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ORIGINAL ARTICLE

Noise protection with N-acetyl-l-cysteine (NAC) using a variety of noise exposures, NAC doses, and routes of administration

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Abstract

Conclusion. These studies extend previous work on N-acetyl-l-cysteine (NAC) and noise, showing protection with NAC against a high-kurtosis noise, showing protection with NAC at low doses, as well as protection by oral gavage. The studies further reveal the potential for the use of NAC in a clinical population exposed to noise. *Objective.* To extend previous work on NAC protection from noise, the current study examined the effectiveness of NAC against a high-kurtosis noise that combined continuous and impact noise, tested the effectiveness of NAC at varying doses, and tested NAC when administered by gavage. *Materials and methods.* Chinchillas were tested for auditory brainstem responses (ABRs) at five frequencies before and at three time points after one of three noise exposures: high-kurtosis (2 h, 108 dB L_{eq}), impulse (75 pairs of 155 dB pSPL impulses), or continuous (4 kHz octave band, 105 dB SPL for 6 h). Animals were treated with NAC or saline vehicle before and after noise. *Results.* The NAC was protective against the high-kurtosis noise both at low doses and when given orally by gavage.

Keywords: *Glutathione, antioxidants, reactive oxygen species, kurtosis, noise-induced hearing loss*

Introduction

Over the last few years, understanding of noise-induced hearing loss (NIHL) has expanded with the increase in knowledge of the molecular mechanisms underlying noise-induced hair cell death and with the demonstration of pharmacological strategies to protect the ear against NIHL. Key among the underlying molecular mechanisms for NIHL is the involvement of oxidative stress in noise-induced outer hair cell (OHC) death. Noise has been linked to increases in a number of different reactive oxygen species (ROS) [1,2] and products of lipid peroxidation [3,4]. The discovery of the involvement of oxidative stress in noise-induced OHC death led to the demonstration of protection from noise with antioxidant supplementation [5–11].

The glutathione pathway is one of the key antioxidant pathways in the body and in the cochlea. In its reduced form (GSH), glutathione can react with the hazardous hydroxyl radical (OH) to form water. Additionally, in a reaction catalyzed by glutathione peroxidase, two molecules of GSH can convert hydrogen peroxide (H_2O_2) into two molecules of water. Increases in the hydroxyl radical have been detected in the cochlea after noise [2] and hydrogen peroxide may represent an intermediate molecule between superoxide (O_2) and the hydroxyl radical. N-acetyl-l-cysteine (NAC) acts as a substrate for glutathione synthesis, as well as having its own antioxidant properties. It has been shown to be effective in protecting the ear from noise trauma. NAC was initially demonstrated as a protective agent against damage from a 6 h continuous octave-band noise when the NAC was combined with salicylate [7]. Noise-induced temporary and permanent threshold shifts, as well as OHC loss, were lower in

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animals that received injections of NAC in the days before and after the noise exposure. It has since been shown to protect against short-term high level impulse noise (155–160 dB pSPL) [10,11]. Additionally, evidence suggests that NAC may be effective in protecting against cisplatin ototoxicity [12,13] and meningitis-related cochlear damage [14].

Approximately 10 million Americans have some form of NIHL [15]. Occupational NIHL alone represents the causes of 7–21% of adult hearing loss worldwide [16]. Military personnel, construction and industrial workers [17], firefighters [18], and farmers [19] are all routinely exposed to potentially hazardous levels of noise during the working day. While hearing protection devices represent the best option for individual hearing protection in the workplace, poor compliance and potential dangers associated with attenuated sound input limit the protective benefits that can be realized with personal hearing protection devices. Therefore, the search for an effective, clinically useful pharmacological intervention to protect against noise is ongoing. NAC has the potential to represent one such intervention tool.

This report describes three experiments with NAC that were performed to further validate NAC's clinical potential and to further elucidate the practical aspects of potential clinical application of the drug. The first experiment tested the protective value of NAC against a high-kurtosis noise, a high-crest factor noise that combines impact noise with a continuous noise to create a noise that is more damaging than either component separately and is a good approximation of many workplace noise exposures. The resulting noise was a simulation of the type of noise found in many factories and processing plants. The second experiment tested the protective value of NAC at doses lower than the typical 325 mg/kg that has been utilized in previous studies [7,10]. The third experiment utilized NAC delivered orally by gavage, as oral dosing may represent the most practical administration of the drug in humans.

Materials and methods

Forty-six adult chinchillas weighing between 400 and 700 g were used in the studies. Before noise exposure and between test times, the animals were housed in a quiet colony. All procedures involving use and care of the animals used in these studies were reviewed and approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee and the Animal Care and Use Committee of the Naval Medical Center San Diego, in accordance with the Declaration of Helsinki.

Experimental design

The animals were divided into three experiments. Experiment 1 tested the effectiveness of NAC in protecting against a high-kurtosis noise exposure. Six animals received NAC and four were controls. Experiment 2 tested the effectiveness of NAC against impulse noise at various doses ($n=24$, 6 controls and 18 experimental animals). Experiment 3 tested the effectiveness of NAC when administered orally by gavage ($n=12$, 6 controls and 6 experimental animals).

Auditory brainstem recording (ABR)

For experiments 1 and 2, 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. The non-inverting electrode was placed at the vertex. The inverting electrode was placed behind the pinna of the test ear. The ground electrode was placed at the shoulder. Acoustic 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-2, Etymotic Research, Inc., Elk Grove Village, IL, USA) which was positioned approximately 5 mm from the tympanic membrane. The output of the insert earphone was calibrated before each testing session. 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 two 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 ABR wave I was seen and the next lowest level, where no response was seen upon visual inspection. For experiment 3, the ABRs were collected with awake animals that had been trained to be still in their restraint tubes during the collection procedure (method detailed in Kopke et al. [8]).

Drug administration

For experiments 1 and 2 NAC was delivered via intraperitoneal injections. The NAC was first dissolved in physiological saline to the desired concentration. The solution was filtered and the pH was adjusted to physiologic pH. For experiment 1, the

NAC was delivered at 325 mg/kg, with controls receiving sterile saline injections. For experiment 2, three doses of NAC were used: 325 mg/kg, 100 mg/kg, and 50 mg/kg. Again, controls received injections of sterile saline.

The dosing schedules for experiments 1 and 2 were the same. NAC was given twice daily for the 2 days preceding the noise exposure, once 1 h before the noise, once 1 h after the noise, and twice daily for the 2 days after the noise. A total of 10 injections were given.

Experiment 3 followed the same dosing schedule as experiments 1 and 2, but the NAC (325 mg/kg dissolved in saline) was delivered by gavage. Control animals received sterile saline by gavage. Fluid was given in a single bolus volume of 4.0 ml/kg. Food and water were made available to the chinchillas ad libitum throughout the study.

Noise exposures

For experiment 1, a high-kurtosis noise was used. The noise consisted of 117–130 dB pSPL impacts superimposed on 98 dB (A) SPL of steady-state Gaussian noise, for a kurtosis value of $\beta(t) = 25$. The noise was played from a CD recording, routed through an attenuator (Hewlett Packard, HP350D), a filter (Krohn-Hite 3550 R), and a power amplifier (NAD 2200). The noise was delivered through an acoustic horn (JBL 2360) hanging directly over the cage. Noise levels were measured using a sound level meter (Larson Davis 800B) and a ½ inch condenser microphone (ACO 7013). The overall level of the 2 h noise was 108 dB L_{eq}. The cage used was a $1 \times 1 \times 1$ foot wire cage. The animals were exposed individually, with food and water available during the duration of the exposure.

For experiment 2, the noise was a series of 75 pairs of 155 dB pSPL impulses of simulated M-16 gunfire, for a total of 150 impulses delivered over a period of 75 s. 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 animals were exposed to the noise in a restraint tube placed 3 inches from the tube through which the sound was delivered. The restraint tube was placed facing the sound source, so both ears were exposed at the same time. The calibration procedure for the impulse noise can be found in a previous report [10].

Experiment 3 was a 105 dB SPL continuous octave band of noise centered at 4 kHz for 6 h duration. The noise was generated by a standard audiometer (GSI 16), selected to white noise, routed through an attenuator (HP 350 D), a band-pass filter (Krohn-Hite 3550R), and a power amplifier (Crown D150A model 716) to an audiometric loudspeaker suspended directly above the animal's cage. The sound spectrum output of the system was confirmed using a Larson and Davis model 800B sound level meter.

Assessment of threshold shift

To assess noise-induced threshold shift in the animals, ABR testing was performed at three time points following noise: 15 min post noise, 1 week post noise, and 3 weeks post noise. Pre-exposure thresholds were subtracted from the three threshold measurements to calculate threshold shift at each time point. The 3-week measurement provided the data for calculation of permanent threshold shift (PTS).

Statistical analysis

For each experiment, three-factor ANOVA (group × frequency × time post noise) was used to analyze differences between the means of the experimental groups across the five different test frequencies at the three different time points post noise. Group and frequency were analyzed as between-subjects variables, and time post noise was analyzed as a repeated measure. If a significant main effect occurred for group or frequency, post hoc testing with Newman-Kuels tests was performed to delineate the nature of the differences. If a significant main effect of, or interaction involving, time occurred, the different days were compared with paired subjects *t* tests.

Results

Pre-exposure thresholds

All animals used in the studies were compared for differences between experimental groups' pre-exposure thresholds. Statistical analysis (two-way ANOVA) revealed a significant main effect of frequency, but no main effect of group or group × frequency interaction. The absence of differences between groups before drug treatment or noise exposure gave confidence that the differences observed in threshold shift between groups were the result of the different noise exposures and NAC treatments the groups received.

Experiment 1

Experiment 1 tested the effectiveness of 325 mg/kg NAC in protecting against a 108 dB L_{eq} high-kurtosis noise that was a mix of 500 Hz continuous octave band noise (OBN) and high-level impact

noise. Figure 1 displays the threshold shift data at the five frequencies tested at 15 min, 1 week, and 3 weeks after the noise for both the treated animals and the saline controls. A three-way ANOVA was performed on the threshold shifts (group × frequency × day post exposure) with day as a repeated measure. There was a significant day × group interaction ($p < 0.0001$). An independent samples t test was run, comparing the two groups (NAC vs controls) at each of the time-frequency points post noise. The NAC group was significantly lower than the controls at 1, 2, 4, and 8 kHz at 15 min, and all five frequencies at 3 weeks. At 1 week, NAC was lower than the controls at 1 kHz only.

Experiment 2

Experiment 2 tested the effectiveness of NAC across three concentrations against impulse noise. The four experimental groups were: saline controls, 325 mg/kg NAC, 100 mg/kg NAC, and 50 mg/kg NAC. Threshold shift data at each frequency and test time are displayed in Figure 2. Three-way ANOVA (group × frequency × day) revealed a significant two-way group × day interaction ($p < 0.001$). A series of one-way ANOVAs at each frequency-time point were run to compare group differences. At 1 kHz, the 325 mg/kg group was lower than the controls at 3 weeks ($p < 0.05$). At 4 kHz, the 325 mg/kg group was lower than the controls at all three time points ($p < 0.05$). Additionally, the 100 and 50 mg/kg groups were lower than the controls at 1 week and 3 weeks. At 6 kHz, the 325 mg/kg group was lower than the controls at all three time points ($p < 0.05$). Additionally, the 50 mg/kg group was lower than the controls at 1 week and 3 weeks, and the 100 mg/kg group was lower than the controls at 3 weeks ($p < 0.05$). At 8 kHz, the 325 mg/kg group was lower than the controls at 15 min and 3 weeks ($p < 0.05$). Additionally, the 100 and 50 mg/kg groups were lower than the controls at 3 weeks ($p < 0.05$).

Experiment 3

Experiment 3 tested the effectiveness of 325 mg/kg NAC, delivered orally by gavage, in protecting against a 105 dB SPL, 6 h, 4 kHz continuous OBN. PTS data at each frequency are displayed in Figure 3. Two-way ANOVA (group × frequency) revealed a significant main effect of group ($p < 0.01$), with the NAC-treated group showing lower PTS at all tested frequencies.

Discussion

Dosing with NAC has consistently resulted in reduced noise-induced threshold shifts in animal models [7,8,10,11]. The current studies were undertaken to further examine the clinical potential of NAC for human patients. The first experiment examined the protective value of NAC against a high-kurtosis noise. The high-kurtosis noise is of interest since it more accurately simulates workplace noise exposures in manufacturing and industrial facilities [20]. The noise consisted of a moderate (95 dB(A) SPL) white noise background, punctuated by frequent high-level impacts. Previous studies of the influence of the kurtosis factor of a noise on the resulting NIHL have shown that noises with equal energy (as measured by dB L_{eq}) can induce significantly different levels of PTS and OHC loss, depending on the kurtosis (β) factor of the noise. The higher kurtosis factor noises ($\beta \geq 25$) induce more PTS and OHC loss than lower kurtosis noises [21]. The use of NAC as a protective strategy against a high-kurtosis noise provided some insight into the

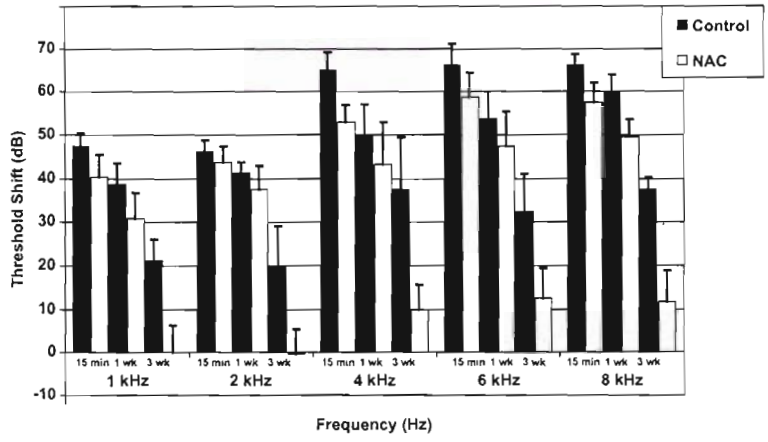

Figure 1. Recovery patterns of the control (black bars) and NAC-treated (white bars) groups for the five frequencies tested at 15 min, 1 week, and 3 weeks after exposure to a 2 h, 108 dB L_{eq} noise exposure with a kurtosis factor of $\beta(t) = 25$, plotted as threshold shift in dB against test time grouped at each test frequency. Error bars are +1 SEM.

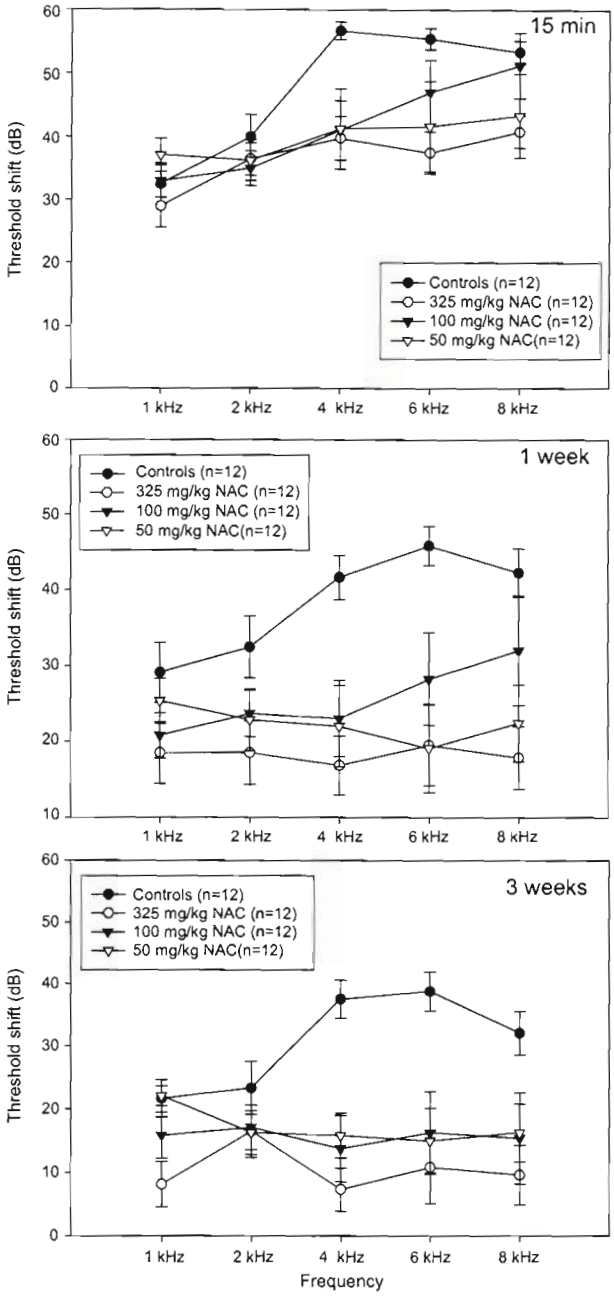

Figure 2. Threshold shifts for each of the groups receiving different doses of NAC treatment at 15 min (top panel), 1 week (middle panel), and 3 weeks (bottom panel) after exposure to 75 pairs of 155 dB pSPL impulses of simulated M-16 gunfire, plotted as threshold shift in dB against test frequency. Error bars are +1 SEM. Data in Figure 2 were presented in part as an abstract at the 2004 Association of Research in Otolaryngology Meeting and in Kopke et al. NAC for noise: from the bench top to the clinic. Hearing Research 2006 (Epub ahead of print).

potential effectiveness for NAC to be used in workplace noise protection. Consistent with previous findings, NAC was effective in reducing PTS from the high-kurtosis noise exposure at all tested frequencies. Since the noise is a combination of continuous noise and high-level impacts, the

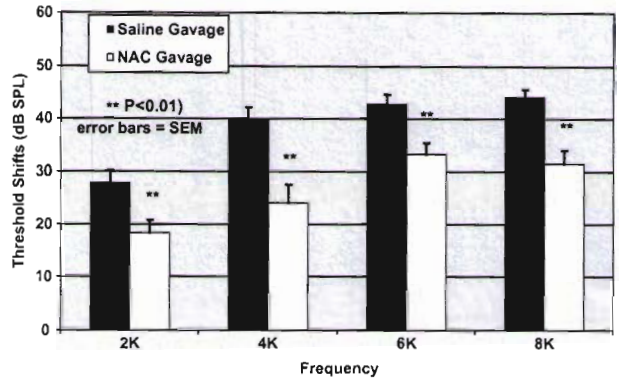

Figure 3. Permanent threshold shifts of control (black bars) and NAC-treated (by oral gavage) (white bars) chinchillas as tested 3 weeks after exposure to a 6 h, 105 dB SPL 4 kHz OBN. Data are plotted as threshold shift in dB against frequency. Error bars are +1 SEM.

cochlear damage process is thought to be a combination of metabolic damage (ROS, decreased cochlear blood flow) and mechanical trauma (broken stereocilia, loss of cell-to-cell connections). NAC is thought to be protective in the cochlea in a number of ways: increasing glutathione, reducing ROS and lipid peroxidation [22], and inhibiting cell signaling for apoptosis [23]. The effectiveness of NAC in protecting against a high-kurtosis noise suggests that NAC's mechanisms of protection are effective in simultaneously protecting against both metabolic and mechanical damage pathways.

The second experiment was undertaken to examine the effectiveness of NAC at doses lower than the 325 mg/kg dose that has been used in previous experiments with the chinchilla [7,10]. While 325 mg/kg was the most effective dose, lower doses of 100 and 50 mg/kg proved to be effective as well. This finding is encouraging for the consideration of NAC clinically. Even in fairly high doses, NAC has shown minimal side effects when given orally in human patient populations [24,25], although some gastrointestinal side effects have been noted in a minority of cases. The use of a lower dose will minimize any potential side effects and allow for greater use of the drug in combination with other pharmacological protection strategies.

To move further toward the goal of clinical application of NAC as a noise protection strategy, the third experiment was performed to test the effectiveness of NAC protection from noise when the NAC is administered orally by gavage. The standard 325 mg/kg dose was used. While the magnitude of protection is lower than when the NAC is administered by intraperitoneal injection (based on comparison of NAC protection from current gavage data with published protection from NAC by intraperitoneal injection [7]), the PTS for

the chinchillas receiving NAC by gavage were significantly lower than controls at 2–8 kHz, with mean differences 7–25 dB.

The primary use of NAC clinically has been to combat acetaminophen overdose. In those cases, NAC is administered orally. Thus, its bioavailability in oral administration has been established. In the cases of noise protection studies, unless there is frequent sampling of cochlear fluids, there is always some uncertainty about the amount of any drug that is reaching the cochlea when the drug is given systemically. Therefore, the demonstration of effective protection with NAC given by gavage provides some confidence that the drug, or its metabolite cystine, is reaching the cochlea when given orally. Further study of the pharmacokinetics of the drug in both the blood and the cochlear fluids after oral administration is warranted to examine the relative bioavailability of the drug when given orally versus intraperitoneally. Such study is also warranted with lower doses of NAC by gavage, to find the optimal concentration and route of administration for use in a clinical setting.

Taken collectively and together with previous published accounts [7,10,11] the current data provide further insight into the effectiveness of NAC in preventing NIHL in laboratory settings. Additionally, the results represent another step toward the eventual goal of developing an effective protection strategy that can be deployed in a clinical patient population.

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