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

Richard D. Kopke, COL MC USA; John K. M. Coleman, PhD; Jianhong Liu, MD; Kathleen C. M. Campbell, PhD; Robert H. Riffenburgh, PhD

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 antagonizing the action of cochlear N-methyl-D-aspartate (NMDA) receptors using carbamathione, which acts as a glutamate antagonist. 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. Average OHC losses were 30% to 40% in carbachamthione-treated animals and 60% in control animals. IHC losses were 5% in the 4- to 6-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 < .01). 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...
### Objective/Hypothesis

Oxidative stress plays a substantial role in the genesis of noise-reduced cochlear injury that causes permanent hearing loss. We present the results of three different approaches to enhance intrinsic cochlear defense mechanism against oxidative stress.

### Subject Terms

Enhancing Intrinsic Cochlear Stress Defenses To Reduce Noise-Induced Hearing Loss
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-CoA, cardiolipin, methionine, oxidative stress.

Laryngoscope 112:1516-1533, 2002

INTRODUCTION

Noise is a pervasive and increasing hazard in all developed or developing nations, with 600 million persons estimated to be working in environments with hazardous levels of noise (50-60 dB 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, and 90% of coal miners are estimated to have a hearing impairment by age 52 years. The U.S. Government spends over $250 million in compensation each year for military-related noise-induced hearing loss (NIHL). 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.4 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.7-13 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 noise inputs suffice to challenge the cochlea (approximately 125 dB SPL, or greater) to cause significant mechanical damage,19,20 much noise exposure is at such a level as to metabolically challenge the cochlea as well.18-20 Acoustic overexposure leads to the production of reactive oxygen species (ROS) and other free radicals in the cochlea.21-23 These ROS are quite capable of inducing cochlear damage as well as loss of function when introduced into the cochlea.24 In recent years, a variety of compounds with antioxidant effects have been shown to ameliorate noise-induced cochlear injury.18,20-27

While it is becoming increasing 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,25 mechanical trauma, oxidative stress resulting from both toxins and noise.18 Experiments were undertaken to strengthen the hypothesis that downregulating the activity of the NMDA receptor by binding with its physiological 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 modulatory site, carboxymethathione 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.60 There is substantial evidence that GSH in the cochlea plays a significant role in protecting the cochlea from oxidative stress due to both toxins and noise.18,20,27 Experiments were undertaken to strengthen the hypothesis that supplementation with an antioxidant, specifically chosen to enhance mitochondrial biogenesis, or self-repair, enhances noise-induced cochlear injury.18 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 biogenesis and self-repair, enhances noise-induced cochlear injury.20 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.

Laryngoscope 112: September 2002

Kopke et al.: Enhancing Noise-Induced Hearing Loss Defenses

branes,19 or reticular lamina21 or glutamate excitotoxicity resulting from excessive H 20 stimulation.18-20 Excessive Ca 2+ influx results 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-inducing mechanical 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).18-20 Mitochondrial injury, which can be caused by excessive ROS generation within mitochondria or as a consequence of glutamate excitotoxicity or NO production 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.18 ALCAR also restores a key mitochondrial lipid known as cardiolipin in oxidatively injured cells, further restoring mitochondrial integrity.20

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.18,52-54 Conversely, general glutamate antagonists such as MK-801 and kynurenate reduce cochlear injury resulting from noise.18-20 Accordingly, carboxymethathione was chosen as a test compound to explore the hypothesis that downregulating the activity of the 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. There is substantial evidence that GSH in the cochlea 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.18 ALCAR also restores a key mitochondrial lipid known as cardiolipin in oxidatively injured cells, further restoring mitochondrial integrity.20

Laryngoscope 112: September 2002

1516
TABLE I: Summary of Site and Mechanism of Action of Experimental Agents (Data in Use at September 2002)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Site of Action</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALCAR</td>
<td>Mitochondria</td>
<td>Improves energy production; restores mitochondria and carotid levels; reduces ROS production; other</td>
</tr>
<tr>
<td>Carbamothine</td>
<td>NMDA receptors</td>
<td>Downregulates NMDA receptor activity</td>
</tr>
<tr>
<td>MET</td>
<td>Cochlea</td>
<td>Provides cysteine for synthesis of GSH; free radical scavenger</td>
</tr>
</tbody>
</table>

METHODS

Twenty-four female adult chinchilla lancers were divided equally into four experimental groups. Animals were fed a standard chinchilla diet (Mazuri Chinchilla Diet, 5M01, PMI Nutrition International Inc., Brentwood, MO). The four groups consisted of a saline control group and three experimental agent (EA) treatment groups (ALCAR, Carbamothine, 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), carmustine (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-carnosine, 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 the injections were given every 12 hours, starting 48 hours before the Noise Exposure. Shortly after the last saline or EA injection before noise exposure, Animals were acclimated to the sound booth and the acoustical environment. The noise exposure conditions, the noise output of the system was monitored before each noise exposure using a sound level meter. Also, a preamplifier (Larson Davis, Provo, UT) sound level meter (A scale, SPL) centered at octave bandwidths of 40-7000 Hz, at octave intervals of 2, 4, 6, and 8 kHz. All acoustic stimuli were presented at a rate of 20 dB per octave 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 comparison. 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.4 Specifically, an octave band noise centered at 4 kHz was generated by a standard audiometer (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 350R; Krohn-Hite Corp., Iron, MA), and a power amplifier (Crown D150A model 716, Crown Audio Inc., Elkhart, IN) to an audiometric loudspeaker (JBL Model 250A, JBL Inc., Northridge, CA) suspended above the animal's cage. The sound spectrum output of the system was confirmed using a Larson and Davis model 825; the sound spectrum output of the system was monitored during each noise exposure using a sound level meter. Also, a preamplifier (Larson and Davis model 825) and a condenser microphone (Larson and Davis, LDL 255R) 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 level of 100 ± 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 libitum 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 infrasound booth to monitor the effect of sound shadowing. The average temperature in the sound booth was 68.9°F (range, 66-71°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 58-64 ± 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 diaphragms placed into the animal housing room) was approximately 40 dB SPL.

Histological Examination

Following each auditory test (i.e., at 3 weeks post-noise exposure), each animal was deeply 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 placed in 10% formaldehyde placed into the animal's head until the cochlea was clear of the bone. Each cochlea was then placed into the sound booth and 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 255R) 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 level of 100 ± 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 libitum 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 infrasound booth to monitor the effect of sound shadowing. The average temperature in the sound booth was 68.9°F (range, 66-71°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 58-64 ± 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 diaphragms placed into the animal housing room) was approximately 40 dB SPL.

Kopke et al.: Enhancing Noise-Induced Hearing Loss Defences 1517
ANOVA ears as replications. Post-hoc tests were performed by the mum asymptotically. The means of the data for all treatments method with significance set at

Statistical Analyses

A three-way (4 x 4 x 4) ANOVA was used to analyse 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 x 4) ANOVA was performed to analyse the effect on HC counts of treatment (saline control, ALCAR, carbonamides, and MIBT), fre­quency, and time, as determined by time intervals 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 mini­ mum 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 antici­ pated, were fit by the exponential model

\[ TS = e^{-b \times x} + c, \]

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., 3.5 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

\[ TS = e^{-b \times x} + c. \]

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 dB of TS.

RESULTS

There were no significant differences in ABR thresh­ olds 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 ex­ posure 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 over­ all reduction in threshold shift for ALCAR-treated ani­ mals 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 < .06) and 6 weeks (P < .03). 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 Rs produced coefficients of determination (R²) in the 0.80 to 0.95 range, indicating adequate agree­ ment 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). OH 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. HOC loss was significantly reduced at 2, 4, and 6 kHz, and was less than 5% com­ pared with an average of about 20% in control cochleae at 8 kHz (Fig. 1C). Mean inner and outer HC counts from

Kopke et al.: Enhancing Noise-Induced Hearing Loss Defenses

Laryngoscope 112: September 2002

1518
noise-exposed animals were significantly different from each other based only on treatment (s–ALCAR, 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 30–50 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 < .01) and almost significant at 6 kHz (P = .039). 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 intercept baseline at approximately 4 to 5 weeks compared with the asymptote reached for the threshold shift recovery curve of control animals (Tables II A and Table IIIB; 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 = .002). The average IHC loss for the animals treated with the glutamate receptor antagonist

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 cochlea in kHz. There was very little OHC loss in the ALCAR-protected cochleae (less than 1%), whereas there was substantial outer hair cell loss in the saline-control noise-exposed group (from 40%–70% loss) in the frequency regions tested for hearing (P < .001). Sample size is 12 for all groups (12 ears, 6 animals). (C) Outer hair cell cytococholekrograms. Data are plotted mean (continuous line) and SEM (shaded area) with respect to the 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 OHC loss in ALCAR-treated animals compared with noise-exposed controls. Maximal IHC loss occurred between the 4- and 16-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 cochlear in the saline-treated animals compared with less than 3% in the ALCAR-treated animals (P < .05). Error bars are ± SEM. Sample size is 12 for all groups (12 ears, 6 animals). Laryngoscope 112: September 2002 Kopke et al: Enhancing Noise-Induced Hearing Loss Defenses 1519
Threshold Shifts (dB SPL)

\[
\begin{array}{c|c|c|c|c|c|}
\hline
\text{Week} & \text{Control} & \text{Carbamathione} & \text{Control} & \text{Carbamathione} & \text{Control} & \text{Carbamathione} \\
\hline
0 & 50 & 50 & 40 & 40 & 30 & 30 \\
1 & 60 & 60 & 50 & 50 & 40 & 40 \\
2 & 70 & 70 & 60 & 60 & 50 & 50 \\
3 & 80 & 80 & 70 & 70 & 60 & 60 \\
\hline
\end{array}
\]

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 (noise-exposed and saline-exposed control) for 1 hour (zero week) and 1-, 2-, 3-week post-noise exposure. Auditory test frequencies are the same as for Figure 1A as in Figure 1A. Mean ± SEM. The Y-axis depicts mean percent missing outer hair cells. The X-axis represents percent distance from the cochlear apex. The upper X-axis depicts the associated frequency range of the cochlea.

YPKope et al: Enhancing Noise-Induced Hearing Loss Defenses

Laryngoscope 112: September 2002

1520
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 treated), time (bars [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 67 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 cochlea (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 cochlea in kHz. There was very little OHC loss in the low dose MET-protected cochlea (less than 10% of total OHCs). OHC loss in the saline-control noise-exposed group (average of approximately 50% 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 < .001). Error bars are ± SEM. Sample (n) size is 12 for all groups (12 ears, 6 animals).
sure environment within a grid of carefully measured noise—dose isolars, 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 death.

The hypoxia of afferent dendrites induced by glutamate and the firing of afferent dendrites induced by glutamate and related free radicals such as peroxynitrite. Activation of protein kinases, activation of phospholipase A2, activation of xanthine oxidase with the subsequent generation of superoxide anion, in addition to mitochondrial injury, may also occur. Glutamate excitotoxicity can be divided into an early phase (up to 30 min) and a late phase (30–24 h) of ROS production consequent to the excessive glutamate production. 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. NMDA and -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. In addition, AMPA and kainate, when infused into the cochlea in high concentration, can induce cochlear injury consisting of destruction of dendrites beneath inner hair cells. This is morphologically similar to what is seen after ocular 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. Glutamate antagonists like MK-801 and others have been reported to reduce NITS and NITTS.

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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency (kHz)</th>
<th>A</th>
<th>b</th>
<th>Weeks to Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALCAR</td>
<td>2</td>
<td>4.11</td>
<td>1.061</td>
<td>3.90</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.56</td>
<td>1.327</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.49</td>
<td>0.859</td>
<td>4.57</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.43</td>
<td>0.753</td>
<td>4.79</td>
</tr>
<tr>
<td>Carbamathione</td>
<td>2</td>
<td>3.84</td>
<td>1.098</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.22</td>
<td>0.878</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.49</td>
<td>0.699</td>
<td>5.12</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.38</td>
<td>0.771</td>
<td>4.50</td>
</tr>
<tr>
<td>MET</td>
<td>2</td>
<td>3.68</td>
<td>1.336</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.37</td>
<td>0.790</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.41</td>
<td>0.771</td>
<td>4.54</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.34</td>
<td>0.716</td>
<td>4.78</td>
</tr>
</tbody>
</table>

*The data were fit to the equation of the form a + bx 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 (0.5 dB) was taken as the time required for the average animal to return to normal hearing and is also tabulated.
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 HIC losses (>96% 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.86 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,87,88 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.89 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.90 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 versus 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,97,98 and that the status of the IHC synapses and other cellular changes may be as important as missing hair cells.99 MET has previously been reported to reduce the oxidative stress-related ototoxicity of the chemotherapeutic agent cisplatin100–102 as well as aminoglycoside antibiotics.103 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 oxidative injury.104 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 salicylate105 and NAC alone.106 NAC is another cysteine-supplying drug shown to reduce noise-induced cochlear lipid oxidation and damage.107,108 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 postnoise exposure, may have been important in that a second burst of ROS production may occur some time after the cessation of noise.104 Also, it has been reported that increased ROS production may continue for at least a number of hours after noise.109,110 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.111 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.112,113 has been shown to play a
key role in cell death. Inhibiting mitochondrial biogenesis enhances noise-induced cochlear injury. Glutamate excitotoxicity or ischemia/reperfusion can lead to a number of mitochondrial deficits, including bioenergetic collapse and loss of redox homeostasis, which can ensue in the opening of the mitochondrial permeability transition pore and cell death. In association with the oxidative stress of aging, ischemia reperfusion, or glutamate excitotoxicity, 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, increased mitochondrial electron "leak" from the electron transport chain leading to increased ROS production, loss of mitochondrial membrane integrity, and reduced energy production.

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. 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. Overall, it appears that ALCAR may enhance the metabolic efficiency of com-
Fig. 6. Individual OHC cytococholeograms from a sample of noise-exposed animals. Depicted are individual outer hair cell (OHC) cytococholeograms 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 cytococholeograms showed fairly extensive OHC loss from 80% to 100%, especially at higher frequencies. Cytococholeograms from ALCAR-treated animals demonstrated little outer hair cell loss, or a narrow band of OHC, similar to the MET-treated animals. The cytococholeograms 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.
promised subpopulations of mitochondria decreasing the rate at which mitochondria-derived oxidants are produced. 101,102

Carbamathione. Many glutamate antagonists available to date are associated with undesirable side effects resulting from the excessive activity of some of these antagonists. 53•100

The latter type of interaction produces partial glutamate antagonism, which yields complete inhibition on interaction with the receptor (e.g., CGS 19760) 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

This difference in hair cell-sparing ability could be the result of methionine's ability to support mitochondrial GSH levels as well as inhibit the 70% to 100% of the major determinants of 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. 104

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. 66•68 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 64 than L-methionine, which could be advantageous for a neuroprotective 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 transysnaptic 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 brain thiol levels and reduce the oxidative stress produced by lead intoxication. 118

The effect of MET in reducing OHC loss was similar to what was previously reported using L-NAC in combination with the free radical scavenger salicylate. 63 However, the hair cell-sparing ability of the MET treatment was superior to that of the NAC/salicylate (50%–60%) combination in these two studies in which the animals were exposed to 8 hours of continuous 1-kHz octave band (OB) noise at 105 dB SPL. 119 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.106,110

Other studies reporting on the effect of modifying anti-
oxidant defenses by modulating GSH or GSH-related en-
yzymes 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 anti-
oxidant enzymes in the cochlea-reducing hearing and hair
cell loss resulting from both continuous122 and impulse121
noise. GSH monomethyl 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
enhance the repair of damage resulting from ROS.122

In the current study, TS in the noise-exposed saline-control ani-
mals 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 mathemat-
ically 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 en-
hanced 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.123

Also, ALCAR is reported to have a restorative capability as well.97

**Clinical Applicability**

As mentioned in the Introduction, HPDs have a num-
er 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, air-
craft, and aircraft engines from heavy industry to lawn mowing exceed safe
levels.

The protective capability of earplugs, muffs, and hel-
mets, as tested in the laboratory, are not representative of
those used in actual use. Paakkonen15 showed that in field conditions
hearing protection is less than needed against weapons
noise. This is underscored by Savolainen and Lehtomäki14 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 colleagues9 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 signifi-
cance. In a study of US Marine recruits, in which training
and use of hearing protection were mandatory and care-
fully 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%), and this is consistent with other
reports.126 In many occupational environments, the need
to hear may contraindicate the use of HPDs.

It should be recognized that devices designed to phys-
ically 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 Barany10
and Tonndorf15 found that external ear canal vibration
induced from the skull is transmitted to the ossicles. An
other potential pathway for damaging acoustic energy is
via the skull through the cerebrospinal fluid into the co-
chlear fluid.124 Hence, for all the above reasons, the addi-
tion of an agent to the protective armamentarium render-
ing 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 MEGT) or some of those criteria
(carbamathione).

ALCAR has been used as both a dietary supplement
and a drug for the treatment of neurodegenerative dis-
eases and diabetes.113,114 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.105,127

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 char-
acteristic of carbamathione is that it is a metabolite
formed from the known drug, disulfiram.129 Unlike many
other glutamate receptor antagonists, carbamathione
should selectively target the redox modulatory site of only
NMDA glutamate receptors.106 As such, it is only a partial
antagonist. Furthermore, carbamathione requires activa-
tion in vivo at the site of action.106 Unlike DETC-MeSO,
carbamathione does not also inactivate aldehyde dehydro-
genase in vitro or in vivo106 and should not induce alcohol
Laryngoscope 112: September 2002 Kople et al: Enhancing Noise-Induced Hearing Loss Defenses 1527
interleukina (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.85,100 The parent drug, disulfiram.

Methionine is a nonessential amino acid in most diets, but in the presence of a low protein diet and/or in developing animals as opposed to adults,111 ·138 - 142 L-methionine deprivation which can increase the conversion of D-methionine to the L-isomer, 115 ·116 which is toxic unless it is converted to the L-isomer. 111 - 114 ·143 Moreover, many of the serious side effects associated with other glutamate antagonists were noted in the placebo group. 145

CONCLUSION
The inner ear possesses intrinsic mechanisms to defend itself against the oxidative stress associated with the human system. Both the D- and L-methionine isomers are present in a wide variety of foods. Methionine comprises 20 mg/g high-quality protein in the diet.109 Methionine is used therapeutically for other purposes such as 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 NMDA-induced 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.

Acknowledgments
The authors thank the Office of Naval Research and US Army for the funding for this research and for the generous support of equipment and time during the conduct of this research by Mr. William W. Corbin, Jr., and Dr. Doug W. Ohlin of the US Army Center for Health Promotion and Preventive Medicine. They also thank Prof. John Schloss (Advanced Therapeutics & Diagnostics, University of Kansas and Kuwait University) for the gift of carbamathione (S-(4-N,N-diethylcarbamoyl)glutathione).
Cyclophosphamide is the ultimate metabolite of disulfiram that is responsible for its antagonistic effect on NMDA receptors (Nagendran et al. / Riol Chem 1977:272:24347-24351; Liu et al. The fate of thiosemicarbazide sulfoxides in vitro and in vivo. In: Frey PA, Nortzop BD, eds. Enzymatic Mechanisms. Arpadatemia: 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, MAJ Alec Hail, LTC Harriet McCarthy, COL V. Kinney, Major Michael V. De Water, Dr. Joseph G. McBride, Mr. Kevin J. Katz, Mr. John W. V. Van Vliet, USN, Mr. S. Qing and Mr. Collin Benfante, 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. Hunker 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. Faghdeli, CAPT (Ret.) Darrell H. Hunker, USN CAPT (Ret.) Dennis K. McBrirde USN, Dr. Yehoah Raphael, Dr. Josef M. Miller, Dr. Robert J. Ruben, and Dr. Leonard P. Rybak.

BIBLIOGRAPHY


100. Scatton


Kopke et al.: Enhancing Noise-Induced Hearing Loss Defenses

*Laryngoscope* 112: September 2002

1531


Kopke et al.: Enhancing Noise-Induced Hearing Loss Defenses