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CANDIDATE'S THESIS

Enhancing Intrinsic Cochlear Stress Defenses to Reduce Noise-Induced Hearing Loss

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Objectives/Hypothesis: **Oxidative stress plays a substantial role in the genesis of noise-induced cochlear injury that causes permanent hearing loss. We present the results of three different approaches to enhance intrinsic cochlear defense mechanisms against oxidative stress. This article explores, through the following set of hypotheses, some of the postulated causes of noise-induced cochlear oxidative stress (NICOS) and how noise-induced cochlear damage may be reduced pharmacologically. 1) NICOS is in part related to defects in mitochondrial bioenergetics and biogenesis. Therefore, NICOS can be reduced by acetyl-L carnitine (ALCAR), an endogenous mitochondrial membrane compound that helps maintain mitochondrial bioenergetics and biogenesis in the face of oxidative stress. 2) A contributing factor in NICOS injury is glutamate excitotoxicity, which can be reduced by antagonizing the action of cochlear *N*-methyl-D-aspartate (NMDA) receptors using carbamathione, which acts as a glutamate antagonist. 3) Noise-induced hearing loss (NIHL) may be characterized as a cochlear-reduced glutathione (GSH) deficiency state; therefore, strategies to enhance cochlear GSH levels may reduce noise-induced cochlear injury. The objective of this study was to document the reduction in noise-induced hearing and hair cell loss, following application of ALCAR, carbamathione, and a GSH repletion drug D-methionine (MET), to a model of noise-induced hearing loss. *Study Design:* This was a prospective, blinded observer study using the above-listed agents as modulators of the noise-induced cochlear injury response in the species *chinchilla laniger*. *Methods:* Adult *chinchilla laniger* had baseline-hearing thresholds determined by auditory brainstem response (ABR) recording. The animals then received injections of saline or saline plus active experimental compound starting before and continuing after a 6-hour 105 dB SPL continuous 4-kHz octave band noise exposure. ABRs were obtained immediately after noise exposure and weekly for 3 weeks. After euthanization, cochlear hair cell counts were obtained and analyzed. *Results:* ALCAR administration reduced noise-induced threshold shifts. Three weeks after noise exposure, no threshold shift at 2 to 4 kHz and <10 dB threshold shifts were seen at 6 to 8 kHz in ALCAR-treated animals compared with 30 to 35 dB in control animals. ALCAR treatment reduced both inner and outer hair cell loss. OHC loss averaged <10% for the 4- to 10-kHz region in ALCAR-treated animals and 60% in saline-injected-noise-exposed control animals. Noise-induced threshold shifts were also reduced in carbamathione-treated animals. At 3 weeks, threshold shifts averaged 15 dB or less at all frequencies in treated animals and 30 to 35 dB in control animals. Averaged OHC losses were 30% to 40% in carbamathione-treated animals and 60% in control animals. IHC losses were 5% in the 4- to 10-kHz region in treated animals and 10% to 20% in control animals. MET administration reduced noise-induced threshold shifts. ANOVA revealed a significant difference (*P* <.001). Mean OHC and IHC losses were also significantly reduced (*P* <.001). *Conclusions:* These data lend further support to the growing body of evidence that oxidative stress, generated in part by glutamate excitotoxicity, impaired mitochondrial function and GSH depletion causes cochlear injury induced by noise. Enhancing the cellular oxidative**

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stress defense pathways in the cochlea eliminates noise-induced cochlear injury. The data also suggest strategies for therapeutic intervention to reduce NIHL clinically. *Key Words:* Noise-induced HL, acetyl-L-carnitine, carbamathione, methionine, oxidative stress.

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INTRODUCTION

Noise is a pervasive and increasing hazard in all developed or developing nations, with 600 million persons estimated to be[1] working in environments with hazardous levels of noise (50–60 million in the United States and Europe). Ten million persons in the United States have permanent, irreversible hearing loss from noise or trauma.[2] Forty-four percent of carpenters and 48% of plumbers reported they had a perceived hearing loss,[3] and 90% of coal miners are estimated to have a hearing impairment by age 52 years.[4] The U.S. Government spends over $250 million in compensation each year for military-related noise-induced hearing loss (NIHL).[5] It is estimated that NIHL is one of the most common military occupational disabilities, even in the era of mandated hearing conservation practices. Mechanical hearing protection is essential and effective; however, inherent limitations allow a significant percentage of permanent hearing loss to occur after relatively short military noise exposures.[6,7] Inherent limitations of mechanical hearing protection devices (HPDs) include: 1) noise exceeding the protective capabilities of the device, 2) skull transmission of damaging acoustic energy, 3) fitting, and 4) compliance issues.[8–17] Hence, a pharmacological preventative or rescue agent for NIHL would be an important element of a comprehensive approach to maintaining inner ear functional integrity in patients exposed to noise.

To develop such a preventative or therapeutic approach, a comprehensive understanding of the molecular pathophysiology of NIHL is required. Over the past decade, evidence has accumulated linking oxidative stress to some forms of noise-induced cochlear injury.[18] While certain high-level noises impart sufficient energy to the cochlea (approximately 125 dB SPL or greater) to cause significant mechanical damage,[19,20] much noise exposure is at such a level so as to metabolically challenge the cochlea as well.[21–23] Acoustic overexposure leads to the production of reactive oxygen species (ROS) and other free radical molecules in the cochlea,[24–26] and these ROS are quite capable of inducing cochlear damage as well as loss of function when infused into the cochlea.[27] In recent years, a variety of compounds with antioxidant effects have been shown to ameliorate noise-induced cochlear injury.[18,28–37]

While it is becoming increasingly apparent that oxidative stress plays a major role in noise-induced cochlear injury, less is known about the possible cochlear generators of the oxidative stress during acoustic overexposure. Postulated generators of ROS and other free radicals in the noise-stressed cochlea include ischemia–reperfusion,[29,30,38–45] metabolically overdriven cochlear mitochondria,[46] and ionic fluxes. The ionic fluxes may be the result of transient noise-induced microlesions in cell membranes,[47] or reticular lamina[48] or glutamate excitotoxicity resulting from excessive IHC stimulation.[49–52] Excessive Ca^{2+} influx resulting from mechanically induced microlesions or ROS-induced damage of Ca^{2+} regulatory proteins can lead to increased intracellular Ca^{2+}. Reactive oxygen species or Ca^{2+} flux-induced molecular cascades may then ensue. These include phospholipase A_2 activation, superoxide generation by proteolytically activated xanthine oxidase, and formation of nitric oxide (NO) and its breakdown products through activation of calmodulin and nitric oxide synthase (NOS).[18,53]

Mitochondrial injury, which can be caused by excessive ROS generation within mitochondria or as a consequence of glutamate excitotoxicity or GSH depletion,[53–56] has been shown to play a key role in cell death. Inhibiting mitochondrial biogenesis, or self-repair, enhances noise-induced cochlear injury.[46] To further test the hypothesis that mitochondrial injury plays an important role in NIHL, ALCAR was chosen as a candidate compound because of its capacity to enhance mitochondrial bioenergetics and repair in the face of oxidative stress. ALCAR serves as a precursor for acetyl-CoA, a mitochondrial energy substrate, and L-carnitine, which can shuttle lipid substrates into mitochondria for β-oxidation and enhance ATP production.[57] ALCAR also restores a key mitochondrial lipid known as cardiolipin in oxidatively injured cells, further restoring mitochondrial integrity.[58]

Besides the effects of noise-induced mitochondrial injury, several lines of evidence suggest that glutamate excitotoxicity plays a role in noise-induced cochlear injury. Glutamate agonists, when infused into the cochlea, mimic the pathological changes at the cochlear afferent nerve endings seen after acoustic overexposure.[49,52,59,60] Conversely, general glutamate antagonists such as MK 801 and kynurenate reduce cochlear injury resulting from noise.[49,51,61,62] Accordingly, carbamathione was chosen as a test compound to explore the hypothesis that downregulating NMDA-receptor activity through modification of the natural redox modulatory site of the receptor would reduce permanent threshold shifts and noise-induced hair cell loss. By downregulating the activity of the NMDA receptor by binding with its physiological redox modulatory site, carbamathione may enhance the intrinsic cellular defenses used to prevent glutamate toxicity.

Reduced glutathione (GSH) is one of the key antioxidant compounds present in all eukaryotic cells,[63] and there is substantial evidence that GSH in the cochlea plays a significant role in protecting the cochlea from oxidative stress resulting from both toxins and noise.[18] Experiments were undertaken to strengthen the hypothesis that supplementation with an antioxidant, specifically chosen to enhance GSH levels, would reduce NIHL. One of the major determinants of GSH levels is the availability of cysteine. Cysteine is derived from several sources including methionine.[63–65] (see Table I for a summary of the experimental agents). These agents were also chosen on the basis of being FDA-approved agents, or in the case of carbamathione, a metabolite of an FDA-approved agent, with potential for use in human clinical trials in the future.

TABLE I.
Summary of Site and Mechanism of Action of Experimental Agents in This Study.

Agent	Site of Action	Mechanism of Action
ALCAR	Mitochondria	Improves energy production; restores cardiolipin and carnitine levels; reduces ROS production; other
Carbamathione	NMDA receptors	Downregulates NMDA receptor activity
MET	Cochlea	Provides cysteine for synthesis of GSH; free radical scavenger

METHODS

Twenty-four female adult *chinchilla laniger* were divided equally into four experimental groups. Animals were fed a standard chinchilla diet (Mazuri Chinchilla Diet, 5MO1, PMI Nutrition International Inc., Brentwood, MO). The four groups consisted of a saline control group and three experimental agent (EA) treatment groups (ALCAR, Carbamathione, and MET). Baseline hearing thresholds obtained through auditory brainstem response (ABR) measurements were taken within 2 days prior to initial noise exposure. Hearing thresholds were repeated several hours after the last saline or EA injection before noise exposure.

Animals received ALCAR (acetyl-L-carnitine, Sigma-Aldrich Co., St. Louis, MO; concentration 25 mg/mL; dose 100 mg/kg), carbamathione (courtesy of Prof. John V. Schloss, Department of Medicinal Chemistry, University of Kansas, Lawrence, KS; concentration 1.4 mg/mL; dose 5.6 mg/kg), and MET (D-methionine, Sigma-Aldrich Co., St. Louis, MO; concentration 50 mg/mL; dose 200 mg/kg), all dissolved in sterile 0.9% saline (pH normalized to 7.2 ± 0.2) or sterile 0.9% (pH 7.2) saline alone (saline control group) by intraperitoneal injection. The volumes of all the injections were standardized so that all animals received the same volume of injection on a milliliter per kilogram basis. All injections were given every 12 hours, starting 48 hours before the noise exposure, 1 hour before noise, and 1 hour after exposure, and then twice per day after exposure the next 2 days. All groups had ABRs performed pre-noise, 1 hour post-noise, and once a week for 3 weeks. Shortly after the last ABR, animals were humanely euthanized, and the temporal bones were harvested and subsequently stained with a vital dye to indicate the presence of living hair cells.

The Laboratory Animal Care and Use Committee of the Naval Medical Center San Diego approved the care and use of the animals in this study.

ABR Measurement

Animals were awake and lightly restrained in a plastic tube during the 30-minute recording procedure. Hearing thresholds were determined by auditory brainstem response (ABR) through subcutaneous needle electrodes placed in the skin of the head and posterior to each ear. Once in place, these electrodes were not removed until the end of testing. The electrodes could be changed at the preamplifier when testing the opposite ear since ports for left and right ear were present on the preamplifier. The needle size was quite small (30-g); thus, sedation or local anesthesia were unnecessary. Animals tolerated the restraint tube so well that the animals rested comfortably during the testing. The person performing the ABR measurements was blinded as to which treatment group the animal belonged.

Digitally generated stimuli consisted of tone pips (4-ms Blackman rise/fall ramp, 0-msec plateau, and alternating phase) at octave intervals of 2, 4, 6, and 8 kHz. All acoustic stimuli were routed through a computer-controlled attenuator to an insert earphone (Etymotic Research ER-2, Elk Grove Village, IL) inserted into the ear canal (closed system). The sound delivery tube of the insert earphone was positioned approximately 5 mm from the tympanic membrane. Earphone sound delivery was calibrated using a coupler attached to the sound level meter approximating the distance from the earphone to the tympanic membrane. Six hundred samples were collected from the recording electrode, amplified (50,000–75,000×), filtered (100–1500 Hz), and fed to an A/D converter computerized on a signal processing board. Stimuli at a rate of 23/sec were varied in 10-dB descending steps until threshold was reached, then 5-dB ascending steps were presented to confirm threshold. Earphone inserts on the tested ear were removed, and control ABR runs during which no sound was presented were determined for comparisons. Threshold was defined as the midpoint between the lowest level at which a clear response was evidenced and the next lower level where no response was observed.

Noise Exposure

The noise exposure protocol was developed from the procedure of Hu et al.[34] Specifically, an octave band noise centered at 4 kHz was generated by a standard audiometer (GSI 16; Grason Stadler Instruments, Milford, NH) selected to white noise and routed through an attenuator (HP 350 D; Hewlett-Packard Corp., Palo Alto, CA), a band-pass filter (Krohn-Hite 3550R; Krohn-Hite Corp., Avon, MA), and a power amplifier (Crown D150A model 716; Crown Audio Inc., Elkhart, IN) to an audiometric loudspeaker (JBL Model 2350A; JBL Inc., Northridge, CA) suspended directly above the animal's cage. The sound spectrum output of the system was confirmed using a Larson and Davis model 800B (Larson Davis, Provo, UT) sound level meter (A scale, SPL), centering the octave bandwidth at 4 kHz. To ensure consistent noise exposure conditions, the noise output of the system was monitored before each noise exposure using a sound level meter. Also, a preamplifier (Larson and Davis model 825) and a condenser microphone (Larson and Davis, LDL 2559) were positioned within the cage at the level of the animal's head for continuous monitoring during the exposure. Each animal was exposed continuously to the noise at a level of 105 ± 0.5 dB SPL for 6 hours. During the noise exposure, the animal was restrained by a breeding collar commonly used for female chinchillas in a small wire cage with ad-lib water access. Two animals were exposed at a time, and after the first 3 hours of noise exposure, their cages were moved to carefully predetermined locations within the sound exposure booth to minimize the potential effects of sound shadowing. The average temperature in the sound booth was 68.9°F (range, 68–71°F) and the average humidity was 65.7% (range, 64–68%), and these values were similar to those outside the booth in the animal facility (temperature 68.8 ± 2°F, humidity 58–68 ± 5%). When the animals were not being exposed to noise, they were housed in a quiet animal colony. The average ambient noise level during a 24-hour period (as measured by two dosimeters placed into the animal holding room) was approximately 49 dB SPL.

Histological Examination

Following final auditory tests (i.e., at 3 weeks post-noise exposure), the animals were heavily anesthetized with 30 mg/kg ketamine and 1 mg/kg xylazine and decapitated. Each temporal bone was quickly removed from the skull. Each cochlea was exposed and slowly perfused through the oval and round windows with a solution of 0.2 mol/L sodium succinate and 0.1% nitrotet-

razolium blue in 0.2 mol/L phosphate buffer (pH = 7.4 at 37°C). Samples were then immersed in the same solution for 1 hour at 37°C. Lastly, the cochlea was rinsed with buffer and fixed with 4% paraformaldehyde for 24 hours. Cochleae were dissected using an Olympus SZ-60 (Olympus Optical Co. Ltd., Japan) dissecting microscope, and sections of the organ of Corti were mounted on glass slides (25 × 75 mm) and examined as surface preparations for hair cell loss under a light microscope (Olympus BH-2; Olympus Optical Co. Ltd., Japan) with a 10 × 10 grid at 200× magnification. Missing (supporting cell scar) or non-viable (absence of blue staining) hair cells were noted by the absence of blue vital stain in the area of inner and outer hair cells. An experienced but experiment-blinded observer counted missing hair cells over the length of the basilar membrane per cochlear turn. Data from each section was input into a worksheet on Origin software (Origin version 6.0, Microcal Software Inc., North Ampton, MA) to construct a continuous range of data from the hook area to the apex of the cochlea. Absolute hair cell counts were converted to percentages of missing inner or outer hair cells by dividing the cell count for the experimental animals by control value cell counts for normative values developed for each cochlear region. Normative values were confirmed by counting all inner and outer hair cells along the basilar membrane from base to apex in 40 ears from 20 healthy non-noise-exposed chinchilla matched for size and age and compared with published data.[66] Each cochlea's length was normalized to 100%. The number of inner and outer hair cells as a function of percent distance from the apex was established using a linear regression model. The percentage of missing hair cells was then plotted as a function of percent distance from the cochlear apex.[31,34,66] A cytocochleogram was developed for inner and outer hair cells for each cochlea, and cytocochleogram means were computed and graphed after smoothing the data set for each 100-point set with a Fast Fourier Transfer filter. The smoothed data were then normalized with an interpolated curve function for 2000 points. The upper horizontal axis for the cytocochleogram corresponds to frequency place along the basilar membrane while the lower horizontal axis represents percent distance from the cochlear base to the apex along the basilar membrane (Figs. 1–3).

Statistical Analyses

A three-way (4 × 4 × 4) ANOVA was used to analyze the effect of treatment on group mean hearing thresholds over time, with time and frequency as repeated measures, and animals and ears as replications. Post-hoc tests were performed by the Scheffé method with significance set at $P < .05$. A two-way (4 × 4) ANOVA was performed to analyze the effect on HC counts of treatment (saline control, ALCAR, carbamathione, and MET), frequencies (2, 4, 6, and 8 kHz), and the interaction between treatment groups and frequencies. Animals and ears were treated as replications. Post-hoc testing was performed by the method of Scheffé.

The time required to return to normal hearing, or the amount of residual hearing loss in the event of no return to normal, was of interest. The physiological response of the auditory system to damaging noise is a partial or full recovery of threshold shift (TS) gradually over time approaching either normal hearing or a residual hearing loss. Thus, the TS would be expected to follow an exponential decline, approaching a minimum asymptotically. The means of the data for all treatments and for all frequencies decline in such a pattern, supporting the theory. The treated ears, for which a return to normal was anticipated, were fit by the exponential model

$$TS = e^{a-b} \times \text{weeks},$$

where the parameters a and b were estimated by linear regression on logarithm (TS). The time at which the residual TS reduced to half the error variability of the measuring instrument, i.e., 2.5 dB, was taken as the time required for the average animal to return to normal hearing. The control animals were not expected to return to normal but to retain a residual hearing loss. The model required for a residual non-zero TS is the exponential decline plus a residual constant c, or

$$TS = e^{a-b} \times \text{weeks} + c.$$

Again, the data means supported this theory. The parameters for this model were estimated by a non-linear least-squares method, yielding the asymptotic limit on hearing recovery as c dB of TS.

RESULTS

There were no significant differences in ABR thresholds when compared before and after injections performed prior to noise exposure (ANOVA, P ranging from .56–1.0 for all frequencies, data not shown). Thus, injection of saline or saline plus experimental agent had no effect on baseline hearing thresholds.

ALCAR administration beginning prior to noise exposure resulted in a substantial reduction in permanent threshold shifts when compared with saline-injected noise-exposed control animals (Fig. 1A). The initial threshold shifts at 1-hour post-noise exposure were not significantly different between the control and treated animals. By 1 week, there was a reduction in the treated animals' thresholds compared with control animals, and this difference continued to increase over time. Three weeks post-noise exposure, there was almost no threshold shift at 2 and 4 kHz, and less than a 10-dB threshold shift at 6 and 8 kHz in contrast to a threshold shift of 30 to 35 dB in control animals. ANOVA showed a significant overall reduction in threshold shift for ALCAR-treated animals compared with saline control animals at 4, 6, and 8 kHz ($P < .001$), but not at 2 kHz ($P > .05$). Based on post-hoc testing, ALCAR treatment did significantly lower threshold shifts at 2 kHz (compared with controls) at 2 ($P < .05$) and 3 weeks ($P < .01$). Of interest is that the slope of the recovery curve for the control animals flattens over time, whereas the treated animals' ABR threshold shifts demonstrate a steep recovery slope that if extrapolated would reach baseline by approximately 4 to 5 weeks post-noise (Table IIA; Fig. 4A). Did the modeled curves of threshold shift (TS) reduction through time adequately fit the data? The fits produced coefficients of determination (R^2) in the 0.80 to 0.95 range, indicating adequate agreement between the data and the model. Also, because the fit for the saline–control animals passed through the data means, any goodness-of-fit test would indicate adequate agreement between the model and the data (Tables IIA and IIB; Fig. 4A, B).

ALCAR treatment also reduced both the inner and outer hair cell loss associated with the noise overexposure (Fig. 1B). OHC loss in the saline-injected noise-exposed controls averaged 60%, whereas the OHC loss in the ALCAR-treated animals averaged less than 10% for the 4- to 10-kHz region of the cochlea. IHC loss was significantly reduced at 2, 4, and 6 kHz, and was less than 5% compared with an average of about 20% in control cochleae at 8 kHz (Fig. 1C). Mean inner and outer HC counts from

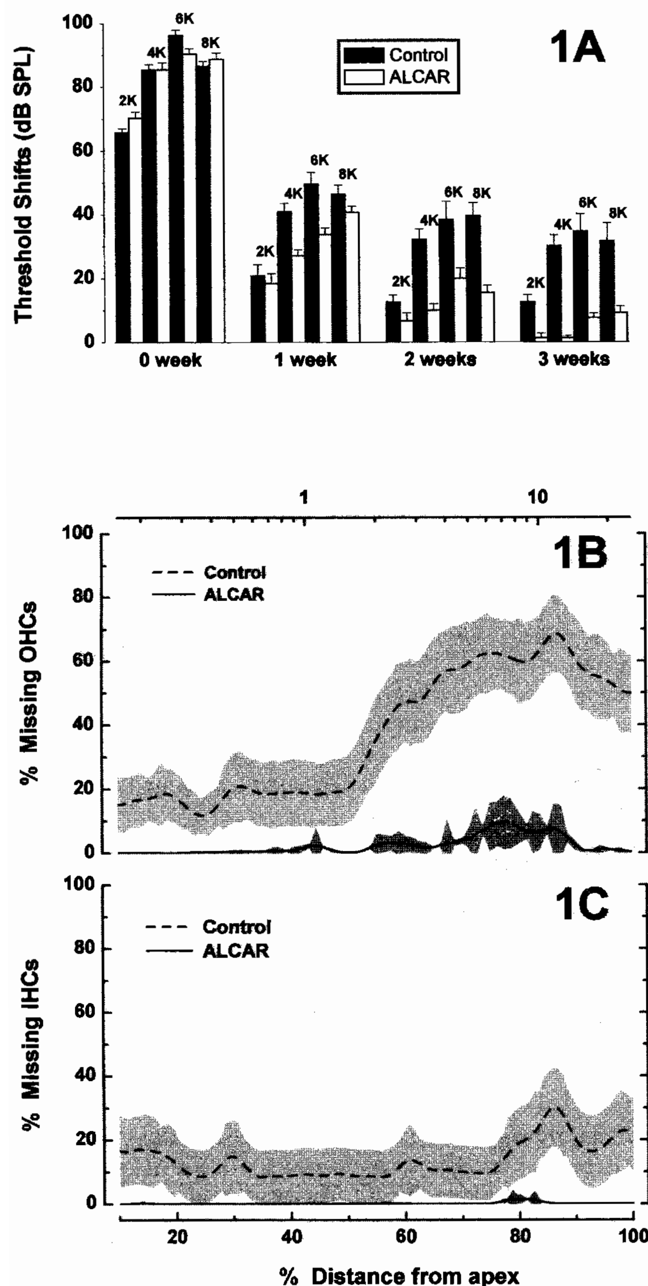


Fig. 1. (**A–C**) Mean threshold shift and cytocochleogram data for noise-exposed animals given saline (control) or saline plus ALCAR (treated). (**A**) Auditory threshold shifts for noise-exposed ALCAR or saline-treated animals. Group mean auditory threshold shifts in dB SPL (post-noise thresholds **minus** baseline thresholds) are plotted as a function of treatment group (saline-noise and ALCAR-noise treatment), over time [week zero (1 hour), or 1-, 2-, 3-week post-noise] and by threshold test frequency for 2, 4, 6, and 8 kHz. Six hours of sound, centered at 4-kHz octave band noise at 105 dB SPL intensity, produced an initial threshold shift ranging approximately 65 to 95 dB for both groups. There was an overall treatment effect for the ALCAR-treated group compared with the saline-noise group ($P < .001$ for 4, 6, and 8 kHz, and $P < .05$ for 2 kHz) beginning at week 1. The threshold shift at 3 weeks for saline-noise-exposed controls ranged between approximately 12.5 and 35 dB SPL from 2 to 8 kHz, whereas PTS for the pretreatment animals was significantly reduced ($P < .01$) to approximately 0 to 10 dB over the same frequencies. Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals). (**B**) Outer hair cell cytocochleogram data. Depicted are mean (continuous line) and SEM (shaded area) cytocochleograms for outer hair cells (OHCs) from ALCAR-pretreated noise-exposed cochleae (solid line) and saline-treated noise-exposed cochleae (dotted line), respectively. The Y-axis depicts percent missing outer hair cells. The lower X-axis represents percent distance from the cochlear apex, and the upper X-axis depicts the associated frequency range of the cochleae in kHz. There was very little OHC loss in the ALCAR-protected cochleae (less than 10%), whereas there was substantial outer HC loss in the saline-control noise-exposed group (from 40%–70% loss) in the frequency regions tested for hearing ($P < .001$). Sample (n) size is 12 for all groups (12 ears, 6 animals). (**C**) Inner hair cell cytocochleogram data. Illustrated are the mean (continuous line) and SEM (shaded area) for inner hair cell (IHC) cytocochleograms, with missing IHC percentages on the Y-axis as a function of the measured percent distance from the cochlear apex. The associated frequency region of the cochlea is also plotted on the upper X-axis. There was very little IHC loss in ALCAR-treated animals compared with noise-saline controls. Maximal IHC loss occurred between the 4- and 10-kHz region of the basilar membrane after 6 hours of 4-kHz octave band noise exposure. Twenty percent to 30% of the IHCs were lost in the region between 3 and 10 kHz of the cochleae in the saline-treated animals compared with less than 3% in the ALCAR-treated animals ($P < .05$). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals).

noise-exposed animals were significantly different from each other based only on treatment (saline control, ALCAR, carbamathione, and MET) ($P < .001$, OHC; $P < .003$, IHC). Post-hoc analysis revealed that mean OHC counts were significantly reduced from saline controls ($P < .001$), as were the mean IHC counts ($P < .05$).

Intraperitoneal injection of carbamathione, an NMDA receptor antagonist, beginning before noise exposure, was also associated with a substantial reduction of noise-induced permanent threshold shift (NIPTS) (Fig. 2A). The carbamathione-treated animals had an initially greater noise-induced temporary threshold shift (NITTS); however, this was not statistically different from controls except at 6 kHz ($P < .05$). However, by week 1 post-noise, the threshold shifts of the treated animals were less than those of control animals and continued to decline further relative to controls over time. Threshold shifts for the treated animals at 3 weeks averaged 15 dB or less at all frequencies (compared with 30–35 dB at 4, 6, and 8 kHz in controls). Two-way ANOVA revealed a significant overall treatment effect. Interaction effect was significant at 4 and 8 kHz ($P < .001$) and almost significant at 6 kHz ($P = .528$). Post-hoc analysis also demonstrated a significant reduction in threshold shifts for the treated animals compared with the control animals for weeks 1 through 3 at 4, 6, and 8 kHz ($P < .01$) and at 3 weeks for 2 kHz ($P < .05$). It appeared that the slope of the threshold shift recovery curve of the treated animals would intersect baseline at approximately 4 to 5 weeks compared with the asymptote reached for the threshold shift recovery curve of control animals (Tables IIA and Table IIB; Fig. 4A, B).

Average OHC losses for the carbamathione-treated animals were 30% to 40% compared with 60% loss seen in control cochleae ($P < .001$) (Fig. 2B). Mean OHC losses for the carbamathione group were greater than those for ALCAR ($P < .05$) and approached significance when compared with MET ($P = .062$). The average IHC loss for the animals treated with the glutamate receptor antagonist

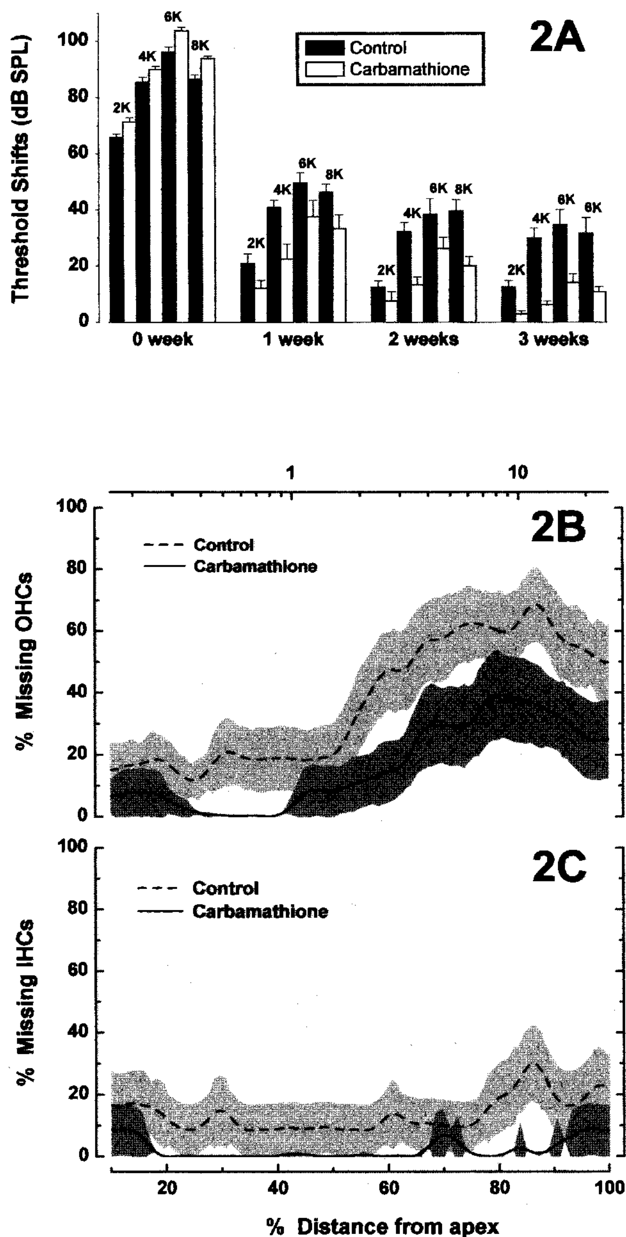


Fig. 2. (**A–C**) Threshold shift and cytocochleogram data for noise-exposed animals given saline (control) or saline plus carbamathione (treated). (**A**) Auditory threshold shifts for carbamathione and saline-treated animals. Threshold shifts (dB SPL) are expressed the same as in Figure 1A. Mean ± SEM for threshold shifts are plotted as a function of treatment group (saline-noise and carbamathione-noise treatment) for 1 hour (zero week) and 1-, 2-, 3-week post-noise exposure. Auditory test frequencies are the same as for Figure 1A (2, 4, 6, and 8 kHz). Each group contained six female chinchillas (12 ears) that were exposed to 6-hour sound (4-kHz octave band; 105 dB SPL). The sound exposure produced an initial threshold shift ranging approximately 65 to 96 dB for saline controls and 71 to 104 for the carbamathione group (statistically similar except at 6 kHz [$P < .05$]). There was an overall treatment effect for carbamathione-treated animals compared with the saline-noise condition at 4, 6, and 8 kHz ($P < .001$) for weeks 1 to 3, and at 3 weeks for 2 kHz ($P < .05$). (**B**) Outer hair cell cytocochleogram data. Depicted are cytocochleograms for outer hair cells (OHCs) from carbamathione-treated noise-exposed and saline-treated noise-exposed cochleae. The Y-axis depicts mean percent missing outer hair cells. The lower X-axis represents percent distance from the cochlear apex, and the upper X-axis depicts the associated frequency range of the cochleae. There was less OHC loss in the carbamathione-protected cochleae (from approximately 30%–40%), whereas the outer hair cell loss in the saline-control noise-exposed group was substantially greater, being approximately 60% over the same frequency regions ($P < .001$). However, ALCAR afforded significantly greater OHC protection than carbamathione ($P < .05$) and the differences with MET approached significance ($P = .062$). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals). (**C**) Inner hair cell cytocochleogram data. Illustrated are the mean inner hair cell cytocochleograms with missing hair cell percentages on the Y-axis as a function of the measured percent distance from the cochlear apex. The associated frequency region of the cochlea is also plotted on the upper X-axis. Carbamathione treatment was associated with significantly less IHC loss than the saline-treated noise-exposed animals ($P < .05$). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals).

was 5% or less in the 4- to 10-kHz region compared with 10% to 20% for the control animals ($P < .05$) (Fig. 2C).

Systemic MET administration beginning before noise exposure was also effective in reducing the NIPTS and OHC loss induced in this model (Fig. 3A). Initial NITTS at week 0 was not different from control animals except at 6 kHz ($P < .05$). ANOVA revealed a significant treatment effect of MET administration for weeks 1 through 3 at all frequencies tested ($P < .001$). An extrapolated regression model of threshold shift recovery intercepted the horizontal axis at approximately the 5-week time point (Table IIA; Fig. 4A).

Mean OHC losses were significantly reduced at all frequencies (Fig. 3B). Mean IHC losses were also reduced (Fig. 3C). These reductions in mean OHC losses ($P < .001$) and IHC losses ($P < .05$) were significant.

Results for noise-exposed saline controls have been contrasted with results from the noise-exposed treated animals above. One aspect of the control results deserves note, namely, the residual hearing loss estimates for the untreated subjects. Table IIB gives the residual hearing loss estimates, approximately 12 dB for 2 kHz, but in the range of 30 to 35 dB for higher frequencies. Figure 4B shows an example of such a result. Did the modeled fit of TS reduction through time adequately fit the data? The fit passes through the data means so that any test of disparity would be based on zero difference. The model fits the data well.

Figure 5 shows low- and high-power micrographs of vital-stained surface preparations of representative cochleae from control and treated animals taken in the 6-kHz region. Characteristically, the saline-injected noise-exposed animals' cochleae demonstrated no staining in OHC rows two and three with scattered residual staining OHCs in row one (Fig. 5, control). IHC loss for the controls was scattered and is consistent with the known increased resistance of IHCs to noise-induced injury.[67,68] In contrast to the control cochleae, the drug-treated noise-exposed cochleae showed significant preservation of the OHCs and the generally uniform staining of the IHCs (Fig. 5—ALCAR, carbamathione, MET). The preservation of OHC, by carbamathione, was noted to be less than that seen with ALCAR or MET (Fig. 5).

Representative individual cytocochleogram data for OHCs are displayed in Figure 6. In general, saline-

injected noise-exposed control animals showed a wide band of loss of 50% to over 90% of OHC in the 4- to 10-kHz region of the cochlea (Fig. 6, control). In contrast to the control ears, the treated ears, while demonstrating individual variability, generally demonstrated less outer hair cell loss, and the width of the band of hair cell loss along the basilar membrane was much narrower in the treated compared with control animals (compare Fig. 6, ALCAR, carbamathione, MET, with Fig. 6, control).

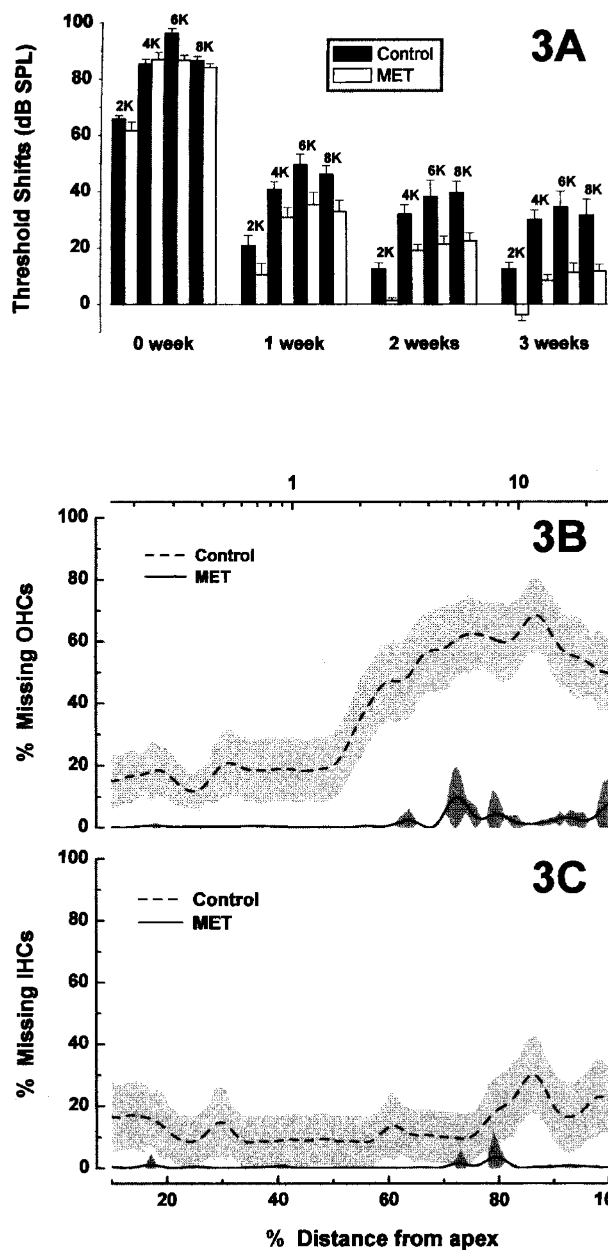


Fig. 3. (**A–C**) Threshold shift and cytocochleogram data for noise-exposed animals given saline (control) or saline plus MET (treated). (**A**) Auditory threshold shifts for saline-treated compared with MET-treated animals. Mean auditory threshold shifts (dB SPL) are expressed the same as in Figure 1A. Means for threshold shifts plotted as a function of treatment group (saline-noise and MET-noise treatment), time (zero [1 hour], or 1, 2, 3 weeks post-noise) and by threshold test frequency for 2, 4, 6, and 8 kHz. Sound exposure was the same as previously described in Figure 1A. Initial threshold shifts (week 0) ranged from approximately 62 to 87 dB SPL for the MET-noise group, which were statistically similar to the saline-treated noise-exposed group ($P > .05$), except for 6 kHz in which the mean threshold was significantly less than that of controls ($P < .05$). There was an overall treatment effect for the MET-treated group compared with the saline-noise group ($P < .001$) for all test frequencies beginning at week 1. Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals). (**B**) Outer hair cell cytocochleogram data. Depicted are mean (continuous line) and SEM (shaded area) cytocochleograms for outer hair cells (OHCs) MET-pretreated noise-exposed cochleae (solid line) and saline-treated noise-exposed cochleae (dotted line), respectively. The Y-axis depicts mean percent missing outer hair cells. The lower X-axis represents percent distance from the cochlear apex and the upper X-axis depicts the associated frequency range of the cochleae in kHz. There was very little OHC loss in the low dose MET-protected cochleae (less than 10%), whereas there was substantial OHC loss in the saline-control noise-exposed group (average of approximately 60% for the 4- to 10-kHz region). These differences were significant ($P < .001$). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals). (**C**) Inner hair cell cytocochleogram data. Illustrated are the mean inner hair cell cytocochleograms with missing inner hair cell (IHC) percentages on the Y- axis as a function of the measured percent distance from the cochlear apex. The associated frequency region of the cochlea in kHz is also plotted on the upper X-axis, and the percent distance from the cochlear apex is depicted on the lower X-axis. MET treatment (solid line) afforded significant protection of IHCs as seen by a reduction to 5% or less of IHC loss with MET treatment verses over 20% in the saline-treated animals ($P < .05$). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals).

DISCUSSION

The chinchilla has been used as a model of noise-induced hearing loss for decades.[34,67,69–73] In the current study, the model of noise-induced hearing loss and obtaining threshold shift measurements was derived from another model.[34] However, the methodology for obtaining the threshold shifts was by ABR with cutaneous placement of the electrodes (rather than by assessment of responses of surgically placed inferior colliculus [IC] electrodes). A total of 144 measurements comparing IC evoked potentials to ABR data were made on 16 different subjects. Auditory responses using the IC-evoked potentials compared with the cutaneous electrode ABR responses were found to be in excellent agreement (<5 dB for 86% of the measurements and <10 dB for 98% of the measurements, data not shown). This approach avoided subjecting the animal to surgery for IC electrode placement. Others have used far-field ABR measurement techniques in chinchillas as well.[74] A continuous noise was chosen in the current experiments, although other types of noise exist, including impulse noise and impact noise, as well as mixtures of continuous and other types of noise leading to complex noise exposures.[73] The method for assessing cochlear injury through the preparation and analysis of cytocochleograms was also derived from a well-established model.[34,66]

Several measures were undertaken to attempt to reduce the well-known variability associated with noise-induced cochlear injury. These measures included selection of only female subjects of similar age and size, habituating the animals to handling and the noise exposure environment, shifting the animals in the noise expo-

TABLE II.
Fits and Extrapolated Return to No Threshold Shift for Three Treatments at Four Frequencies.*

Treatment	Frequency (kHz)	*A*	*b*	Weeks to Normal
ALCAR	2	4.11	1.061	3.80
	4	4.56	1.327	4.44
	6	4.49	0.859	4.54
	8	4.43	0.753	4.79
Carbamathione	2	3.84	1.098	4.77
	4	4.22	0.878	3.77
	6	4.49	0.699	5.12
	8	4.38	0.771	4.50
MET	2	3.68	1.336	2.08
	4	4.37	0.780	4.44
	6	4.41	0.771	4.54
	8	4.34	0.716	4.78

*The data were fit to the model $e^{a-b\times weeks}$ by a non-linear regression method, yielding *a* and *b* values as tabulated. The time value at which the threshold shift reduced to half the accepted measurement error (2.5 dB) was taken as the time required for the average animal to return to normal hearing and is also tabulated.

sure environment within a grid of carefully measured noise–dose isobars, use of a breeding collar restraint to help avoid shielding of an ear by the animal, and careful measurement of noise energy output before, during, and after each exposure. In addition, one investigator performed over 90% of the ABR measurements. These precautions explain the reduced variability and the small SE of means shown on the threshold shift grafts (Figs. 1A–3A).

It was hypothesized that ALCAR given before and after the noise exposures would decrease the amount of hair cell and permanent hearing loss induced by the continuous noise. Others have shown that impairing mitochondrial biogenesis increases noise-induced cochlear injury where such inhibition increased noise-induced hearing loss by 80%.[46] In addition, age-related hearing loss, thought to be related to chronic oxidative stress, was attenuated by administration of ALCAR in rats.[75] In the current study, intraperitoneal administration of ALCAR was highly effective in attenuating noise-induced PTS and hair cell loss. Threshold shifts were reduced to 10 dB or less compared with 30 to 35 dB in controls, and IHC and OHC losses were reduced to less than 10% in the ALCAR-treated animals, compared with 20% or over 60% IHC and OHC losses, respectively, in saline controls. This suggests that loss of mitochondrial integrity plays an important role in noise-induced cochlear injury, and that strategies to maintain mitochondrial integrity are apt to be effective in ameliorating NIHL.

Glutamate is thought to be a major neurotransmitter between the inner hair cell and the afferent cochlear nerve ending.[76] It is postulated that with excessive sound stimulation, excessive synaptic glutamate concentrations are developed leading to overstimulation of glutaminergic receptors invoking metabolic cascades resulting in cell injury and death. Some of the potentially harmful cascades set in action by glutamate excitotoxicity may include increases in intracellular calcium with the activation by calcium-dependent calmodulin nitric oxide synthase (NOS) and the excessive production of nitric oxide (NO) and related free radicals such as peroxynitrite. Activation of protein kinases, activation of phospholipase A_2, activation of proteases such as calpain, activation of xanthine oxidase with the subsequent generation of superoxide anion, in addition to mitochondrial injury[18,77] may also occur. Glutamate excitotoxicity can be divided into an early phase (up to 30 min) and a late phase (3–24 h) of ROS production consequent to the excessive glutamate production.[77] A later phase of glutamate-induced ROS production occurs as a self-propagating process in which damaged mitochondria become both the source of additional ROS production and further cell damage.[77]

NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate ionotropic receptors are present in the cochlea associated with afferent neurons based on detection of receptor mRNA as well as immunohistochemical studies.[78–81] AMPA and kainate, when infused into the cochlea in high concentration, can induce cochlear injury consisting of destruction of dendrites beneath inner hair cells.[52,59,60] This is morphologically similar to what is seen after cochlear ischemia or acoustic overexposure. This damage can be reduced by prior application of a selective AMPA receptor antagonist (ischemia) or a more broad-spectrum glutamate receptor antagonist, kynurenate, for noise.[49,60,62,82] Glutamate antagonists like MK-801 and others have been reported to reduce NIPTS and NITTS.[49,51,61,62]

Although the preponderance of evidence thus far implicates the non-NMDA ionotropic glutaminergic receptors in the pathogenesis of noise-induced afferent dendrite injury,[83] it appears that NMDA receptors may be involved with some of the radial dendrites contacting the modiolar side of inner hair cells.[60] Others have reported that local application of specific NMDA receptor antagonists blocked the firing of afferent dendrites induced by glutamate and NMDA.[84,85] In addition, in the central nervous system, glutamate toxicity is mediated primarily by the NMDA receptor.[77]

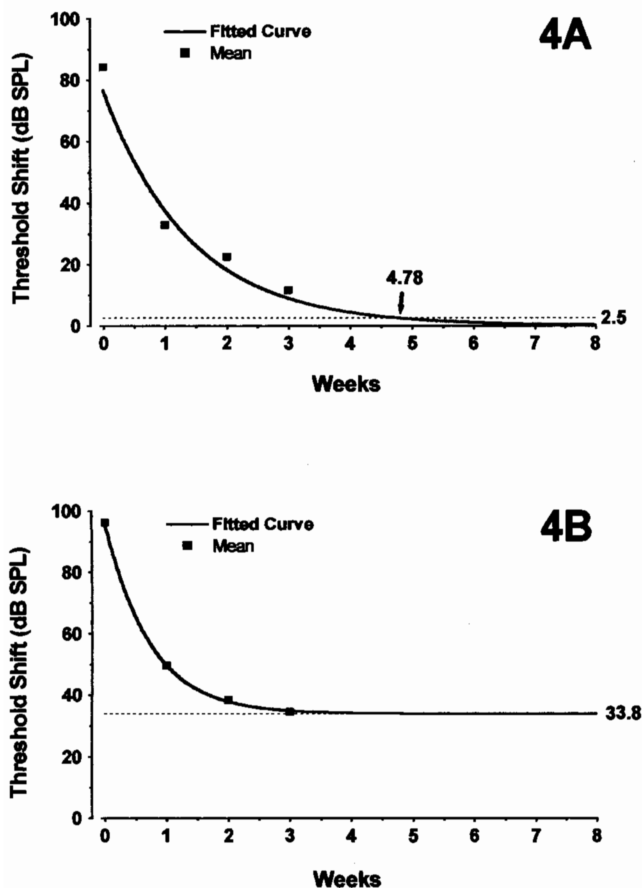


Fig. 4. (**A** and **B**) Extrapolated recovery curves for noise-exposed saline-treated (control) animals and noise-exposed saline plus experimental compound-treated animals. (**A**) The fit to the model of Table IIA is shown for MET at 8 kHz as an example. The 4.78-week return-to-normal time is shown. The means of the data are superimposed as dots. (**B**) The fit to the model of Table IIB is shown for the control animals at 6 kHz as an example. The asymptotic approach to sustained hearing loss of 33.8 dB can be seen. The data means for weeks 0, 1, 2, and 3 lie on the curve. For all frequencies, the hearing loss stabilized to within 2.5 dB of the asymptotic sustained loss by 3 weeks.

In the current study, it was hypothesized that the NMDA glutamate antagonist carbamathione would attenuate NIPTS and hair cell loss when given systemically before and after noise exposure. This effect was found to reduce NIPTS to 15 dB or less compared with 35 dB for saline-injected controls. OHC losses were reduced by over 50% compared with saline controls. The reductions in hair cell loss and threshold shift were less than that seen with ALCAR, but the milligram per kilogram dose of carbamathione was also over 15-fold less than for the ALCAR. It was interesting to note that carbamathione appeared to more effectively attenuate IHC losses (>90% reduction at 2, 6, and 8 kHz) than the outer hair cell losses (approximately 50%). This more selective protection of IHCs is consistent with the bulk of afferent glutaminergic synapses being associated with inner hair cells in the cochlea.[60] This also may be a reason why the reduction in PTS was relatively robust despite the modest OHC losses. It is noteworthy that other investigators have reported that the broad-spectrum glutamate antagonist kynurenate was able to provide about 50% protection from damage resulting from noise,[49,62] similar to the findings in the current study. Other explanations for the less robust protective effect seen with carbamathione, besides dosage, might be that AMPA/kainate receptors play an important role in glutamate-induced cochlear injury, and these receptors were not downregulated by the compound.[83] Another explanation is that OHC injury may occur through non-glutamate-related mechanisms or be the result of mechanisms involving glutamate and not mediated by ionotropic receptors.[86] In the current study, the residual threshold shifts for carbamathione were similar to those of MET and ALCAR, yet there was greater OHC loss for carbamathione verses MET and ALCAR. One explanation might be that carbamathione more effectively protected IHC structure and/or their synapses relative to the other two compounds, morphology not examined by the methodology of the current study. Others have noted a relatively poor correlation between OHC losses and TS,[87,88] and that the status of the IHC synapses and other cellular changes may be as important as missing hair cells.[89]

MET has previously been reported to reduce the oxidative stress-related ototoxicity of the chemotherapeutic agent cisplatin[90–92] as well as aminoglycoside antibiotics.[93] D-methionine was chosen rather than the naturally occurring L isomer because of the data indicating that the D isomer is metabolized differently, giving it increased bioavailability as a result of an increased serum half-life. In humans, the dextro isomer is less quickly used in protein formation, and thus may have a longer period of bioavailability than L-methionine.[94]

MET, in the current study, effectively reduced the HC loss and NIPTS in the chinchilla model. This effect of cochlear protection from noise resulting from MET was comparable to similar studies using the antioxidant combination of L-*N*-acetylcysteine (NAC) and salicylate[31] and NAC alone.[95] NAC is another cysteine-supplying drug[64] shown to reduce noise-induced cochlear lipid oxidation and damage.[95,96] NAC given systemically or across the round window membrane increased NAC levels in the cochlea (Zou et al., 2001, submitted to *Hearing Research*).

The dosage schedule used in the current study, in which treatment agents were continued for 48 hours post-noise exposure, may have been important in that a second burst of ROS production may occur some time after the cessation of noise.[24] Also, it has been reported that increased ROS production may continue for at least a number of hours post-noise.[25] Additionally, early- and late-phase (up to 24 h post-insult) glutamate toxicity and related mitochondrial injury can occur in some models of oxidative injury.[77] Thus, the continued injection of the protective agents may have reduced the potential damage from these ongoing processes.

Biochemistry, Mechanism of Action

ALCAR. Mitochondrial injury, which can be caused by excessive ROS generation within mitochondria or result as a consequence of glutamate excitotoxicity, ischemia–reperfusion, or GSH depletion,[54,55] has been shown to play a

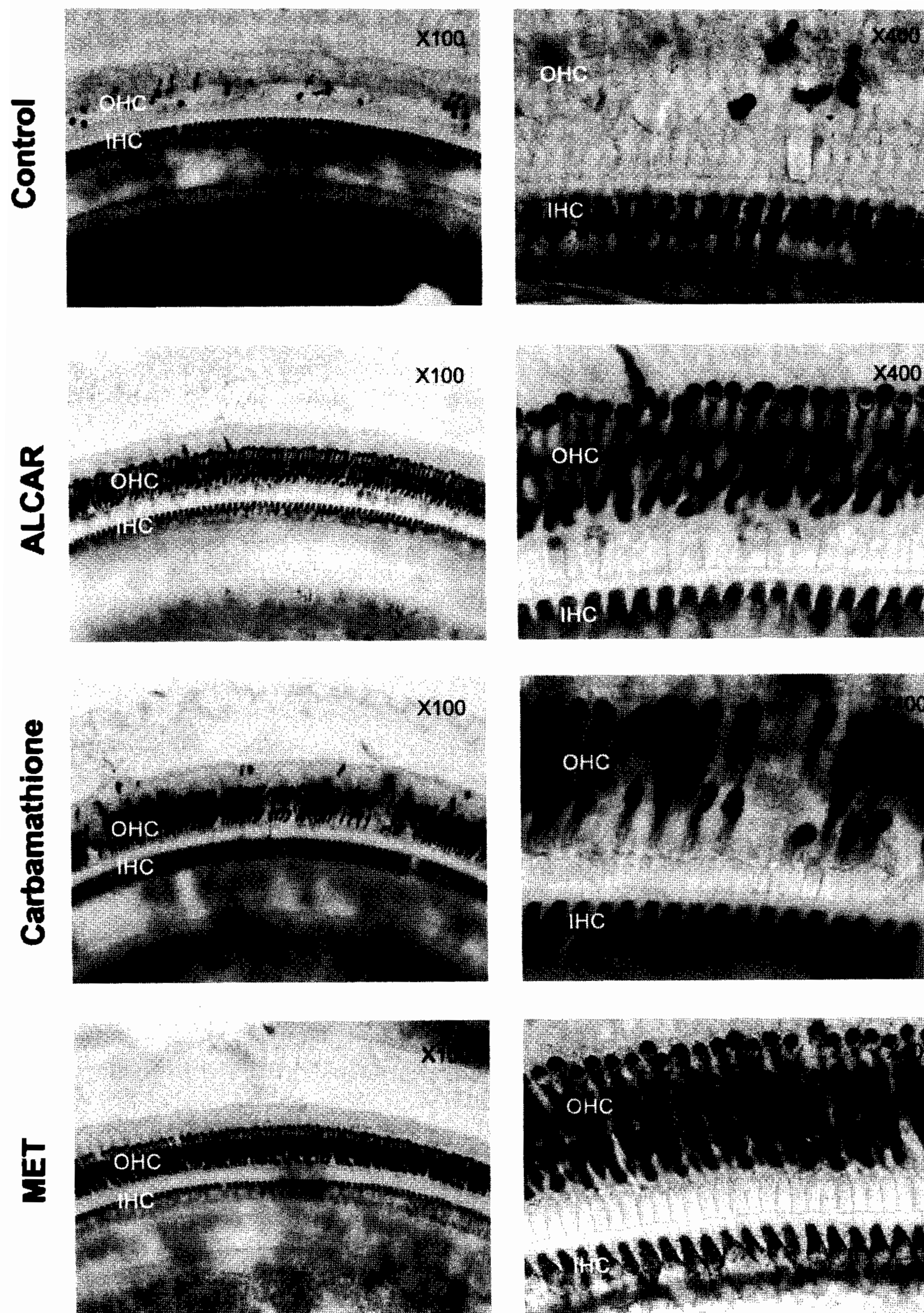
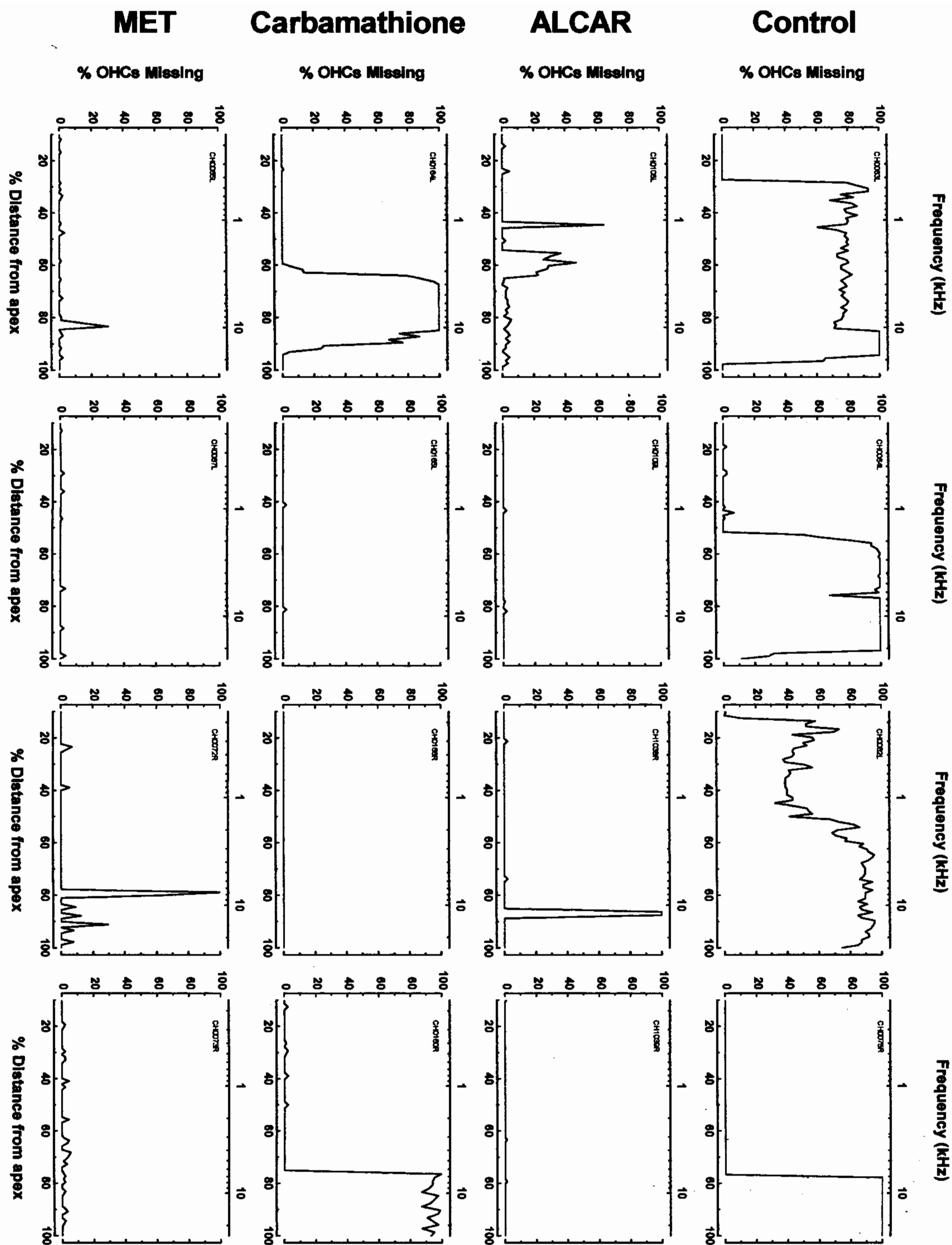


Fig. 5. Low- and high-power photomicrographs of surface preparations of cochleae from noise-exposed animals. A sample of photomicrographs of surface preparations of cochleae stained with sodium succinate histochemistry methodology is depicted in this figure. The micrographs in the left column are low-power (100×), and the micrographs in the right column are high-power (400×), views from the 6-kHz region. All photomicrographs were taken from noise-exposed animals treated with saline (control) or saline plus ALCAR, carbamathione, or MET, depicted in rows from top to bottom. The single row of inner hair cells (IHC) is oriented toward the bottom, and the three rows of outer hair cells (OHC) are oriented toward the top of each micrograph. The control animal photomicrographs demonstrate almost total loss of viable (stained) OHCs with scattered IHC loss. In contrast to this, micrographs from MET- and ALCAR-treated animals demonstrate three complete rows of viable OHCs, similar to non-noise-exposed cochleae (not shown). Carbamathione-treated animals demonstrated cochleae with an intermediate level of OHC loss.

key role in cell death. Inhibiting mitochondrial biogenesis enhances noise-induced cochlear injury.[46]

Glutamate excitotoxicity or ischemia/reperfusion can lead to a number of mitochondrial deficits, including bioenergetic collapse and loss of redox homeostasis, which can eventuate in the opening of the mitochondrial permeability transition pore and cell death.[54] In association with the oxidative stress of aging,[57,75,97] ischemia reperfusion, or glutamate excitotoxicity,[77] a number of harmful cellular and molecular consequences occur. There is a reduction in several key mitochondrial molecules, including cardiolipin and carnitine, decreased cytochrome oxidase activity with its disassociation from the mitochondrial inner membrane complex,[98] increased mitochondrial electron "leak" from the electron transport chain leading to increased ROS production, loss of mitochondrial membrane integrity, and reduced energy production.[97,99]

ALCAR can serve as a precursor for acetyl-CoA and L-carnitine which can shuttle lipid substrates into mitochondria for β-oxidation and to enhance ATP production.[57] ALCAR can also increase ATP production by supplying acetyl CoA to the tricarboxylic acid cycle as an energy substrate. It can also restore carnitine and cardiolipin levels, enhance the activity of cytochrome c oxidase, enhance mitochondrial DNA transcription, restore the transport of key mitochondrial metabolites, and protect mitochondrial membrane integrity.[58] Overall, it appears that ALCAR may enhance the metabolic efficiency of com-



Individual OHC cytocochleograms

Fig. 6. Individual OHC cytocohleograms from a sample of noise-exposed animals. Depicted are individual outer hair cell (OHC) cytocochleograms from cochleae of noise-exposed saline-treated (control) animals and from animals exposed to noise and treated with saline plus ALCAR (ALCAR), carbamathione (Carbamathione), and D-methionine (MET). On the vertical axes are plotted percentages of missing OHCs. On the upper horizontal axes are plotted frequency, and on the lower horizontal axes percent distance from the cochlear apex. The plots depict unsmoothed data. Control cytocochleograms showed fairly extensive OHC loss from 80% to 100%, especially at higher frequencies. Cochleograms from ALCAR-treated animals demonstrated little outer hair cell loss, or a narrow band of OHC, similar to the MET-treated animals. The cytocochleograms from carbamathione-treated animals tended to show either almost no hair cell loss or a large degree of loss over a narrower width of cochlea compared with controls. The data for the carbamathione-treated animals suggested that a threshold for effective dose was being approximated.

TABLE III.
Fits and Asymptotic Convergence to Mean Residual Hearing Loss (threshold shift) of Control Animals for Four Frequencies.*

Frequency (kHz)	*a*	*B*	*c*: Residual Hearing Loss (dB)
2	3.99	1.80	11.73
4	4.02	1.60	29.66
6	4.13	1.37	33.84
8	3.99	1.28	32.41

The data were fit to the model $e^{a-b \times weeks} + c$ by a non-linear least squares method, yielding *a*, *b*, and *c* values as tabulated. The value *c* represents the mean threshold shift below which there is no further return to normal.

promised subpopulations of mitochondria decreasing the rate at which mitochondria-derived oxidants are produced.[57,97]

Carbamathione. Many glutamate antagonists available to date are associated with undesirable side effects resulting from the excessive activity of some of these blocking agents.[53,100] Carbamathione (*S*-[*N,N*-diethylcarbamoyl] glutathione, also known as DETC-GS), unlike classic glutamate antagonists which yield complete inhibition on interaction with the receptor (e.g., CGS 19755) or directly at receptor-linked, calcium ion channels (e.g., phencyclidine or MK 801), is thought to impart its inhibitory effects through interaction with the redox modulatory site of the NMDA receptor.[101–103] The latter type of interaction produces partial glutamate antagonism, is selective for the NMDA subtype of glutamate receptor, and should produce fewer side effects. Carbamathione can be produced from DETC-MeSO (*S*-methyl-*N,N*-diethylthiolcarbamate sulfoxide) in vivo. Both carbamathione and DETC-MeSO are metabolites of disulfiram, a drug that has been used to treat alcoholism over the past 50 years.[104,105]

DETC-MeSO protects against seizures induced by NMDA, methionine sulfoximine (a glutamate analogue), ammonia, alcohol withdrawal, or hyperbaric oxygen.[101,102] One of the consequences of oxidative damage can be the release of excessive glutamate leading to toxicity.[106] Blockage of presynaptic NMDA receptors by carbamathione in the context of noise-induced oxidative stress may allow the molecule to perform a dual function in reducing presynaptic release of glutamate as well as prevent the consequences of glutamate release postsynaptically.[101] Of interest, GSH, GSSG, and the nitric oxide adduct of GSH formed by the reaction of NO with GSH S-nitrosoglutathione (GSNO) in an aqueous aerobic environment function as NMDA and kainate glutamate receptor antagonists, reducing the activation of these receptors, diminishing calcium influx through receptor-gated ionophores with a consequent reduction of calcium-induced activation of nitric oxide synthase and NO formation.[107] In the current study, carbamathione given intraperitoneally before and after noise exposure significantly reduced NIPTS and OHC losses. MET, serving as a source for GSH, may owe its otoprotective effects, in part, to reduction of glutamate excitotoxicity through modulation of receptor activity by GSH, GSSG, and GSNO.

MET. A critical amino acid, methionine, is known to enhance the synthesis of the important antioxidant, glutathione (GSH). One of the major determinants of GSH levels is the availability of cysteine. Cysteine is derived from several sources, including methionine. The conversion of this amino acid to cysteine involves several enzymatic steps. The first step involves the transformation of methionine to S-adenosylmethionine using ATP and catalyzed by an adenosyltransferase followed by six more steps during which the amino acid glutamate is incorporated into the structure of the glutathione molecule. The final step to form glutathione involves the addition of the amino acid glycine by glutathione synthetase.[64]

Like L-*N*-acetyl-cysteine (NAC), a supplier of cysteine for GSH synthesis, D-Met is another potentially effective candidate because it acts as an ROS scavenger as well as a neuroprotective agent by increasing intracellular GSH.[64,108] Unlike NAC, MET can increase mitochondrial GSH.[109] Enhancing mitochondrial biogenesis in this way may be important in preventing cellular loss resulting from noise-induced oxidative stress.[46,55] Finally, methionine is capable of enhancing intracellular GSH in yet another way by reducing the injury-induced transport-mediated efflux of GSH from the injured cell.[110]

D-methionine, the isomer used in the current study, is substantially safer than L-methionine. Several studies suggest that D-methionine itself is not toxic unless it is converted to the L-isomer.[111–114] In the human, D-methionine results in higher plasma levels[94] than L-methionine, which could be advantageous for a protective agent. In humans, 60% to 70% of D-methionine is excreted without conversion to the L isomer,[115,116] except in L-methionine deprivation, which can increase the conversion.[117]

Studies evaluating the change in inner ear GSH and cysteine in response to MET administration are ongoing and not completed as yet. However, systemic or transtympanic administration of L-*N*-acetyl cysteine (NAC), another cysteine precursor compound, has been found to enhance NAC levels in the cochlea. NAC given orally to guinea pigs can achieve a perilymphatic concentration of 5 μg/mL 3 hours after administration and still persist at a level of 0.4 μg/mL in the endolymph 6 hours after administration (Zou et al., 2001 submitted to *Hearing Research*). Furthermore, a metabolite of methionine metabolism in the GSH synthesis pathway, S-adenosylmethionine, given systemically was able to increase CSF S-adenosylmethionine and GSH[118] and brain GSH.[119] Also, systemically administered L-methionine was able to enhance brain thiol levels and reduce the oxidative stress produced by lead intoxication.[120]

The effect of MET in reducing PTS was similar to what was previously reported using L-NAC in combination with the free radical scavenger salicylate.[31] However, the hair cell-sparing ability of the MET (over 90%) was superior to that of the NAC/salicylate (50%–60%) combination in these two studies in which the animals were exposed to 6 hours of continuous 4-kHz octave band (OB) noise at 105 dB SPL.[31] This difference in hair cell-sparing efficacy could be the result of methionine's ability to support mitochondrial GSH levels as well as inhibit the

injury-induced GSH efflux from the injured hair cell.[109,110]

Other studies reporting on the effect of modifying antioxidant defenses by modulating GSH or GSH-related enzymes have been reported. An initial approach used the round window membrane (RWM) application of *R*-phenylisopropyl adenosine (R-PIA) to enhance cochlear antioxidant defenses by increasing the activity of key antioxidant enzymes in the cochlea-reducing hearing and hair cell loss resulting from both continuous[34] and impulse[121] noise. GSH monoethyl ester is rapidly taken up by cells and converted to intracellular GSH available to augment the cell's antioxidant defenses. In this approach, the GSH ester was placed on the RWM and was found to effectively prevent NIPTS and hair cell loss from impulse noise.[121] Another GSH precursor molecule, 2-oxothiazolidine-4-carboxylate (OTC), has also been shown to reduce noise-induced threshold shifts when administered systemically prior to noise.[28]

Effect on TS and Recovery of TS

With few exceptions, the NITTS values were not significantly different between control and treated ears (see time point zero in Figs. 1–3). This is consistent with the notion that the molecular mechanisms of NITTS are different from those producing NIPTS.[74] Recently, Nordmann and colleagues[74] reported that TTS may be the result of temporary buckling of the pillar cells and the subsequent uncoupling of OHC stereocilia bundles from the tectorial membrane. This process may not depend on the molecular consequences of oxidative stress. In the current study, TS in the noise-exposed saline-control animals recovered logarithmically over time to a predicted PTS of 30 to 34 dB from 4 to 8 kHz. In contrast to this, the noise-exposed treated animals were predicted mathematically to recover logarithmically to no TS. This suggests that the treatment reduced the initial injury to the extent that hair cells were not lost or that the treatments enhanced the repair of the injury. GSH has been reported to play a role in not only reducing oxidative stress, but also in enhancing the repair of damage resulting from ROS.[122] Also, ALCAR is reported to have a restorative capability as well.[97]

Clinical Applicability

As mentioned in the *Introduction*, HPDs have a number of inherent limitations and prevention of noise trauma is restricted by those inherent limitations. Noise levels in many occupations and environments exceed the protective capability of both insert and external hearing protection devices. In the military environment, weapon noise far exceeds safe levels.[8] In addition, heavy machinery, aircraft carriers, and tanks impose additional exposure to high levels of noise. Other occupational and recreational environments present similar levels of exposure, so that activities from heavy industry to lawn mowing exceed safe noise levels.

The protective capability of earplugs, muffs, and helmets, as tested in the laboratory, are not representative of actual use. Paakkonen[10] showed that in field conditions hearing protection is less than needed against weapons noise. This is underscored by Savolainen and Lehtomaki[11] who found in a prospective study of 449 military subjects that 87% suffered acute acoustic trauma (AAT) during combat training, and that 41% suffered AAT from a single shot or detonation. Labarere and colleagues[9] found that 57% of military personnel with acute acoustic trauma were wearing hearing protection when the hearing loss causing the accident took place. Poor fit of the protector will further degrade protection.

These issues are more than of theoretical significance. In a study of US Marine recruits, in which training and use of hearing protection were mandatory and carefully controlled, 33% (93 of 283) reported more than one instance of accidental noise exposure without protection during routine weapons training. Hearing loss rates were similar whether protection was worn all the time (14%) or most of the time (18%),[7] and this is consistent with other reports.[6,123] In many occupational environments, the need to hear may contraindicate the use of HPDs.

It should be recognized that devices designed to physically block acoustic energy transfer through the external meatus do not address the transmission of sound through the skull itself. Clinical procedures demonstrate that sound can be transmitted from one site on the skull to another with negligible attenuation.[12] Bekesy,[14] Barany[13] and Tonndorf[15,17] found that external ear canal vibration induced from the skull is transmitted to the ossicles. Another potential pathway for damaging acoustic energy is via the skull through the cerebrospinal fluid into the cochlear fluid.[124] Hence, for all the above reasons, the addition of an agent to the protective armamentarium rendering the cochlea more resistant to noise damage would be useful.

The ideal protective agent would be an effective oral agent with a long half-life that is free of side effects and relatively inexpensive. In addition, if the compounds were available and had an already-established safety record, this would effectively reduce costs of development. The agents tested in the current study meet many of those criteria (ALCAR and MET) or some of those criteria (carbamathione).

ALCAR has been used as both a dietary supplement and a drug for the treatment of neurodegenerative diseases and diabetes.[125–127] A number of placebo-controlled, randomized prospective studies using doses in adults of 1.5 to 3 g per day for as long as a year demonstrate that ALCAR is very well tolerated. Reported side effects were usually not different from those seen in the placebo control subjects.[125,127]

Carbamathione has not yet been used clinically and would have to be developed through customary Federal Drug Administration procedures for a new drug before being clinically available. In this regard, one helpful characteristic of carbamathione is that it is a metabolite formed from the known drug, disulfiram.[128] Unlike many other glutamate receptor antagonists, carbamathione should selectively target the redox modulatory site of only NMDA glutamate receptors.[102] As such, it is only a partial antagonist. Furthermore, carbamathione requires activation in vivo at the site of action.[103] Unlike DETC-MeSO, carbamathione does not also inactivate aldehyde dehydrogenase in vitro or in vivo[129] and should not induce alcohol

intolerance (a disulfiram-ethanol reaction or DER) like disulfiram. For these reasons, carbamathione would appear to be a useful compound with the potential to be free of many of the serious side effects associated with other glutamate antagonist agents[53,100] or the parent drug, disulfiram.

Methionine is a micronutrient, and thus it is not alien to the human system. Both the D- and L-methionine isomers are present in a wide variety of foods. Methionine comprises 26 mg/g high-quality protein in the diet.[130] Methionine is used therapeutically for other purposes and at relatively high doses. The World Health Organization lists methionine as an essential drug for treating acetaminophen overdose.[131] As an oral antidote, methionine is administered initially at 2.5 g, followed by three more 2.5-g doses at 4-hour intervals, for a total dose of 10 g over 12 hours. Monteagudo et al.[132] noted that methionine is "remarkably free of side effects," including nausea and vomiting. Di Rocco et al.[133] administered 3 g L-methionine twice a day for 6 months to treat vacuolar myelopathy in 12 HIV-infected human adults. Patients tolerated it well, other than one complaint of some nausea. DiRocco et al.[133] further report that even 20 g per day for an adult is safe for chronic administration. Methionine has been available for decades as an over-the-counter orally administered preparation to reduce urinary odor and dermatitis. For adults, the recommended dosing is 200 to 400 mg orally three to four times per day.[134]

Most human studies using methionine reported no side effects.[135–137] However, methionine toxicity can occur for very high dosing of racemic or L-methionine, particularly in the presence of a low protein diet and/or in developing animals as opposed to adults.[111,138–142]

D-methionine is substantially safer than L-methionine, and several studies suggest that D-methionine itself is not toxic unless it is converted to the L-isomer.[111–114,143] In the human, D-methionine results in higher plasma levels[94] than L-methionine, which could be advantageous for a protective agent. In humans, 60% to 70% of D-methionine is excreted without conversion to the L-isomer,[115,116] except in L-methionine deprivation which can increase the conversion.[117] Unlike L-methionine, D-methionine is metabolized by 2-keto-methylthiobutyrate,[112] which is non-toxic even at high levels.[143] However, before being administered in high doses over a lengthy period of time, appropriate toxicologic and clinical studies assessing side effects should be performed in accordance with FDA guidelines. These studies should be followed by well-controlled clinical trials to determine efficacy. NAC, another cysteine prodrug and otoprotectant alluded to earlier, also has had a long-standing safety record over many decades.[144] It has also been used in high doses (8 g) per day to treat patients with AIDS over a period of months and side effects were not different from those experienced in the placebo group.[145]

Should a clinically effective and safe compound be found, a number of potentially useful clinical scenarios would pertain. In those working in extremely noise-hazardous work environments, an agent could be taken daily during working hours to augment mechanical hearing protection where noise levels exceeded the protective capability of the device. In other noisy work environments, a drug or supplement could be taken to ensure protection during episodes, when for practical reasons protection could not be worn, or when the seal for the device was compromised. In addition, some individuals are inherently susceptible to NIHL, and if they could be identified, they might benefit from the combination of drug therapy and HPD use. Workers, who are reasonably compliant with HPD use yet show initial mild NIPTS, could be started on a protective agent as they continue their work-related noise exposure to prevent further hearing loss. Use of an effective protective agent could also be envisioned to safely increase a worker's allowable noise exposure dose, avoiding early termination of work shifts resulting from noise exposure limits.

CONCLUSION

The inner ear possesses intrinsic mechanisms to defend itself against the oxidative stress associated with acoustic overexposure, including protection for stressed mitochondria (ALCAR), a potent antioxidant (GSH), and a means to downregulate glutamate responses (redox modulatory site of the NMDA receptor). These intrinsic mechanisms may be augmented to reduce NIHL.

ALCAR, an intrinsic molecule that maintains mitochondrial energy substrate and enhances mitochondrial repair, significantly attenuated NIPTS and hair cell loss in a chinchilla model.

Carbamathione, a molecule that specifically downregulates the activity of the NMDA receptor, effectively reduced the NIPTS and hair cell loss in this model of NIHL.

MET, a compound, which can enhance cellular GSH levels by increasing cysteine bioavailability, was very effective as an agent to reduce NIPTS and cochlear hair cell loss.

These data strengthen the hypothesis and are consistent with reports of other laboratories, indicating that oxidative stress plays a major role in the genesis of one type of noise-induced cochlear injury.

These data support previously reported data suggesting that mitochondrial injury and consequent inefficient energy production, NMDA-induced glutamate excitotoxicity, and GSH depletion all play a role in the development of injury to the cochlea resulting from excessive continuous noise.

These data, in demonstrating successful protection from acoustic overexposure through the strategy of augmentation of intrinsic cochlear oxidative stress defense mechanisms, suggest approaches for reducing noise-induced deafness in clinical populations.

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Carbamathione is the ultimate metabolite of disulfiram that is responsible for its antagonist effect on NMDA receptors (Nagendra et al. *J Biol Chem* 1977;272:24247–24251; Liu et al. The fate of thiocarbamate sulfoxides in vitro and in vivo. In: Frey PA, Northrop DB, eds. *Enzymatic Mechanisms*. Amsterdam: IOS Press, 1998:107–115; Ningaraj et al. *J Biomed Sci* 2001;8:104–113]. The authors also express their deep appreciation for the expert and sustained technical and scientific assistance of LTC Ronald L. Jackson, USAR, Dr. Eve A. Williams, Mr. Gavin E. G. Jones, CDR Michael E. Hoffer, USN, Miss Kimberly Wood, and MAJ Alec Hail, USAR, of Naval Medical Center San Diego. The primary author wants to express his sincere gratitude to Dr. J. V. D. Hough and CAPT (Ret.) Darrell H. Hunsaker for their sponsorship into the Triological Society and their advice on the manuscript. The first author also is deeply appreciative of the invaluable clinical or research mentorship provided over a number of years by COL (Ret.) Donald W. Yim, USA, Dr. J. V. D. Hough, Dr. Thomas R. Van De Water, Dr. Joseph G. Feghali, CAPT (Ret.) Darrell H. Hunsaker, USN CAPT (Ret.) Dennis K. McBride USN, Dr. Yehoash Raphael, Dr. Josef M. Miller, Dr. Robert J. Ruben, and Dr. Leonard P. Rybak.

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