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Free Radical Biology & Medicine 44 (2008) 1772–1784
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Original Contribution

# Effectiveness of 4-hydroxy phenyl *N-tert*-butylnitrone (4-OHPBN) alone and in combination with other antioxidant drugs in the treatment of acute acoustic trauma in chinchilla

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Received 20 November 2007; revised 31 January 2008; accepted 11 February 2008
Available online 20 February 2008

**Abstract**

Acute acoustic trauma (AAT) results in oxidative stress to the cochlea through overproduction of cellular reactive oxygen, nitrogen, and other free radical species appearing from 1 h to 10 days after noise exposure. It has been shown that *N*-acetyl-L-cysteine (NAC), a glutathione prodrug, and acetyl-L-carnitine (ALCAR), a mitochondrial biogenesis agent, are effective in reducing noise-induced hearing loss. Phenyl *N-tert*-butylnitrone (PBN), a nitrone-based free radical trap, appears to suppress oxidative stress in a variety of disorders and several biological models. In this study, we tested whether 4-hydroxy PBN (4-OHPBN), a major metabolite of PBN, administered 4 h after noise exposure is effective in treating noise-induced hearing loss and whether a combination of antioxidant drugs (4-OHPBN plus NAC and 4-OHPBN plus NAC plus ALCAR) provides greater efficacy in attenuating AAT since each agent addresses different injury mechanisms. Chinchilla were exposed to a 105 dB octave-band noise centered at 4 kHz for 6 h. 4-OHPBN and combinations of antioxidant drugs were intraperitoneally administered beginning 4 h after noise exposure. Hearing threshold shifts in auditory brainstem responses and missing outer hair cell counts were obtained. 4-OHPBN reduced threshold shifts in a dose-dependent manner while both drug combinations showed greater effects. These results demonstrate that 4-OHPBN and combinations of antioxidants can effectively treat acute acoustic trauma and drug combinations may increase the effectiveness of treatment and decrease the required individual medication dose.


*Keywords:* Cochlea; Acute acoustic trauma; Free radical; Hearing loss; Antioxidant; 4-OHPBN; NAC; ALCAR; Auditory brainstem response

## Introduction

Acute acoustic trauma (AAT) may result in a sudden permanent sensorineural hearing loss occurring within a short time after exposure to an intense sound in both military and civilian work environments. In military conditions this is most often caused by such things as explosions, blasts, or loud noises from vehicles ranging from 100 to 140 dB [1] and military weapons generating approximately 140–185 dB peak sound pressure levels (SPL) [2]. More than 30 million Americans are potentially exposed to hazardous noise levels in occupations such as transportation, construction, and coal mining, as well as recreationally [3]. To protect workers from continuous noise,

*Abbreviations:* ABR, auditory brainstem response; ALCAR, acetyl-L-carnitine; AAT, acute acoustic trauma; DMSO, dimethyl sulfoxide; IHCs, inner hair cells; iNOS, inducible nitric oxide synthase; NAC, *N*-acetyl-L-cysteine; OBN, octave band noise; OHCs, outer hair cells; 4-OHPBN, 4-hydroxy PBN; PBN, phenyl *N-tert*-butylnitrone; PEG, polyethylene glycol; PTS, permanent threshold shift; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPL, sound pressure levels.

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doi:10.1016/j.freeradbiomed.2008.02.005

the National Institute for Occupational Safety and Health (NIOSH) recommends permissible daily exposure time in terms of noise levels with the A-weighting sound level (dBA) reflecting the shape of the human audiogram. The current recommendation is that exposure to 85 dB of noise be limited to 8 h and that above this noise level, a "3 dB halving rule" be applied. For every 3 dB increase in the noise level, the noise duration time should be halved. Although mechanical hearing protection devices are essential in these noisy environments, AAT may not be prevented due to inherent limitations of hearing protection devices resulting in permanent hearing loss [4]. Therefore, pharmacological interventions have been considered as adjuncts for preventing or treating impairments in auditory function caused by AAT.

Generally, acoustic trauma has been thought to mainly damage the sensory hair cells of the cochlea through mechanical and metabolic mechanisms. On the basis of the morphological appearance of hair-cell nuclei following acoustic trauma, two basic cell death pathways have been identified. Necrosis, known as a passive unprogrammed cell death, is characterized by swollen and pale-staining cytoplasm resulting in rupture of the cell, spillage of the cell contents, and evocation of an inflammatory response [5,6]. Apoptosis, known as active programmed cell death, is characterized by shrunken dark cytoplasm with a pyknotic nucleus, remaining intact cell membrane with no inflammatory response [5,6]. Although both necrosis and apoptosis are associated with acoustic trauma-induced cell death pathways, the apoptotic process is a primary cell death pathway known to occur through the activity of specific enzymes (caspase-3, -8, -9), cytochrome *c*, c-jun, and c-jun NH2-terminal kinase (JNK) [7,8].

Hair cell death induced by acoustic trauma arises through the overproduction of free radicals such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radicals. Although the direct relationship between the formation of free radicals and the cell death pathway is not fully understood, many studies have reported increases of free radicals in the cochlea immediately after noise exposure extending to 7–10 days after noise exposure, which can lead to hair cell death [9–13]. Recent studies have shown that excessive formation of free radicals following acoustic trauma involves different mechanisms such as mitochondrial injury, glutamate excitotoxicity, and ischemia/reperfusion [5,14–16].

Pharmacological approaches to preventing or treating hair cell damage and hearing loss caused by AAT can be developed by increasing cochlear antioxidant defenses in the cochlea. NAC (*N*-acetyl-L-cysteine), a scavenger of ROS, was effective in reducing noise-induced hearing loss and increasing intracellular glutathione (GSH) by providing cysteine for synthesis of GSH [14,16,17]. An endogenous mitochondrial membrane compound, acetyl-L-carnitine (ALCAR), was effective in reducing age-related and noise-induced hearing loss by enhancing mitochondrial bioenergetics and biogenesis in the face of oxidative stress, restoring key mitochondrial molecules known as carnitine and cardiolipin, restoring mitochondrial membrane integrity, and reducing ROS production [14,15,18–20].

Another family of free radical species, RNS, is also produced by noise exposure [9,10]. An example of a RNS is nitric oxide (NO). Overproduction of NO can be cytotoxic. Phenyl *N*-*tert*-butylnitrone (PBN) is a nitrone-based spin trapping agent of free radical species including hydroxyl radicals, superoxide anions, and other secondary free radicals. PBN has been reported to inhibit reactive oxygen and/or nitrogen species-induced stress and signal transduction abnormalities in a variety of disorders [21–26]. Currently, NXY-059, a disulfate ester of PBN, has been examined in Food and Drug Administration (FDA) phase III clinical trials for treatment of stroke [27]. PBN appears to suppress oxidative stress in several biological models reducing ROS and RNS formation in neurological disorders and reducing exacerbated signal transduction processes and excessive ROS production by mitochondria [28,29]. PBN decreased the signal transduction process in neurodegenerative diseases if given after the toxic insult [30]. It has been shown that 4-hydroxy PBN (4-OHPBN), a major metabolite of PBN formed in the liver microsomal system, may play an important role in the pharmacological action of PBN [31].

Since each antioxidant agent described above has a distinctly different mechanism and site of action, combinations of antioxidant agents may provide a stronger synergistic effect than single agents in preventing or treating noise-induced hearing loss [16,17,32–34]. Therefore, the present study was conducted to test the hypotheses that 4-OHPBN administered shortly after AAT is effective in treating noise-induced hearing loss and a combination of antioxidant drugs would provide greater efficacy in attenuating AAT. 4-OHPBN and NAC were used in a two-drug combination and 4-OHPBN and NAC and ALCAR were used in a three-drug combination.

## Materials and methods

### *Animals and synthesis of 4-OHPBN*

The experimental procedures in the study were reviewed and approved by the Institutional Animal Care and Use Committees of the Office of Naval Research and the Oklahoma Medical Research Foundation (OMRF).

Forty-eight female adult *Chinchilla laniger* (Moulton Chinchilla Ranch, Rochester, MN) weighing 500–850 g were used in this study because of their similarity to human audiograms. The ages of animals ranged from 3 to 5 years old. Animals were housed in plastic cages in the OMRF animal facility. Free access to a standard chinchilla diet (Mazuri Chinchilla Diet, 5MO1, PM1 Nutrition International Inc., Brentwood, MO) and tap water was allowed throughout the experimental periods. The ambient noise levels measured in the animal facility were 68–72 dB SPL at low frequencies (70–80 Hz) and less at higher frequencies. Chinchilla were randomized into seven groups ($n=12$ for the control group and $n=6$ for each experimental group): (1) control group; (2–5) 4-OHPBN groups (10, 20, 50, and 75 mg/kg); (6) 4-OHPBN plus NAC; (7) 4-OHPBN plus NAC plus ALCAR. 4-OHPBN was synthesized using a straightforward chemical reaction, extraction, and crystallization procedure [35] that has been done in our laboratory for over 20 years. 4-OHPBN utilized 4-OH-benzaldehyde in place of benzaldehyde utilized in PBN synthesis and 2-methyl-2-nitro

propane as starting chemicals. The starting chemicals were reacted in the presence of Zn metal catalyst under an acidic ethanol/water solvent and slowly reacted at a temperature of 2–10 °C to produce 4-OHPBN in good yield. After filtration, clean up, and dissolution in ethanol with minimal water, 4-OHPBN was then crystallized out using an excess water/minimal ethanol solvent at 2 °C overnight. A second crystallization step was usually done. These synthesized nitrones had previously been characterized by mass spectroscopy, NMR, and melting point and found to be superior in terms of purity to the few commonly used nitrones that can be purchased commercially. 4-OHPBN synthesized at OMRF was first dissolved in dimethyl sulfoxide (DMSO, 0.8 ml per 100 mg, Sigma-Aldrich Inc., St. Louis, MO) at 37°C and then polyethylene glycol (PEG) 400 (0.8 ml per 100 mg, Sigma-Aldrich Inc.) was added. Sterile saline (0.4 ml per 100 mg) was added before injection. The two-drug combination group received 50 mg/kg of 4-OHPBN and 100 mg/kg of NAC (Hospira Inc., Lake Forest, IL). Animals in the three-drug combination group received 20 mg/kg of 4-OHPBN, 50 mg/kg of NAC, and 20 mg/kg of ALCAR (Sigma-Aldrich Inc.). These agents were intraperitoneally administered. Experimental animals received the initial injection 4 h after noise exposure and continual injections twice daily for the next 2 days. In the control group, equal volumes of carrier solution (DMSO, PEG 400, and sterile saline, 2:2:1 ratio) were injected at the same time points as in the experimental groups. The carrier solution control groups were paired with the treatment groups during noise exposure.

### *Noise exposure*

For noise exposure, two animals at a time were placed in two small wire restraint cages on a wooden plate. They were exposed to a 105 dB SPL octave-band noise centered at 4 kHz for 6 h in a sound isolation booth [Industrial Acoustics Company (IAC), New York, NY]. The noise was digitally generated by a Tucker Davis Technologies (TDT, Alachua, FL) device, passed through a real-time attenuator (TDT, RP2), filtered, amplified with a preamplifier (QSC audio power, Costa Mesa, CA), and transduced with a high-frequency acoustic driver and an acoustic speaker (JBL 2350, Northridge, CA) suspended from the ceiling of the sound booth and positioned directly above the wire cages. Before noise exposure, the sound spectrum output of the system was calibrated with a sound level meter centered at an octave bandwidth of 4 kHz. A condenser microphone (B&K 2804, Norcross, GA) coupled to the preamplifier was placed between the two wire cages at the level of the animals' heads to monitor the noise level. During noise exposure, the noise level was continually and visually monitored using the PULSE software system [B&K Sound & Vibration Measurement (version 10.0)] including FFT Analysis Type 7770 and CPB Analysis 7771.

### *Auditory brainstem responses*

Auditory brainstem response (ABR) was measured for the right ear of each animal within 3 days before initial noise exposure (baseline threshold), immediately after, and then 21 days after noise exposure for each frequency. Permanent threshold shift (PTS) was obtained by subtracting the baseline threshold from the final hearing threshold measured at 21 days after noise exposure. ABR recordings were done under light ketamine (20 mg/kg) and xylazine (1 mg/kg) anesthesia. Small supplemental doses (1/3 of initial dose) were given if needed. ABR thresholds were recorded from subcutaneous needle electrodes placed under the skin of the head. An active needle electrode and a reference electrode were placed proximal to the right ear and the left ear, respectively, while a ground electrode was placed at the vertex. A computer-aided system (Intelligent Hearing Systems, Miami, FL) coupled to high-frequency transducers was used to generate auditory stimuli. Acoustic stimuli were tone pips (5 ms duration and 1 ms Blackman rise and fall) at frequencies of 0.5, 1, 2, 4, 6, and 8 kHz. All acoustic stimuli were transduced through the computer-controlled attenuator to a 3A insert earphone [Etymotic Research (ER)-3A, Etymotic Research Inc., Elk Grove Village, IL] placed about 5 mm from the tympanic membrane. The insert earphone was calibrated with a coupler mounted to the sound level meter approximating its placement. The electrical responses obtained from the electrodes were amplified (×100,000), filtered (100–3000 Hz), and digitized through an A/D converter on a signal processing board. They were averaged at a sample rate of 1024 for each level. Hearing thresholds were tested in 10 dB descending steps until near the threshold, and then 5 dB ascending steps were taken to determine the threshold. Threshold was defined as the midpoint between the lowest level of a clear response and the next level where no response was observed. The investigators performing the ABR measurements were blinded as to the identity of the animal groups.

### *Histological examination*

After the final auditory tests performed at 3 weeks after noise exposure, the animals were humanely euthanized with transcardial injection of sodium pentobarbital under anesthesia with a mixture of ketamine (20 mg/kg) and xylazine (1 mg/kg). The temporal bones were immediately removed from the skull and the cochleae were perfused from the oval window or the round window through a hole made at the apex with a solution of either 0.2 M sodium succinate and 0.1% nitrotetrazolium blue in 0.1 M phosphate buffer (pH 7.4) for 30–40 min at 37 °C or 4% paraformaldehyde in phosphate buffer for 24 h. For the former, the cochleae were washed with buffer and fixed with 4% paraformaldehyde for 24 h. For the latter, the cochleae were washed with PBS, permeabilized with 0.3% Triton X-100 for 10 min, and stained for actin with 1% rhodamine phalloidin for 30 min. Cochleae were dissected and sections of the organ of Corti were mounted on a glass slide as surface preparations and examined for hair cell counts under a light microscope (Olympus BH-2; Olympus Optical Co. Ltd.). Absolute hair cell counts were transformed into percentages of missing inner hair cells (IHCs) or outer hair cells (OHCs) by dividing the cell count for the experimental animals by control value hair cell counts for normative values developed for each cochlear section [36]. Data from each cochlear section were entered as inputs into a worksheet to construct continual data points along the whole cochlear length. Each cochlear length was normalized to 100%. Finally, the

percentage of the missing IHCs and OHCs was plotted as a function of percentage distance from the apex, called a cytocochleogram [14,16,36]. To change the percentage distance from the cochlear apex in the cytocochleogram into the specific frequency regions, the cochlear frequency-place map was obtained by an equation of $F=125e^{0.051d}$, where $F$ is the frequency in Hz and $d$ is percentage distance from the apex [37,38].

*Statistical analysis*

All data values used in the text and figures are mean±SE. Statistically significant differences in threshold shifts and percentage of missing hair cells between the control group and each of the treatment groups were tested via ANOVA (SPSS 14.0 for Windows). Frequency was treated as the within-subject factor while treatment was the between-subjects factor. When a main effect was found, post hoc tests, such as LSD and Tukey, were performed for mean comparisons among different groups. A $p$ value less than 0.05 was considered a statistically significant difference.

## Results

*ABR threshold shift*

There were no significant differences between all the groups in temporary thresholds measured immediately after noise exposure (data not shown). The ABR threshold shifts varied with 4-OHPBN treatment group ($p<0.001$) and frequency ($p<0.001$). However, there was no significant interaction of 4-OHPBN treatment group × frequency ($p>0.05$). As shown in Fig. 1, mean ABR threshold shifts in the control group ranged from about 10 dB at low frequencies to 38 dB at high frequencies. In all four 4-OHPBN treatment groups, the threshold shifts were reduced, although the degree of the reduction was different among the groups. The higher the dose of 4-OHPBN, the greater the reduction in threshold shifts. The administration of 4-OHPBN showed greater effects at high frequencies than at low frequencies. In the lower dose groups (10 and 20 mg/kg), the mean threshold shifts were approximately 5 dB at lower frequencies and 23 dB at higher frequencies. No significant differences in ABR threshold shifts between the control group and the 4-OHPBN 10 mg/kg treatment group were found across frequencies. However, significant differences in ABR threshold shifts between the control group and the 4-OHPBN 20 mg/kg treatment group were shown at both 1 and 8 kHz ($p<0.05$ and $p<0.01$, respectively). The amount of the reduction was 8 dB at 1 kHz and 23 dB at 8 kHz. At the 4-OHPBN dose of 50 mg/kg, the threshold shifts were significantly reduced at 0.5, 1, 6, and 8 kHz compared to the control group ($p<0.01$, $p<0.05$, $p<0.05$, and $p<0.01$, respectively). The amount of the reduction was 10 dB at 0.5 kHz and 22 dB at 8 kHz. At the 4-OHPBN dose of 75 mg/kg, the threshold shifts were around 10 dB across frequencies. With the highest dose of 4-OHPBN, the threshold shifts were significantly reduced at all frequencies except at 1 kHz compared to the control group ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.01$, and $p<0.001$, respectively). The amount of the reduction was 8 dB at 0.5 kHz and 29 dB at 8 kHz.

While ABR threshold shifts varied with drug combination treatment groups ($p<0.001$) and frequency ($p<0.01$), there was no significant interaction of drug combination treatment group × frequency ($p>0.05$), indicating that ABR threshold shifts were equivalent across all test frequencies. Fig. 2 shows the results of different drug combination treatment groups. The two-drug combination group significantly decreased the

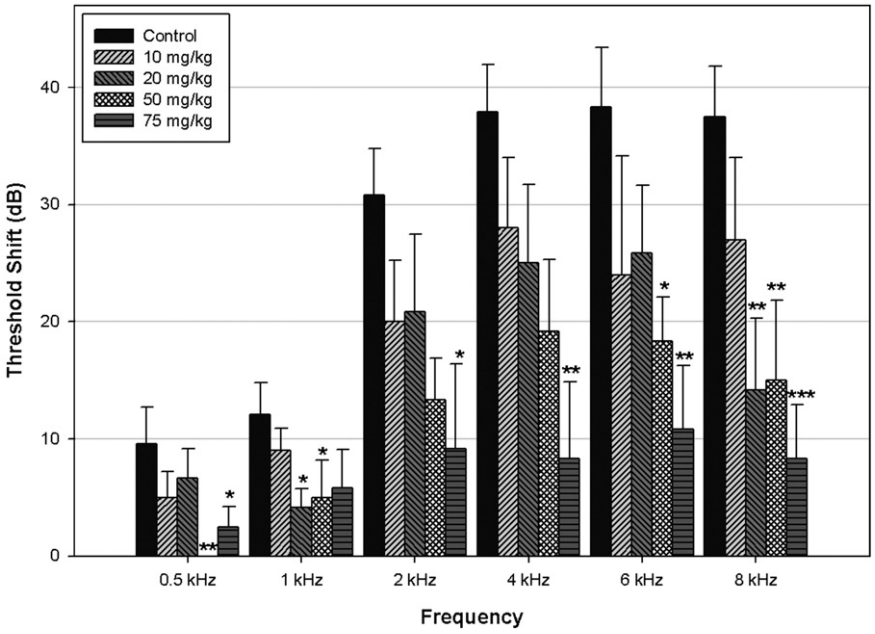


Fig. 1. ABR threshold shifts of the 4-OHPBN treatment groups compared with the control group. The threshold shifts at high doses of 4-OHPBN were significantly reduced compared to the control group. Asterisks *, **, and *** represent statistically significant differences in ABR threshold shifts between the control group and each 4-OHPBN treatment group at each frequency at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

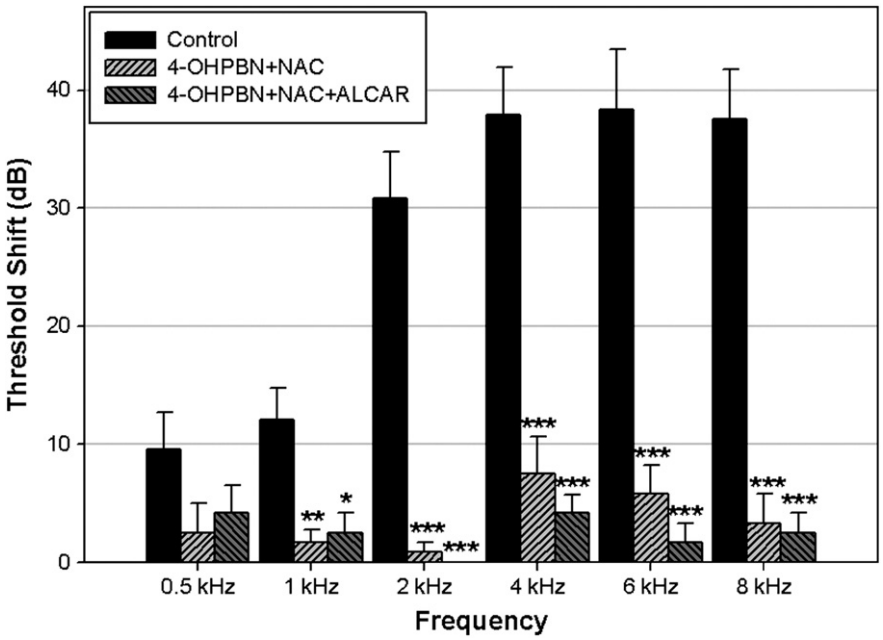


Fig. 2. ABR threshold shifts of the two- and three-drug combination treatment groups compared with the control group. The threshold shifts of both the two- and the three-drug combination treatment groups were significantly reduced compared to the control group. Asterisks *, **, and *** indicate statistically significant differences in ABR threshold shifts between each combination treatment group and the control group at each frequency at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

threshold shift at all frequencies except 0.5 kHz compared to the control group ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively). The amounts of reduction ranged from 10 dB at 1 kHz to 33 dB at 8 kHz. Compared to the effects of 4-OHPBN (75 mg/kg) alone, this combination group with a lower dose of 4-OHPBN (50 mg/kg) showed better effects. The three-drug combination treatment group showed significant reductions in threshold shifts at all frequencies except 0.5 kHz compared to the control group ($p<0.05$, $p<0.001$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively). The amounts of reduction were 9 dB at 1 kHz and 36 dB at 6 kHz. The amounts of reduction for the three-drug combination treatment group appeared to be greater than that of the two-drug combination treatment group at all frequencies except 0.5 and 1 kHz, although there were no statistically significant differences between the two-drug combination treatment group and the three-drug combination treatment group. For significant reductions in threshold shifts, 4-OHPBN required a lower dose as the number of drugs in the combination increased.

Fig. 3 displays ABR threshold shifts averaged at higher frequencies (2–8 kHz) among the control group, the 4-OHPBN treatment group, and the drug combination treatment group. The amount of reduction in ABR threshold shifts for the 4-OHPBN treatment group became greater as the dose of 4-OHPBN increased. The mean threshold shifts at 10, 20, 50, and 75 mg/kg were significantly decreased compared to those of the control group ($p<0.05$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively). For 10 mg/kg of 4-OHPBN, the mean threshold shift of the control group was reduced by 31% (approximately 11 dB). The amount of reduction in PTS increased to 39% (14 dB), 56% (20 dB), and 75% (27 dB) when the doses of 4-OHPBN were increased to 20, 50, and 75 mg/kg, respectively. Compared to the 4-OHPBN treatment groups of 10 and 20 mg/kg, the threshold shifts at 75 mg/kg were significantly decreased. Furthermore, the ABR threshold shifts for the drug combination treatment groups were significantly reduced compared to the control group and the 4-OHPBN treatment groups at 10, 20, and 50 mg/kg. The amount of reduction increased from 86 to 92% when the treatment increased from a two-drug combination to a three-drug combination. This indicates that drug combination treatment groups have higher therapeutic effects on the treatment of AAT than a single 4-OHPBN treatment group.

### *Hair cell counts*

There were no significant differences in inner hair cells counts among the control group, the 4-OHPBN treatment group, and the drug combination treatment groups (data not shown). Significant differences were found only in outer hair cells counts. Fig. 4 illustrates a cytocochleogram relating mean percentage of missing OHCs to the measured percent distance from the cochlear apex. Average OHC losses for the 4-OHPBN treatment group were 22, 6, and 4% for 10–20, 50, and 75 mg/kg, respectively, compared with 43% loss seen in the control group. The OHC losses between each 4-OHPBN treatment group and the control group were significantly different ($p<0.001$). The amount of reduction in OHC loss became greater as the dose of 4-OHPBN increased. The OHC losses of the 4-OHPBN 50 and 75 mg/kg group were significantly decreased compared to the 4-OHPBN 10–20 mg/kg treatment group ($p<0.001$). The equation of the cochlear frequency-place map [37,38] was used to determine whether there were significant differences in OHC loss at specific cochlear regions corresponding to frequencies of 2, 4, 6, and 8 kHz between the control group and the 4-OHPBN treatment group. The

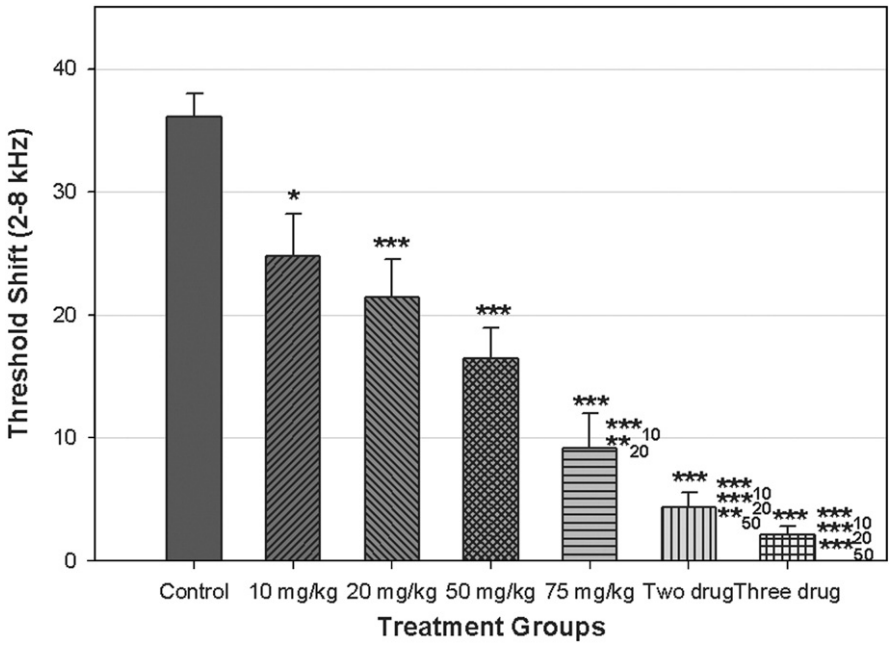


Fig. 3. ABR threshold shifts averaged at frequencies of 2–8 kHz for the control group, the 4-OHPBN treatment group, and the drug combination treatment groups. The reductions in threshold shifts were directly associated with both increases in 4-OHPBN dosage and the number of drug combinations. Asterisks * and *** represent statistically significant differences in ABR threshold shifts between the control group and each treatment group at $p<0.05$ and $p<0.001$, respectively. Asterisks ***10, **20, ***20, **50, and ***50 indicate statistically significant differences in ABR threshold shifts between each treatment group (75 mg/kg, two- and three-drug combinations) and each different dosage of 4-OHPBN (10, 20, and 50 mg/kg) at $p<0.001$, $p<0.01$, $p<0.001$, $p<0.01$, and $p<0.001$, respectively.

OHC losses varied only with the 4-OHPBN treatment group ($p<0.001$). Fig. 5 represents the percentage of missing OHC as a function of frequency. The OHC losses of the 4-OHPBN 50 and 75 mg/kg groups were significantly reduced at frequencies of 2, 4, 6, and 8 kHz compared to the control group ($p<0.05$, respectively). No significant differences in OHC loss were found between other doses of 4-OHPBN.

Fig. 6 displays significant differences in the percentage of missing OHCs between the control group and the drug combination treatment groups. Average OHC losses were 6.88

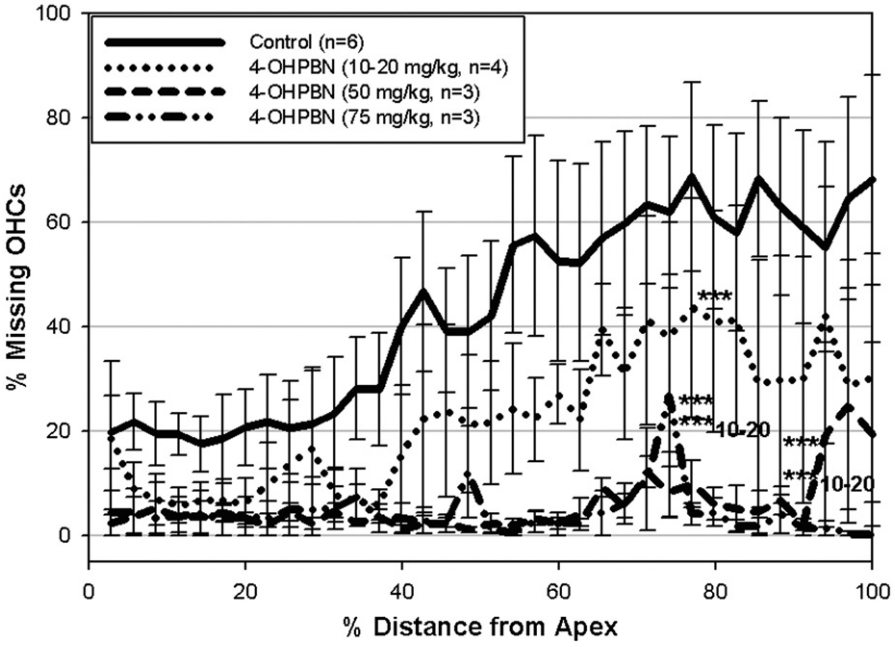


Fig. 4. OHC counts of different 4-OHPBN treatment groups compared with the control group. These cytocochleograms represent missing OHC percentage as a function of percentage distance from the apex. Average OHC losses were reduced with increases in 4-OHPBN dosage. Asterisks *** indicate a statistically significant difference in OHC loss between each 4-OHPBN treatment group and the control group at $p<0.001$ while asterisk ***10–20 represent a significant difference in OHC loss between different 4-OHPBN treatment groups and 4-OHPBN treatment group of 10–20 mg/kg at $p<0.001$.

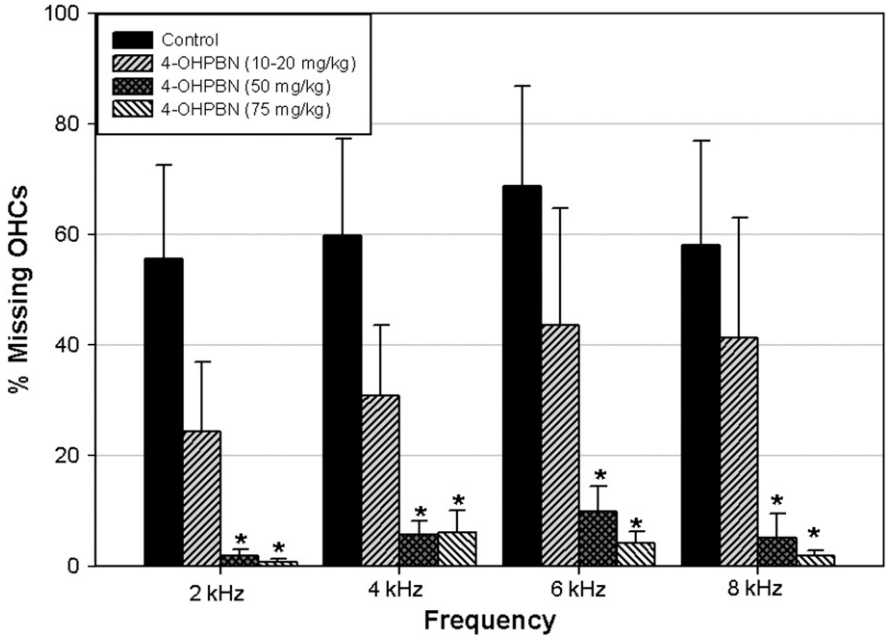


Fig. 5. Mean percentage of missing OHC corresponding to cochlear frequency regions ranging from 2 to 8 kHz for different 4-OHPBN treatment groups and the control group. Asterisks * indicate a statistically significant difference in OHC loss between different 4-OHPBN treatment groups and the control group at each cochlear frequency region of 2, 4, 6, and 8 kHz at $p<0.05$.

and 6.65% for the two- and three-drug combination treatment groups, respectively. The OHC losses of the two- and three-drug combination treatment groups were significantly reduced compared to the control group ($p<0.001$). In specific frequency regions, the OHC loss varied with treatment group ($p<0.001$). Fig. 7 represents that significant differences in OHC loss were observed between the control group and the two/three-drug combination treatment groups at frequencies of 2, 4, 6, and 8 kHz ($p<0.05$, respectively). However, there were no significant differences between the two and three-drug combination treatment groups.

Fig. 8 illustrates the percentage of missing OHCs averaged at higher frequencies (2–8 kHz) among the control group, the 4-OHPBN treatment group, and the drug combination treatment

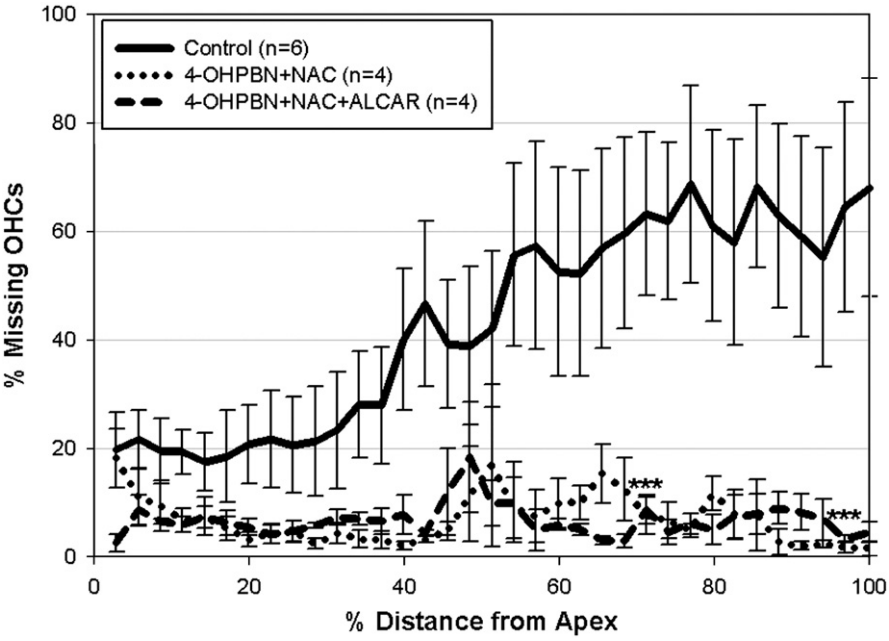


Fig. 6. OHC counts of different drug combination treatment groups compared with the control group. These cytocochleograms represent missing OHC percentage as a function of percentage distance from the apex. The OHC losses at both drug combination treatment groups were significantly reduced compared to the control group. Asterisks *** indicate a statistically significant difference in OHC loss between different drug combination treatment groups and the control group at $p<0.001$.

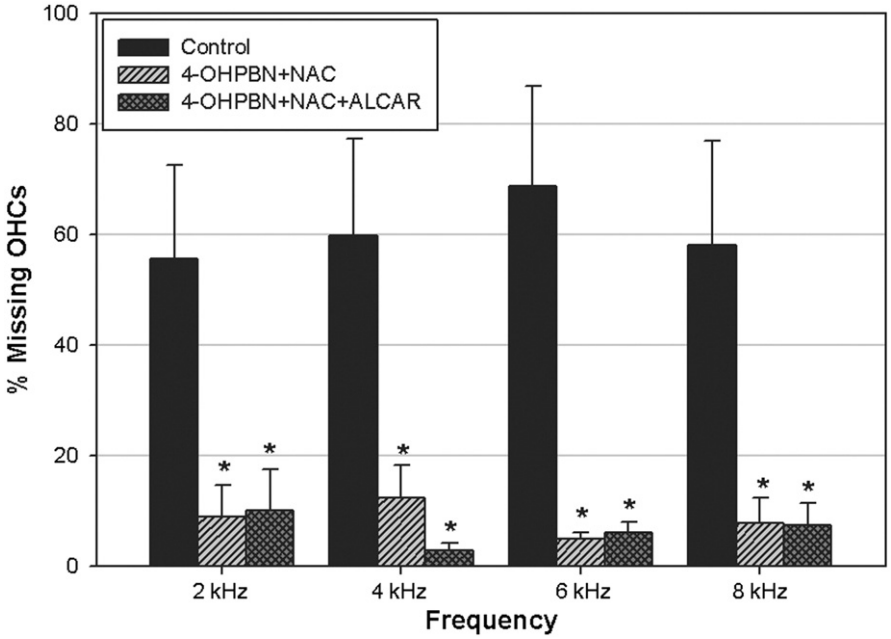


Fig. 7. Mean percentage of missing OHC corresponding to cochlear frequency regions ranging from 2 to 8 kHz for different drug combination treatment groups and the control group. Asterisks * indicate a statistically significant difference in OHC loss between different drug combination treatment groups and the control group at each cochlear frequency region of 2, 4, 6, and 8 kHz at $p<0.05$.

groups. The amount of reduction in OHC loss for the 4-OHPBN treatment groups became greater as the dose of 4-OHPBN increased. The mean OHC losses of the 4-OHPBN 10–20, 50, and 75 mg/kg groups were significantly decreased compared to the control group ($p<0.01$, $p<0.001$, $p<0.001$, respectively). For 10–20 mg/kg of 4-OHPBN, the mean OHC loss of the control group was reduced by 44%. The amount of reduction in PTS increased to 92 and 95% when the doses of 4-OHPBN were increased to 50 and 75 mg/kg, respectively. The OHC losses at doses of 50 and 75 mg/kg were significantly decreased compared to doses of 10–20 mg/kg ($p<0.05$, each). For the drug combination treatment groups, the OHC loss was significantly

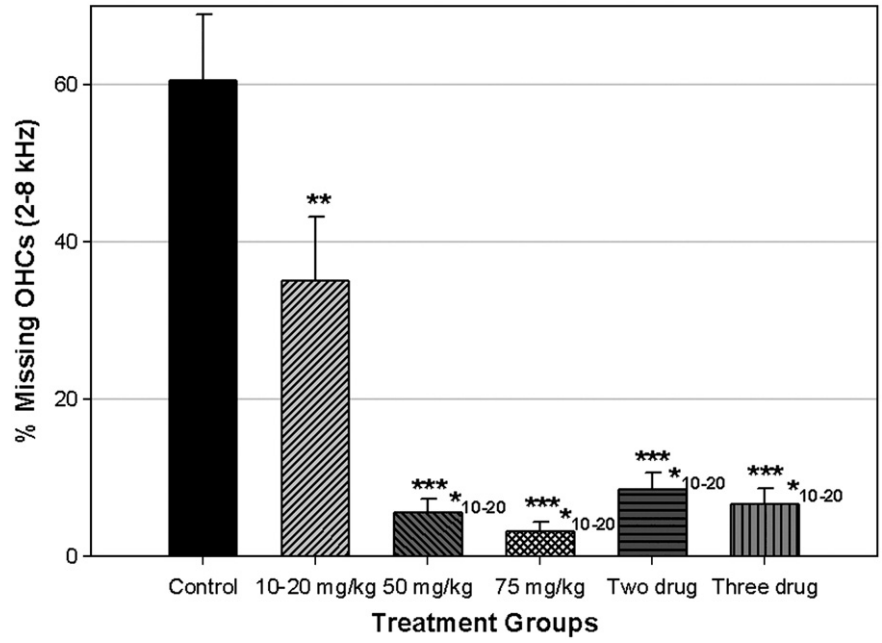


Fig. 8. Percentage of missing OHC averaged at cochlear frequency regions corresponding to 2–8 kHz for four 4-OHPBN treatment groups, different drug combination treatment groups, and the control group. The OHC losses were reduced with both increases in 4-OHPBN dosage and the number of drug combinations. Asterisks ** and *** represent statistically significant differences in OHC loss between each treatment group and the control group at $p<0.01$, and $p<0.001$, respectively. Asterisk *10–20 indicates statistically significant differences in OHC loss between each treatment group and 4-OHPBN of 10–20 mg/kg at $p<0.05$.

reduced compared to the control group ($p<0.001$) and the 4-OHPBN dose of 10–20 mg/kg ($p<0.05$). The amount of reduction increased from 89 to 90% when the treatment increased from the two-drug combination to the three-drug combination. No significant differences in OHC losses between the 4-OHPBN doses of 50 and 75 mg/kg and the drug combination treatment groups were observed.

## Discussion

The present study used an octave-band noise centered at 4 kHz because a classic pattern of noise-induced hearing loss in an audiogram shows a notch centered at 4 kHz of approximately 30–50 dB [5,39]. The noise level used in our present study is categorized as an extremely loud noise equivalent to the sound level of a woodshop, chainsaw, firecrackers, or bulldozer. Usually, conversational speech is measured at a noise level of 60 dB while a siren at 10 m distance and the noise levels of discotheques and close to loudspeakers at rock concerts measure approximately 110 dB. Based on the 3 dB halving rule recommended in the safety guideline of the NIOSH [40], our noise level of 105 dB is limited to less than 3.75 min of exposure time before becoming very dangerous to the human ear. The present study showed that the noise exposure of 6 h at 105 dB caused permanent hearing loss of approximately 38 dB and damaged about 50% of outer hair cells. These results are consistent with previous studies using the same noise level and duration [3,14,17,40].

Exposure to excessive noise can damage the cochlea through mechanical and metabolic mechanisms [3,41–43]. Exposure to noise higher than 115 dB SPL mainly leads to mechanical damage including the disruption of the Reissner membrane and basilar membrane [44], damage or loss of stereocilia bundles and cuticular plates [45,46], detachment of stereocilia tips from the tectorial membrane [46], damage to pillar cells [47], damage of cell junctions between hair cells and supporting cells [13], holes in the reticular lamina [48], swelling and rupturing of the dendritic terminals of the auditory nerve fibers [5,49], disruption of subcellular organelles [3,50], and death of hair cells [50]. Exposure to noise at 115 dB or less can result in metabolic damage including disruption of ion balance in cochlear fluids [7] and reduction in blood flow leading to a cochlear ischemia–reperfusion injury similar to that occurring during a stroke in the brain [3].

When a high level of noise is presented to the inner ear, free radicals, molecules with an unpaired electron, form in the cochlea causing substantial damage to molecular structures. The formation of free radicals exceeds the capacity of the cellular defense mechanisms inducing oxidative stress. Many studies have shown that exposure to acoustic trauma induces increases in the production of free radicals such as hydroxyl [51,52], superoxide [53], lipid peroxidation [54], nitric oxide [55,56], nitrotyrosine (NT), and 4-hydroxy-2-nonenal (4-HNE) [57–59] and a decrease in antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [59–61] in the cochlea. Increased free radical production may be due to excessive release of glutamate from afferent neurons, ischemia/reperfusion, or mitochondrial damage [5]. Oxidative damage leads then to hair cell death by apoptosis or to a lesser extent necrosis resulting in a hair cell lesion that then leads to hearing loss. Successful pharmacological approaches to the prevention and treatment of sensorineural hearing loss caused by AAT have been developed to target one of the known molecular mechanisms associated with hearing loss induced by acoustic trauma. The overproduction of free radicals was reduced by inhibiting ROS production with allopurinol [62]; scavenging free radicals with salicylate [16], mannitol [63], and PBN [21–24]; up-regulating antioxidant enzyme activity with *R*-phenylisopropyladenosine [64,65]; treating with exogenous antioxidants such as glutathione monoethyl ester [66], PBN [25,26], and NXY-059 [27]; enhancing GSH homeostasis with NAC and methionine [14,16,17]; and protecting and repairing mitochodria with ALCAR [14,15,18–20]. Increases in glutamate excitotoxicity induced by acoustic trauma were decreased by glutamate antagonists such as carbamathione, MK 801, kynurenic acid, caroverine, and magnesium [14,67–69]. Lipid peroxidation was inhibited by a lazaroid [70]. Cochlear blood flow was increased by blood flow promoting drugs such as pentoxifylline [71], hydroxyethyl starch [72], sarthran [73], and a combination of vitamins A, C, and E plus magnesium [34] that preserved cochlear microcirculation. Additionally, otoprotective effects were shown by specific cell signal inhibitors of cochlear apoptosis such as CEP-1347, an inhibitor of c-Jun N-terminal kinase activation [74] and KX1-004, a Src-PTK inhibitor [75].

The present study is the first attempt to evaluate the therapeutic effect of 4-OHPBN on AAT. The administration of 4-OHPBN produced a significant reduction in both permanent threshold shifts and OHC loss. The reduction was correlated with the dose of 4-OHPBN. When the dose of 4-OHPBN was increased from 10 to 75 mg/kg, the PTS decreased from 31 to 75% while OHC loss decreased from 44 to 95%. These results are consistent with previous studies reporting that AAT can be reduced by other antioxidant drugs, such as NAC and ALCAR [13–15,20]. This indicates the possibility of clinical application of 4-OHPBN to treat AAT.

However, the findings of the present study are not consistent with some previous studies showing that a different but related compound, PBN, does not reduce auditory damage induced by noise alone [76–80]. This difference may result from differences in intensity and duration of noise and the animal models used in those studies. Previous studies used a 100 dB octave band noise (OBN) centered at 13.6 kHz for 2 h [76] and 97 or 95 dB OBN centered at 8 kHz for 4 h [80]. Although one study used the same intensity as in the present study [79], they used a different duration (4 h) and animal model (rat). In addition, an intrinsic difference between 4-OHPBN and PBN may contribute to the differences. When 4-OHPBN was compared to PBN and its other derivatives, such as 3-hydroxy PBN (3-OHPBN), 2-hydroxy PBN (2-OHPBN), and 2-sulfoxy PBN (2-SPBN), the effect of 4-OHPBN on inhibiting hepatocarcinogenesis was stronger than that of the parent compound (PBN) and the other derivatives [81]. Thus, 4-OHPBN may have a stronger biological effect than PBN and its other derivatives.

The pharmacokinetics of PBN examined in other species can provide clinically useful information in preventing or treating

hearing loss induced by AAT. After intravenous (10 mg/kg), oral (20 mg/kg), and subcutaneous (30 mg/kg) administration to rats, the plasma concentration of PBN increased quickly after administration and then declined reaching its baseline within 24 h in an exponential fashion [82]. In more detail, PBN showed a total plasma clearance of 12.37±3.82 ml/min/kg and volume of distribution at steady state of 1.74±0.51 l/kg, resulting in an elimination half-life of 2.01±0.35 h. After oral (20 mg/kg) and subcutaneous (30 mg/kg) administration, PBN demonstrated a peak plasma concentration of 7.35±1.92 and 3.56±0.66 μg/ml, area under the concentration–time curve from 0 to infinity of 23.89±5.84 and 15.96±3.10 μg/h/ml, and a mean oral bioavailability of 85.63±20.93 and 38.13±7.40%, respectively. The pharmacokinetic properties and the clearance mechanism of PBN in rats were consistent with those observed with intravenous (5 mg/kg) injection in monkey [83].

The underlying mechanisms of PBN may involve the reduction of oxidative stress [28]. PBN was reported to protect cochlear function from combined exposure to noise and carbon monoxide by reducing the formation of free radical species in the cochlea [76–78,84]. Carbon monoxide damages the inner hair cell-Type I spiral ganglion synapse that uses glutamate as the neurotransmitter as opposed to outer hair cells that have much less afferent innervation. The function of PBN may be related to glutamate toxicity because PBN, like the NMDA receptor blocker MK-801, can reduce hearing loss induced by carbon monoxide ototoxicity [85]. Additionally, PBN can attenuate cochlear damage from combined exposure to noise and acrylonitrile. The protective effects of PBN on acrylonitrile-induced damage appear to be related to oxidative stress reduction by preventing the depletion of GSH caused by combined noise and acrylonitrile exposure and also reducing reactive epoxide binding to cytochrome *c* oxidase [79,80]. PBN may play an important role in increasing the Cu/Zn form of superoxide dismutase in the cochlea [86]. The Cu/Zn form of superoxide dismutase has been shown in other organs to restore the same type of antioxidant defense mechanisms associated with cochlear protection [87].

In addition to its role as a neuroprotectant, PBN has been shown to function as a potent anti-inflammatory agent in nature. PBN is very effective in inhibiting the activation/mRNA level of inflammatory mediators such as inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX2), COX catalytic activity, and nuclear factor kappaB [88]. Recently, it was shown that expression of iNOS synthase and nitric oxide-related ROS production was increased in the wall of the blood vessels of stria vascularis and marginal cells in the cochlea after exposure to 110–120 dB broadband noise at 3 h/day for 3 consecutive days [55,56]. Thus, iNOS is associated with inflammation and ischemia–reperfusion injury of the cochlea.

The results of NAC and ALCAR administered at 325 and 100 mg/kg, respectively, 4 h after identical steady-state AAT [40] were relatively comparable with those of our study shown in Fig. 3. The PTS with NAC (325 mg/kg) and ALCAR (100 mg/kg) averaged at 2–8 kHz was reduced up to 44 and 56%, respectively, compared to the control group. Although the degree of PTS reduction for the control group in the present study was larger (4 dB) than that of the control group (32 dB SPL) reported by Coleman et al. [40], there were no statistically significant differences between the control groups. Fig. 9 shows that the mean PTS of the two- and three-drug combinations were significantly

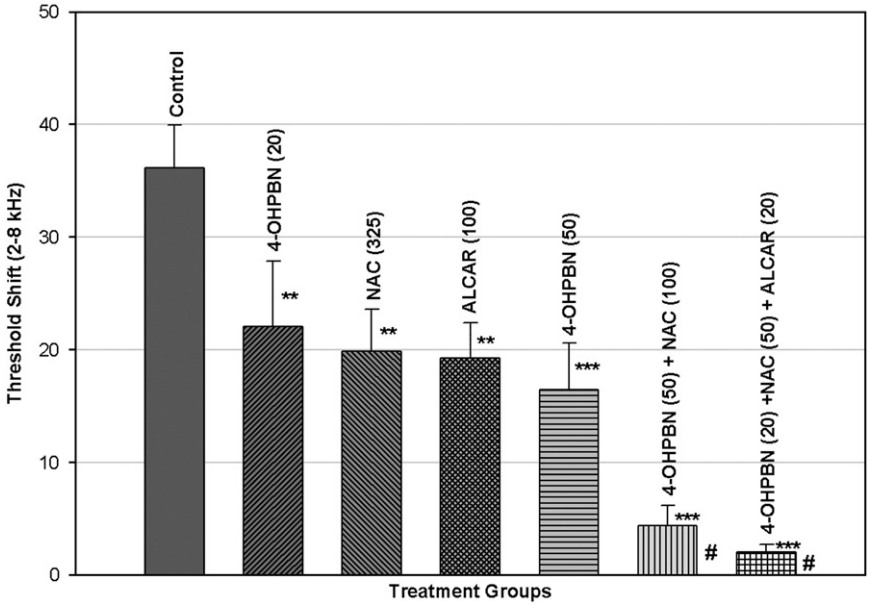


Fig. 9. ABR threshold shifts averaged at frequencies of 2–8 kHz for each antioxidant drug and the drug combination treatment groups. Asterisks ** and *** indicate statistically significant differences between the control group and each treatment group at $p<0.01$ and $p<0.001$, respectively. The symbol # shown in the two-drug combination treatment group indicates significant differences in averaged ABR threshold shifts between the two-drug combination treatment group and other treatment groups [4-OHPBN(20), NAC(325), ALCAR(100)] at $p<0.001$ while the symbol # shown in the three-drug combination treatment group shows significant differences between the three-drug combination treatment group and other treatment groups [4-OHPBN(20), NAC(325), ALCAR(100)] at $p<0.001$ and the three-drug combination treatment group and another treatment group [4-OHPBN(50)] at $p<0.05$. Data for NAC (325 mg/kg) and ALCAR (100 mg/kg) were excerpted from Coleman et al. [40].

reduced compared to each experimental group while there was no significant difference between the two-drug combination and the three-drug combination treatment groups. The mean PTS of the two-drug combination decreased by 75% compared to 4-OHPBN alone (50 mg/kg) while the mean PTS of the three-drug combination decreased by 91% compared to 4-OHPBN alone (20 mg/kg). The present study suggests that the two-drug and three-drug combination treatment groups have greater efficacy when compared to each antioxidant drug alone and at lower dosages based partly on historical data. The findings of this study are consistent with those of a combination of NAC and salicylate, a hydroxyl radical scavenger, reporting a 17 dB PTS reduction [16] and a combination of salicylate and Trolox, a water soluble analog of α-tocopherol and an inhibitor of peroxynitrite-mediated tyrosine and guanine nitrosylation, showing approximately 18 and 15 dB reductions in threshold shifts for treatment groups beginning 1 h and 1 day, respectively, after noise exposure (average at 4–16 kHz) [32]. This suggests that AAT is a result of multiple injury pathways and that a combination of antioxidant drugs can produce a synergistic effect in the treatment of AAT since each antioxidant drug may address different injury mechanisms [5,16,17,32].

NAC may be involved in preventing a variety of mechanisms associated with AAT [5,15–17,40]. NAC has been known to act as a ROS scavenger and function as a neuroprotective agent by increasing levels of glutathione in the face of oxidative stress. Additionally, NAC has been shown to reduce mitochondrial injury, reduce caspase, mitogen-activated protein kinase/C-Jun kinase, and Src protein tyrosine kinase activation, and inhibit glutamate excitotoxicity, lipid peroxidation, programmed cell death, inflammation, and necrosis [17]. The preclinical data reviewed by Kopke et al. demonstrated that NAC functions as a strong protectant from AAT [17]. However, the protective effects of ALCAR are mainly related to mitochondrial injury. ALCAR has been reported to prevent mitochondrial injury that may result from either overproduction of ROS or other damage such as glutamate excitoxicity, ischemia–reperfusion, or GSH depletion by oxidative stress [14,15,40,89]. ALCAR can serve as a precursor for acetyl-CoA, a mitochondrial energy substrate, and L-carnitine that can shuttle lipid substrates into mitochondrial substrates for energy generation and oxidation [15,40,90]. Furthermore, ALCAR can achieve its protective effect on mitochondria by reducing mitochondrial injury [14], restoring important mitochondrial lipids, such as carnitine and cardiolipin [14,40], increasing reduced GSH and coenzyme Q10 [40], maintaining normal morphological and structural integrity of mitochondrial membranes and mitochondrial density [40], enhancing the activities of cytochrome *c* oxidase and DNA transcription [91], and mimicking acetylcholine, a neurotransmitter [12,35,92,93]. It has been reported that the administration of ALCAR significantly reduced noise-induced PTS [14,15,40].

Kopke et al. [16] and Yamashita et al. [32] provide important insight on the timing of drug administration which will be applicable to future clinical studies of 4-OHPBN alone and in combinations with other antioxidant drugs. These authors suggest that early administration of certain antioxidant drugs prior to noise exposure as well as up to 3 days following noise exposure was effective in reducing PTS. Coleman et al. reported that the initial administration of NAC and ALCAR could be given 1–12 h after 6 h of steady-state noise exposure [40]. The greatest reduction in threshold shift was observed when the administration was initiated 1 h after noise exposure. In addition, there was a significant reduction in PTS when the first treatment was given 4 h after noise exposure. However, there was no significant PTS reduction when the initial treatment was started 12 h after noise exposure. Therefore, the initial administration started 12 h after noise exposure may be too late to counter the first oxidative burst. Yamashita et al. showed the possibility of a secondary oxidative burst in 7–10 days after noise exposure [12,32]. If so, pharmacological treatment extended up to 10 days may counter the secondary oxidative burst. The use of 4-OHPBN alone or in combination with other antioxidant drugs extended over the period of 7–10 days after noise exposure may provide clinically useful information of its treatment effect on the secondary oxidative burst.

It should be noted that the reductions of ABR threshold shifts shown in the 4-OHPBN treatment groups and drug combination treatment groups were not directly correlated with OHC loss at the cellular level. The drug combination treatment groups showed a greater reduction in ABR threshold shifts than the 4-OHPBN treatment groups (Fig. 3) whereas more OHC loss was observed in the drug combination treatment groups than the high doses of 4-OHPBN (Fig. 8). This discrepancy may occur due to hair cell injury or other cochlear impairment such as damage to stereocilia, changes in F-actin level, and reduction in energy metabolism [15,16,41,93], or enhanced cochlear function in the remaining hair cells in the drug combination treatment groups [66,94–96], or the relatively different contributions of NAC and ALCAR to the functions of OHCs and IHCs [15].

## Acknowledgments

This study is supported by grants from the Office of Naval Research and the INTEGRIS Baptist Medical Center, Oklahoma City, Oklahoma, and by the research fund provided to Dr. Floyd who holds the Merrick Foundation Chair in Aging Research from the Merrick Foundation.

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