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Research paper

N-Acetyl L-cysteine does not protect against premature age-related hearing loss in C57BL/6J mice: A pilot study ☆

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Abstract

A compound capable of preventing age-related hearing loss would be very useful in an aging population. *N*-acetyl-L-cysteine (L-NAC) has been shown to be protective against noise exposure, a condition that leads to increased oxidative stress. Not withstanding environmental factors, there is evidence that age-related hearing loss (AHL) in the mouse is linked to more than one genetic loci and, by extension, in humans. Our hypothesis is that AHL defect results in increased sensitivity to oxidative stress and L-NAC would be able to protect the hearing of a mouse model of pre-mature AHL, the C57BL/6J (B6) mouse strain. L-NAC was added to the regular water bottle of B6 mice (experimental group) and available *ad lib*. The other group received normal tap water. Hearing was tested monthly by the ability to generate the auditory brainstem response (ABR). After the final ABR test, mice were sacrificed by an overdose of Avertin, ears were harvested and hair cell loss was quantified. There was no difference in ABR thresholds or in histopathology between the control group and the group receiving L-NAC in their drinking water. In contrast to the protective effects of L-NAC against noise-induced hearing loss, the lack of protective effect in this study may be due to (i) the dosage level; (ii) the duration of treatment; (iii) the biochemical mechanisms underlying age-induced hearing loss; or (iv) how the mouse metabolizes L-NAC.

Keywords: Presbycusis; Age-related; Hearing loss; Mouse; Reactive oxygen species; Cochleogram; Auditory brainstem response

1. Introduction

The inbred mouse strain C57BL/6J (B6) begins developing age-related hearing loss (AHL) by six months of age and has provided a useful model for studying AHL (Erway et al., 1993). This phenotype has been traced to a mutation of the gene coding for cadherin 23, *Cdh23* (Noben-Trauth et al., 2003). *Cdh23* was previously known as *Ahl* (Erway et al., 1993). The locus maps to mouse chromosome 10 (Noben-Trauth et al., 2003). Both inner and outer hair cell loss is observed in B6 beginning between three and six months of age compared to the CBA/CaJ mouse strain, which shows no hair cell loss until 18 months of age (Spongr et al., 1997). Prosen et al. (2003) have shown behavioral threshold shifts as early as three months of age in B6. Auditory brainstem responses (ABRs) of mice

☆ *Disclaimer:* The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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homozygous for *Cdh23* have measurable age-related, high-frequency hearing impairment. In our laboratory, we have detected elevated thresholds in B6 at 32 kHz as early as four-weeks of age (Nolte, 2005).

Staecker et al. (2001) demonstrated that the antioxidant systems of C57BL/6J mice have a number of differences when compared with normal hearing strains (i.e., CBA/CaJ and a congenic B6 strain with the *Ahl* allele replaced with the wild-type *Castaneous* allele). Using immunohistochemical techniques they showed that qualitative levels of super-oxide dismutase, glutamyl transferase and 4-hydroxynonenal vary between 3-month-old and 9-month-old B6 mice; and differ from the levels detected in normal hearing CBA/CaJ mice. Using semi-quantitative PCR analyses, levels of messenger RNA for copper/zinc and magnesium super-oxide dismutase and catalase in B6 mice were statistically greater than levels expressed in 3-month-old CBA/CaJ mice. On the other hand, the level of glutathione peroxidase did not differ statistically from the CBA/CaJ baseline in B6. One could argue that the oxidative stress system of a B6 mouse ear is not impaired by the *Cdh23* mutation and in some cases may be enhanced.

One possible prophylactic agent could be *N*-acetyl L-cysteine (L-NAC). L-NAC is extremely safe and has been used for many years to protect the liver from the toxic effects of acetaminophen overdose (Marzullo, 2005; Smilkstein et al., 1989). This liver damage is known to be caused by over production of a highly reactive free radical, *N*-acetyl-*p*-benzoquinoneimine (Marzullo, 2005).

L-NAC (at 325 mg/kg) has been shown, in chinchillas, to protect the cochlea from the damaging effects of noise when combined with salicylate (at 50 mg/kg) and injected prior to noise exposure (Kopke et al., 2001). Also, though not yet published in the peer-reviewed literature, Kopke's study of the protective effects of oral L-NAC in a cohort of US Marines prior to firearms training is of great interest (Kopke, 2005). They report a "small but significant" protective effect on hearing in marines pre-treated with L-NAC prior to small arms fire.

There have been three pathways proposed for the action of L-NAC. First, L-NAC is a precursor for glutathione, the body's natural reactive oxygen species (ROS) scavenger. L-NAC crosses the blood-cochlea barrier while glutathione does not. Presumably, infusion of L-NAC increases the cochlea's store of glutathione. Second, L-NAC has been shown to have basic protective properties independent of glutathione: both L- and D-isomers of NAC were able to protect cells *in vitro* from ROS. Since only L-NAC is enzymatically converted to glutathione, this strongly suggests that the protective effects of NAC can be independent of glutathione, probably through cell cycle regulation (Ferrari et al., 1995). Third, in cell culture, L-NAC has been shown to block apoptosis probably through inducing specific gene expression (Yan et al., 1995).

Our hypothesis is that the inner ear in B6 mice is weakened and thus more sensitive to normal ROS and free radicals due to *Cdh23* defects. The administration of L-NAC should provide protection against this sensitivity by boosting the free-radical scavenging mechanism of the cochlea.

2. Materials and methods

All animal procedures were approved by the University of Cincinnati Institute Animal Care and Use Committee. Twelve, 4-week-old female mice of the C57BL/6J strain were purchased from The Jackson Lab (TJL), Bar Harbor, ME. The mice were divided into two groups. One group received 500 mg of L-NAC (Sigma–Aldrich, Inc. #A7250, CAS 616-91-1) dissolved into 500 ml of tap water. The control group received 500 ml of plain tap water. L-NAC is stable at room temperature (RT) for at least 24 h (Kenneth Cheever, NIOSH, personnel communication) and there is no concern that L-NAC will become inactive in water bottles stored at RT. L-NAC treatment was initiated at four weeks of age in the experimental group.

Water was changed daily. First, the previous day's bottles were weighed on a triple beam balance to 0.1 gm accuracy and weights recorded. L-NAC was then dissolved into water in a 500 ml beaker. Clean polyethylene bottles with clean stoppers and sipper tubes were filled with either L-NAC solution or tap water. Before placement in the cages these bottles were weighed to obtain a base weight. The 24 h difference provided an indication of consumption. Only three days data were lost due to leakage during the seven month experiment. Because the mice were housed three per cage there may have been individual differences in oral ingestion of L-NAC. To reduce stress to the mice, body weights were not taken. C57BL/6J strain growth and body weight information is available on the Jackson Lab website (www.jax.org).

2.1. Auditory brainstem response

Beginning at eight weeks of age, mice were tested monthly for the ability to generate the auditory brainstem response (ABR). Mice were anesthetized with an i.p. injection of Avertin (tribromoethanol, 0.4 mg/gm). Body temperature was not monitored.

Auditory brainstem responses (ABR) were generated to 4, 8, 16 and 32 kHz tone pips (tested in ascending order). Tone pips consisted of a three millisecond envelope: 1 ms ramp onset, 1 ms plateau and 1 ms decay. Tone pips were generated by Tucker-Davis Technologies (TDT) System 2 hardware (Alachua, FL, USA) running BioSig® Software on a Pentium class computer. The tone pip was presented binaurally through specula attached to supertweeters. The ABR was recorded through Grass® stainless steel needle electrodes placed subcutaneously at the vertex (active), right cheek (inverting) and left cheek (common). The resulting signal was band-pass filtered (100–3000 Hz), amplified (10,000×) and digitized by a TDT Bioamp. Responses were collected and averaged at 30 presentations per second for up to 512 times. The stimulus was presented at 100 dB SPL and progressed downward in 5 dB steps

until no response was identifiable. Presentations were halted early if the characteristic ABR was noted. A second trace was collected and compared with the first if there was some question whether a response was recorded.

Tone pips were calibrated by extending the tone burst plateau to one minute and measuring the output of the speculum via an 1/8'' Brüel & Kjær (B&K) microphone and a Brüel & Kjær 2608 Measuring Amplifier. A short piece of polyethylene tubing was connected between the speculum of the supertweeter and the 1/8'' microphone, similar to the technique described by Pearce et al. (2001). The microphone was calibrated by a B&K microphone calibrator.

2.2. Cytocochleograms

At month seven, immediately after the final ABR was completed, each mouse was given an overdose of Avertin. The mouse was decapitated, the cochleae dissected out, the oval and round windows opened and RT 4% paraformaldehyde in phosphate buffer was infused through the round window. Cochlear surface preparations were made for the quantification of hair cell loss by removing the surrounding bone and permeabilizing the tissue with 0.3% Triton X-100 in PBS for 20 min and then incubating the cochlea in phalloidin (TRITC, Molecular Probes 1:80 concentration) for 60 min. Phalloidin is a mushroom toxin which binds to actin filaments. After the cochleae were stained with phalloidin they were dissected into half-turns and each piece was further dissected in a drop of Citifluor AF1 (Agar Scientific) and as much as possible of the modiolus was removed. Hair cells throughout the cochlea were examined using a Zeiss Axiovert light microscope and a 40× oil objective. The criterion for identification of hair cell loss was made by a scar formation. The percent loss of hair cells for each 0.25 mm segment was calculated depending on the total length. The percent loss of the inner and outer hair cells (IHCs and OHCs) was determined throughout the cochlea and was plotted on a cochleogram.

2.3. Statistical methods

A mixed models statistical analysis was used to analyze the data. For ABR threshold data, the model included the independent variables of group (L-NAC vs control), frequency (4, 8, 16 and 32 kHz), and month (1–7), and all of their two-way and three-way interactions. For the cochleograms the dependent variables included per cent missing inner hair cells (ihc), outer hair cells row 1 (ohc1), row 2 (ohc2), row 3 (ohc3) and outer hair cell mean (ohcm). The effects for the cochleogram data were group (L-NAC vs control), basilar membrane distance (mm), and the interaction of group × distance.

All calculations were done with SAS® (Version 9.1, SAS Institute, Inc., Cary, North Carolina), particularly the MIXED procedure.

3. Results

The control mice consumed a daily average of 4.07 ml of tap water (standard deviation ± 1.02), while the L-NAC mice consumed a daily average of 4.42 ml (standard deviation ± 1.58). The difference was not statistically significant. Assuming each mouse weighs about 20 g, the equivalent L-NAC dosage for the experimental group was 221.25 mg/kg/day (standard deviation ± 79.33 mg/kg/day). No attempt was made to factor in weight changes with growth over the 7 month period.

Fig. 1 displays the mouse ABR threshold changes over the six months for the four test frequencies. Filled circles represent those mice receiving regular tap water (controls) while the open circles represent those mice consuming L-NAC in their water supply. Overall, graphically there is no difference between the control mice and the L-NAC mice at any frequency. The greatest change over 6 months occured at the highest frequency, 32 kHz. At 16 kHz the first change appears to have occured at 5 months and at 8 kHz the first change appears at 6 months. No change was noted at 4 kHz.

Table 1 shows the results of the mixed model statistical analysis for the ABR data. The analysis did detect a small but statistically significant main effect for ABR threshold between the two groups. This 4 dB overall difference between the groups, while statistically significant, is probably not functionally significant.

The analysis detected a significant main effect for ABR test frequency. This analysis responds to the shape of the mouse ABR audiogram and indicates a difference in frequency when data are held constant across groups and across ages. This is not experimentally important. The analysis detected a significant main effect for month indicating a difference in ABR thresholds as the B6 mice age. This was also expected since it is well-known that B6 mice develop age-related hearing loss. The analysis found no statistically significant interaction for group × frequency (both groups have equivalent ABR thresholds), group × month (both groups age at the same rate), and group × frequency × month (both groups thresholds age at the same rate). More important, the lack of significance of these interactions indicate no differences between the L-NAC and control groups for hearing loss.

Fig. 2 shows differences in mean outer and inner hair cells between L-NAC treated mice and control mice. There do not appear to be any major differences either in outer hair cell loss and inner hair cell loss between the two groups. There is major loss in outer hair cells beginning at about 4.5 mm extending to complete loss at the base. Inner hair cells are lost at about 5 mm and extend to almost complete loss in the base.

Table 2 provides statistical support of the graphical results. Table 2 shows only the results of the mean of the three rows of outer hair cells but statistical analysis of inner and individual rows of outer hair cells were the same. The main effect of treatment group was not significant.

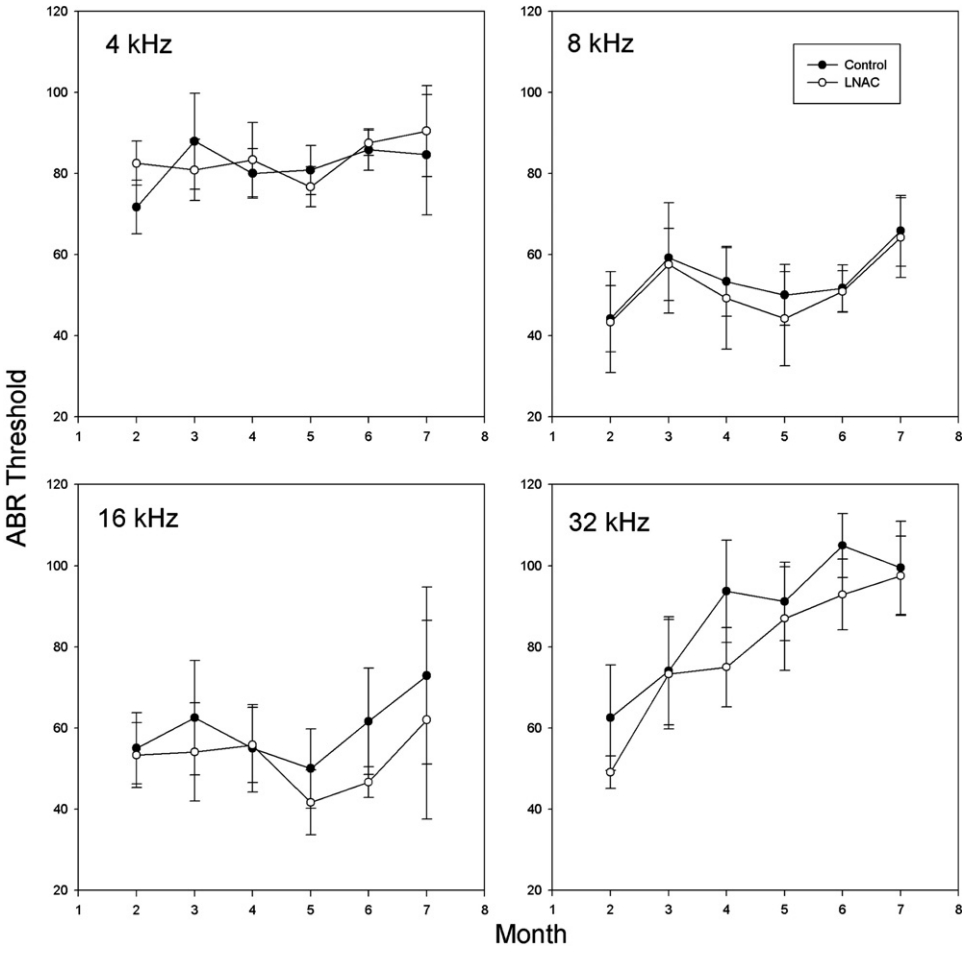

Fig. 1. Mean Auditory Brainstem Response (ABR) thresholds in decibels (dB) for 4, 8, 16 and 32 kHz for each month of the study. Filled circles are control group data, open circles are animals receiving L-NAC in their water. Error bars indicate ±1 standard deviation.

Table 1
Results of the mixed model statistical analysis for ABR thresholds

Source	df	*F*	*p*
Group	1, 10	5.57	<0.04*
Frequency	3, 30	127.13	<0.0001*
Month	5, 50	12.89	<0.0001*
Group × frequency	3, 30	2.53	0.075
Group × month	5, 50	0.25	0.939
Frequency × month	15, 150	10.28	<0.001*
Group × freq × month	15, 150	1.64	0.070

The main effects group (control versus NAC), month (1–6), and frequency (4, 8, 16 and 32 kHz).
* Indicates statistically significant result.

4. Discussion

Our data suggest that L-NAC does not protect hearing from the early aging effects in B6 mice. Our prediction that a ROS scavenger L-NAC would protect the hearing of the mice was not upheld.

We believe that the failure to protect the C57BL/6J mice may be related to one of the following: dose level, mechanism of *Cdh23* age-related hearing loss not being related to ROS, liver binding of L-NAC, insufficient duration of exposure, or poor stability of L-NAC in circulation. These hypotheses provide directions for further research.

The average dose level of 221 mg/kg/day might be below the protective threshold. Drinking water was chosen as the vehicle for administering the compound because we know that all subjects were exposed to the compound in a relatively constant amount. Also, a protective compound such as L-NAC would probably be administered orally to humans.

One might argue that the form of AHL demonstrated by the B6 strain is mediated by the abnormal *Cdh23* allele, rather than by mechanisms associated with oxidative stress, and therefore is not amenable to L-NAC treatment. More likely, however, is that B6 mice have weakened hair cells due to the abnormal *Cdh23* allele. The dysfunctional hair cells in this strain could make them particularly vulnerable to environmental insults or age-related mitochondrial defects, both of which induce oxidative stress in the cochlea. For example, Davis et al. (1999) have shown that the B6 mouse strain is significantly more vulnerable to the effects of noise.

Few studies have examined the role of oxidative stress in the development of age-related hearing loss or noise sus-

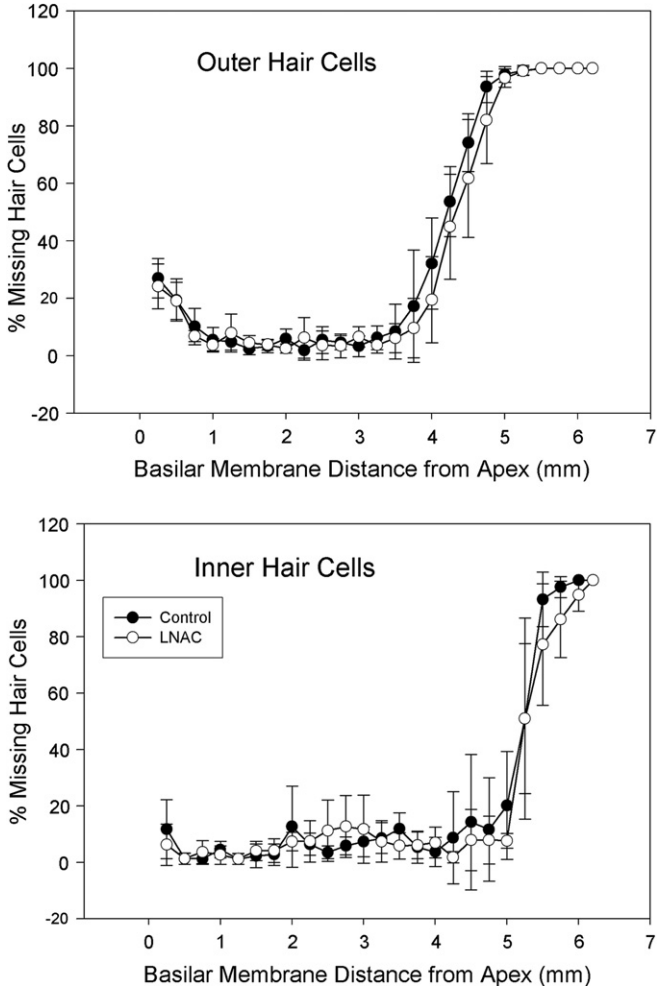

Fig. 2. Mean cochleograms for outer hair cells (top) and inner hair cells (bottom). Data points indicate percent hair cells missing at each 0.25 mm of basilar membrane. Apex is on the left at origin. Filled circles indicate control mouse group, open circles indicate group receiving L-NAC in water. Error bars indicate ±1 standard deviation.

Table 2
Results of the mixed model statistical analysis for mouse cochleograms

Source	df	*F*	*p*
Group	1, 10	1.31	0.27
Distance	22, 211	351.76	<0.0001*
Group × distance	22, 211	1.46	0.0917

This displays the results of the averages of the three rows of outer hair cells. The main effects are group (control versus NAC) and cochlear distance (0–5.5 mm).

* Indicates statistically significant result.

ceptibility in the B6 strain, or the particular influence of the *Ahl* locus in the generation of ROS. Staecker et al. (2001) compared age-matched B6 with normal hearing CBA/CaJ mice and noted increased labeling for super-oxide dismutase, glutamyl transferase and 4-hydroxynonenal in the lateral wall and spiral ganglia of B6 mice, which varied with age between 3 and 9 months of age. Expression levels of antioxidant enzyme mRNA were also elevated, with peak expression varying with age. Levels of messenger RNA for copper/zinc and magnesium super-oxide dismutase increased gradually up to 9 months of age, and were greater than levels expressed in 3- or 9-month-old CBA/CaJ mice. Catalase expression peaked at 6 months of age in the B6 mice, but was much higher at 9 months of age in the CBA/CaJ group. On the other hand, the level of glutathione peroxidase did not differ statistically from the CBA/CaJ baseline. The time course of these changes parallels the hearing threshold shifts and cochlear degeneration which begin at 3–6 months of age. Although cochlear degeneration and hearing loss in B6 mice was originally attributed to the *Ahl* locus harboring the cadherin 23 mutation, the picture is now more complicated.

A third explanation is that the livers of these mice were absorbing the L-NAC before it could make its way to the ear. The present study did not measure levels of L-NAC in the blood. Liver trapping would argue against using L-NAC in human studies since injection of the compound before exposure to noise would not be very feasible. A recent article by Kramer et al. (2006) attempted to reduce temporary threshold shift in humans by administering a single 900 mg oral dose of NAC prior to their attending a 2 h nightclub session. Auditory threshold shifts and distortion product otoacoustic emission shifts of subjects receiving NAC did not differ significantly from subjects receiving a placebo prior to noise exposure.

It is possible that L-NAC is only protective against short, intense noise exposures. It may not be protective against a longer, lower level free-radical insult such as aging.

A compound which displays prophylactic protection against presbycusis in humans would be welcomed by the aging population. A compound which protects against both aging and noise damage would be doubly welcome. Presently there are no compounds identified which meet these needs.

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