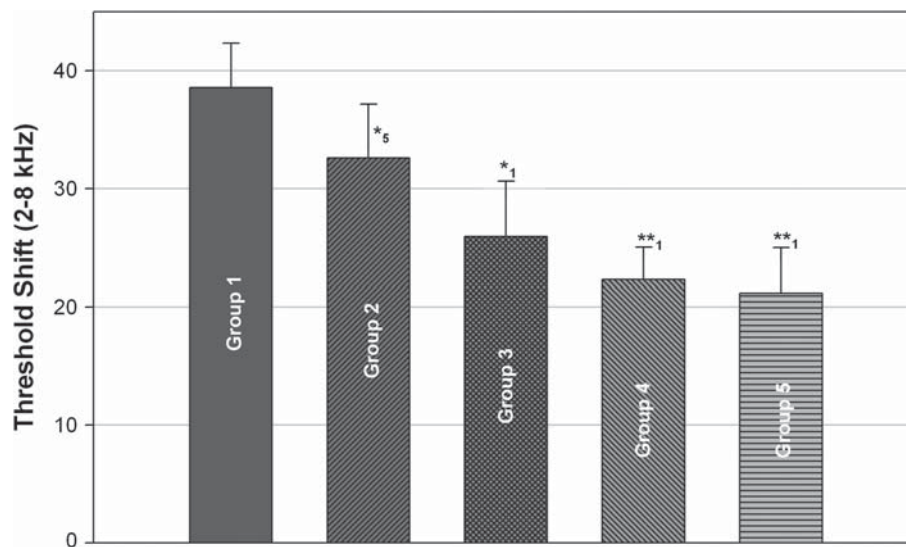


Figure 2. Permanent ABR threshold shifts averaged at frequencies of 2–8 kHz for different groups. Compared to group 1, significant reductions in the average threshold shifts were found for groups 3, 4 and 5. Another significant reduction was found between group 2 and group 5. The number after the asterisk represents the groups compared for statistical analysis as shown in Table 1.

OHCs to the measured percent distance from the cochlear apex (Figure 3). Average percentages of OHC losses at 55–90% distance from the apex (high frequency regions) were approximately 50%, 37%, 40% and 10%, respectively for group 2 ($n=6$), group 3 ($n=6$), group 4 ($n=6$) and group 5 ($n=5$) after noise exposure, compared to a 65% average loss in group 1 ($n=6$). The OHC losses of groups 3, 4 and 5 were significantly reduced, compared to groups 1

and 2. The OHC losses of group 5 were significantly reduced, compared to group 4. Figure 4 illustrates the percentage of missing OHCs averaged at higher frequencies (2–8 kHz) among specific groups (Figure 4). The mean OHC losses of groups 3, 4 and 5 were significantly reduced, compared to group 1. The mean OHC losses of group 5 were significantly reduced, compared to groups 2, 3 and 4. Based on these results, the percentage of missing OHCs could be averaged

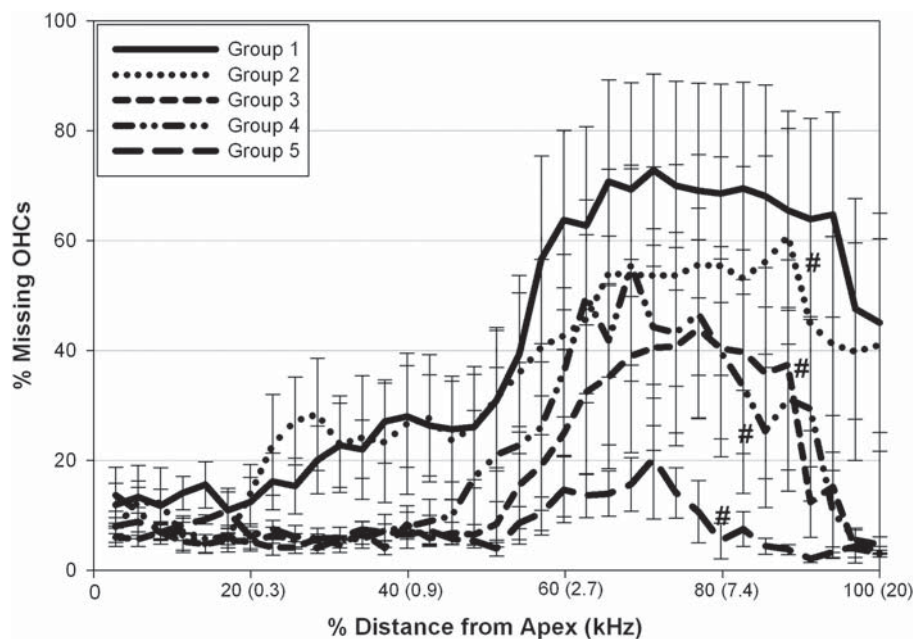


Figure 3. A cytochocleogram representing percentage of missing OHCs at the measured percent distance from the cochlear apex in different groups and the corresponding frequency regions. Compared to group 1, significant reductions in OHC loss were found in different groups, especially at the 55–100% distance from the cochlear apex. These ranges correspond to the frequency regions of 2–20 kHz. The symbol # indicates statistically multiple significances among different groups. There were significant differences between group 2 and group 3 (***), group 2 and group 4 (***), group 2 and group 5 (***). There were also significant differences between group 3 and group 1 (***), and group 3 and group 2 (**). Finally, there were significant differences between group 5 and group 1 (***), group 5 and group 2 (***), and group 5 and group 4 (**).

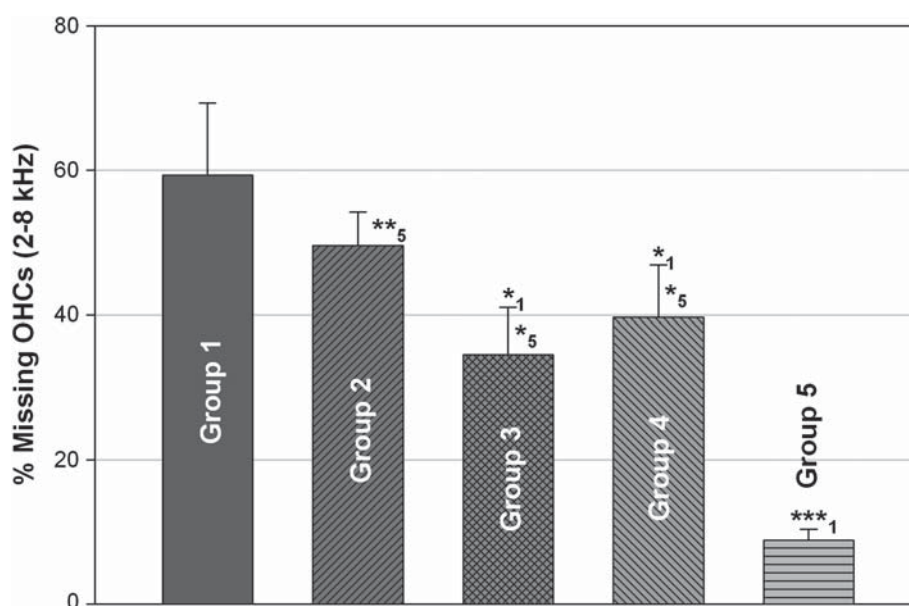


Figure 4. Mean percentages of OHC loss averaged at high frequency regions (2–8 kHz) for different groups. Compared to group 1, significant reductions in the average threshold shifts were found for groups 3, 4 and 5. Compared to group 5, other significant reductions were found compared to groups 2, 3 and 4. The number after the asterisk represents the group compared for statistical analysis as shown in Table 1.

at higher frequencies (2–8 kHz) for the experimental groups with treatment delays (24 and 48 h) and different treatment durations (3 and 8/9 days). The mean OHC losses for the experimental groups with a 24 h treatment delay were significantly reduced compared to those of group 1. The amount of reduction was 58%. Although there was no significant difference, the mean OHC loss of the experimental groups with a 48 h treatment delay was reduced, compared to group 1. The amount of reduction was 28%. The mean OHC losses for the experimental groups with 8/9 days treatment duration were significantly reduced compared to those of group 1 and the experimental group with 3-day treatment duration. The amount of reduction was 25% and 63%, respectively ($p < 0.05$).

Immunohistochemistry

All sections for 4-HNE or NT assessment were taken from the middle basal turn (8–10 kHz area, approximately 86% distance from the apex) and the middle second turn (2 kHz area, approximately 57% distance from the apex) of the cochlea. There were no significant differences in NT staining between specific pairs of groups (data not shown). However, significant differences were found in 4-HNE staining. Figure 5 shows 4-HNE immunolabeling results for the normal control group (no noise exposure), the noise + saline group (group 1) and the noise + treatment group treated for 9 days with injections starting at 24 h (group 5) (Figure 5). For the normal control group, 4-HNE immunolabeling was not observed in the organ of Corti.

However, for group 1, 4-HNE immunolabeling was found in the inner hair cells and the Deiters' cells at the basal turn; and in the inner hair cells, the Deiters' cells and the Hensen's cells at the middle turn. For group 5, 4-HNE immunolabeling was found in only the inner hair cells at the basal turn and not observed in the organ of Corti at the middle turn. The 4-HNE staining for this experimental group was weaker than those of the noise-exposed group (group 1).

There were statistically significant differences among groups in the relative fluorescence intensity at the middle turn of the cochlea (Figure 6). Significant differences were found between the normal control and group 1 and between group 1 and the noise + treatment groups (3 and 9 days), but not between the normal control and the noise + treatment groups (3 and 9 days) and between the noise + treatment group (3 days) and the noise + treatment group (9 days).

Discussion

The benefits of combination therapies

The three-drug combination used in this study consisted of 4-OHPBN, NAC and ALCAR. The nitron, 4-OHPBN has been known to have a variety of properties as follows: its ability to spin-trap free radicals, its antioxidant properties, its action on important membrane enzymes including ion transport proteins and its function as a potent anti-inflammatory agent modulating inducible nitric oxide synthase and inducible cyclooxygenase activity [24]. NAC has shown its

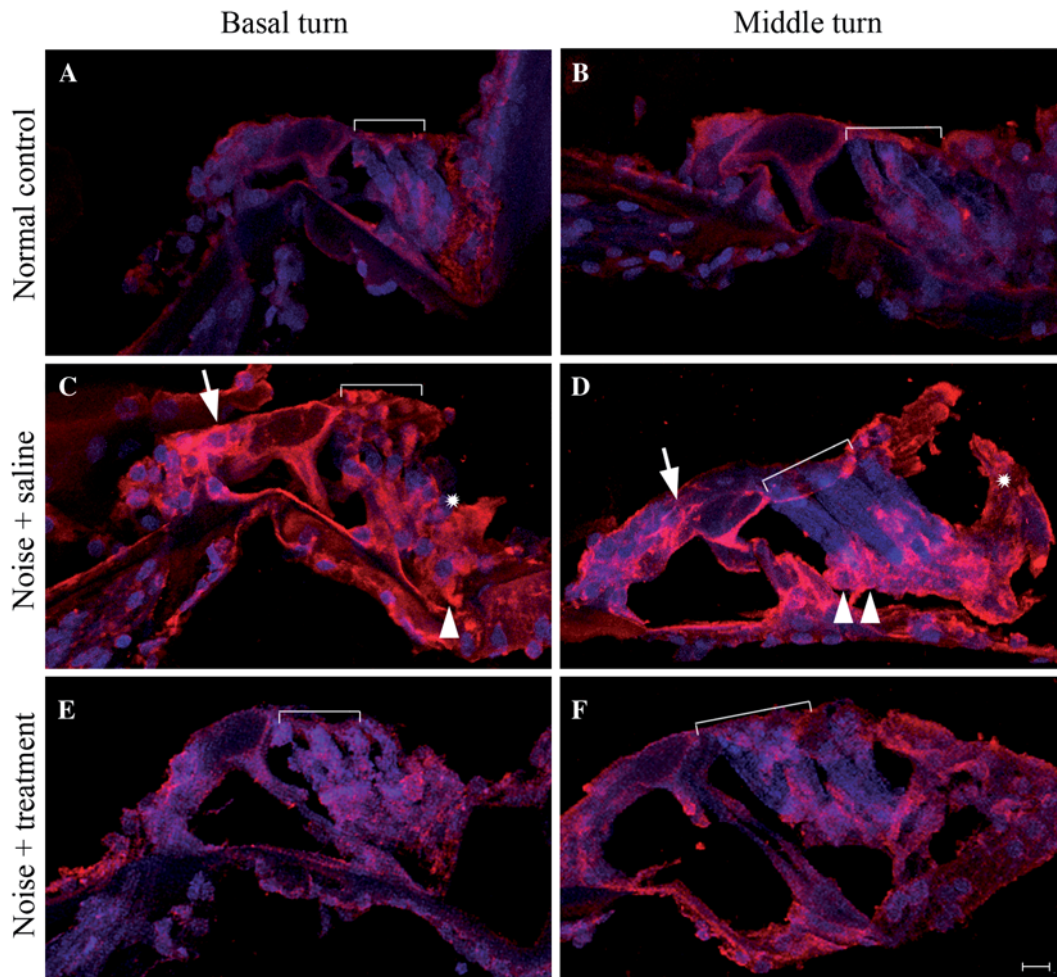


Figure 5. 4-HNE immunocytochemical staining in the organ of Corti in different groups. For the normal control group (no noise exposure), no positive 4-HNE staining was found in the organ of Corti in the basal (panel A) and middle turns (panel B). However, for the noise + saline group, positive 4-HNE staining was found in the basal and middle turns. At the basal turn, strong positive 4-HNE staining was shown in the inner hair cells (arrow in panel C), the Deiters' cells (arrowhead in panel C) and the Hensen's cells (starburst in panel C), while most of the outer hair cells and the Hensen's cells were missing. In the middle turn, positive 4-HNE staining was found in the inner hair cells (arrow in panel D), the Deiters' cells (arrowheads in panel D) and the Hensen's cells (starburst in panel D). For the noise + treatment group treated for 9 days with injections starting at 24 h, positive 4-HNE staining was only found in the inner hair cells at the basal turn (panel E), staining was weaker than that of the noise + saline group, positive staining was not seen in the organ of Corti in the middle turn (panel F). Brackets indicate the outer hair cell region in A–F. Scale bar = 10 μ m for panels (A–F).

ability to inhibit lipid peroxidation and scavenge ROS and functions as a neuroprotective agent and a cysteine donor to increase GSH synthesis [2,4,5,25]. ALCAR functions mainly to protect oxidative stress-induced mitochondrial injury by enhancing mitochondrial bioenergetics and biogenesis [2,3,26,27].

Although each antioxidant agent targets and affects different injury mechanisms, its effectiveness may be maximized in combination with other antioxidant drugs in preventing or treating AAT [6,11,28,29]. The synergistic effect of combination of antioxidant drugs has been supported by many other studies. A combination of 4-OHPBN and NAC decreased PTS by 30 dB, while a combination of 4-OHPBN and NAC and ALCAR given 4 h after noise exposure reduced PTS by 37 dB in chinchilla [6]. NAC and salicylate, a hydroxyl radical scavenger, decreased PTS by 17 dB PTS in chinchilla [5]. A combination

of salicylate and Trolox, a water-soluble analogue of α -tocopherol and an inhibitor of peroxynitrite-mediated tyrosine and guanine nitrosylation, reduced PTS by approximately 15–18 dB in guinea pigs [15]. Given an iron chelator, a free radical scavenger in combination with deferoxamine mesylate and mannitol, the three-drug combination reduced PTS by about 20 dB in guinea pigs [30]. Another combination of vitamins A, C and E (free radical scavengers) plus magnesium was effective in reducing both PTS (about 30 dB) and cell death [29], while folate, vitamin E and ALCAR provided synergistic protection against oxidative stress [28].

The optimal timing for treatment

The present study shows that the noise used in our study induced permanent hearing loss of about 39 dB

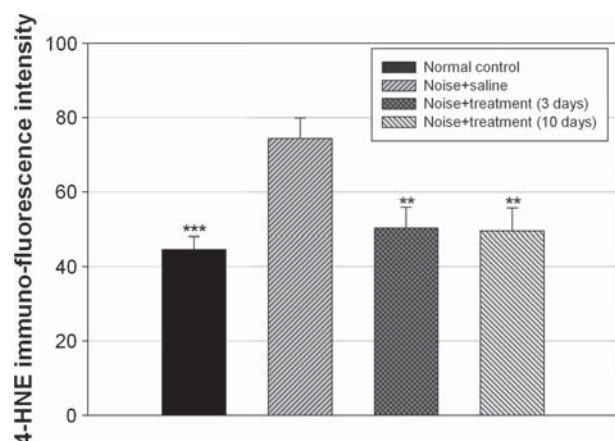


Figure 6. Relative fluorescence intensity for 4-HNE in the organ of Corti 21 days after noise exposure. Compared to experimental group 1 (noise + saline), significant reductions were found in the normal control (no noise exposure) and noise + treatment (3 and 10 days) groups.

with approximately 41% loss of OHCs as well as the distinct formation of 4-HNE. Large losses (about 70%) induced by noise were observed at 55–90% distance from the apex, indicating high frequency regions (2–10 kHz). The functional and morphological consequences of noise trauma were significantly attenuated by a combination of 4-OHPBN plus NAC plus ALCAR. The present study found that the effectiveness of the three-drug combination depends on the time of initial treatment and the length of treatment after noise exposure. When the treatment delay after noise exposure decreased from 48 to 24 h, the average threshold shift and OHC loss significantly decreased ($p < 0.05$). When the length of treatment increased from 3 days to 8/9 days, the average threshold shift and OHC loss significantly decreased ($p < 0.05$). Maximal effectiveness of treatment was observed in the experimental group treated for 9 days with injections starting at 24 h after noise exposure. These data demonstrate that a pharmacological therapy started within 24 h and continuing for 10 days after noise exposure can effectively treat AAT in a chinchilla model using steady-state noise exposure.

Although the present study emphasizes effective therapeutic intervention extended up to 10 days after noise exposure, it is necessary to mention that early administration of antioxidant drugs prior to or shortly after noise exposure is effective in preventing or treating AAT. Coleman et al. (2007) investigated the timing (1, 4, 12 h) of the initial administration of NAC or ALCAR after noise exposure on the effectiveness of treatment for AAT [10]. The greatest reduction was observed with the initial administration at 1 h after noise exposure. The initial administration at 4 h after noise exposure was still effective in treating AAT because it countered the first oxidative burst. There was no efficacy in treating AAT for the initial administration at 12 h after noise exposure. The present

study provides different results. This difference may result from the different drugs used in this study and/or different timing and duration of treatment of drug. Other studies have shown that a combination of antioxidant drugs had greater efficacy when given before noise exposure than when given up to 3 days after noise exposure [5,14].

The temporal relation between ROS production and optimal timing of treatment

In general, after noise exposure, the threshold shifts gradually decrease and morphological damage increases. Although these changes were found to continue up to 30 days after noise exposure [25], the first 10 days after noise exposure are an important time period in treating AAT. At day 10 after noise exposure, continued formation of ROS/RNS reaches a maximum and PTS and OHC loss plateau [14,15]. Furthermore, markers of ROS and RNS, 4-hydroxynonenal (4-HNE) and NT are elevated 7–10 days after noise exposure [15] and the free radical content of the cochlear tissue is increased 3–6 days and also 12 days after noise exposure [31]. Due to these increases in free radicals 7–10 days after noise exposure, it is not surprising to observe the benefit of antioxidant treatment extending up to 10 days after noise exposure because it may prevent or reduce cumulative damage resulting from the delayed production of free radicals. The increase of NT shown by Yamashita et al. (2005) was not observed in our current study [15]. The difference may result from different noise levels used in each study.

Another important finding of the current study is that the formation of 4-HNE remains but NT was not found up to 21 days after noise exposure. At approximately 21 days after noise exposure in chinchilla, compound threshold shift becomes permanent hearing loss and progressive hair cell loss stabilizes [3–5]. The 4-HNE results of the present study are consistent with the findings of Yamashita et al. (2004) showing that the immunoactivity of 4-HNE in inner hair cells and supporting cells of the organ of Corti remains up to 14 days after noise exposure [14,15]. However, the NT results of the present study are not consistent with those of Yamashita et al. (2004) showing that the immunoactivity of NT in OHC and Deiter's cells reaches a maximum by days 7–10 after noise exposure [14]. Generally, since 4-HNE is an indicator of oxidative damage formed as an abundant product of polyunsaturated fatty acid oxidation and decomposition, it reacts extensively with DNA and proteins, depletes intracellular GSH and many cell signalling cascades [21]. NT, a marker of nitric oxide production in the body, is formed by nitration of a tyrosine residue in proteins [22]. The formation of 4-HNE begins immediately after noise exposure and stays up to 21 days after noise exposure, while the

formation of NT begins immediately after noise exposure and disappears up to 21 days after noise exposure. Significantly lower expression of 4-HNE found at 21 days after noise exposure in noise + treatment groups suggests that antioxidant drugs inhibit the formation of 4-HNE and promote hair cell survival. This indicates that inhibition of 4-HNE formation in the inner ear may be one of the major reasons to explain the therapeutic effects of the combination of 4-OHPBN plus NAC plus ALCAR. However, strong NT staining was observed in the spiral ligament immediately or few hours after noise exposure, while the relatively weak expression of NT was found in the same region after antioxidant drug treatment [32,33]. We did not find any significant NT staining in the organ of Corti or in the spiral ganglion 21 days after noise exposure in the current study. This may indicate that the formation of ROS in AAT is more important than RNS in the organ of Corti. However, the relationship between free radical formation in the lateral wall of the cochlea and hair cell death is still unclear [34]. In addition, each of the antioxidant drugs used in this study has a different target and site of action as follows: 4-OHPBN scavenges free radicals and reduces inflammation [1–6], NAC provides cysteine for GSH synthesis [3–5] and ALCAR decreases ROS production and preserve mitochondria [2,7–9]. Therefore, each antioxidant drug may contribute differently to the attenuation of HNE and NT production in the cochlea induced by AAT. However, our current study could not differentiate the unique site and mechanism of action for each antioxidant drug.

It should be noted that the PTS data did correspond to those of 4-HNE intensity, but did not correspond to those of percentage of missing OHCs. In addition, the difference in PTS between 3-day treatment and 10-day treatment was also shown in the percentage of missing OHCs but not in the 4-HNE intensity. It may indicate that although the OHCs of 10-day treatment survived longer than those of 3-day treatment, they did not function well. Finally, we want to mention that our data are based on chinchilla and these data may not generalize to other species.

Conclusion

This study shows that the combination of antioxidant drugs (4-OHPBN plus NAC plus ALCAR) can be effectively used in treating AAT if the duration of treatment is extended over the period of 7–10 days after noise exposure and the initial treatment up to 1 day after noise exposure altered outcomes is delayed.

Acknowledgements

The authors thank Anita Montgomery for her help in the preparation of this manuscript. In addition, we

like to thank Angelica Vasquez-Weldon for her help in the preparation of 4-OHPBN.

Conflict of interest

None. This study was supported by grants from the Office of Naval Research and INTEGRIS Health, Oklahoma City, Oklahoma.

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Departments of the Navy, Army, Defense or Government of USA.

References

- [1] Kotake Y, Sang H, Miyajima T, Wallis GL. Inhibition of NF-kappaB, iNOS mRNA, COX2 mRNA, and COX catalytic activity by phenyl-N-tert-butyl-nitron (PBN). *Biochem Biophys Acta* 1998;1448:77–84.
- [2] Kopke RD, Bielefeld E, Liu J, Zheng J, Jackson R, Henderson D, Coleman JK. Prevention of impulse noise-induced hearing loss with antioxidants. *Acta Otolaryngol* 2005;125:235–243.
- [3] Kopke RD, Coleman JK, Liu J, Campbell KC, Riffenburgh RH. Candidate's thesis: enhancing intrinsic cochlear stress defenses to reduce noise-induced hearing loss. *Laryngoscope* 2002;112:1515–1532.
- [4] Kopke RD, Jackson RL, Coleman JK, Liu J, Bielefeld EC, Balough BJ. NAC for noise: From the bench top to the clinic. *Hearing Res* 2007;226:114–125.
- [5] Kopke RD, Weisskopf PA, Boone JL, Jackson RL, Wester DC, Hoffer ME, et al. Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla. *Hearing Res* 2000;149:138–146.
- [6] Choi CH, Chen K, Vasquez-Weldon A, Jackson RL, Floyd RA, Kopke RD. Effectiveness of 4-OHPBN alone and in combination with other antioxidant drugs in the treatment of acute acoustic trauma in chinchilla. *Free Radic Biol Med* 2008;44:1772–784.
- [7] Hagen TM, Ingersoll RT, Wehr CM, Lykkesfeldt J, Vinarsky V, Bartholomew JC, et al. Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci U S A* 1998;95:9562–9566.
- [8] Gadaleta MN, Cormio A, Pesce V, Lezza AM, Cantatore P. Aging and mitochondria. *Biochimie* 1998;80:863–870.
- [9] Seidman M, Van de Water TR. Pharmacological manipulation of the inner ear. *ENT Journal* 2003;82:276–288.
- [10] Coleman JKM, Kopke RD, Liu J, Ge X, Harper EA, Jones GE, et al. Pharmacological rescue of noise induced hearing loss using N-acetylcysteine and acetyl-L-carnitine. *Hearing Res* 2007;226:104–113.
- [11] Yamane H, Nakai Y, Takayama M, Konishi K, Iguchi H, Nakagawa T, et al. The emergence of free radicals after acoustic trauma and strial blood flow. *Acta Otolaryngol Suppl* 1995;519:87–92.
- [12] Ohlemiller KK, Dugan LL. Elevation of reactive oxygen species following ischemia-reperfusion in mouse cochlea observed in vivo. *Audiol Neurotol* 1999;4:219–228.
- [13] Ohlemiller KK, Wright JS, Dugan LL. Early elevation of cochlear reactive oxygen species following noise exposure. *Audiol Neurotol* 1999;4:229–236.
- [14] Yamashita D, Jiang HY, Schacht J, Miller JM. Delayed production of free radicals following noise exposure. *Brain Res* 2004;1019:201–209.
- [15] Yamashita D, Jiang HY, Le Prell CG, Schacht J, Miller JM. Post-exposure treatment attenuates noise-induced hearing loss. *J Neurosci* 2005;134:633–642.

- [16] Greenwood DD. A cochlear frequency-position function for several species—29 years later. *J Acoust Soc Am* 1990;87: 2592–2605.
- [17] Eldredge DH, Miller JD, Bohne BA. A frequency-position map for the chinchilla cochlea. *J Acoust Soc Am* 1981;69: 1091–1095.
- [18] Harding GW, Bohne BA, Ahmad M. DPOAE level shifts and ABR threshold shifts compared to detailed analysis of histopathological damage from noise. *Hearing Res* 2002;174: 158–171.
- [19] Cassandro E, Sequino L, Mondola P, Attanasio G, Barbara M, Filipo, R. Effect of superoxide dismutase and allopurinol on impulse noise-exposed guinea pigs—electrophysiological and biochemical study. *Acta Otolaryngol* 2003;123:802–807.
- [20] Karlida T, Yalçın S, Öztürk A, Ustünda B, Gök U, Kaygusuz I, Susaman N. The role of free oxygen radicals in noise induced hearing loss: effects of melatonin and methylprednisolone. *Auris Nasus Larynx* 2002;29:147–152.
- [21] West JD, Marnett LJ. Alterations in gene expression induced by the lipid peroxidation product, 4-hydroxy-2-nonenal. *Chem Res Toxicol* 2005;18:1642–1653.
- [22] Ohshima H, Friesen M, Brouet I, Bartsch H. Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem Toxicol* 1990;28:647–652.
- [23] Weaver CV, Liu SP, Lu JF, Lin BS. The effects of benzene exposure on apoptosis in epithelial lung cells: localization by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) and the immunocytochemical localization of apoptosis-related gene products. *Cell Biol Toxicol* 2007;23:201–220.
- [24] Floyd RA, Kopke RD, Choi C-H, Foster SB, Doblas S, Townner RA. Nitrons as therapeutics. *Free Radic Biol Med* 2008;45:1361–1374.
- [25] Henderson D, Spongr V, Subramanian M, Campo P. Anatomical effects of impact noise. *Hearing Res* 1994;76: 101–117.
- [26] Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A* 1994;91:10771–10778.
- [27] Fischel-Ghodsian N, Kopke RD, Ge X. Mitochondrial dysfunction in hearing loss. *Mitochondrion* 2004;4:675–694.
- [28] Dhitavat S, Ortiz D, Rogers E, Rivera E, Shea TB. Folate, vitamin E, and acetyl-L-carnitine provide synergistic protection against oxidative stress resulting from exposure of human neuroblastoma cells to amyloidbeta. *Brain Res* 2005;1061: 114–117.
- [29] Le Prell CG, Hughes LF, Miller JM. Free radical scavengers vitamins A, C, and E plus magnesium reduce noise trauma. *Free Radic Biol Med* 2007;42:1454–1463.
- [30] Yamasoba T, Schacht J, Shoji F, Miller JM. Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. *Brain Res* 1999;815:317–325.
- [31] Liu, Z. Experimental study on the mechanism of free radical in blast trauma induced hearing loss. *Chin J Otorhinolaryngol* 1992;27:24–26.
- [32] Ohinata Y, Yamasoba T, Schacht J, Miller JM. Glutathione limits noise-induced hearing loss. *Hear Res* 2000;146:28–34.
- [33] Kujawa SG, Liberman MC. Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. *J Neurosci* 2006;26:2115–2123.
- [34] Du X, Choi C-H, Chen K, Cheng W, Floyd RA, Kopke RD. Reduced formation of oxidative stress biomarkers and migration of mononuclear phagocytes in the cochleae of chinchilla after antioxidant treatment in acute acoustic trauma. *International J Otolaryngol* 2011; in press.

This paper was first published online on Early Online on 10 August 2011.