



injury, as impulse noise differs in physical nature from continuous noise and consequently results in acute and more severe cochlear damage [5]. In this study, we extend the above findings by looking at two promising compounds (ALCAR and NAC) that may be effective in protecting cochlear hair cells against impulse noise-induced injury in an effort to provide insight into the most effective agents for protection from acute acoustic damage induced by impulse noise.

Mitochondrial injury plays an important role in NIHL based on a putative mechanism of cell death in the face of excessive ROS generation within mitochondria or from glutamate excitotoxicity or GSH depletion [1,6–9]. ALCAR, an endogenous mitochondrial membrane compound, was selected as a candidate otoprotectant because of its capacity to enhance mitochondrial bioenergetics and repair oxidative stress injury to mitochondria. It serves as a precursor for acetyl-CoA, a mitochondrial energy substrate, and L-carnitine, which can shuttle lipid substrates into mitochondria for  $\beta$ -oxidation and enhance adenosine triphosphate production. ALCAR also restores a key mitochondrial lipid known as cardiolipin in oxidatively injured cells, further restoring mitochondrial integrity [10]. NAC was chosen as another potentially effective candidate because this AO protects cells by various mechanisms. The most well-known actions of NAC are that it acts as an ROS scavenger as well as a neuroprotective agent (by increasing intracellular GSH) [11–14]. The aim of this study was to examine the protective capacity of utilizing these two nutritional supplements, NAC and ALCAR, by administering them just before and after exposure to damaging impulse noise to prevent hearing loss in a chinchilla model.

## Material and methods

The Animal Care and Use Committees of the Naval Medical Center, San Diego and the State University of New York at Buffalo approved the care and use of the animals in this study, in accordance with the guidelines of the Declaration of Helsinki.

Eighteen female adult chinchilla laniger were divided into two experimental groups and one control group (six animals in each group). Data were collected from both ears of the animals (i.e.  $n = 12$  for each group). To investigate protective effects, the AOs NAC (Cat. #100098; 325 mg/kg; ICN Biomedicals, Inc., Aurora, OH) and ALCAR (A-1509; 100 mg/kg; Sigma Chemical Co., St. Louis, MO) were freshly mixed in sterile normal saline (pH 6.8–7.2) daily and administered by i.p. injection twice daily (b.i.d.) for 48 h and 1 h prior to

and 1 h following noise exposure, and then b.i.d. for the following 2 days. In the control group, equal volumes of sterile normal saline were injected at the same time points as in the experimental groups and the animals were exposed to the same impulse noise.

Hearing thresholds were obtained by recording auditory brainstem responses (ABRs). Thresholds were obtained according to the following schedule: prior to injections of AOs,  $\approx 15$  min post-noise exposure and 1 and 3 weeks post-noise exposure. Previous work demonstrated that when these agents were injected ABR thresholds did not change (unpublished observations). The person performing the ABR measurements was blinded as to which treatment group the animal belonged. Upon completion of the final threshold measurements, the animals were humanely euthanized, and the cochleae harvested. Cochleae were then examined for hair cell loss using the vital dye (succinate dehydrogenase) uptake method, in which viable hair cells are stained blue and missing or dead hair cells fail to stain. The mean hair cell counts were then compared between the groups based on a computer-generated cytochrome program. The investigators performing the hair cell counts were blinded as to the origin of the tissue.

## ABR recording

Animals were lightly anesthetized with ketamine (40 mg/kg)/xylazine (1 mg/kg) and lightly restrained in a plastic tube during the recording procedure. ABR thresholds were measured via subcutaneous needle electrodes placed in the skin of the head and proximal to the ear. Acoustically generated stimuli consisted of tone pips generated using the Blackman protocol and ramped at 1, 2, 4, 6 and 8 kHz. The stimulus was routed through a computer-controlled attenuator to an insert earphone (Etymotic Research ER), which was positioned  $\approx 5$  mm from the tympanic membrane. The output of the insert earphone was calibrated by measuring the SPL in a chamber of equivalent volume to that of the external ear canal of the animal. The electrical response from the recording electrode was amplified ( $\times 100\,000$ ), filtered (100–3000 Hz) and fed to an analog-to-digital converter on a signal processing board in the computer. At threshold, at least 2 repetitions of at least 600 samples were recorded at each level. Stimuli were presented at a rate of 21/s, and the stimulus level was varied in 5-dB descending steps, until threshold was reached, and then in 5-dB ascending steps for confirmation. Threshold was defined as the midpoint between the lowest level at which a clear response was seen and the next lowest level, where no response was seen.

### Noise exposure

Alert animals were placed in a restraint tube and exposed to impulses simulating M-16 rifle fire. The impulses were generated with a Spectrum TMS 320c25 signal processing board, amplified with an NAD 2200PE amplifier and transduced with a high-frequency JBL L455J acoustic driver. The alert animals were placed in front of the sound source, so that each ear received equal intensity exposure of 75 pairs (at a rate of 1 pair/s) of 155-dB peak SPL (pSPL) impulses, for a total of 150 impulses. The impulses were calibrated with a 0.25-in. microphone (Brüel & Kjaer 4136) attached to a pistonphone (Larson Davis CA 250). The signal was run through a pre-amplifier (Larson Davis 2200C) to an oscilloscope (Tektronix TDS3012). The pistonphone level of 114 dB SPL was used to calculate the voltage on the oscilloscope for a 155-dB SPL sound source. The microphone was placed at a distance and height from the sound source corresponding to the location of the animals' ears. Impulses were captured on the oscilloscope, and the level was manipulated until the peak of the impulse was 155 dB pSPL.

### Histological examination

After the final ABR measurement, the animals were deeply anesthetized with ketamine (40 mg/kg)/acepromazine (1 mg/kg) and decapitated. Each bulla was quickly removed from the skull, opened and slowly perfused from the oval window to the round window with 0.2 M sodium succinate and 1.0% nitroretazolium blue in 0.2 M phosphate buffer (pH 7.4) and immersed in the same solution for 1 h at 37°C. Cochleae were rinsed with PBS and then perfused and stored in cold 4% paraformaldehyde for 24 h. The cochleae were dissected, and sections of the organ of Corti from the entire length of the cochlea were mounted on a slide as surface preparations. All specimens were examined for loss of hair cells under a light microscope (Olympus BH-2; Olympus Optical Co. Ltd.). Data from each section were input into a worksheet in Origin software (version 6.0; Micro Software Inc.) 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 hair cells (IHCs) or outer hair cells (OHCs) by dividing the cell count for the experimental animals by control value cell counts for normative values developed for each cochlear region. Each cochlea's length was normalized to 100%. The numbers of IHCs and OHCs as a function of percent distance from the apex were established using a linear regression model, and the percent distance from the apex was converted to a frequency corresponding to the

relative location. The percentage of missing hair cells was then plotted as a function of frequency. A cytochleogram was developed for IHCs and OHCs for each cochlea, and cytochleogram means were computed and graphed after smoothing the data set for each 100-point set with a fast Fourier transform filter. The smoothed data were then normalized with an interpolated curve function for 2000 points [1].

### Statistical analyses

Mean hearing threshold shifts in decibels were compared using a repeated-measures two-way ANOVA with interaction model for three agents (control, ALCAR, NAC), one strategy (protection) and five frequencies (1, 2, 4, 6 and 8 kHz), with frequency as a repeated measure. Only two treatments were compared at a time. This ANOVA was repeated over three time periods (0, 1 and 3 weeks) for impulse noise exposure. Each ear was treated as a separate data point ( $n = 12$ ).

A two-way ANOVA with interaction model was also used to analyze the effect on hearing thresholds of frequency for one of three time points. Hearing threshold shifts for each treatment at Weeks 1 and 3 were compared with those at Day 0. For all analyses where a significant effect included more than two levels, post-hoc pair-wise comparisons were conducted using Fisher's least squares differences (LSD) method. A two-way ANOVA was performed to analyze the effect on hair cell counts of treatment (saline control, NAC and ALCAR), frequency (1, 2, 4, 6 and 8 kHz) and the interaction between treatment groups and frequencies. Data from one ear were considered as one data point. Hence the results from 6 chinchillas resulted in a sample size of 12 (ears). Post-hoc testing was performed using the LSD method.  $p < 0.05$  was considered statistically significant. Statistical comparisons were made using the GB-STAT (School Pak Version™) software package.

## Results

### ABR threshold shift

Baseline ABR thresholds for both left and right ears at 1, 2, 4, 6 and 8 kHz were obtained. All animals showed normal ABR thresholds across all five frequencies (data not shown). Hearing loss was observed immediately after impulse noise exposure (NE) in all groups. As shown in Figures 1 and 2, at Day 0, the impulse NE (150 impulses at 155 dB pSPL) produced initial threshold shifts ranging from  $\approx 30$  dB at 1 kHz to  $\approx 60$  dB at the higher frequencies (4–8 kHz). In all three groups recovery

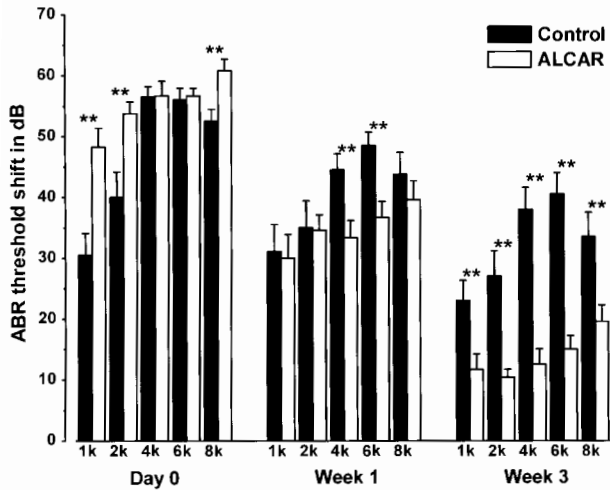


Figure 1. ABR threshold shifts for the ALCAR group ( $n = 12$  ears) and the control group ( $n = 12$  ears) at 3 time points (Day 0, Weeks 1 and 3) after impulse NE. The label "Day 0" represents the time point immediately ( $\approx 15$  min) after the exposure. Error bars represent the SE of the means.  $**p < 0.01$  for LSD post-hoc comparisons made between control and ALCAR-treated animals at each frequency and time point.

of threshold shift occurred; however, the degree of recovery differed among the groups. In the control group, hearing recovered slowly and to a small extent over the 3-week post-exposure period (Figure 3). In contrast, the NAC group showed greater and statistically significant improvements in hearing at Week 1 for most of the frequencies, and then a relatively slower recovery afterwards, while hearing thresholds recovered more rapidly for the ALCAR group between Weeks 1 and 3 (Figure 3). Overall, at Week 3, in comparison with the control group, recovery of hearing in the ALCAR ( $F = 25.83$ ;  $n =$

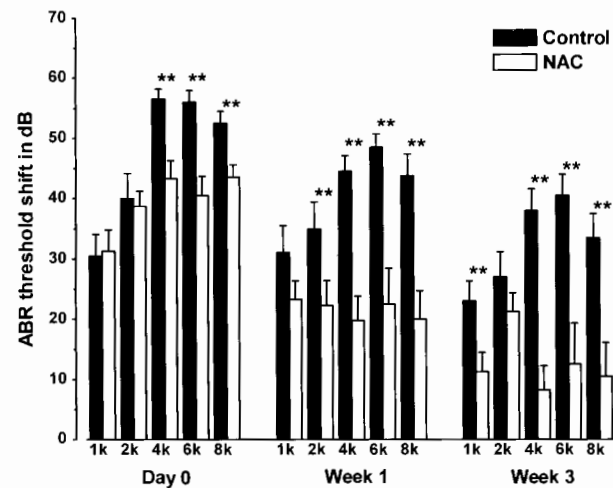


Figure 2. ABR threshold shifts for the NAC group ( $n = 12$  ears) and the control group ( $n = 12$  ears) at 3 time points (Day 0, Weeks 1 and 3) after impulse NE.  $**p < 0.01$  for LSD post-hoc comparisons made between control and NAC-treated animals at each frequency and time point. Other details as for Figure 1.

12;  $p < 0.01$ ) and NAC ( $F = 14.49$ ;  $n = 12$ ;  $p < 0.01$ ) groups was significantly greater. It was noted that at the time point immediately after exposure, i.e. Day 0, the threshold shift in the ALCAR group exhibited greater hearing loss (temporary threshold shift) than that in the control group, with a statistically significant difference ( $p < 0.01$ ) at 1, 2 and 8 kHz (Figure 1). Meanwhile, at the same time point, a smaller threshold shift compared to the control group was seen in the NAC group, with a statistically significant difference at 4, 6 and 8 kHz ( $p < 0.01$ ) (Figure 2). In addition, the progression of hearing recovery for the ALCAR group was different from that for the NAC group (Figure 3). Although the rates of recovery for the ALCAR and NAC groups between Day 0 and Week 1 were roughly parallel, the recovery of hearing for the NAC-treated animals was slower than that for the ALCAR-treated animals in the period between Weeks 1 and 3. Despite these differences, at 3 weeks after exposure, both treatment groups displayed similar threshold shifts at most frequencies ( $p > 0.05$ ), except for at 2 kHz, where the difference in threshold shift between these groups was significant ( $p < 0.05$ ). The threshold shifts ranged approximately between 10 and 20 dB, which were much smaller than those in the control group, and this difference was statistically significant (ALCAR:  $F = 25.83$ ,  $n = 12$ ,  $p < 0.01$ ; NAC:  $F = 14.49$ ,  $n = 12$ ,  $p < 0.01$ ), therefore indicating an effect of hearing protection against impulse noise injury.

#### Hair cell counts

Figures 4 and 5, panels (A) and (C), illustrate mean hair cell counts in a cytochrome, which portrays mean missing OHC and IHC percentages, respectively, as a function of frequency place in the cochlea. Panels (B) and (D) are derived from panels (A) and (C), demonstrating the mean percentages of OHCs and IHCs missing, respectively, in cochlear regions corresponding to frequencies ranging from 2 to 8 kHz. In the control group, substantial OHC loss was seen which was mainly distributed in the area on the basilar membrane corresponding to frequencies  $> 0.6$  kHz (Figure 4A). The amount of hair cell loss was as much as 76–98% in areas corresponding to frequencies between 2 and 8 kHz (Figure 4A and B). Significant IHC loss was also seen in the control group (Figure 4C and D); however, IHC damage was much less severe than OHC damage (Figures 4 and 5). Application of either ALCAR or NAC significantly reduced OHC loss, the effect of NAC being more pronounced: only 23–33% OHC loss in the NAC group (Figure 5B), compared to 46–61% in the ALCAR group in the

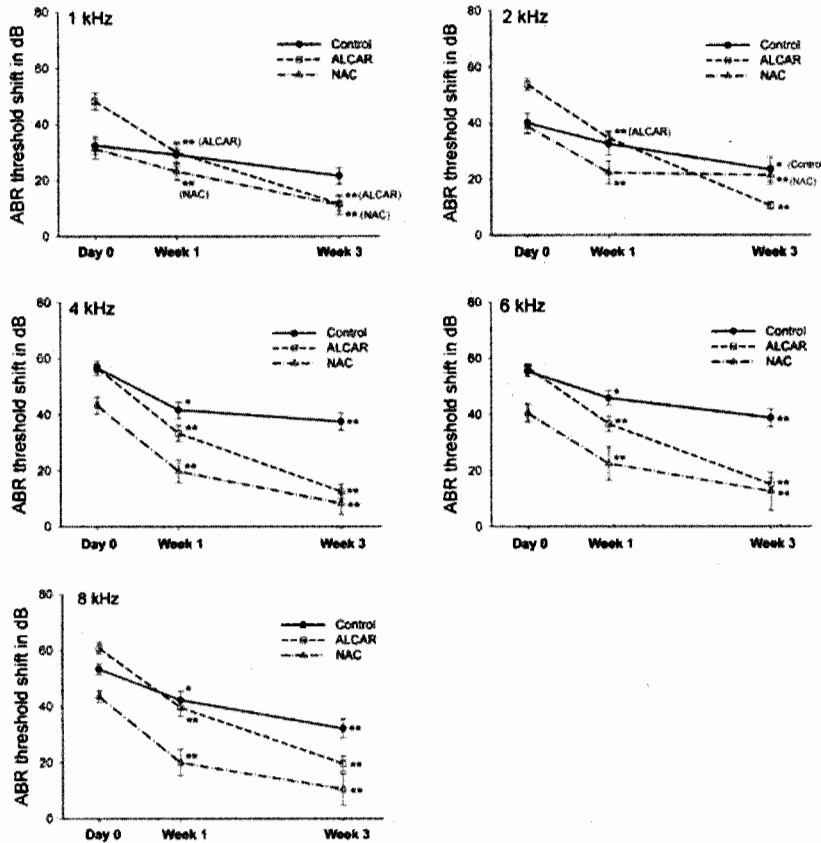


Figure 3. Hearing recovery process of the three groups after impulse NE. Hearing threshold shifts (mean  $\pm$  SE;  $n = 12$  ears) are presented as a function of time after exposure. The vertical bars represent the SE of the means. The asterisks represent the statistical significance of the hearing threshold shifts for each group at Weeks 1 and 3 compared with that at Day 0 (LSD post-hoc test; \* $p < 0.05$ , \*\* $p < 0.01$ ). ABR threshold shifts for the ALCAR group were significantly greater than those for the NAC group at Week 1 for the 2-, 4-, 6- and 8-kHz frequencies ( $p < 0.01$ ).

areas corresponding to 2–8 kHz (Figure 4B). Statistical analysis showed that the differences in the amount of OHC loss between the NAC and control groups were significant across the areas corresponding to 2, 4, 6 and 8 kHz, as shown in Figure 5B ( $p < 0.01$ ). The differences between the ALCAR and control groups were also statistically significant ( $p < 0.01$  at 2 and 8 kHz and  $p < 0.05$  at 4 and 6 kHz). A comparison between the ALCAR and NAC groups also revealed a significant difference in the amount of OHC loss ( $F = 2.24$ ,  $n = 12$ ,  $p < 0.05$  at 2, 4 and 6 kHz). This provides evidence that NAC exerts a better protective effect on OHCs than ALCAR under the condition of impulse NE at the dosages used. Less IHC loss was observed in both the ALCAR and NAC groups than that in the control group (Figures 4C, D and 5C, D); however, only the ALCAR group showed a statistically significant difference in IHC loss compared with the control group (Figure 4C and D). Therefore, in contrast to the OHC results, greater IHC loss was observed in the NAC group than in the ALCAR group ( $F = 1.70$ ,  $p = 0.21$ ,  $n = 12$ ,  $p < 0.05$  at 2 and

6 kHz). The percentage of IHC loss was higher, and the range of IHC loss wider, in the NAC group (Figure 5C and D).

## Discussion

The increase of ROS in the cochlea following loud sound exposure and the resultant hair cell damage are assumed to be treatable with AO therapy [3,4]. In a series of studies, we have shown that administration of the AO agents NAC and ALCAR reduces hearing loss as well as hair cell loss induced by continuous intense NE [1]. Consistent with the observations of other authors [15–18], these results open promising possibilities for the clinical application of AOs to protect the ear from acoustic trauma. In this study, we extended the application of the AO agents NAC and ALCAR to impulse NIHL and demonstrated a protective effect of these drugs on hearing and sensory cells in the cochlea in a chinchilla model of acute acoustic injury to the inner ear. The ABR threshold data showed that ALCAR and NAC were able to reduce the impulse

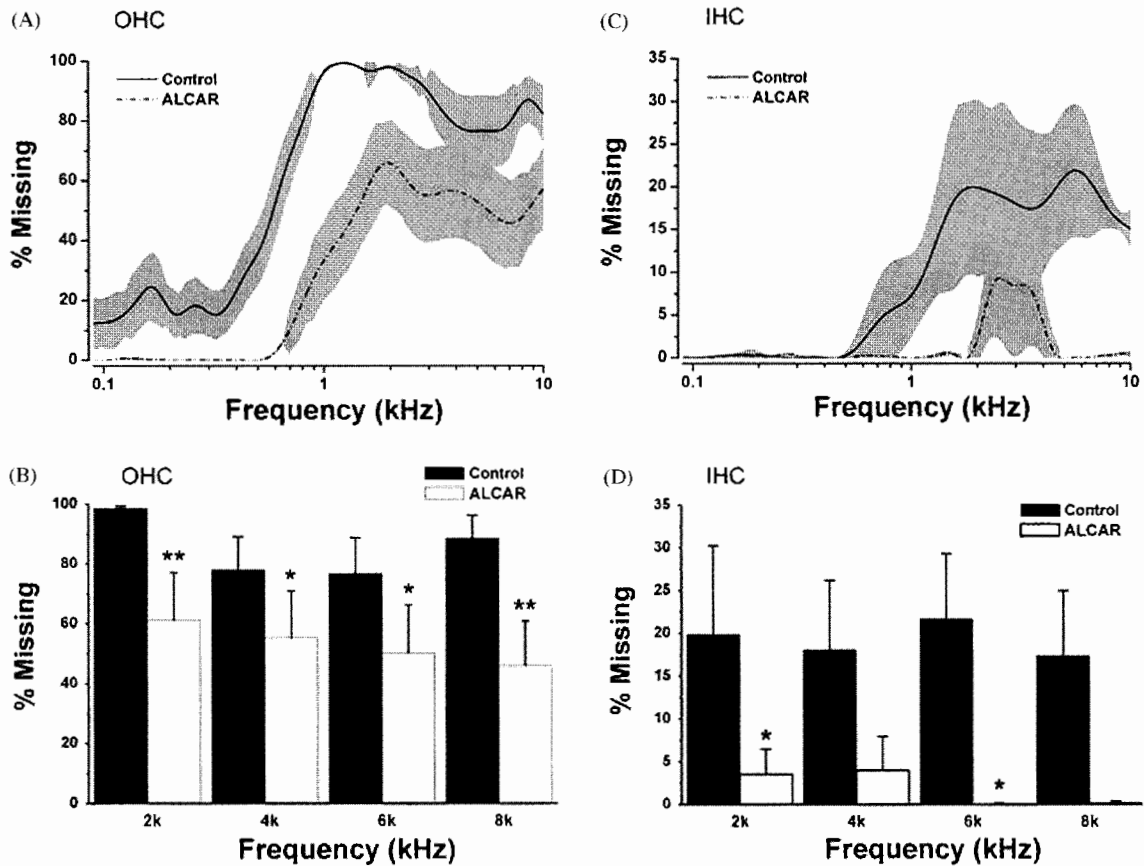


Figure 4. Hair cell counts for the ALCAR group ( $n=12$  ears) and the control group ( $n=12$  ears) after impulse NE. (A, C) Cytochleograms portraying mean missing hair cell percentage as a function of frequency. The gray areas represent the SE of the means. (B, D) The mean percentages of hair cells missing in cochlear regions corresponding to frequencies ranging from 2 to 8 kHz. The data are derived from panels (A) and (C). \* $p < 0.05$ ; \*\* $p < 0.01$  for LSD post-hoc comparisons made between control and ALCAR-treated animals at each frequency.

noise-induced PTS by up to 25–30 dB (Figures 1–3 at Week 3). The smaller amount of cochlear hair cell loss observed in the ALCAR and NAC groups also suggests the efficacy of these drugs in protecting the hair cells from acoustic injury (Figures 4 and 5). These results are consistent with those of our previous studies on continuous NE, and further support the hypothesis that AOs are able to protect cochlear sensory cells and hence hearing under conditions of intense NE, even with the acute acoustic injury caused by impulse noise. The results also suggest that the hair cell damage mechanisms caused by ROS following impulse NE are likely similar to those involved in continuous NE.

Although the effect on ABR threshold shifts of ALCAR and NAC at Week 3 following impulse NE revealed the similar potency of these two drugs, the patterns of hair cell loss between the ALCAR and NAC groups were different (Figures 4 and 5). The differences in hair cell loss may result from different protective mechanisms of ALCAR and NAC or may be related to dosage differences. Specifically, loud sound-induced oxidative damage interferes with the

electron transport system and further increases superoxide production. The resulting damage to the mitochondria compromises their ability to meet cellular demand [6]. Observations show that NE results in damage of the crista and internal and external membranes of the mitochondria, leading to loss of integrity of the mitochondrial outer membrane (unpublished data). The mitochondrial damage occurred as early as 2 h after sound overstimulation and continuously progressed with time (for up to 7 months afterwards) [7]. ALCAR helps protect mitochondria from oxidative stress by maintaining normal electron transport and reducing ROS production by way of its effects on the mitochondrial membrane. This is believed to occur via maintenance of a key phospholipid, cardiolipin, an important co-factor of a number of critical transport proteins [10]. In contrast, NAC protects cells by means of various molecular mechanisms: (i) acting as an ROS scavenger; (ii) replenishing intracellular GSH; (iii) inhibiting activation of caspase-3 and c-Jun N-terminal kinase to reduce apoptosis; (iv) reducing lipid peroxidation; and (v) improving

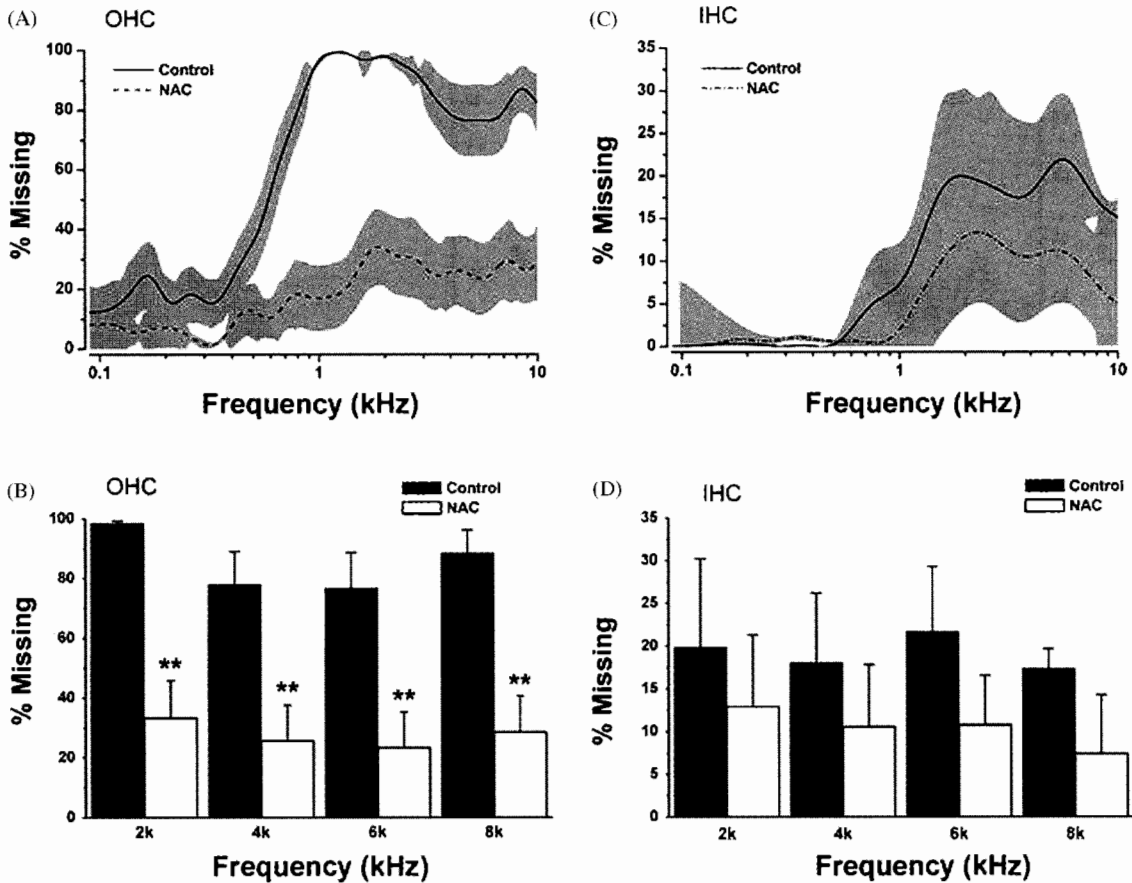


Figure 5. Hair cell counts for the NAC group ( $n = 12$  ears) and the control group ( $n = 12$  ears) after impulse NE. For details, see Figure 4.

microcirculation [11–14]. The elevation of GSH in the cochlea plays a significant role in protecting it from oxidative stress resulting from loud noise [3,18]. Data from the current experiment indicate that, despite their different cellular mechanisms of action, both ALCAR and NAC are capable of protecting the cochlea from impulse noise-induced damage by reducing hair cell injury and hence reducing the PTS, although the degree and pattern of hair cell loss and the pattern of threshold shift after NE in animals treated with these drugs are different. These differences could be evidence of the different protective mechanisms of ALCAR and NAC or may be due to the different dosages used in this study and this will require further study. Because these agents protect cells via different mechanisms, their use in combination may have additive or synergistic effects.

Despite the pattern of IHC and OHC loss in ALCAR- and NAC-treated animals, i.e. the ALCAR-treated animals showed a greater degree of OHC loss than the NAC-treated animals, whereas less IHC loss was observed in the ALCAR group, the hearing threshold shifts in the ALCAR and NAC groups were similar at Week 3. A report [9] showed that, after impulse NE, the apoptotic OHCs

maintained a normal level of succinate dehydrogenase (SDH) and so did some of the necrotic OHCs. This may partly account for the discrepancies between the hair cell count and ABR results in the ALCAR and NAC groups. It is possible that in the NAC group of the current experiment, a proportion of the SDH-stained hair cells were in the process of apoptosis or necrosis and were non-functional. This may also have occurred in the animals in the ALCAR group. Other authors [19] have noted a discrepancy between functional impairment of auditory sensitivity and cellular damage. A certain degree of NIHL can be observed without hair cell loss at related cochlear locations [20]. This may be due to non-lethal hair cell injury leading to hair cell functional impairment, such as damaged stereocilia, F-actin derangement or reduced energy metabolism [16,21]. We did not evaluate these possibilities using the histological methods employed in the current study. Furthermore,  $\approx 20$ –40% of noise-induced OHC loss or up to 10% of IHC loss can be seen without corresponding hearing loss [18,20]. Thus relatively intact auditory threshold function can occur without survival of the entire hair cell population as long as the remaining hair cells are functioning normally.

Another possibility could be the dependence of hearing on the combined function of both the IHCs and OHCs. We hypothesize that, after NE, the residual IHCs and OHCs form a new balance of interaction. Hearing is thus determined by the concerted action of the IHCs and OHCs. For example, both IHCs and OHCs appear to contribute to the summing potential of the cochlea [22]. Thus the ALCAR group had greater survival and function of IHCs whereas the NAC group had greater survival of OHCs, each compensating to some degree to yield similar ABR thresholds. This could be another interpretation of the observed discrepancies between the hair cell count and ABR results in the ALCAR and NAC groups in this study. It was noted that, immediately after impulse NE, the threshold shift in the ALCAR group was significantly greater than that in the control group at most of the frequencies measured. This phenomenon was also observed in continuous NE in animals treated with ALCAR or carbamathione (a glutamate antagonist), but without statistical significance [1]. Hagen et al. [10] demonstrated that older rats, when supplemented with ALCAR, displayed increased cellular oxygen consumption than untreated older rats. The authors stated that ALCAR supplementation may have increased mitochondrial cellular respiration. They found that cellular GSH and ascorbate levels were 30% and 50% lower, respectively in the cells of ALCAR-treated rats, thereby increasing oxidative stress. The ALCAR treatment in our study may have added to the noise-induced oxidative stress in the initial stages post-NE. ALCAR may have increased mitochondrial activity, stimulating cellular respiration and resulting in an increased rate of ROS production. This effect, along with the lowering of cellular GSH and ascorbate levels, may initially cause greater oxidative stress to hair cells immediately after an acute acoustic injury. Although ALCAR may reverse many aspects of mitochondrial dysfunction, it may affect an increase in the oxidative-induced injury initially and for some short period of time after acute acoustic trauma. This may explain in part the difference in threshold shifts between the NAC- and ALCAR-treated groups measured immediately after impulse NE.

ALCAR and NAC are clinically used medicines with long-standing safety records over decades. ALCAR has been used as both an oral dietary supplement and as a drug for the treatment of neurodegenerative diseases and diabetes. It has been used in adults at doses of 1.5–3 g/day for as long as a year without harmful side-effects, the side-effects which were reported were both mild and uncommon, including nausea and skin irritation [23]. NAC is a cysteine prodrug and otoprotectant.

It has also been used to treat patients with AIDS at high oral doses of up to 8 g/day over a period of months without significant side-effects; again, while uncommon and mild, these included gastrointestinal distress [24]. NAC is a Food and Drug Administration (FDA)-approved drug for acetaminophen overdose, countering the (sometimes fatal) liver damage induced by oxidative stress and GSH depletion [25].

Both agents ameliorated the effects of impulse NE on PTS when compared to the control group. The recovery curves seen with NAC and ALCAR appeared to be different, suggesting differing underlying mechanisms of recovery. The efficacy of ALCAR and NAC in protecting the hair cells from loud sound and the pharmacological safety of these drugs suggest a promising prospect for clinical use in ear protection from both continuous and impulse noise-induced damage to the cochlea. Despite progress in engineering approaches for reducing noise in the workplace and/or improving individual hearing protection devices (HPDs) as well as noise conservation programs, noise-induced cochlear injury remains a common and costly disability. While HPDs are always being improved, they have certain inherent limitations, which will be difficult, if not totally impractical, to overcome. Pharmacological intervention with agents such as NAC and ALCAR may be used as an adjunct to current HPDs to enhance, but not replace, them. These current pharmacologic and hearing protector technologies are not mutually exclusive but complementary. The pharmacological approach may obviate some of the intrinsic limitations of HPDs or protect individuals who are constitutionally more susceptible to noise injury, forming an important complement to HPDs in the hearing conservation program. In environments where the noise hazard exceeds the protective capability of the HPD or where relatively short periods of non-compliance with HPDs are unavoidable, a protective agent could be taken to supplement protection or if compliance with HPDs has failed.

Of course, the questions of the safety and efficacy of taking these agents need to be addressed in scientifically and ethically performed clinical trials where they are used as an adjunct to HPDs. NAC, a thiol-containing amino acid derivative, is used in the US as a nutritional supplement with proven potent AO properties. It is a main ingredient in many over-the-counter nutraceutical preparations, but is also a drug which has passed the stringent safety requirements for FDA approval.

The safety profiles of NAC and ALCAR are well understood, i.e. they have favorable side-effects profiles that are devoid of serious adverse events. The most common side-effects associated with the

oral regimens of these agents are gastrointestinal and dermatological in nature and some of these side-effects can be avoided by administering these compounds with meals or by adjusting the doses given.

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