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Research paper

Oxidative stress pathways in the potentiation of noise-induced hearing loss by acrylonitrile

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Abstract

We hypothesize that the disruption of antioxidant defenses is a key mechanism whereby chemical contaminants can potentiate noise-induced hearing loss (NIHL). This hypothesis was tested using acrylonitrile (ACN), a widely used industrial chemical whose metabolism is associated with glutathione (GSH) depletion and cyanide (CN) generation. CN, in turn, can inhibit Cu/Zn superoxide dismutase (SOD). We have shown previously that ACN potentiates NIHL, even with noise exposure approaching permissible occupational levels. However, the relative involvement of GSH depletion and/or CN production in this potentiation is still unknown. In this study, we altered these metabolic pathways pharmacologically in order to further delineate the role of specific antioxidants in the protection of the cochlea. We investigated the effects of sodium thiosulfate (STS), a CN inhibitor, 4-methylpyrazole (4MP), a drug that blocks CN generation by competing with CYP2E1, and L-*N*-acetylcysteine (L-NAC), a pro-GSH drug, in order to distinguish between GSH depletion and CN production as the mechanism responsible for potentiation of NIHL by ACN. Long-Evans rats were exposed to an octave-band noise (97 dB SPL, 4 h/day, 5 days) and ACN (50 mg/kg). Separate pre-treatments with STS (150 mg/kg), 4MP (100 mg/kg) and L-NAC (4×400 mg/kg) all dramatically reduced blood CN levels, but only L-NAC significantly protected GSH levels in both the liver and the cochlea. Concurrently, only L-NAC treatment decreased the auditory loss and hair cell loss resulting from ACN + noise, suggesting that GSH is involved in the protection of the cochlea against reactive oxygen species generated by moderate noise levels. On the other hand, CN does not seem to be involved in this potentiation.
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Keywords: Acrylonitrile; L-*N*-acetylcysteine; Sodium thiosulfate; 4-methylpyrazole; Noise; Hearing loss; Hair cell loss; Ototoxicity; Reactive oxygen species; Oxidative stress; Glutathione; Cyanide; Superoxide dismutase

1. Introduction

Oxidative stress has been identified as a mechanism involved in cochlear hair cell death caused by noise exposure. A review of the recent literature on this topic shows that most studies investigated the effects of very intense noise exposures, which are rarely encountered by humans. For example, Yamane et al. (1995) observed increases in superoxide ($O_2^{\cdot-}$) in the stria vascularis after an exposure to 120–125 dB SPL rock music. Ohlemiller et al. (1999b) reported increases in the hydroxy radical ($OH^{\cdot}$) after exposure to 110–113 dB broadband noise. Ohinata et al. (2000) showed evidence of increased lipid peroxidation, one consequence of oxidative stress, in cochleae exposed to 115 dB SPL 4-kHz broadband noise. These results suggest that the cochlea can neutralize reactive oxygen species (ROS) efficiently, and that considerable noise stress is needed to overwhelm this buffering system.

Abbreviations: NIHL, noise-induced hearing loss; STS, sodium thiosulfate; 4MP, 4-methylpyrazole; L-NAC, L-*N*-acetylcysteine; ACN, acrylonitrile; OHCs, outer hair cells; GSH, glutathione; ROS, reactive oxygen species; CYP2E1, cytochrome P450 2E1; SOD, superoxide dismutase; CN, cyanide

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However, the use of genetic animal models with reduced antioxidant potential showed that, if the intrinsic free radical buffering system is altered or inefficient, then the cochlea can become much more vulnerable to noise. For instance, Ohlemiller et al. showed that knockout mice with targeted deletion of either Cu/Zn SOD (1999a) or glutathione peroxidase (2000) were significantly more sensitive to a 1-hour exposure to 110 dB broadband noise. In addition, recent data from studies using combined exposure to selected chemical contaminants with noise suggest that reducing intrinsic cochlear antioxidant defenses may lead to oxidative stress, even for moderate noise exposures (Pouyatos et al., 2005). Acrylonitrile (ACN; vinyl cyanide) is one of these compounds. This industrial chemical is used extensively in the plastic, butyl rubber and textile industries (SRI, 1984; IARC, 1999). Approximately 125,000 workers are exposed to ACN daily in the US (Kirschner, 1996). While the NIOSH recommended permissible exposure level to ACN is quite low (1 ppm), exposure can quickly reach high levels via skin contact in case of accidental exposure. ACN has a metabolism associated with substantial potential for oxidative stress (Fig. 1). One metabolic pathway involves the direct conjugation of ACN with reduced glutathione (GSH), which plays a prominent role in buffering reactive oxygen species (ROS). A second pathway entails the formation of an intermediate epoxide (2-cyanoethylene oxide, CEO) via a specific cytochrome P450, CYP2E1. Subsequent metabolism of CEO occurs via conjugation with GSH or via hydrolysis to yield cyanide (CN) and other metabolites. CN is known to inhibit the Cu/Zn form of superoxide dismutase (SOD) in the cochlea (Pierson and Gray, 1982) as in other organs (Weisiger and Fridovich, 1973). SOD plays a significant role in the degradation of the superoxide anion into hydrogen peroxide and thence via catalase and glutathione peroxidase into water and molecular oxygen. CN can also disrupt hearing transiently when administered systemically as potassium cyanide (KCN) (Tawackoli et al., 2001). KCN causes a profound reduction in the endocochlear potential indicative of dysfunction in the stria vascularis. However, KCN does not seem to cause any permanent hearing impairment.

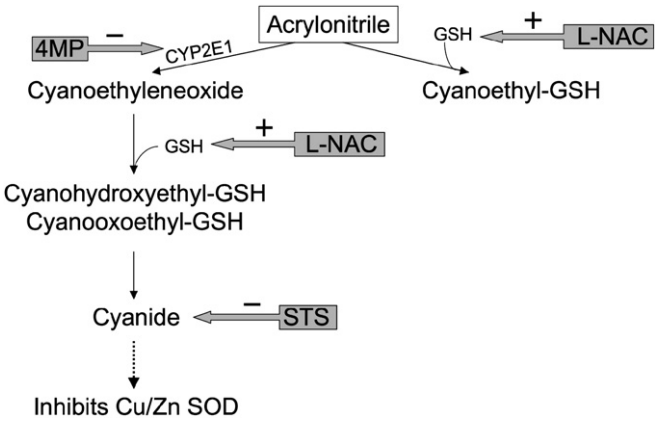


Fig. 1. Simplified acrylonitrile metabolism with the action of the three antioxidants used in this study. ACN, acrylonitrile; STS, sodium thiosulfate; 4MP, 4-methylpyrazole; L-NAC, L-*N*-acetylcysteine; GSH, glutathione; CYP2E1, cytochrome P-450 2E1; +, increases; –, decreases.

Fechter et al. (2003, 2004) have shown previously that ACN (50 mg/kg *sc*/day) can potentiate permanent NIHL for noise levels ranging from 105 dB SPL for 5 days (4 h/day) to 108 dB for 8 h, and that this potentiation can be prevented by the administration of α-phenyl-*N*-*tert*-butylnitrone, a spin-trap agent that sequesters ROS. Subsequently, Pouyatos et al. (2005) demonstrated that ACN could even promote NIHL for octave bands of noise presented at 95–97 dB SPL for 5 days (4 h/day) and that the auditory impairment correlated with loss of outer hair cells (OHCs). The results from these two studies suggested that oxidative stress involvement in the potentiation was likely, but the precise mechanism remained unknown.

The present investigation was therefore designed to determine the respective involvements of GSH depletion and cyanide production in the potentiation of NIHL by ACN. To achieve this purpose, three different drugs were used to alter ACN metabolism: sodium thiosulfate (STS), 4-methylpyrazole (4MP) and L-*N*-acetylcysteine (L-NAC).

- STS, a sulfur-containing molecule, is routinely administered in case of accidental/industrial smoke inhalation in order to accelerate cyanide detoxification. STS donates sulfur that combines with CN forming the inactive metabolite, thiocyanate, hence reducing the amount of CN available to impair cellular cytochrome oxidase and SOD.
- The drug 4MP is metabolized by CYP2E1 preventing ACN from being oxidized by this specific cytochrome, likely by a mechanism of competition. Our pilot studies showed that when administered in conjunction with ACN, this drug blocks the ACN metabolism through the oxidative pathway, and therefore prevents CN generation.
- L-NAC is a pro-GSH drug used in clinical practice to enhance the conjugation of GSH with acetaminophen in toxic overdoses. It has also been used in cases of ACN overdoses in humans (Leng and Lewalter, 2002). L-NAC is also a thiol compound, and a direct scavenger of free radicals.

The ability of daily pre-treatments with STS (150 mg/kg), 4MP (100 mg/kg) or L-NAC (cumulative daily dose 1600 mg/kg) to block the potentiation of NIHL (octave-band noise centered at 8 kHz at 97 dB SPL for 4 h/day for 5 days) by ACN (50 mg/kg) was investigated by measuring temporary and permanent auditory loss, and assessing the resultant cochlear hair cell loss caused by these exposures.

2. Methods

2.1. Subjects

A total of 148 male Long-Evans rats (225–249 g, 7–8 weeks old) obtained from Harlan (Indianapolis, IN) were employed in these experiments. The subjects were housed with free access to food and water in their home cages. Temperature was maintained at 21 ± 1 °C and lights were on from 6:30 am to 6:30 pm. The Loma Linda Veteran Medical Center Institutional Animal Care and Use Committee (IACUC) approved all the experimental protocols. All exposures and testing were performed during the daytime.

2.2. Procedures

The experiment described here was designed to determine whether STS, 4MP or L-NAC pre-treatments could protect the cochlea against the potentiation of NIHL by ACN. For this purpose, 65 Long-Evans rats were exposed to different combinations of ACN, STS, 4MP, L-NAC and/or noise. Experimental groups and treatment schedules are detailed in Table 1.

Distortion Product Otoacoustic Emissions (DPOAEs) and Compound Action Potentials were used to assess auditory impairment. DPOAEs were measured prior to experimental treatment, 3 days post-exposure and again 4 weeks later. CAP thresholds were determined following the last DPOAE measurement. Following CAP testing, cochleae were harvested for histological analysis. Additional subjects (Table 1) were used to perform cochlear and liver GSH measurement ($n = 64$) as well as blood CN level determination ($n = 19$) at different time points after ACN, STS + ACN, 4MP + ACN and L-NAC + ACN injections.

2.3. Acrylonitrile and antioxidant exposures

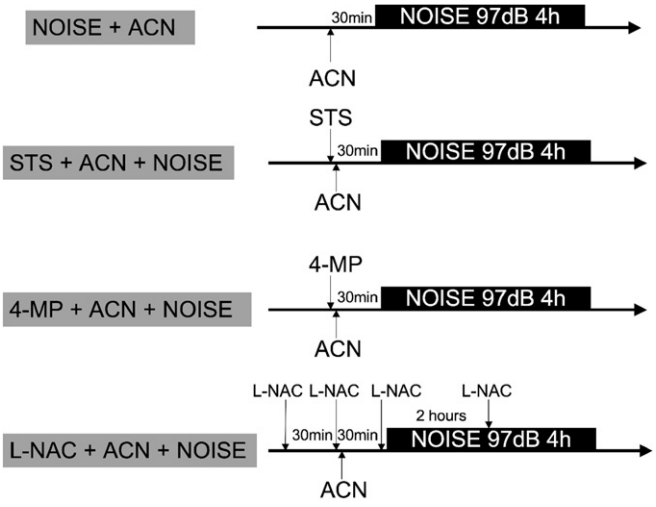
Stabilized ACN (99%), STS and 4MP were purchased at Sigma–Aldrich (St. Louis, MO). The 20% L-NAC solution was manufactured by Hospira, Inc. (Lake Forest, IL). This solution also contained 0.05% EDTA and sodium hydroxide for pH adjustment to pH 7.0 (6.0–7.5). Treatment schedules are described in Table 1.

ACN (50 mg/kg) was injected subcutaneously 30 min prior to the daily noise exposure in order to provide an adequate interval for CN production and GSH depletion prior to noise exposure (Fechter et al., 2003). Dose–effect relationships of STS and 4MP on blood CN levels generated by ACN metabolism were determined (unpublished data), and doses that reduce CN generation by at least 75% were selected. Thus, STS and 4MP were administered by intra-peritoneal injections at respective concentrations of 150 mg/kg (Breen et al., 1995; Vesey et al., 1985) and 100 mg/kg (Feierman and Cederbaum, 1986) in saline just before the daily ACN injection. The choices of the dose and the mode of administration for L-NAC were based on results from a previous dose–effect study (unpublished data): 4 × 400 mg/kg daily was the lowest dose tested that replenished liver GSH by at least 50% compared to ACN alone. We chose to administer L-NAC by gavages, in accordance to the directions of use of our commercial product (20% acetylcysteine, Hospira, Inc, Lake Forest, IL). L-NAC dose was given by 4 successive gavages at a dose of 400 mg/kg (cumulative dose

Table 1
Experimental groups and treatments

Functional data	*n*
Controls	7
ACN 50 mg/kg sc + STS 150 mg/kg *ip*	4
ACN 50 mg/kg sc + 4-MP 100 mg/kg *ip*	4
ACN 50 mg/kg sc + L-NAC 4 × 400 mg/kg *po*	2
97 dB OBN/8 kHz	4
97 dB OBN/8 kHz + STS 150 mg/kg *ip*	4
97 dB OBN/8 kHz + 4-MP 100 mg/kg *ip*	5
97 dB OBN/8 kHz + L-NAC 4 × 400 mg/kg *po*	6
97 dB OBN/8 kHz + ACN 50 mg/kg *sc*	12
97 dB OBN/8 kHz + ACN 50 mg/kg *sc* + STS 150 mg/kg *ip*	4
97 dB OBN/8 kHz + ACN 50 mg/kg *sc* + 4-MP 100 mg/kg *ip*	7
97 dB OBN/8 kHz + ACN 50 mg/kg *sc* + L-NAC 4 × 400 mg/kg *po*	6
Blood cyanide data	***n***
Controls	1
ACN 50 mg/kg sc	5
ACN 50 mg/kg sc + STS 150 mg/kg *ip*	5
ACN 50 mg/kg sc + L-NAC 4 × 400 mg/kg *po*	4
ACN 50 mg/kg sc + 4-MP 100 mg/kg *ip*	4
Cochlear and liver glutathione data	***n***
Controls	8
ACN 50 mg/kg sc	10
ACN 50 mg/kg sc + STS 150 mg/kg *ip*	14
ACN 50 mg/kg sc + 4-MP 100 mg/kg *ip*	15
ACN 50 mg/kg sc + L-NAC 4 × 400 mg/kg *po*	17

ACN, acrylonitrile; STS, sodium thiosulfate; 4-MP, 4-methylpyrazole; L-NAC, L-*N*-acetylcysteine; dB, decibel; OBN/8 kHz, octave band noise centered at 8 kHz; DPOAEs, distortion product otoacoustic emissions; CAPs, compound action potentials.



1600 mg/kg; see Table 1 for detailed schedule). We did not witness any sign of adverse health effect after the 4 daily gavages, representing a total volume of approximately 4 mL/day.

2.4. Blood CN determination

Rats were exposed to ACN alone (50 mg/kg *sc*; $n = 5$), STS (150 mg/kg *ip*) + ACN ($n = 5$), 4MP (100 mg/kg *ip*) + ACN ($n = 4$) or L-NAC (4×400 mg/kg *po*) + ACN ($n = 4$). Fifteen minutes after the injection of ACN, animals were anesthetized with ketamine (87 mg/kg *im*) and xylazine (13 mg/kg *im*). The anesthesia was maintained for 5 h by injecting the same anesthetic mixture at 1/5th of the original dose every 30 min. 400 μL of blood was drawn by cardiac puncture at 30 min, 1, 2, 3, 4 and 5 h after treatments. The blood was immediately placed in a Conway cell and was acidified with sulfuric acid (0.25 M). The Conway cells were gently shaken for 3 hours so that liberated CN could be trapped in 0.1 M NaOH solution contained in a separate compartment within the cell. Standards and blanks were utilized by adding known concentrations of KCN to a blood sample from one untreated control subject (control blood contains no CN). Blood CN levels were determined using methods of Feldstein and Klendshoj (1954) as described in Baselt (1988). The NaOH samples containing unknown amounts of CN liberated from the blood were reacted with chloramine T and pyridine-barbituric acid solution, yielding a color reaction measured spectrophotometrically at 580 nm.

2.5. Liver and cochlear GSH levels

Rats were exposed to ACN alone (50 mg/kg *sc*), STS (150 mg/kg *ip*) + ACN, 4MP (100 mg/kg *ip*) + ACN (50 mg/kg *sc*) or L-NAC (4×400 mg/kg *po*) + ACN (50 mg/kg *sc*). At 1 h or 3h post-exposure, rats were anesthetized (ketamine/xylazine: 87/13 mg/kg *im*) and liver was harvested and immediately frozen in liquid nitrogen. For some of those animals, cochleae were simultaneously harvested and frozen for GSH analysis. GSH was measured independently in each pair of cochleae, and in ~150-mg liver samples.

Total levels of GSH in liver and cochleae were determined by using the method of Tietz (1969), modified for microplate use. Tissue was homogenized in phosphate buffer (0.5 M, pH 7.2), and aliquots were added to a reaction mix containing 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) as a chromogenic substance. Absorbance was determined at 412 nm. Content of GSH in the tissue was determined by comparing their absorption to a standard curve with known amounts of GSH. The protein concentration was determined using Quick Start™ Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Data are expressed as μmol/mg of protein.

2.6. Noise exposure

Exposures were conducted in a ventilated reverberant 40 L Plexiglas cylinder. The subjects were placed within small wire-cloth enclosures ($15 \times 13 \times 11$ cm) within the chamber. They were conscious and free to move within the enclosures. Broadband noise was generated by a function-generator (Stanford Research System, Model DS335, Menlo Park, CA) and bandpass filtered (Frequency Devices, 9002, Haverhill, MA) to provide an OBN with center frequency of 8 kHz. The roll-off for the filter system was 48 dB/octave. This signal was amplified by a SAE 2200 Power Amplifier (Scientific Audio Electronics Inc., Los Angeles, CA) and fed to speakers (Vifa D25AG-05, Videbaek, Denmark) located approximately 5 cm above the subjects' wire-cloth enclosure. Sound intensity measured at the level of the rats' pinnae by a Quest Type 1 sound pressure meter with 1/1 octave filter set (models 1700 and OB300, Oconomowoc, WI) was 97 dB in the octave band centered at 8 kHz. The noise spectrum was comparable to the one published in Pouyatos et al. (2005). Sound level in the exposure chamber was maximal and essentially flat between 6.3 and 10 kHz. The levels were approximately 7 dB lower at 5 and 12.5 kHz. The acoustic intensity was approximately 20 dB below maximum at 4 and 16 kHz. Noise levels varied less than 1 dB within the chamber.

2.7. Auditory assessment

2.7.1. Distortion Product Otoacoustic Emissions (DPOAEs)

The primary functional measurement of the present work was the DPOAE ($2f1 - f2$). The $f1$ and $f2$ primary tones were generated by a dual-channel synthesizer (Hewlett Packard Model 3326A) and attenuated, under computer control, using customized software. The $f1$ and $f2$ primaries ($f2/f1 = 1.25$) were then presented through two separate earphones (Radio Shack, Realistic Dual Radial Horn Tweeters, Tandy Corp., Ft. Worth, TX) and delivered to the outer-ear canal through a probe, where they acoustically mixed to avoid artifactual distortion. Ear-canal sound pressure levels, measured by an emissions microphone assembly (Etymotic Research, ER-10B+, Elk Grove Village, IL) embedded in the probe, were sampled, synchronously averaged, and Fourier analyzed for geometric mean (GM) frequencies $[(f1 \times f2)^{0.5}]$ ranging from 5.6 to 19.7 kHz (i.e., $f2 = 6.3$–22.5 kHz) by a computer-based DSP board. Corresponding noise floors were computed by averaging the levels of the ear-canal sound pressure for five frequency bins above and below the DPOAE frequency bin (±54 Hz).

For test frequencies above 20.1 kHz, a computer-controlled dynamic-signal analyzer (Hewlett Packard Model 3561A) was used. The related noise floors were estimated by averaging the levels of the ear-canal sound pressure for the two FFT frequency bins below the DPOAE frequency (i.e., for 3.75 Hz below the DPOAE). No artifactual DPOAEs were ever measured in a hard-walled cavity

that approximated the size of the rat outer-ear canal, which was used to calibrate the tonal stimuli. For both stimulus protocols, DPOAEs were considered to be present when they were at least 3 dB above the noise floor. Between $F2 = 20$ and $F2 = 25$ kHz, the DP-grams display an artifactual notch due to the resonance of the rat's outer auditory meatus, that prevents the primaries from reaching the eardrum with adequate intensities, and increases the noise floor obtained within this range. Candreia et al. (2004) previously observed a similar notch in the mouse. This phenomenon was recently described in detail by Martin et al. (2006).

DPOAEs were measured as DP-grams. Specifically, DP-grams described emission levels in response to primary tones at $L1 = L2 = 75$ dB SPL as a function of the GM frequencies, which ranged from 2.9 to 56.3 kHz ($f2 =$ 3.2–63 kHz), in 0.1-octave increments.

The animals were lightly anesthetized by injection of xylazine (7 mg/kg *im*) and ketamine (44 mg/kg *im*), and placed on a heating table in order to maintain the body temperature at 38 °C. The probe was inserted in the right auditory canal; the same ear subsequently used for CAP determination. Subjects received DPOAE testing prior to experimental treatment, 3 days after the end of this treatment, and again 4 weeks post-exposure.

2.7.2. Compound action potentials (CAPs)

CAP threshold assessment was performed 4 weeks following the end of experimental exposures. CAPs recorded from the round window were elicited with pure-tone bursts at 2, 4, 6, 8, 12, 16, 20, 24, 30, 35 and 40 kHz. Auditory thresholds were assessed in a double walled sound booth. The subjects were anaesthetized with xylazine (13 mg/kg *im*) and ketamine (87 mg/kg *im*) and normal body temperature was maintained using a heating table. The temperature of the cochlea was maintained using a low voltage high-intensity lamp. The auditory bulla was opened via a ventrolateral approach to allow the placement of a fine (od 0.1 mm) Teflon-coated silver wire electrode (A-M system, Inc., Carlsborg, WA) onto the round window. A silver chloride reference electrode was inserted into neck musculature. The CAP signals evoked by pure tones were amplified 1000× between 0.1 and 1.0 kHz with a Grass A.C. preamplifier (Model P15, W. Warwick, RI). The sound level necessary to generate a visually detectable CAP response averaged over 4 sweeps on a digital oscilloscope (approximate response amplitude of 1 mV) was identified.

2.8. Hair cell counts

Immediately after CAP measurements, rats were decapitated and the cochleae harvested. Within 2 min, the cochleae were fixed by perilymphatic perfusion with 1 ml of a trialdehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% dimethyl sulfoxide in phosphate buffered saline, pH 7.4). Following the primary 24 h-fixation, the tissue was first washed with 0.1 M phosphate buffered saline, post-fixed with 2% osmium tetroxide in water for 2 h, and finally washed again with 0.1 M phosphate buffered saline. The organ of Corti was dissected in 70% ethanol and mounted in glycerin to allow counting of the hair cells. Cells were counted as present either when the stereocilia, the cuticular plate or the cell nucleus could be visualized. No attempt was made to assess the degree of possible cellular damage to surviving cells. The frequency-place map established by Muller (1991) was used to superimpose the frequency coordinates on the length coordinates of the organ of Corti. This "map" reflects the fact that the cochlea is organized in a tonotopic fashion with high frequency sound producing maximum stimulation of cells in the base, and low frequency sound in the apex. A cochleogram showing the percentage of hair cell loss as a function of distance from the base of the cochlea was plotted for each animal. The results were averaged across each group of subjects for comparison between groups. The custom programs used for counting cochlear hair cells, plotting, and averaging cochleograms, were developed by R. Lataye and Dr. P. Campo from the "Institut National de Recherche et Sécurité", Nancy, France.

2.9. Statistical analysis

The DPOAE and CAP data were analyzed using two-way ANOVA with Bonferroni's post test performed using GraphPad Prism (version 4.0b for Mac, GraphPad Software, San Diego, CA, www.graphpad.com).

Baseline, 1 day and 4 week post-exposure DPOAE amplitudes were analyzed with separate repeated measures ANOVAs using the experimental treatment as between subject factor and $f2$ frequency as within subject factor. Planned post hoc comparisons were performed between treatment groups using the Bonferroni's test. $P = 0.05$ was considered as the significance threshold.

CAP thresholds and OHC loss were analyzed with two-way ANOVAs to evaluate the effects of experimental treatment (between subject factor) at different frequencies or cochlear locations (within subject). Bonferroni's post hoc tests were performed between experimental groups. $P = 0.05$ was considered as the significance threshold.

Liver and cochlear GSH levels were analyzed by Student's t-test with Microsoft Excel 2004 for Mac (V11.1, Microsoft Corporation, WA). No statistical analysis was performed on subjects with undetectable levels of GSH.

3. Results

3.1. Functional data

3.1.1. DPOAEs

Fig. 2 shows DP-grams obtained (a, b, c) before, (d, e, f) 3 days and (g, h, i) 4 weeks after the different experimental treatments. For sake of clarity, the experimental groups are

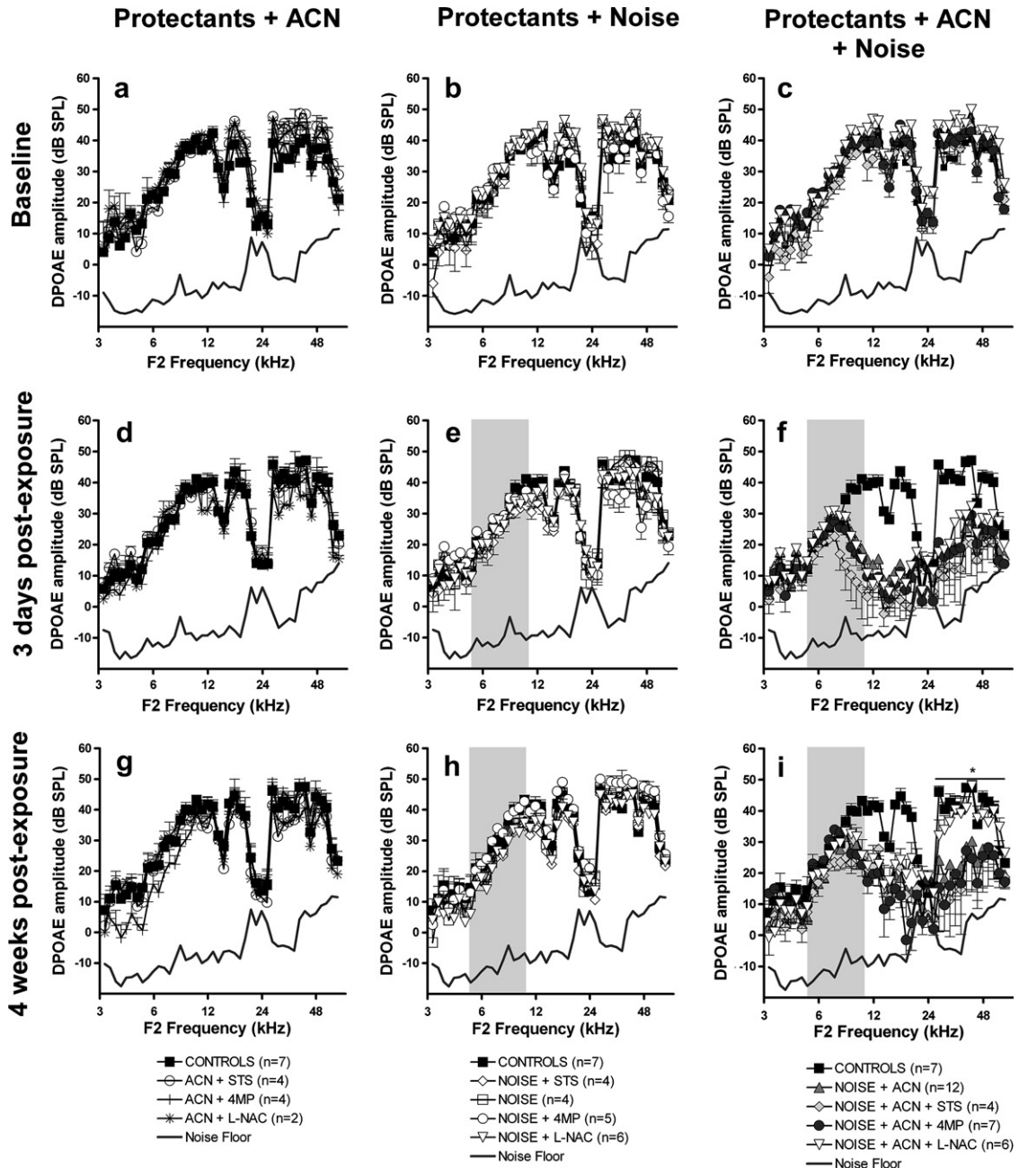


Fig. 2. DPOAE amplitudes obtained (a, b, c) before, (d, e, f) 3 days and (g, h, i) 4 weeks after the different experimental treatments. For sake of clarity, the experimental groups are divided in three categories, which are displayed in three different columns: Left column: Protectants + ACN (STS + ACN, 4MP + ACN and L-NAC + ACN); center column: Protectants + Noise (noise, STS + noise, 4MP + noise, L-NAC + noise); right column: Protectants + ACN + Noise (ACN + noise, STS + ACN + noise, 4MP + ACN + noise, L-NAC + ACN + noise). For reference, DP-grams obtained in controls were included in each of the graphs. DP-grams were obtained with the levels of the primaries $f1$ and $f2$ set at 75 dB SPL, and with $f2/f1 = 1.25$. The tested $f2$ frequencies ranged from 3.2 to 63 kHz (geometric mean frequencies: 2.9–56.3 kHz), in 0.1 octave increments. The gray area represents the noise frequency range. Error bars: ±SEM.

divided in three categories, which are displayed in three different columns:

- Left column: Protectants + ACN (STS + ACN, 4MP + ACN and L-NAC + ACN)
- Center column: Protectants + Noise (noise, STS + noise, 4MP+noise, L-NAC + noise)
- Right column: Protectants + ACN + Noise (ACN+ noise, STS + ACN + noise, 4MP + ACN + noise, L-NAC + ACN + noise). For reference, DP-grams obtained in controls were included in each of the graphs.

The baseline DP grams (Figs. 2a–c) obtained in the different groups were remarkably similar.

At 3 days post-exposure, no DPOAE amplitude decrement could be observed in the animals exposed to protectants + ACN (Fig. 2d) or protectants + noise (Fig. 2e). By contrast, DPOAE amplitudes were profoundly reduced between 7 and 55 kHz in animals exposed to ACN + noise, STS + ACN + noise, 4MP + ACN + noise and L-NAC + ACN + noise (Fig. 2f). The amplitude decrements were quite similar in these 4 groups, the maximum shifts averaging 25–30 dB between 12 and 32 kHz when compared to their respective baselines (except in the notch region).

After the recovery period, 4-week post-exposure (Fig. 4i), the animals that received combined exposure to ACN + noise, STS + ACN + noise and 4MP + ACN + noise showed little change in their DPOAE amplitudes compared to the 3 day post-exposure readings (Fig. 2f). For example, the permanent DPOAE drop was still 22 dB in ACN + noise animals, 31 dB in L-NAC + ACN + noise, and 37 dB in 4MP + ACN + noise rats at 19 kHz relative to their baselines. By contrast, the DPOAEs obtained in the animals exposed to L-NAC + ACN + noise (Fig. 2f) recovered to baseline (control) levels above 25 kHz. However, at lower frequencies, L-NAC failed to prevent the reduction in DPOAE amplitude caused by ACN + noise treatment: indeed, for frequencies of 20 kHz and below, DPOAEs obtained in L-NAC + ACN + noise-treated animals were comparable to DPOAEs measured in ACN + noise, STS+ACN + noise and 4MP + ACN + noise treated rats. The permanent loss for the L-NAC + ACN + noise experimental group was therefore restricted to the frequency range neighboring the upper limit of the noise exposure range.

DP-grams obtained in animals exposed to protectants + ACN (Fig. 2g) and protectants + noise (Fig. 2h) were still similar to controls.

The DPOAE amplitudes were analyzed by repeated measure ANOVAs performed at the different time points. The ANOVAs showed a significant effect of treatment at 3 days post-exposure [$F_{(11/53)} = 28.69$, $p < 0.0001$], as well as 4 weeks post-exposure [$F_{(11/53)} = 35.24$, $p < 0.0001$]. No significant effect of treatment was observed at baseline. The ANOVAs also revealed significant effects of treatment x frequency interaction [3 days: $F_{(473/2279)} = 3.981$, $p < 0.0001$; 4 weeks: $F_{(473/2279)} = 2.581$, $p < 0.0001$], pointing out that the effect of treatment was dependent on the frequency considered.

Post hoc comparisons using Bonferroni's multiple comparison tests showed that, at 3 days post-exposure, DPOAE amplitudes measured in animals that received ACN + noise, STS + ACN + noise, 4MP + ACN + noise and L-NAC + ACN + noise were not significantly different from each other [$p > 0.05$], but significantly lower than DPOAEs measured in all the remaining experimental groups [$p < 0.05$]. At 4 week post-exposure, DPOAEs measured in the L-NAC + ACN + noise animals were significantly higher than in ACN + noise subjects between 27 and 58.82 kHz [$p < 0.05$]. L-NAC + ACN + noise DPOAE amplitudes were not different from control DPOAEs between 20 and 63.3 kHz [$p > 0.05$]. ACN + noise,

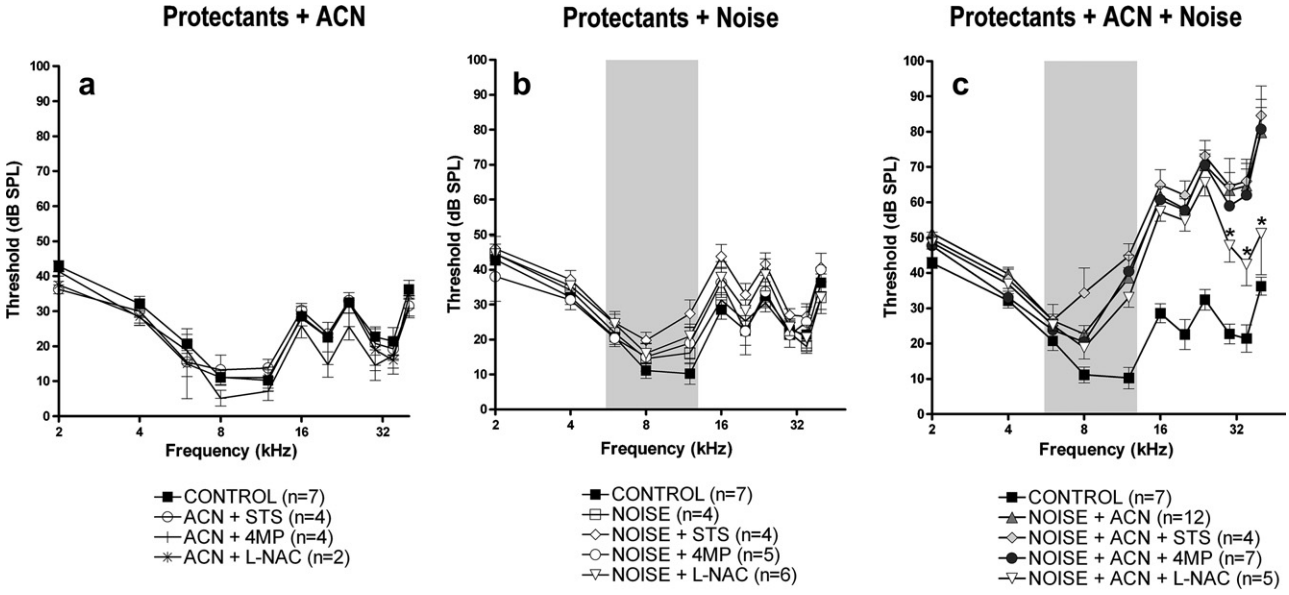


Fig. 3. Effects of the different experimental treatments on compound action potential (CAP) thresholds measured 4 weeks post-exposure for frequencies ranging from 2 to 40 kHz. For sake of clarity, the experimental groups are divided in three categories, which are displayed in three different graphs: (a) Protectants + ACN (STS + ACN, 4MP + ACN and L-NAC + ACN), (b) Protectants + Noise (noise, STS + noise, 4MP + noise, L-NAC + noise) and (c) Protectants + ACN + Noise (ACN + noise, STS + ACN + noise, 4MP + ACN + noise, L-NAC + ACN + noise). For reference, CAP thresholds obtained in controls were included in each of the graphs. The gray area represents the noise frequency range. Error bars: ±SEM.

STS + ACN + noise and 4MP + ACN + noise DPOAEs were significantly lower than controls between 10 and 58.8 kHz [$p < 0.05$].

3.1.2. CAPs

Fig. 3 presents the disruption of CAP thresholds measured 4 weeks post-exposure in the different experimental

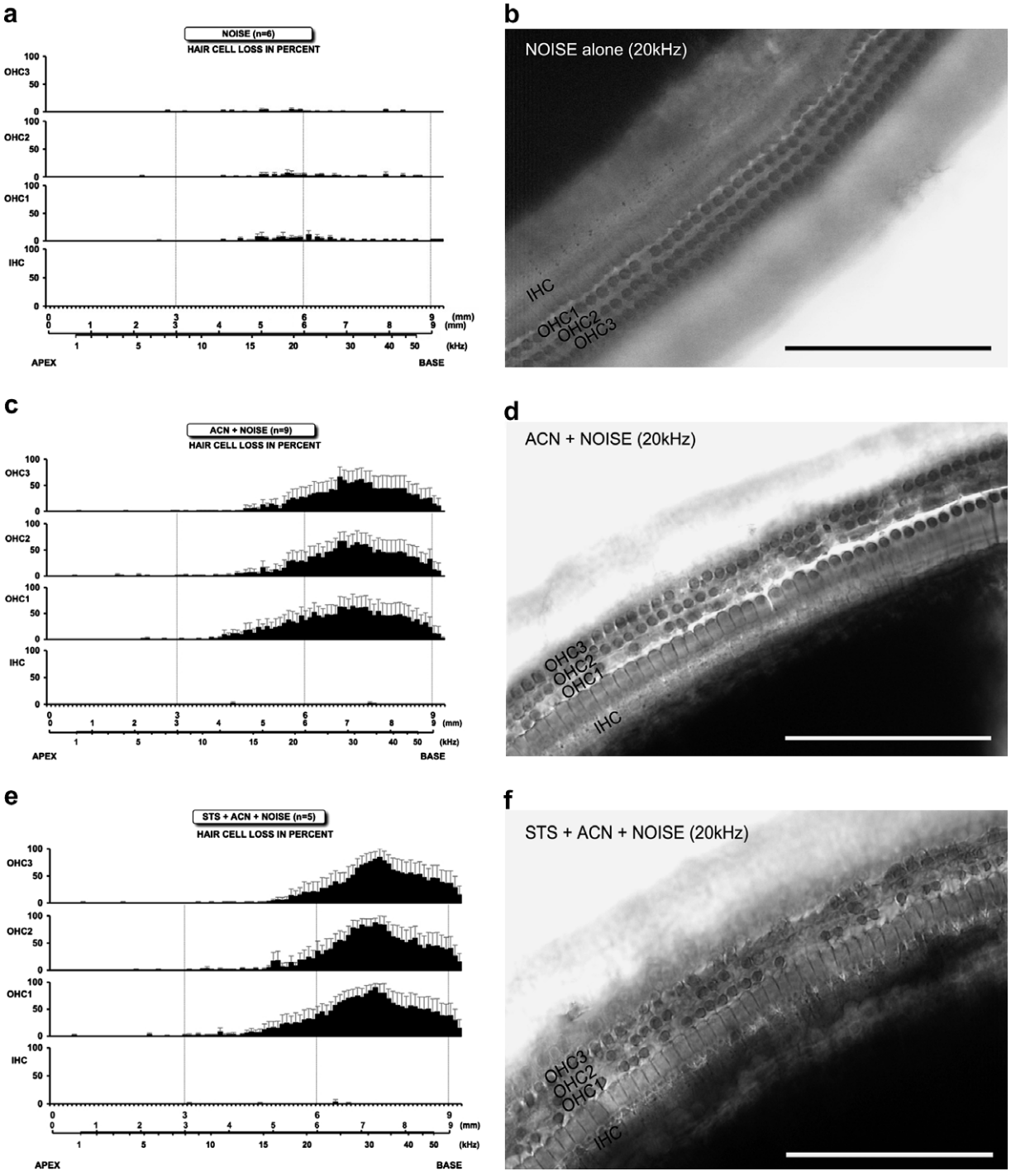


Fig. 4. Average cytocochleograms (left panels) and microphotographs of the organ of Corti (right panels) showing hair cell loss in rats exposed to (a,b) noise alone, (c, d) ACN + noise, (e, f) STS + ACN + noise, (g, h) 4MP + ACN + noise or (i, j, k) L-NAC + ACN + noise. Treatment doses and schedules are detailed in Table 1. All microphotograph were taken around the 20 kHz cochlear location, except (k) that illustrates the 40–50 kHz region. Abscissa – upper trace: length (mm) of the entire spiral course of the organ of Corti from the bottom of the hook. – lower trace: frequency-map according to Muller (1991). Ordinate: hair cell loss in percent. IHC: inner hair cells; OHC1: first row of outer hair cells; OHC2: second row; OHC3: third row. Error bars in the cytocochleogram represent the standard error. The scale bars represent 100 μm.

groups. For sake of clarity, the experimental groups are divided in 3 categories, which are displayed in three different graphs: (a) Protectants + ACN (STS + ACN, 4MP + ACN and L-NAC + ACN), (b) Protectants + Noise (noise, STS + noise, 4MP + noise, L-NAC + noise) and (c) Protectants + ACN + Noise (ACN + noise, STS + ACN + noise, 4MP + ACN + noise, L-NAC + ACN + noise). For reference, CAP thresholds obtained in controls were included in each of the graphs. One animal from the L-NAC + ACN + noise group died during the surgical procedure, which decreased to $n = 5$ the number of subjects in this group.

Animals that received Protectants + ACN (Fig. 3a) and Protectant + Noise (Fig. 3b) did not show any significant threshold shift compared to controls.

The animals that received combined exposure to ACN + noise, STS + ACN + noise and 4MP + ACN + noise (Fig. 3c) showed significant threshold shifts, ranging

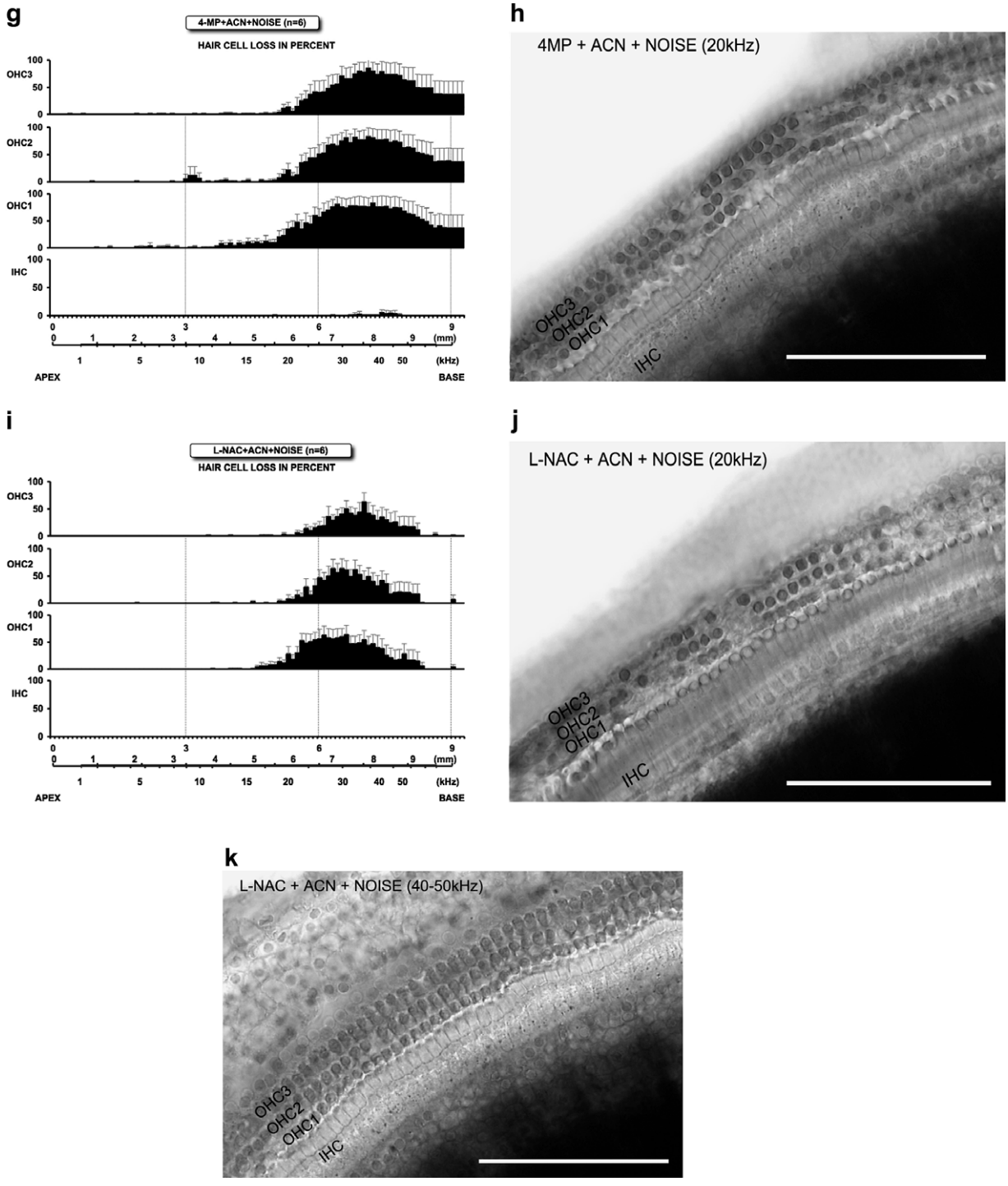


Fig. 4 (*continued*)

from 21 to 50 dB in the 16–40 kHz frequency range compared to the controls. L-NAC pre-treatment significantly reduced the threshold shift caused by ACN + noise exposure at the three highest frequencies: CAP thresholds measured in L-NAC + ACN + noise animals were 13, 20 and 26 dB lower than those obtained in ACN + noise animals at 30, 35 and 40 kHz, respectively. The addition of L-NAC, hence, protected auditory sensitivity against the impairment caused by ACN + noise exposure.

CAP thresholds were analyzed by two-way ANOVA with Bonferroni's post test. The overall effect of "treatment" was significant [$F_{(11/52)} = 12.15$, $p < 0.0001$]. Bonferroni's post test revealed that the thresholds from the ACN + noise, STS + ACN + noise, 4MP + ACN + noise and L-NAC + ACN + noise were different from controls [$p < 0.05$]. L-NAC + ACN + noise thresholds were significantly lower than ACN + noise thresholds at 30, 35 and 40 kHz [$p < 0.05$]. At 40 kHz, CAPs measured in L-NAC + ACN + noise were not different from control CAPs [$p > 0.05$].

3.2. Histopathological data

To assess the magnitude of cochlear damage, hair cells were counted from cochleae harvested from the same animals used for physiological studies. The hair cell loss is presented as cochleograms that display the percentage of hair cell loss as a function of distance from the apex of the cochlea. Results obtained in controls, L-NAC + ACN STS + ACN, and 4MP + ACN animals are not shown because no significant hair cell loss was observed. For sake of clarity, cochleograms from STS + noise, 4MP + noise and L-NAC + noise groups are not presented because hair cell loss was similar to noise alone animals.

Fig. 4 shows the mean cochleograms (left panels) and photographs of the organ of Corti (right panels) obtained in animals exposed to noise alone (a, b), ACN + noise (c, d) STS + ACN + noise (e, f), 4MP + ACN + noise (g, h) and L-NAC + ACN + noise (i, j, k).

The cochleae from subjects exposed to noise alone (Figs. 4a and b) – as well as STS + noise, 4MP + noise, L-NAC + noise (not shown) – displayed very limited damage, less than 1.5% OHC loss on average.

By contrast, the cochleae from rats exposed to both ACN and noise (Figs. 4c and d) exhibited substantial damage in the basal (or high frequency) half of the organ of Corti. The mean OHC loss averaged 35% in the three OHC rows in the region corresponding to frequencies above 12 kHz. As expected, neither STS (Figs. 4e and f) nor 4MP (Figs. 4g and h) pre-treatments reduced OHC loss caused by ACN + noise: above 12 kHz, the average OHC losses were 41% and 47% in STS + ACN + noise and 4MP + ACN + noise groups, respectively.

Consistent with physiological results, the pre-treatment with L-NAC (Figs. 4i, j and k) reduced the OHC loss caused by ACN + noise in the region corresponding to

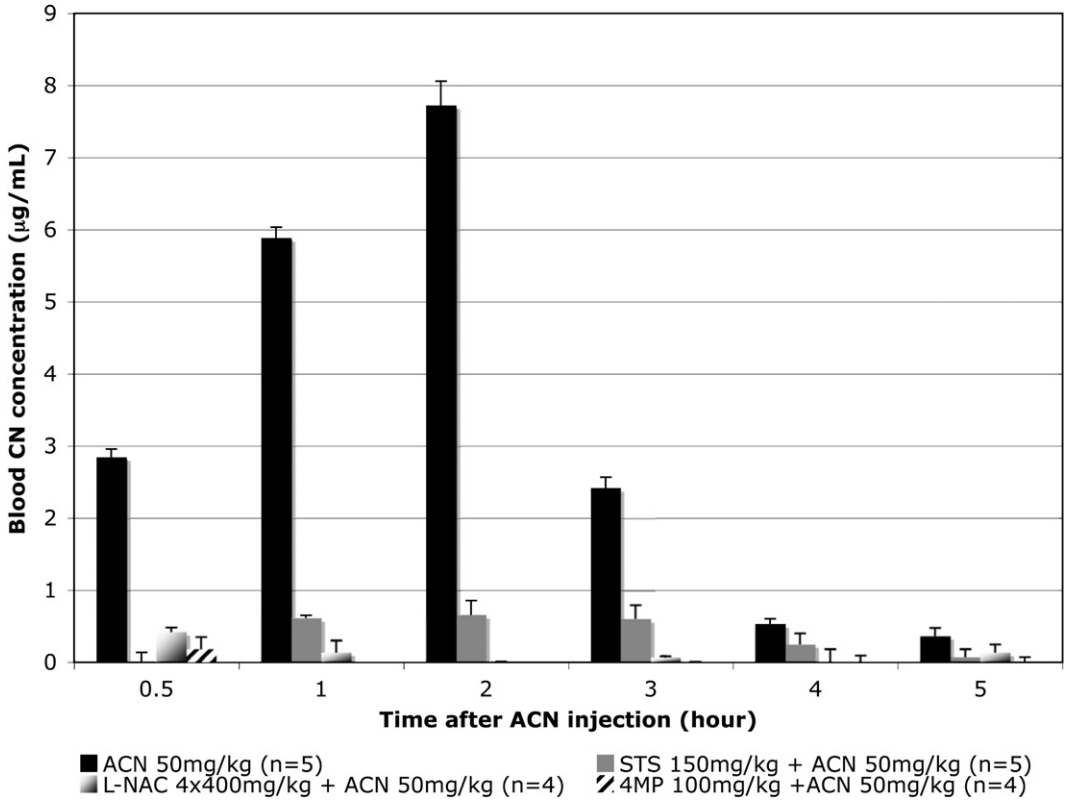


Fig. 5. Accumulation of cyanide in systemic blood following injection with ACN, STS + ACN and L-NAC + ACN. Values at each time point are the means ± SEM.

25 kHz and above: In this area, the cochleae exposed to ACN + noise exhibited 42% loss, compared to 20% for L-NAC + ACN + noise animals (Fig. 4k). Between 12 and 25 kHz, OHC loss was similar in those two groups, 22% (Fig. 4j).

The OHC loss observed in noise, ACN + noise, STS + ACN + noise, 4MP + ACN + noise and L-NAC + ACN + noise treated animals was analyzed with a two-way ANOVA. The overall effect of "treatment" was significant [$F(4,33) = 4.069$; $p < 0.001$]. Bonferroni's post test confirmed that treatments with ACN + noise, STS + ACN + noise, 4MP + ACN + noise and L-NAC + ACN + noise induced significant OHC loss between 19.4 and 68.5 kHz, 18.3 and 68.5 kHz, 17.7 and 68.50 kHz, 18.3 and 33.8 kHz, respectively [$p < 0.05$]. OHC loss observed in L-NAC + ACN + noise-treated cochleae was significantly smaller than the OHC loss counted in ACN + noise cochleae between 29.9 and 68.5 kHz [$p < 0.05$].

3.3. Blood CN levels

Fig. 5 presents the blood CN levels measured at different times following ACN, STS + ACN, 4MP + ACN or L-NAC + ACN administrations. In the rats injected with ACN, blood CN level increased rapidly to reach a peak 2 h after injection (7.72 μg/mL). Two hours later, CN was virtually eliminated from the blood (0.53 μg/mL). Both STS and 4MP administrations dramatically reduced blood CN levels below 0.6 μg/mL at all time points. L-NAC administration also reduced CN concentrations in the blood. Measured concentrations were just above detectable levels at all time intervals.

3.4. GSH levels

Total liver and cochlear GSH levels measured 1 and 3 h after ACN, STS + ACN, 4MP + ACN and L-NAC + ACN treatments, as well as in untreated controls, are presented in Table 2. Prior to treatment, control livers contained approximately 10 times more GSH (69.6 ± 2.3 nmol/mg protein) than control cochlear tissue (7.21 ± 0.54 nmol/mg protein).

3.4.1. Liver GSH levels

One hour after ACN injection, GSH was depleted by 63% compared to control levels ($p < 0.01$). GSH levels remained stable during the next two hours.

Co-treatment with STS induced an 83% decrease of GSH levels (11.8 ± 2.0 nmol/mg protein) compared to control livers at 1 h post-exposure ($p < 0.001$), followed by a recovery to 33.2 ± 5.0 nmol/mg protein at 3 h (−52% compared to controls; $p < 0.05$).

Pre-treatment with 4MP had virtually the same effect on GSH levels as STS at 1 h (−81% compared to controls; $p < 0.001$). At 3 h, GSH levels in 4MP + ACN animals were still 53% lower than controls ($p < 0.001$).

As opposed to the two previous pre-treatments, L-NAC did significantly protect GSH at both time points. GSH depletion was only −23 and −20% compared to controls at 1 and 3 h post-treatment, respectively. GSH levels were significantly higher in L-NAC + ACN than in ACN alone animals at both time points ($p < 0.05$).

3.4.2. Cochlear GSH levels

One hour after ACN administration, GSH levels were depleted down to undetectable levels (<2 nmol/mg pro-

Table 2
Total (a) liver and (b) cochear glutathione content in control, ACN, 4-MP + ACN, STS + ACN and L-NAC + ACN ($n = 4$ per time point) exposed rats measured 1 and 3 h post-treatment

Treatment	GSH (nmol/mg protein)	Time after ACN administration			
		1 hour		3 hours	
		GSH (nmol/mg protein)	% Change compared to controls	GSH (nmol/mg protein)	% Change compared to controls
(a) Liver					
Controls	69.6 ± 2.3 ($n = 8$)				
ACN		25.4 ± 9.1* ($n = 4$)	−63%	25.4 ± 2.3* ($n = 6$)	−63%
STS + ACN		11.8 ± 2.0* ($n = 7$)	−83%	33.2 ± 5.0* ($n = 7$)	−52%
4-MP + ACN		13.1 ± 2.7* ($n = 7$)	−81%	32.1 ± 2.45* ($n = 8$)	−53%
L-NAC + ACN		53.3 ± 9.3*# ($n = 9$)	−23%	55.5 ± 5.6*# ($n = 8$)	−20%
(b) Cochlea					
Controls	7.21 ± 0.54 ($n = 7$)				
ACN		n.d. ($n = 4$)	–	2.82 ± 0.91* ($n = 5$)	−61%
STS + ACN		n.d. ($n = 3$)	–	n.d. ($n = 3$)	–
4-MP + ACN		3.65 ± 0.81* ($n = 3$)	−49%	3.89 ± 2.45* ($n = 3$)	−46%
L-NAC + ACN		24.85 ± 7.59* ($n = 4$)	+244%	16.40 ± 3.73# ($n = 4$)	+127%

Values at each time point are the means ± SEM.
* Significantly different from controls ($p < 0.05$).
# Significantly different from the ACN-treated group at the same time point ($p < 0.05$).

tein). At the 3-h time point, GSH was back above the detection threshold but still depleted by 61% compared to control cochleae ($p = 0.002$).

STS pre-treatment depleted GSH levels below detectable levels at both 1 and 3 h post ACN.

4MP cochleae showed slightly reduced depletion since GSH levels were measurable at both 1 and 3 h post exposure, 3.65 nmol/μg protein (−49% versus control, $p = 0.01$) and 3.89 nmol/mg protein (−46% versus controls, $p = 0.01$), respectively. At the 3-h time point, 4MP + ACN and ACN alone GSH data were not significantly different from each other ($p > 0.05$).

Surprisingly, L-NAC pre-treatment not only protected GSH, but induced an increase of GSH levels above control levels: +244% at 1 h and +127% at 3 h. The difference in GSH levels between L-NAC + ACN and ACN alone treated animals was significant at 1 and 3 h post-treatment ($p < 0.05$).

4. Discussion

In combination with the earlier paper of Pouyatos et al. (2005), this investigation confirms that combined exposure to ACN and noise levels as low as 97 dB OBN for 4 h can induce auditory impairment and OHC loss. That a true potentiation of NIHL occurs in the presence of ACN is demonstrated by the fact that exposure to ACN alone or to noise alone each failed to induce auditory loss or cochlear damage. We found that the animals that received a combined exposure to ACN and noise showed considerable permanent hearing deficits along with massive OHC loss from the 12-kHz region to the extreme base of the cochlea. This damage is therefore surprisingly extended towards the high frequencies, much more than predicted by the half-octave shift theory (12–24 kHz damage for a 8-kHz-centered octave band) (McFadden, 1986). This observation underlines the fact that the noise exposure used in this experiment includes a cochlear stimulation that is not limited to the frequency range of the noise, but which extends from the 8-kHz region to the basal end of the cochlea. We hypothesize that, in our experimental conditions, ACN exacerbates noise-induced damage in the organ of Corti by decreasing antioxidant defenses of hair cells. The OHC-loss shift towards high frequencies could be due to the fact that the cochlea has different susceptibility to ROS depending on the tonotopic location: Sha et al. (2001) observed that cochlear organotypic cultures of basal OHCs were more vulnerable to free-radical damage than apical OHCs, and that basal OHC survival was improved by the addition of L-NAC or GSH. Also, Clerici and Yang (1996) showed that direct perilymphatic generation of ROS (by instillation of artificial supplemented perilymph) induced a rapid degradation in high-frequency CAP threshold sensitivity, the pattern of which being surprisingly similar to threshold shift obtained in our ACN + noise treated animals (Fig. 3). According to these data, the decrease of antioxidant defenses caused by ACN would therefore render the basal OHCs more susceptible to noise than the apical OHCs, and explain the high-frequency shift.

This phenomenon was previously observed in Pouyatos et al. (2005) with the same exposure parameters. However, the potentiation obtained in the present experiment was much more extensive. The reason for this difference is not known, however one can suggest that the younger age of the rats used in the most recent experiment – 7–8 weeks old versus 9–10 weeks in the previous report – could explain the increased cochlear damage.

The use of diverse drugs modifying ACN metabolism allowed us to identify GSH depletion as a mechanism involved in this interaction. Specifically, the administration of L-NAC prior to ACN, which caused GSH replenishment and decreased CN generation, did partially protect against cochlear impairment resulting from ACN + noise treatment specifically at high frequencies. In contrast, STS and 4MP pre-treatments were ineffective in protecting cochlear cells from the deleterious effect of ACN + noise co-exposure. Both of these drugs blocked or reduced blood CN but did not significantly protect GSH levels.

GSH has previously been found to protect the inner ear from stress related damages. Kopke et al. (2002) found that D-methionine, a free radical scavenger and cysteine donor that helps neo-synthesis of GSH, protected the cochlea against noise trauma. Yamasoba et al. (1998) demonstrated that 1-buthionine-[*S*,*R*]-sulfoximine (BSO), an inhibitor of GSH synthesis, significantly increased the thresholds induced by continuous noise trauma. In contrast, oxothiazolidine-4-carboxylate (OTC), which promotes the restoration of GSH, significantly decreased the threshold shifts induced by noise trauma. Henderson et al. (1999) found that antioxidants such as glutathione-monoethylester protected the chinchilla cochlea from impulse noise trauma.

Several reports showed that L-NAC has a protective effect against high-level noise exposures. Kopke et al. (2000, 2005) showed that 350 mg/kg L-NAC reduced both functional and histological damages caused by 155 dB peak SPL noise exposure in chinchillas. Ohinata et al. (2003) demonstrated that 500 mg/kg L-NAC attenuated hearing loss and hair cell loss caused by a 5-h-long 115 dB continuous noise exposure in the guinea pig. Also, Duan et al. (2004) showed that the cochlear trauma caused by 160 dB peak SPL pulses was reduced by 1050 mg/kg L-NAC (3×350 mg/kg).

It is particularly noteworthy that the protection generated by L-NAC was effective only for the extreme base of the cochlea, coding for frequencies above 25 kHz. Once again, this finding is in agreement with the report by Sha et al. (2001) and Clerici and Yang (1996): it suggests that the cochlea has variable susceptibility to ROS depending on the tonotopic location, and that GSH may play a more critical role in protecting against ROS in the basal region of the cochlea.

Despite the fact that L-NAC elevated GSH above control levels in the cochlea, the protection against ACN +

noise was only partial, suggesting that other mechanisms may be involved in the cochlear defense against ROS. One can hypothesize that the auditory loss in the basal part of the organ of Corti may be the result of GSH depletion induced by ACN + noise exposure, while the damage observed in the more apical sections might be the result of a mechanical damage caused by noise. In the 12-25 kHz section of the cochlea, the damage caused by the noise itself may not have been enough to cause the death of the hair cells, but sufficient to prevent a total recovery of hearing threshold, even with the supplemental GSH provided by L-NAC.

The use of 4MP and STS in the present work did not allow us to identify a role for CN as a NIHL promoter and, by extension, cast doubt upon the role of Cu/Zn SOD in protecting against noise and whether superoxide anion is a prominent contributor to NIHL. Yet, because we did not monitor potential inhibition of Cu/Zn SOD by CN, we cannot conclude that this enzyme plays no role in protecting the cochlea from ROS generation.

Pierson and Gray (1982) showed higher levels of SOD in the organ of Corti than in cerebellum, retina, cortex, or lung. 74% of cochlear SOD was inhibited by CN, suggesting that the Cu/Zn isozyme of SOD was predominant in cochlea. Perhaps the ACN exposure did not generate sufficient amount of CN to inhibit the enzyme, or the noise level was too low to generate sufficient quantities of superoxide anion. By contrast to our results obtained with modest noise exposures, several authors have suggested that Cu/Zn SOD is actually involved in the antioxidant defense against ROS generated by high-intensity or long-term noise exposure: For example, Ohlemiller et al. (1999a) have shown that animals with a genetic deficiency in Cu/Zn SOD are more sensitive to 1-h exposure at 110 dB than WT subjects. The administration of SOD-polyethylene glycol, which increases the half-life of SOD, was shown to reduce cochlear impairment caused by exposure to 90 dB SPL noise for 60 h (Seidman et al., 1993).

Based upon the 5 dB Occupational Safety and Health Administration (OSHA) exchange rate, the noise levels used in this study are equivalent to 97.5 dB = 92 dB or 90 dB_A for 8 h. Our exposure parameters were therefore within the 90 dB_A TWA permissible exposure limit on the workplace. While the noise level used here is relevant to human exposure, this is not the case for the ACN exposure. Clearly, the route is different from that experienced by human workers, and the dose and frequency of ACN exposure used in this study are far greater (Thier et al., 2000). However, because the wide use of ACN in industrial settings where noise exposure is also present, our investigation identified a synergic mechanism that may be of considerable public health importance.

L-NAC has been used in previous studies in order to protect the cochlea from oxidative stress at doses ranging from 325 mg/kg (Kopke et al., 2005) to 1750 mg/kg (Duan et al., 2004). The authors did not observe any toxicity, even at the highest dose above.

L-NAC is a clinically used medicine with an extensive safety record over decades. L-NAC has also passed the stringent safety requirement for Food and Drug Administration approval for acetaminophen overdose, countering the liver damage induced by GSH depletion and the consequent oxidative stress. In addition, L-NAC is almost devoid of serious adverse side-effects (Sorbi et al., 2000). L-NAC has therefore been logically proposed as a preventive treatment against NIHL, as an adjunct to hearing protection devices (Kopke et al., 2005). The positive effect of L-NAC observed in the present study suggests that the use of this drug could be broadened to the prevention/treatment of combined effects of pro-oxidant chemicals/drugs and noise. For example, L-NAC has previously been proved effective in protecting liver against gentamicin-induced nephropathy (Mazzon et al., 2001). Since gentamicin can potentially induce ROS in the cochlea (Lopez-Gonzalez et al., 1999) and promote NIHL at sub-ototoxic dose, L-NAC might be a good contender for preventing its ototoxicity.

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