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Research paper

The effectiveness of *N*-acetyl-L-cysteine (L-NAC) in the prevention of severe noise-induced hearing loss

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Abstract

Three groups of chinchillas were exposed to a nonGaussian continuous broadband noise at an Leq = 105 dB SPL, 8 h/d for 5 d. One group ($N = 6$) received only the noise. A second group ($N = 6$) received the noise and was additionally treated with L-NAC (325 mg/kg, i.p.). Treatment was administered twice daily for 2 d prior to exposure and for 2 d following the exposure. During exposure the animals received the L-NAC just prior to and immediately after each daily exposure. The third group ($N = 4$) was exposed to the noise and received saline injections on the same schedule as the L-NAC treated animals. Auditory evoked potential recordings from the inferior colliculus were used to estimate pure tone thresholds and surface preparations of the organ of Corti quantified the sensory cell population. In all three groups PTS exceeded 50 dB at 2.0 kHz and above with severe sensory cell loss in the basal half of the cochlea. There was no statistically significant difference among the three groups in all measures of noise-induced trauma. Treatment with L-NAC did not reduce the trauma produced by a high-level, long duration, broadband noise exposure.

Keywords: L-NAC; Hearing loss; Noise trauma

1. Introduction

The increased metabolic activity associated with high-level noise exposure is known to result in the excessive production of reactive oxygen species (ROS) and other free radicals in various cell populations of the cochlea including the sensory cells. A number of substances with antioxidant properties have been shown to reduce the permanent threshold shifts (PTS) in hearing and cochlear sensory cell loss following noise exposure in animal model systems. Based on the Department of Defense Technology Readiness Level (TRL) criteria (2005), *N*-acetyl-L-cysteine (L-NAC), Ebselen, D-Methionine (D-MET), Acetyl-L-Carnitine (ALCAR) and a Src Inhibitor are at an advanced TRL and are being considered for application to humans routinely exposed to high levels of noise in the military. Comprehensive and up to date reviews on the effects that these and other drugs have on noise-induced hearing loss (NIHL) can be found in the proceedings of the symposium "Pharmacological strategies for prevention and treatment of hearing loss and tinnitus" (Canlon et al., 2007).

L-NAC, an antioxidant that also affects the production of the endogenous antioxidant glutathione and may have anti-apoptotic activity has been shown, in a number of cases, to be effective in reducing the effects of a NIHL (Coleman et al., 2007; Kopke et al., 2005 and others). L-NAC has been studied in several mammalian species including humans (Kramer et al., 2006). Noise exposures used in these studies have usually been of relatively short duration (<6 h) and have included either octave band noise centered at 4 kHz, impulse noise, or combinations of impact and continuous broadband noise.

While human data are very limited, there are a number of studies in animal model systems that have used L-NAC in both rescue and prevention paradigms. In the rescue mode, where drug treatment is initiated after an acute acoustic trauma there is a single study by Coleman et al.

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(2007). In this study three groups of chinchillas ($N = 6$/group) were exposed to a 4 kHz octave band of noise (OBN) at 105 dB SPL for 6 h. Animals were treated with L-NAC (325 mg/kg, i.p.) twice daily starting at 1, 4 or 12 h postexposure. Starting treatment at 1 h postexposure was the most effective with statistically significant average reductions in auditory brainstem response (ABR) estimates of PTS relative to the control group of 6–22 dB in the 2–8 kHz test frequency region. Corresponding reductions in the percent outer hair cell (OHC) losses were 67–80%. The group treated 12 h after exposure showed no statistically significant reduction in PTS relative to the untreated control but treatment did produce large and statistically significant reductions in the percent OHC loss of 27–46%. In this L-NAC treated group PTS was similar to that measured in the untreated control group that showed more than 95% OHC loss in the 4–8 kHz region and a PTS of 30–40 dB. Treatment with L-NAC 12 h postexposure appears to have rescued OHCs but not to have affected PTS suggesting that the rescued cells were not functional.

In the prevention mode, treatment is initiated prior to noise exposure and may continue during exposure and for some time after cessation of the noise. Kopke et al. (2005) treated chinchillas ($N = 6$) with L-NAC (325 mg/kg, i.p.) twice daily for 2 d prior to exposure to 150 noise impulses, 2/s at 155 dB peak SPL. Treatment continued for 2 d postexposure. Compared to the control group the L-NAC treated animals showed 12–30 dB statistically significant less PTS between 1.0 and 8.0 kHz as estimated using the ABR and 53–80% less percent OHC loss. Bielfeld et al. (2007), using the chinchilla in an experimental paradigm similar to that of Kopke et al. (2005), varied the dose of L-NAC (325 mg/kg, 100 mg/kg, and 50 mg/kg, i.p.) and found very similar results for PTS at the 325 mg/kg (i.p.) dose. Reducing the amount of L-NAC to 50 mg/kg did not appear to affect the magnitude of the protective effect. Continuing with the same treatment paradigm, Bielfeld et al. (2007) also exposed chinchillas to a high kurtosis noise [i.e., a combination of impact (117–130 dB peak SPL) and continuous noise (98 dB SPL) for 2 h]. Treatment with L-NAC (325 mg/kg, i.p.) resulted in 20–28 dB reductions in PTS at the 1.0–8.0 kHz ABR test frequencies. In this same study L-NAC (325 mg/kg) was administered on the same schedule by gavage to chinchillas ($N = 6$) that were exposed to a 4 kHz OBN at 105 dB SPL for 6 h. The oral treatment resulted in statistically significant 6–24 dB reductions in PTS across the 2–8 kHz ABR test frequencies relative to the control group. For all experimental conditions in the Bielfeld et al. (2007) study no cell loss data was presented. In a related experiment Bielefeld et al. (2005) exposed chinchillas ($N = 6$) to a 4 kHz OBN at 100 dB SPL for 4 d, 6 h/d. The animals were treated with L-NAC (325 mg/kg, i.p.) 1 h prior to each daily exposure. A 17 dB statistically significant reduction in PTS was measured only at 8 kHz. However, significant reductions in the percent OHC loss of 26–37% were reported between 2 and 8 kHz. This reduction in the percent OHC loss relative to the control group resulted in a near normal OHC population across the 2–8 kHz region of the cochlea despite 25–30 dB PTS.

Ohinata et al. (2003) exposed guinea pigs to a 4 kHz OBN at 115 dB SPL for 5 h. The animals ($N = 7$) were treated with a single 500 mg/kg, i.p. dose of L-NAC, 0.5 h prior to exposure. PTS was reduced 16–24 dB between 2 and 8 kHz and an average 5% reduction in the total percent OHC loss between 9 and 13 mm from the apex was reported. In another study (Duan et al. 2004), rats ($N = 10$) exposed to 50, 160 dB peak SPL noise impulses (0.33/s) were treated with L-NAC (350 mg/kg, i.p.) 1 d and 1 h preexposure, immediately following exposure and at 3 and 24 h postexposure. Relative to the untreated control group, the L-NAC treated rats showed up to a 24 dB increase in PTS across the 4–32 kHz frequency range. Sensory cell loss was severe with up to 80% OHC and 50% IHC loss across much of the cochlea. This cell loss was also more severe than that found in the untreated control group. A second group of rats ($N = 10$) received the same exposure but the treatment schedule was changed. The animals were given the 350 mg/kg, i.p. dose at 1 h preexposure, immediately postexposure and 3 h postexposure. There was a significant reduction in PTS of 10–15 dB at 4, 6 and 32 kHz. The actual PTS was less than 10 dB and the cochleas had about a normal sensory cell population. Thus in comparing these two treated groups, it would appear that the additional treatment 24 h postexposure and the absence of a treatment at 1 d preexposure had a very large and difficult to explain effect on the extent of trauma.

Kopke et al. (2000) combined L-NAC (325 mg/kg, i.p.) with salicylate (50 mg/kg, i.p.) in both a rescue and prevention mode of treatment. Two groups of chinchillas ($N = 5$/group) were exposed to a 4 kHz OBN at 105 dB for 6 h. The rescue group was treated 1 h postexposure and then twice daily for 2 d. The prevention group received the same treatment in addition to a dose 1 h preexposure. Relative to the untreated control group, the PTS between 1 and 8 kHz in the prevention paradigm was significantly reduced 24–33 dB and the percent OHC loss was reduced by 20–50% across the entire basilar membrane. The reductions in PTS at 1 and 2 kHz were similar for both the rescue and prevention groups but at 4 and 8 kHz pretreatment in the prevention mode yielded as much as 15 dB more protection than did the rescue mode treatment. The rescue mode treatment, however, showed no significant reduction in OHC loss across the basilar membrane despite the 18–35 dB reductions in PTS between 1.0 and 4.0 kHz.

To date, military personnel and nightclub attendees have been treated with L-NAC. In the former study, data (personal correspondence) are not yet available in the literature while in the latter (Kramer et al., 2006) no effect on temporary threshold shift (TTS) was found following oral administration of L-NAC. In the Kramer et al. (2006) study, 31 normal hearing subjects were given a 900 mg effervescent L-NAC tablet or a placebo 0.5 h prior to expo-

sure. The subjects spent 2 h in a nightclub where the noise levels were between 93 and 103 dB SPL. Mean TTS varied from 4 dB at the lower frequencies to 14 dB at 4 kHz. There were no statistically significant differences between the group that received the L-NAC and the one that received the placebo.

The noise exposure paradigms in the studies described above were limited to either short duration acute exposures or in one instance to a longer exposure (4 d). The type of noise used was also limited in most studies to a 4.0 kHz OBN. In all of these studies, except for one group in the Duan et al. (2004) paper, L-NAC was shown to be effective in reducing trauma. However, military and industrial noise exposures are typically broadband and of an extended duration. In addition, military exposure environments can have unusually high levels of both impulse and continuous noise (i.e., a complex high kurtosis noise). Since L-NAC is being seriously considered for human prophylactic treatment by the military, this study was designed to determine the value of L-NAC treatment in the prevention of NIHL from exposure to complex high-level, long-term and broadband noise environments capable of producing severe PTS and sensory cell loss.

2. Materials and methods

2.1. Noise exposures

Each animal was exposed to a broadband high kurtosis [$\beta(t)$] noise (Hamernik et al., 2003) at an Leq = 105 dB(A) SPL, 8 h/d for 5 d. The nonGaussian, $\beta(t) = 33$ noise exposure was created by inserting impacts, having randomly varied peak levels (119 dB < peak SPL < 132 dB) and inter-impact intervals, into a Gaussian background noise [97 dB(A) SPL] as described by Hsueh and Hamernik (1990, 1991). The impacts had a probability of occurring in a 750 ms window of 0.6. The approximately flat broadband spectrum of the noise between 0.125 kHz and approximately 15 kHz is shown in Fig. 1 along with a 15 s sample of the nonGaussian waveform.

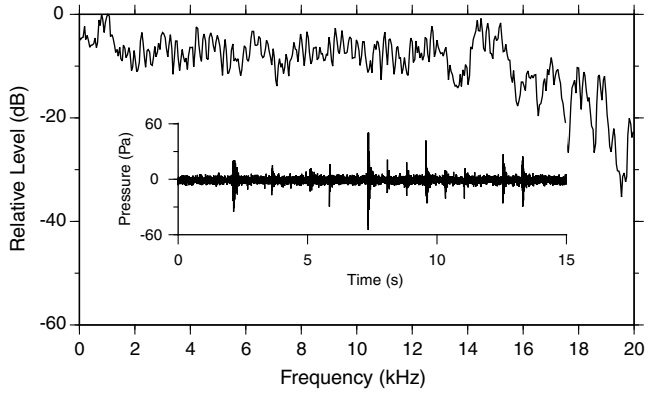

Fig. 1. The relative spectrum level of a 40 s sample of the unweighted 105 dB(A) SPL nonGaussian noise. The inset shows a 15 s sample of the nonGaussian waveform.

2.2. Noise measurement and analyses

During the exposures the noise field was monitored with a Larson Davis 814 sound level meter equipped with a ½-inch microphone. The computer generated acoustic signal, produced by the Electro-Voice Xi-1152/94 speaker system, was transduced by a Brüel and Kjær ½-inch microphone (Model 4134), amplified by a Brüel and Kjær (Model 2610) measuring amplifier and fed to a Windows PC-based analysis system. The signal was sampled at 48 kHz with a recording duration of 5.5 min. The design and digital generation of the acoustic signal is detailed in Hsueh and Hamernik (1990, 1991). Calibration of the exposure was accomplished 'off line' by analyzing 2–8, 5.5 min segments of the digitized acoustic waveform. A mean value of the kurtosis, $\beta(t) = 33$ [$22 < \beta(t) < 37$], was calculated by averaging the $\beta(t)$ from 8, 5.5 min samples of the exposure wave form.

2.3. Auditory evoked potential

Sixteen (16) male chinchillas were used as subjects. The age and weight of each animal in the experimental groups is shown in Table 1. Each animal was anesthetized [i.m. injection of Ketamine (35 mg/kg) and Xyalazine (1 mg/kg)] and made monaural by the surgical destruction of the left cochlea. During this procedure a bipolar electrode was implanted, under stereotaxic control, into the left inferior colliculus and the electrode plug cemented to the skull for the recording of auditory evoked potentials (Henderson et al., 1973; Salvi et al., 1982). The auditory evoked potential (AEP) was used to estimate pure tone thresholds. The animals were awake during testing and restrained in a yoke-like apparatus to maintain the animal's head in a fixed position within the calibrated sound field. AEPs were collected to 20 ms tone bursts (5 ms rise/fall time) presented at a rate of 10/s. Each sampled waveform was

Table 1
The age (months) and weight (g) of each chinchilla (#) in the three experimental groups

Treatment	#	Age	Weight
Noise (Group G105)	28	20	628
	29	64	652
	33	8	692
	38	39	531
	39	39	552
	40	39	673
Noise + saline (Group G106)	44	18	539
	45	21	653
	47	19	615
	48	19	607
Noise + L-NAC (Group G107)	52	20	667
	53	20	556
	55	20	600
	58	21	601
	61	21	605
	62	21	673

analyzed for large-amplitude artifact, and, if present, the sample was rejected from the average and another sample taken. Averaged AEPs were obtained from 250 presentations of the 20-ms tone bursts. Thresholds were measured using an intensity series with 5 dB steps at octave intervals from 0.5 to 16 kHz. Threshold was defined to be one-half step size (2.5 dB) below the lowest intensity that showed a "response" consistent with the responses seen at higher intensities.

2.4. Experimental protocol

Following a two-week post surgical recovery, three AEP preexposure audiograms were obtained (on different days) on each animal at octave intervals between 0.5 and 16.0 kHz. If the mean of the three audiograms fell beyond one standard deviation (s.d.) of laboratory norms, in the direction of poorer thresholds at more than one test frequency, the animal was rejected. The animals were randomly assigned to one of three experimental groups. Group G105 ($N = 6$) was exposed to noise, group G106 ($N = 4$) served as a saline treated and noise exposed control, and group G107 ($N = 6$) was treated with L-NAC and exposed to noise.

The animals were exposed four (or less) at a time to the noise detailed above for 8 h/d for 5 d. During exposure, animals were given free access to food and water and were rotated through a bank of six cages daily. The SPL across cages and in the middle of each cage, varied within less than ± 1 dB. Pure tone AEP thresholds were measured each day immediately following the noise exposure. The procedure took less than 0.5 h after which the animals were returned to the vivarium. The mean of the five audiograms thus obtained defined asymptotic threshold (AT) or shift (ATS). Thresholds were also obtained on all animals at 2, 4, 8, and 16 d after termination of the last day of the exposure. Thirty days following the last exposure day three more audiograms were collected on each animal on different days and the mean used to establish permanently shifted thresholds or permanent threshold shift (PTS).

2.5. L-NAC treatment protocol

N-acetyl-L-cysteine (Sigma) was dissolved in normal saline and the final pH was adjusted to pH 7.2 ± 0.2 with NaOH (1 M) and/or HCl (1 N). Animals were treated with i.p. doses equivalent to 325 mg/kg body weight. The freshly prepared drug was administered twice daily at approximately 8:30 am and at 4:30 pm starting 2 d before the 5 d exposure and continuing through 2 d after the exposure terminated. Each animal received a total of 18 i.p. injections.

2.6. Histology

Following the last AEP test protocol, each animal was euthanized under anesthesia and the right auditory bulla removed and opened to gain access to the cochlea for perfusion. Fixation solution consisting of 2.5% glutaraldehyde in veronal acetate buffer (final pH 7.3) was perfused through the cochlea. After 24 h of fixation the cochlea was postfixed in 1% OsO_4 in veronal acetate buffer. Surface preparation mounts of the entire organ of Corti were prepared (Engstrom et al., 1966) and inner hair cell (IHC) and outer hair cell (OHC) populations were plotted as a function of frequency and location using the frequency-place map of Eldredge et al. (1981). Missing cells were identified by the presence of a characteristic phalangeal scar. For purposes of this presentation, sensory cell population data are presented as group averages (in percent missing) taken over octave band lengths of the cochlea centered on the primary AEP test frequencies.

2.7. Statistical analysis

The threshold shifts and the percent sensory cell loss in octave-band lengths of the cochlea were compared among the groups of animals using a mixed model analysis of variance (ANOVA) with repeated measures on one factor (frequency). The probability of a type 1 error was set at 0.05. Statistically significant main effects of frequency were expected and found in all of the following analyses because of the frequency-specific nature of the audibility curve of the chinchilla and the noise exposure stimulus. For this reason main effects of frequency are not addressed in the following presentation of the results.

3. Results

3.1. Preexposure thresholds

The group mean preexposure thresholds for each experimental group are shown as symbols in Fig. 2. The shaded area represents ± 1 standard deviation of laboratory norms

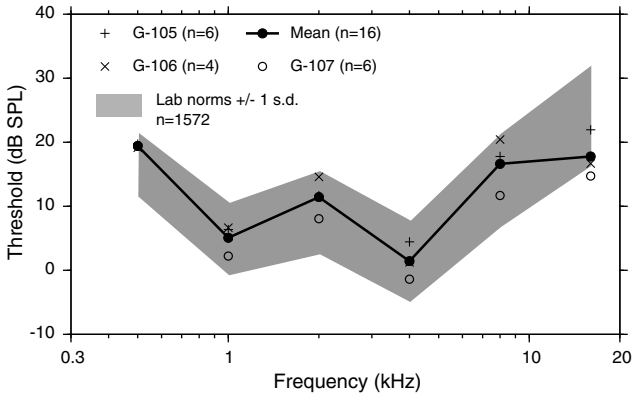

Fig. 2. Group mean preexposure thresholds for the three experimental groups (G105, noise only; G106, noise + saline; G107, noise + L-NAC). The shaded area represents the mean preexposure thresholds ± 1 s.d. from the laboratory norm based on 1572 chinchillas. The bold line represents the mean preexposure thresholds of the 16 chinchillas included in this study.

based on 1572 chinchillas. An ANOVA indicated a statistically significant effect of group ($F = 9.42$, df = 2/65) and no interaction between group and frequency ($F = 1.56$, df = 10/65). Despite this significant group effect, the maximum difference between the best and poorest thresholds is approximately 8 dB at 8.0 kHz for groups G107 (drug treated) and G106 (saline control). At a number of frequencies the drug treated group G107 exhibited the lowest thresholds.

3.2. Summary of the effects of the noise exposure/drug treatment

Group mean preexposure thresholds, asymptotic thresholds and 30 d post-exposure thresholds along with group mean sensory cell losses for the three experimental groups (i.e., G105-noise only; G106-noise plus saline control; G107-noise plus L-NAC treatment) are shown in Figs. 3–5. In these figures the bars on the data points represent the standard error (s.e.) of the mean. If a bar is not present, the s.e. was smaller than the size of the datum symbol. The nonGaussian noise exposure produced a severe hearing and sensory cell loss across the entire test frequency range for all three groups. Asymptotic thresholds (AT) were at or within 10 dB of the limit (~90 dB SPL) of the AEP system at each test frequency. By 30 d postexposure thresholds had recovered in all three groups by 12–40 dB. The actual amount of recovery at frequencies of 2 kHz and above can only be estimated as a result of the AEP ceiling effect. In all

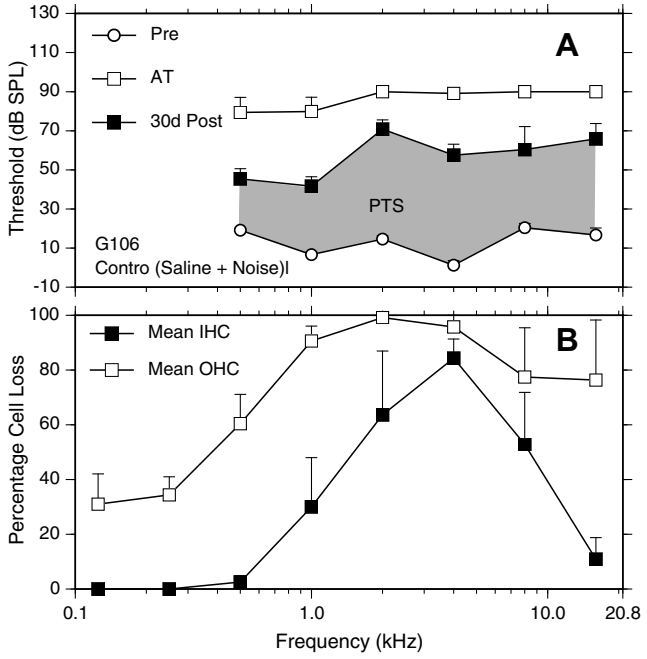

Fig. 4. Summary data from the drug control group G106 ($N = 4$) exposed to the 105 dB(A) nonGaussian noise and treated with saline. Panel (A) shows the group mean preexposure thresholds, asymptotic thresholds (AT), and 30 d postexposure thresholds. Panel (B) shows the group mean outer and inner hair cell (IHC and OHC) losses averaged across octave band lengths of the basilar membrane. (bar = 1 standard error of the mean).

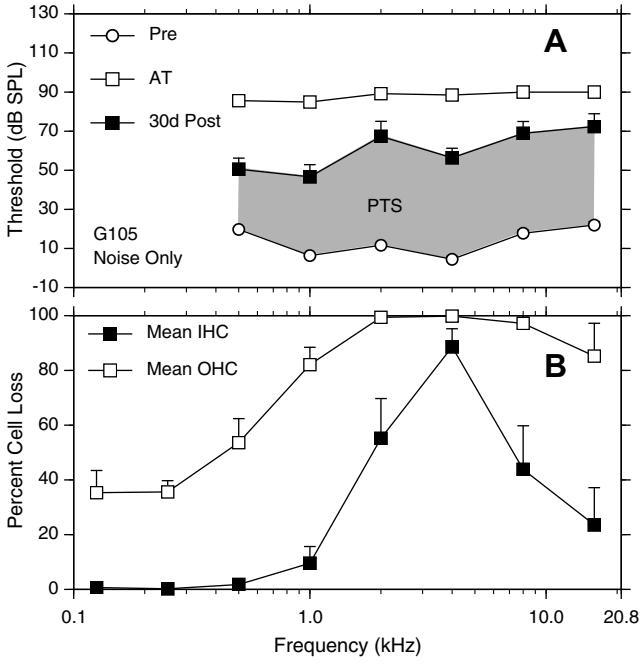

Fig. 3. Summary data from the untreated group G105 ($N = 6$) exposed to the 105 dB(A) nonGaussian noise. Panel (A) shows the group mean preexposure thresholds, asymptotic thresholds (AT), and 30 d postexposure thresholds. Panel (B) shows the group mean outer and inner hair cell (IHC and OHC) losses averaged across octave band lengths of the basilar membrane. (bar = 1 standard error of the mean).

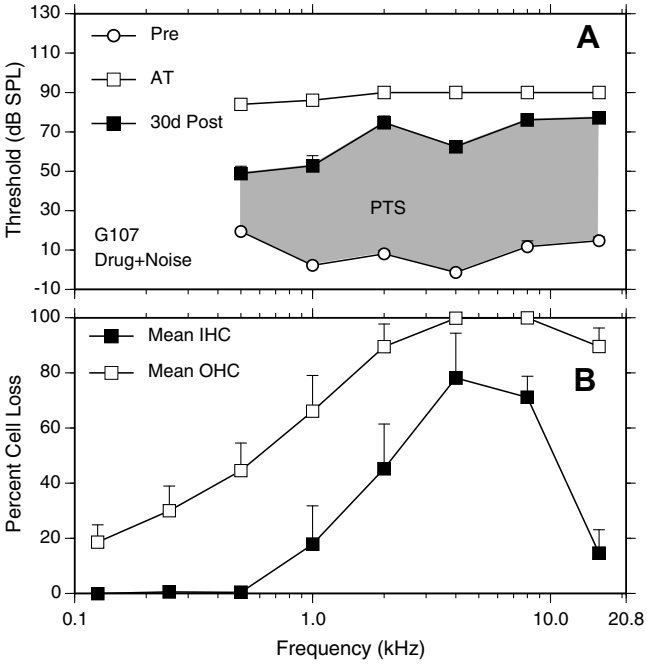

Fig. 5. Summary data from the L-NAC treated group G107 ($N = 6$) exposed to the 105 dB(A) nonGaussian noise. Panel (A) shows the group mean preexposure thresholds, asymptotic thresholds (AT), and 30 d postexposure thresholds. Panel (B) shows the group mean outer and inner hair cell (IHC & OHC) losses averaged across octave band lengths of the basilar membrane. (bar = 1 standard error of the mean).

three groups PTS exceeded 50 dB at 2 kHz and above with a maximum PTS measured in the drug treated group G107 of about 63 dB at 2 kHz. Upon inspection of these figures, the similarity of the effect on AT, PTS, and sensory cell loss that the noise exposure had on all three groups is clear. A comparison of threshold shifts at their asymptotic values and those measured at 2, 4, 8, 16 and 30 d postexposure for the three groups is shown in Fig. 6. Fig. 7 shows a comparison of IHC and OHC losses across the three experimental groups. In all three groups loss of OHC and IHC is extensive. The ANOVA indicated no statistically significant effect of group and no interaction of group and frequency in any of the threshold shift or sensory cell loss data sets. Thus, all three groups sustained the same noise-induced trauma and treatment with L-NAC had no measurable effect on the outcome of the noise exposure. A summary of the ANOVA results is presented in Table 2.

4. Discussion

The results presented above indicate that treatment with the antioxidant L-NAC did not reduce the extent of the trauma produced by a long duration, broadband, high kurtosis noise exposure. The trauma produced by this exposure was substantially more severe than that reported in

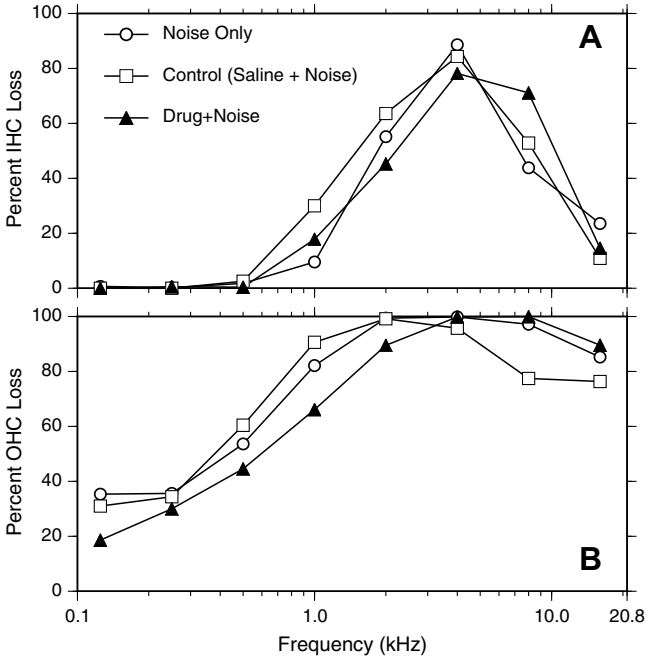

Fig. 7. A comparison of the percent inner hair cell (IHC) loss (panel A) and outer hair cell (OHC) loss (panel B) for the three experimental groups. Sensory cell losses were averaged over octave band lengths of the basilar membrane.

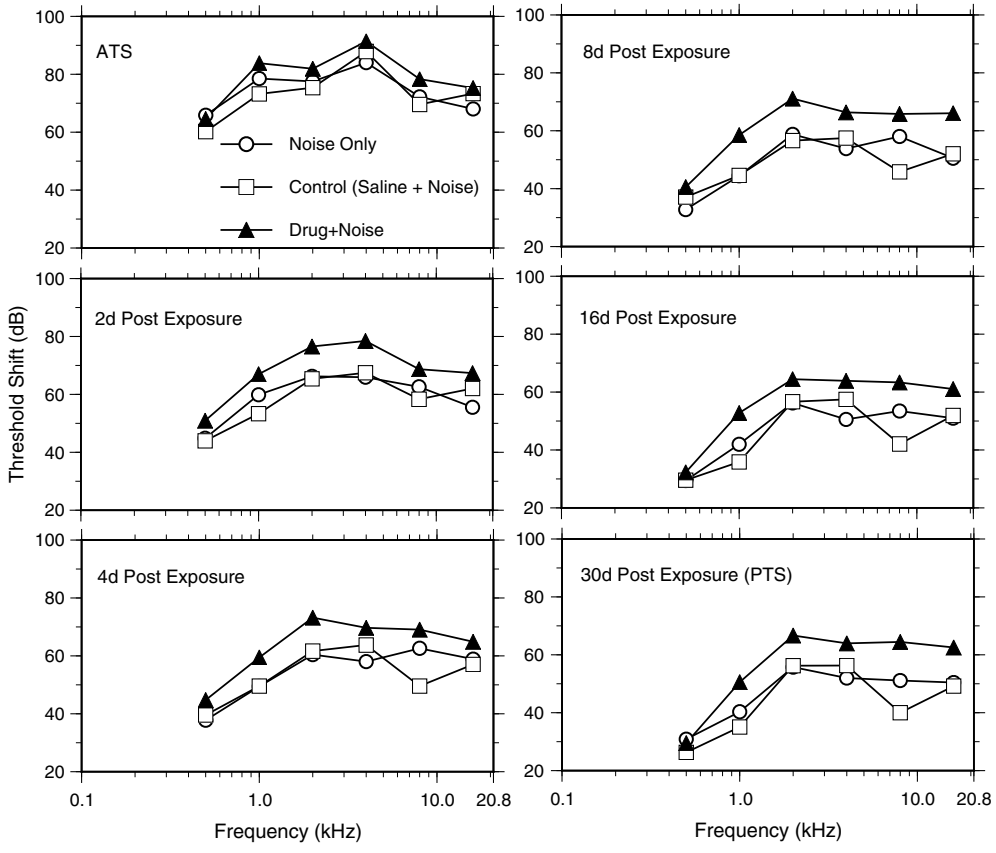

Fig. 6. A comparison of the group mean threshold shifts for the three experimental groups measured over the course of the 5 d noise exposure, i.e., the asymptotic threshold shift (ATS), and threshold shifts measured 2, 4, 8, 16 and 30 d postexposure. The 30 d data represents the PTS.

Table 2
ANOVA results for the evaluation of inner and outer sensory cell loss (IHC, OHC), asymptotic threshold shift (ATS) and the indicated post exposure threshold shifts across the three experimental groups; G105, G106 and G107. Results for the main effect of group and the interaction of group and frequency are shown

Category	Effect	df	*F*-value	*p*-value
ATS	Group effect	2/65	3.323	.0683
	Group * frequency	10/65	.819	.6116
2 d post-exposure	Group effect	2/65	1.757	.2111
	Group * frequency	10/65	.370	.9552
4 d post-exposure	Group effect	2/65	1.437	.2730
	Group * frequency	10/65	1.602	.1258
8 d post-exposure	Group effect	2/65	2.187	.1518
	Group * frequency	10/65	.964	.4830
16 d post-exposure	Group effect	2/65	1.490	.2615
	Group * frequency	10/65	1.135	.3508
30 d post-exposure (PTS)	Group effect	2/65	1.730	.2157
	Group * frequency	10/65	1.573	.1348
IHC	Group effect	2/91	.081	.9229
	Group * frequency	14/91	.564	.8859
OHC	Group effect	2/91	.385	.6881
	Group * frequency	14/91	1.05	.4128

the studies reviewed in the introduction where L-NAC was shown to be effective in reducing noise trauma.

The only studies in the literature that used L-NAC to reduce NIHL in exposure paradigms that produced somewhat comparable levels of sensory cell loss in the control group were the Kopke et al. (2005) prevention study and the Coleman et al. (2007) rescue study both of which used a short duration acute exposure paradigm. In both of these studies, OHC loss in the basal half of the cochlea varied from 75% to 99% with a maximum PTS of 40 dB. Treatment with L-NAC resulted in statistically significant and substantial reductions in PTS and sensory cell loss. The only L-NAC treatment study that used a long-term exposure was that of Bielefeld et al. (2005) where the 4 kHz OBN at 100 dB SPL lasted 6 h/d for 4 d. Between 2.0 and 8.0 kHz, PTS was 25–30 dB in the control group with 30–40% OHC loss in the corresponding regions of the cochlea. While noise-induced trauma in the control group was modest, the L-NAC treated group showed a statistically significant reduction in PTS at only 8.0 kHz. OHC loss was, however, significantly reduced to almost normal levels between 2.0 and 8.0 kHz. Cell loss data for the remainder of the cochlea was not presented.

The results of the current study would suggest that there is a limit to the effectiveness of L-NAC treatments designed to reduce or prevent NIHL. Long duration exposures at levels that lead to PTS in excess of 50 dB or more with severe loss of OHCs and IHCs in the basal half of the cochlea may lead to the chronic production of excessively high levels of ROS or other free radicals that not only overwhelm the cells endogenous defense mechanisms but also the ability of antioxidant drugs to combat the ROS assault. Unresolved, however, is the question of whether it is the severity of the noise-induced trauma or the duration of the exposure that is instrumental in reducing or eliminating the effectiveness of L-NAC as a therapeutic agent.

Acknowledgments

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