Research paper

Antioxidant treatment reduces blast-induced cochlear damage and hearing loss

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Abstract

Exposure to blast overpressure has become one of the hazards of both military and civilian life in many parts of the world due to war and terrorist activity. Auditory damage is one of the primary sequela of blast trauma, affecting immediate situational awareness and causing permanent hearing loss. Protecting against blast exposure is limited by the inability to anticipate the timing of these exposures, particularly those caused by terrorists. Therefore a therapeutic regimen is desirable that is able to ameliorate auditory damage when administered after a blast exposure has occurred. The purpose of this study was to determine if administration of a combination of antioxidants 2,4-disulfonyl \( \text{-phenyl tertiary butyl nitrone} \) (HPN-07) and \( \text{N-acetylcysteine} \) (NAC) beginning 1 h after blast exposure could reduce both temporary and permanent hearing loss. To this end, a blast simulator was developed and the operational conditions established for exposing rats to blast overpressures comparable to those encountered in an open-field blast of 14 pounds per square inch (psi). This blast model produced reproducible blast overpressures that resulted in physiological and physical damage to the auditory system that was proportional to the number and amplitude of the blasts. After exposure to 3 consecutive 14 psi blasts 100% of anesthetized rats had permanent hearing loss as determined at 21 days post exposure by auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) testing. Animals treated with HPN-07 and NAC after blast exposure showed a significant reduction in ABR threshold shifts and DPOAE level shifts at 2–16 kHz with significant reduction in inner hair cell (IHC) and outer hair cell (OHC) loss across the 5–36 kHz region of the cochlea compared with control animals.

The time course of changes in the auditory system was documented at 3 h, 24 h, 7 day and 21 day after blast exposure. At 3 h after blast exposure the auditory brainstem response (ABR) threshold shifts were elevated by 60 dB in both treated and control groups. A partial recovery of to 35 dB was observed at 24 h in the controls, indicative of a temporary threshold shift (TTS) and there was essentially no further recovery by day 21 in the treatment group. Distortion product otoacoustic emission (DPOAE) reached a maximum level shift of 25–30 dB measured in both control and treated groups at 3 h after blast exposure. These levels did not change by day 21 in the control group but in the treatment group the level shifts began to decline at 24 h until by day 21 they were 10–20 dB below that of the controls. Loss of cochlear hair cells measured at 21 day after blast exposure was mostly in the outer hair cells (OHC) and broadly distributed across the basilar membrane, consistent with the distribution of loss of frequency responses as measured by ABR and DPOAE analysis and typical of blast-induced damage. OHC loss progressively increased after blast exposure reaching an average loss of 32% in the control group and 10% in the treated group at 21 days. These findings provide the first evidence that a combination of antioxidants, HPN-07 and NAC, can both enhance TTS recovery and prevent PTS by reducing damage to the mechanical and neural components of the auditory system when administered shortly after blast exposure.

Abbreviations: ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emission; SNHL, sensorineural hearing loss; NAC, N-acetylcysteine; HPN-07, 2,4-disulfonyl \( \text{-phenyl tertiary butyl nitrone} \); OHC, outer hair cell; IHC, inner hair cell; SPL, sound pressure level; PTS, permanent threshold shift; TTS, temporary threshold shift.

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1. Introduction

Experimental studies of blast injury in animals have indicated that auditory system is one of the most vulnerable systems to blast overpressure. The extreme physical force of the blast can rupture the tympanic membrane (TM), disarticulate the ossicular chain, fracture the ossicles, tear the IHCs and OHCs away from the basilar membrane and distort and break the ciliary projections of the hair cells (Patterson and Hamernik, 1997; Roberto et al., 1989; Hamernik, 1987). The incidence of TM rupture in studies of blast-exposed patients ranged from 17 to 29% (Garth, 1994; Jensen and Bonding, 1993; Patterson and Hamernik, 1997; Xydakis et al., 2007; Gondusky and Reiter, 2005). However, despite the potential for extensive cochlear damage, blast-induced hearing loss resulting from exposure to terrorist or military explosions resulted in moderate to severe sensorineural hearing loss (SNHL) not anacusis (Nageris et al., 2008). Permanent SNHL is the most prevalent type of auditory impairment associated with blast trauma of military personnel, with rates ranging from 33% to 78% (Hoffer and Balaban, 2010; Gondusky and Reiter, 2005; Cave et al., 2007; Fausti et al., 2009; Nageris et al., 2008).

The mechanisms of blast-induced hearing loss are not well understood. In addition to the mechanical damage to components of the auditory system, molecular and cellular processes are triggered by the blast that initiate molecular cascades that are responsible for much of the long-term damage (Patterson and Hamernik, 1997). Activation of cell death pathways and molecular mediators of inflammation cause secondary damage to the hair cells and supporting cells of the cochlea, as evidenced by cellular degeneration in the cochlea and accumulation of lymphocytes and macrophages in the scala tympani and perilymph (Patterson and Hamernik, 1997; Hoffer and Balaban, 2010). Increased oxidative stress is associated with both steady state noise-induced and impulse noise-induced hearing impairment (Ohlemiller et al., 1999; Henderson et al., 2006).

Administration of antioxidants has been shown to block some of the non-auditory pathological outcomes of exposure to blast overpressure or other impact injury in animal models: 1) N-acetylcysteine amide was found to reduce the inflammatory response and infiltration of neutrophils in the lungs of chinchillas (Chavko et al., 2009); 2) N-acetylcysteine (NAC) was shown to restore mitochondrial electron transfer, energy coupling capacity, calcium uptake activity and reduced calcium content absorbed to brain mitochondrial membranes of rats following cortical impact (Xiong et al., 1999); 3) NAC was shown to attenuate the inflammatory response in the injured rat brain (Chen et al., 2008); and 4) 2,4-disulfonyl n-phenyl tertiary butyl nitrone (HXY-059, now called HPN-O7) was found to reduce loss of injured brain tissue and improve cognitive function when administered to rats after percussion induced brain injury (Clausen et al., 2008).

Oxidative stress has been associated with pathological processes involved in auditory trauma including: mitochondrial injury, activation of cell death pathways, activation of mediators of inflammation, glutamate excitotoxicity, and increased levels of lipid peroxidase (Abi-Hacehm et al., 2010; Clausen et al., 2008; Haase et al., 2011; Elsaayed et al., 2000; Elsaayed and Gorbunov, 2003; Kopke et al., 2007; Chavko et al., 2009; Wu et al., 2006). These findings suggest that antioxidants have the potential to block the molecular cascades that are triggered by the auditory trauma which induces oxidative stress and results in permanent threshold shifts (PTS) and hearing loss.

Studies of auditory physiology have demonstrated a protective effect of antioxidants against hearing loss resulting from auditory trauma. Administering three antioxidant compounds, 4-hydroxy phenyl N-tert-butyl nitrone (4-OHPBN) and NAC, and acetyl-L-carnitine (ALCAR) 4 h following acute steady state noise [105 dB, sound pressure level (SPL)], prevented noise-induced hearing loss (NIHL) in 100% of the test animals (Choi et al., 2008). Similarly, treatment with two antioxidants, either ALCAR or NAC, significantly reduced PTS in chinchillas exposed to impulse noise, 155 dB SPL (Kopke et al., 2005). The level of exposure in these studies was comparable to repeated exposure to rifle fire.

The question addressed here is whether a similar combination of antioxidants could decrease damage to the auditory system caused by blast overpressure levels (>14 psi, corresponding to 194 dB SPL) which exceed that of steady state noise or impulse noise in previous experiments. We chose a combination of two antioxidants for this study, NAC and HPN-O7. NAC, which functions to increase the intra-cellular pool of the antioxidant glutathione, has been approved by the FDA for more than two decades to treat liver necrosis from acetaminophen overdose (Kopke et al., 2007). HPN-O7 is a free radical spin-trapping agent that has shown activity as a neuroprotectant, and inhibits iNOS, decreases glutamate excitotoxicity, and may decrease cell death (Floyd et al., 2008). HPN-O7 has been tested in multiple clinical stroke trials and found to be very safe (Chen et al., 2008; Lyden et al., 2007; Nilsson et al., 2007).

2. Methods

2.1. Blast simulator

To study the effects of antioxidants on blast-induced hearing loss a blast simulator was developed to simulate the expanding blast wave of an open-field blast. The blast wave generator consists of a steel pressure reservoir with a film inserted between it and a one inch blast nozzle. A pneumatic piston pushes the chamber against the film, sealing the chamber. Compressed nitrogen is delivered to the pressure reservoir until the burst strength of the plastic film is exceeded, rupturing the film and producing a blast shock/pressure wave. The pressure chamber was retracted and the ruptured film replaced with pre-cut sheets of film and the process repeated for subsequent exposures. The interval between each consecutive exposure was set at 1.5 min to allow adequate time to replace the film and recharge the system. The polycarbonate film (McMaster-Carr, Atlanta, GA) produced highly reproducible blast pressure waves that scale linearly with film thickness. Pressures from the blast were monitored using a high-frequency piezoelectric pressure transducer with the face of the sensor facing the blast which was amplified by a Piezotronic 482A21 Signal Conditioner (PCB Piezotronics, Depew, NY) and analyzed by a PicoScope 3000 oscilloscope (PCB Piezotronics, Depew, NY). The magnitude of the resulting blast pressure wave is a function of the thickness and composition of the film and thus the pressure required to rupture the film. The 0.005 in. polycarbonate plastic film used in these studies produced a blast with a peak pressure of approximately 14 psi (13.7 ± 0.8 SD) lasting 1.0 (±0.1 SD) microseconds at 10 cm from the face of the blast nozzle. These values were based on 15 independent tests using a pressure sensor (PCB Piezotronics, Depew, NY) positioned normal to the blast source and at the same position, 10 cm from the face of the blast nozzle, as the head of the experimental subjects. A typical pressure-time history is shown in Fig. 1. These conditions closely simulate those of an open-field blast in that the pressure waves radiate from the site of blast initiation into an open space where they strike the subject.

2.2. Animals/blast exposure

Male Long-Evans pigmented rats (with body weights between 360 and 400 g, Harlan Laboratories, Indianapolis, Indiana) were used in this study. Animals were evaluated by the attending
A holding tube was used to protect the body of the rat, below the neck from the force of the blast and to position the head of each rat in the opening to the blast chamber.

Fig. 1. Pressure-time history recorded 10 cm from the face of the blast chamber. The surface of the piezoelectronic pressure sensor was oriented normal to and centered on the opening to the blast chamber.

veterinarian upon arrival and monitored on a daily basis by a trained staff of laboratory animal technicians. The animals were kept on a normal day/night cycle at 21 °C with free access to food (Laboratory Rodent Diet 5001, LabDiet/Purina Mills Inc., Richmond, IN) and water and allowed to acclimate to the environment before being used for the experiments. The animals were housed in a vivarium at the Oklahoma Medical Research Foundation (OMRF) which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and routinely inspected by the US Department of Agriculture. All procedures regarding the use and handling of animals were reviewed and approved by the OMRF Institutional Animal Care and Use Committee and the US Department of the Navy Office of Naval Research. Variability in hair cell density, ear canal length and volume, and other physical differences that may exist in outbred rats was assumed to be randomly distributed between experimental groups. Additionally, rats for each paired experimental group (control and treated) were from the same birth date and rats was assumed to be randomly distributed between experimental groups. Repeated blast exposures (2, 3 or 4) were given at 1.5-minute intervals. Following the final exposure the outer ears of each rat were examined using a surgical microscope to assess the condition of the TM. Ears having ruptured TMs were excluded from the study.

2.3. Auditory brainstem responses (ABRs)

ABR thresholds were obtained prior to blast exposure and at 3 h, 24 h, 7 d, and 21 d after exposure. Prior to testing, the rats were anesthetized with an intramuscular injection of 50 mg/kg of ketamine and 6 mg/kg of xylazine. Throughout the duration of the recording, supplemental injections of anesthetic agents at approximately half the induction dose were provided as required to maintain sedation. While under anesthesia, the rats were placed on a heating pad and their temperature monitored with a rectal thermistor probe and automatically maintained at 37 °C by a HC temperature controller (HFC-40908, HFC, Bowdoinham, ME). ABR testing was conducted in a sound-attenuated, electrically shielded booth. The stimulus presentation, ABR acquisition, equipment calibration and data management were coordinated using a computerized Intelligent Hearing Systems (IHS) with the Smart-EP software 3.96. ABRs were recorded using three stainless steel needle electrodes (M019258, IHS, Miami, FL) placed subdermally over the vertex (noninverting electrode) and the ipsilateral and contralateral mastoids (inverting and ground electrodes) of the animal. The biological signals were amplified 100,000 times and band-pass filtered between 100 and 3000 Hz with an artifact rejection of 15—20 μV. The amplified responses were averaged and displayed on the computer screen with a recording epoch of 10 ms following stimulus onset. Acoustic stimuli were generated by the PC-based IHS and presented via two high-frequency transducers (M014600, IHS, Miami, FL), the end of which was fitted with a rubber tip that could allow the ABR stimuli to be delivered into the sealed ear canal. ABR thresholds for tone bursts (5-ms duration with 0.5-ms rise/fall times gated using a trapezoidal envelope) in one-octave steps ranging from 2 to 16 kHz were determined visually by reducing the stimulus intensity from the suprathreshold level in 5 dB SPL steps and were defined as the lowest dB level at which peak 2 of the ABR waveform could be positively identified. Each ABR waveform represented the average response to 256 stimulus presentations at a rate of 11/s, and the final analyzable ABR waveform was determined by averaging the waveforms from two consecutive ABR trials. ABR threshold shifts were calculated by subtracting pre-exposure from post-exposure thresholds.

2.4. Distortion product otoacoustic emissions (DPOAEs)

DPOAE levels (amplitudes in dB SPL) were measured prior to blast exposure and at 3 h, 24 h, 7 d, and 21 d after exposure. DPOAE testing was conducted in the same booth, and the rats were prepared for the procedure as they were for ABR testing. By using the PC-based IHS with the Smart OAE software 4.54, DPOAE measurements were performed for pure tones from 2 to 16 kHz. A single ear probe contained an Etymotic ER-10B + ear canal microphone for DPOAE recording and had a soft rubber tip that served to seal the ear canal from external noise; while the ear probe was connected to the two IHS high-frequency transducers for delivery of the stimulus into the ear canal. Cubic 2f1–f2 DPOAE levels were recorded using two primary tones, f1 and f2 (ratio (f2/f1) = 1.22), presented with f1 and f2 primary tone levels of L1/L2 = 70/60 dB SPL. Suprathreshold DPOAE levels were defined as the signal/noise ratio of the 2f1–f2 distortion product for the 70 dB...
and 60 dB SPL f1 and f2 primaries, respectively, and were calculated by subtracting the 2f1−f2 distortion product from the surrounding noise. The noise was defined as the average of the 10 neighboring frequencies (5 above and 5 below the 2f1−f2 distortion product). Each distortion product was the average response of 50 stimulus pair presentations. DP-grams (DPOAE level vs. stimulus frequency) were recorded with a resolution of five points per octave in the f2 frequency range of 2–16 kHz. DPOAE level shifts were calculated by subtracting post-exposure from pre-exposure levels.

2.5. Histological examination

The rats were euthanized with Euthasol solution (Virbac AH, Inc., Fort Worth, Texas) under deep anesthesia 3 weeks after blast exposure. Cardiac perfusion was performed with normal saline followed by the fixative 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.2. Cochleae were then removed and post-fixed in the same fixative overnight at 4 °C, and then transferred to PBS and stored in the buffer until cochleae were prepared for surface preparation and hair cell counts. Cochlear microdissection was accomplished under a light microscope. After treating with 0.3% Triton X-100 in PBS for 10 min, the organ of Corti was incubated with 30 μg/ml solution of phalloidin (Cayman Chemicals, Ann Arbor, MI) at room temperature for 30 min in the dark; each OC specimen was flat mounted in an anti-fade medium on glass slides and was examined under a fluorescence microscope (Olympus BX51, Melville, NY) with 40× magnification. The IHCs and OHCs were counted from the apex to the base in 500 μm segments and the percentage of missing hair cells plotted as cytocochleograms.

2.6. Drug administration

A 20% solution of NAC was purchased from Hospira, Inc. (Lake Forest, IL) and HPN-07 was synthesized and provided by APAC Pharmaceuticals LLC (Columbia, MD). Animals in the treated group were injected intraperitoneally (i.p.) with a combination of 300 mg/kg of NAC plus 300 mg/kg of HPN-07, which were both dissolved in 5 ml/kg of physiological saline solution. Drug administration was started 1 h after blast overexposure and then continued twice a day for the following two days. Rats in all groups underwent ABR and DPOAE testing before blast exposure and at 21 days post blast exposure. These preliminary experiments indicated that 3-blast exposures produced a significant level of permanent ABR threshold shifts that was sufficient to test the effects of drug treatment with minimal incidence of TM rupture.

Having established the experimental blast exposure conditions a study was undertaken to examine the time course of the effects of blast overpressure on auditory physiology and cochlear structure and to determine the effects that antioxidant treatment has on any observed changes. After ABR and DPOAE testing and exposure to three 14 psi blasts rats were randomly assigned to the treatment group or the control group. Each group of control and treated rats was designated for terminal analysis at 3 h, 24 h, 7 or 21 days at which time ABR and DPOAE analysis was performed. Following the final recording session all animals were euthanized and intracardially perfused for cochlear collection. Six cochleae from each terminal group were processed for hair cell counting and the remainder for biomarker studies (results to be reported elsewhere). Rats in groups beyond the 3 h point underwent ABR and DPOAE testing at all interim time points before their terminal 21 day testing.

2.8. Statistical analysis

Means ± standard error of the mean (SEM) were calculated for all parameters measured. Data analyses were based upon analysis of variance (ANOVA). Comparisons of group means were performed via separate two-way ANOVA (treatment or number of blast exposures × test frequency) for an analysis of the main effect of drug treatment or blast exposure number on the overall hearing sensitivity in the range of 2–16 kHz, as assessed by ABR and DPOAE testing. When a significant between-group difference in ABR threshold shift or in DPOAE level shift was found with the overall frequency range analyses, the Bonferroni post-hoc test was performed for a pairwise comparison at each test frequency. Hair cell counting data was analyzed by one-way ANOVA followed by Tukey HSD post hoc comparisons. Statistical analyses were accomplished using commercially available software GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) and p < 0.05 was considered significant.

3. Results

3.1. Blast exposure conditions

Preliminary studies showed that a single exposure with high-level blast overpressures (>20 psi) consistently produced permanent hearing loss as well as rupture of the TM. At moderate blast levels (<15 psi) ABR threshold shifts at 21 d post exposure as well as the frequency of TM ruptures correlated with the number of exposures in a dose dependent manner. Rats were subjected to repeated (2, 3, and 4 times), blast overpressures of 14 psi with a 1.5 min interval between exposures. The incidence of tympanic membrane (TM) rupture at 2, 3, and 4 blasts was 0% (0/20 ears), 10% (2/20 ears) and 50% (10/20 ears) respectively. ABR measurements of threshold shifts averaged across 2–16 kHz were performed at 21 days after blast exposure on ears that had not sustained a ruptured TM (Fig. 2). The level of threshold shifts was proportional to the number of blast (3 or 4) exposures at 14 psi. No significant threshold shift was produced by two blast exposures. A 3-blast exposure caused an averaged 30-dB shift and a 4-blast exposure resulted in an averaged 42-dB shift. These threshold shift values were significantly different (p < 0.05, F(1,72) = 6.708). Based on the ability to consistently produce permanent hearing loss in rats but with a low incidence of damage to the TM it was decided to use the 3-blast exposure condition at 14 psi to study the effect of
antioxidant treatment at intervals during the 21 days following blast exposure.

3.2. ABR threshold shifts

ABR threshold shifts from treated and control ears were plotted to show the time course of the functional recovery and drug effect after exposure to three, 14 psi blasts (Fig. 3). Initial threshold shifts measured 3 h after acoustic trauma were elevated to the same level, 60 dB, in both control and drug treatment groups indicating that animals in both groups had a similar amount of blast-induced hearing loss, and that no effects of treatment could be detected by ABR analysis at this time point. ABR threshold shifts decreased by 25 dB—35 dB at 24 h in the control group, indicative of a temporary threshold shift (PTS). The threshold shifts remained unchanged at the terminal 21 day time point indicative of a permanent threshold shift (PTS) and hearing loss in blast-exposed untreated control animals. In contrast, the ABR threshold shifts in the drug treatment group were 10 dB lower than that of the control group at 24 h and continued to decline reaching a maximum threshold shift reduction of about 20 dB by day 7 post blast exposure. ABR threshold shifts at 2, 4, 8 and 16 kHz show that initial threshold shifts as well as the rate and effect of drug treatment on recovery of threshold shifts relative to controls were similar across of all measured frequencies (Fig. 4). These results suggest that NAC/HPN-07 was able to increase the rate of functional auditory recovery (TTS) and significantly reduce blast-induced permanent hearing loss (PTS).

3.3. DPOAE loss and DPOAE level shifts after blast exposure

DPOAE level shifts in response to the two primary tones at L1, L2 = 70, 60 dB SPL and at F2/F1 = 1.22 in the range of 2—16 kHz were recorded in both treated and control groups. The bar graph (Fig. 5) shows the percentage of ears with complete loss of DPOAE response in ears at 21 days following exposure to 3 and 4 blasts. In the control group 3 exposures to 14 psi blast overpressure caused DPOAE loss in 56% of ears and 4 exposures caused a 75% loss. In contrast, only 20% of ears treated with the antioxidants showed a complete loss in DPOAE response after 3 or 4-blast exposures.

The frequency distribution of DPOAE level shifts at 3hr, 24hr, 7 day and 21 day in ears with detectable DPOAE responses is shown in Fig. 6. Initially no significant differences were seen in DPOAE level shifts between treated and control ears. However, at seven days after exposure, rats in the treatment group showed a significant decrease in level shift in the higher frequency range of 4—16 kHz, and 21 days after blast exposure a significant reduction in shift was observed in the treatment group for all test frequencies. There was no indication of recovery of DPOAE levels in the control group between the initial 3 h reading and 21 days after blast exposure. The broad distribution and level of DPOAE level shifts across all measured frequencies did not appreciably change in the control groups from the initial measurement at 3 h to 21 days post blast exposure. Antioxidant treatment significantly reduced the amount of DPOAE level shifts by an average of 16 dB across all frequencies.

3.4. Histology

Upon initial dissection bleeding was observed in 40% of the untreated and 10% of the treated blast-exposed ears at the apical or middle turns. The blood was most often seen in the areas of the scala vestibule and less often in the scala media. The cochlear pathology included scar formation replacing dead hair cells, fused and damaged stereocilia, and in some extreme cases, separation of the organ of Corti from the basilar membrane. Most of the hair cell loss was in the OHC region and generally in the middle or the basal turn. Although hair cell loss was found in the apical turn, the damage was less. Cytocochleograms show missing OHCs (Fig. 7) and IHCs (Fig. 8) along the length of the basilar membrane in surface preparations of each cochlea collected at 21 days after exposure to 3 blasts. In the control cochleae, 28—53% OHC loss for exposure to 3 blasts was found in the area located between 21 and 83% of the distance from the apex. This area corresponded to hair cells responding to 5—36 kHz frequencies. Consistent with the physiological data, significantly fewer OHCs were missing in animals treated with NAC plus HPN-07 compared to controls (both p-values < 0.001, from F (1, 72) = 266.1 for 3 blasts and F (1, 84) = 245.4 for 4 blasts, respectively): with an average of 0.3—5% loss for 3 blasts and 2—16% loss for 4 blasts in treated animals compared to 40% and 50% respectively for the controls (see Figs. 7 and 9). Although relatively few IHCs were found missing, the difference between the average number of missing cells in the treated ears was found to be significantly lower than in control ears (p < 0.001, F (1,108) = 19.1) (Fig. 8). Ears exposed to 4 blasts showed...
an average higher level of hair cell loss at 21 days post exposure and higher variability between ears (Fig. 9) than ears exposed to 3 blasts. However, as in the 3 blast group, a significant reduction of hair cell loss was observed in the treated animals. Photomicrographs of the cochlear hair cells show the concentration of hair cell loss in the OHC region (Fig. 10A, B) and the almost normal appearance of the hair cells (Fig. 10C, D) in the treated cochlea.

4. Discussion

4.1. Blast conditions producing permanent hearing loss

A variety of methods have been used to generate blast overpressure ranging from the use of actual weaponry (Ylikosi, 1987; Price et al., 1989), or explosive charges (Cheng et al., 2010), or pressure discharges from ruptured film (Jaffin et al., 1987) either in an open space (Long et al., 2008; Svetlov et al., 2010), or an enclosure (shock tube) (Hoffer and Balaban, 2010). Published reports on blast pressure-time history recordings vary in the suddenness of the onset of pressure rise, magnitude of the pressure peak and the duration of the peak overpressure and the presence of a trailing underpressure before returning to ambient pressure. However, the reports have in common the distinguishing characteristic of a wave of blast overpressure (above atmospheric pressure) that suddenly rises and falls, lasting for less than a few milliseconds. These characteristics of a blast overpressure overlap with those of impulse noise which typically have overpressures of less than 1 psi, lasting about 1 ms (Hamernik, 1987; Kopke et al., 2005).

Our primary goal was to produce blast conditions that produced reproducible damage to the auditory system so that the effects of therapeutic intervention could be detected. The blast simulator used in this report produces a signature pressure-time history with overpressure lasting about 1 ms followed by a slight underpressure phase before returning to ambient pressure, similar in form to what has been reported in the literature (Fig. 1) (Hoffer and Balaban, 2010; Hamernik et al., 1991; Svetlov et al., 2010). Both the duration 1.0 ms (±0.1 SD) and amplitude 14 psi (13.7 ± 0.8 SD) of the pressure wave were highly reproducible.

Although control of the physical parameters of the blast is an essential requisite for these studies, the animal itself can be a major source of variability. As in real life situations the orientation of the head and distance from the blast source can affect the impact of the blast overpressure on the auditory system and contribute to experimental variability. Previous studies showed that the animals were tethered (Kopke et al., 2005) placed in a restraint tube (Hoffer and Balaban, 2010), secured to a rigid platform (Hamernik, 1987), or had no restraint (Jaffin et al., 1987), or the type of restraint was not mentioned (Ylikosi, 1987; Roberto et al., 1989). We attempted to minimize this source of variability by placing the anesthetized animals in a restraining tube that allowed each rat to be positioned with its head and ears in the same orientation relative to the blast source.

For the purpose of this study the focus was on the primary source of damage from vestibular transmission or cranial transmission of the blast pressure to the cochlea. It is possible that the cochlear hair cells could be affected by increased venous pressure or inflammatory cytokines produced by blast overpressure in the
tissues in the torso that are transmitted to the cochlea via the circulatory system. By placing the rat in a restraining tube that shielded its torso from the blast overpressure this source of potential damage to the cochlea was minimized. Less than 0.05 psi of blast pressure could be detected at the level of the abdomen of an animal in the tube exposed to a blast wave of 14 psi. Also, damage to organs such as the lungs, kidneys and heart that have been shown to result from whole body exposures to blast overpressures (Elsayed et al., 2000; Elsayed and Gorbunov, 2003) was minimized. Another potential source of variability was eliminated by excluding ears with ruptured TMs from the study, as discussed above. The break-point of each TM may vary depending on the physical condition of the membrane, which can be affected by inflammation, or mechanical damage (e.g., prior blast exposure). Therefore, restricting the physiological and structural analyses to ears with intact TMs eliminated a source of variability in the conduction of the blast force to the cochlea between rats. Restricting the study to ears with intact TMs also permitted analysis of the contribution of both conductive and sensorineural transmission of sound by ABR and DPOAE analysis beginning at 3 h after blast exposure. Otherwise testing could not be performed until the TM had healed in approximately 2 weeks.

In conclusion, the blast model described herein meets the criteria described for good models by Cernak and Noble-Hauesslein (2010) for head trauma: 1) The mechanical force (blast) is controlled, reproducible and quantifiable; 2) The injuries as measured by ABR threshold shifts, DPOAE level shifts and cochlear hair cell loss are reproducible under controlled and quantifiable conditions; 3) The physical properties of the blast (amplitude and number of blast exposures) correlate with severity and nature of injury.

4.2. Blast damage to cochlea

The damage to the physical structure of the auditory system observed in these studies is consistent with that observed in other species exposed to multiple exposures of impulse noise (Ylikosi, 1987; Garth, 1994; Maj and Chait, 1989; Richmond et al., 1989; Patterson and Hamernik, 1997; Roberto et al., 1989; Hamernik, 1987). The incidence of damage to the TM and damage and loss of cochlear hair cells reported here was proportional to the intensity of the blast and the number of exposures. The majority of hair cell loss we observed was in the OHC region and distributed across the length of the cochlear basilar membrane (5–44 kHz) consistent with findings in studies of sheep and pigs exposed to repeated (100) pulses of 160 dB SPL impulse noise (Roberto et al., 1989), and chinchillas exposed to 150 repetitions of 155 dB SPL impulse noise (Kopke et al., 2005), or chinchillas exposed to 100 impulses of 147 dB SPL impulse noise (Hamernik, 1987). It is not surprising that the latter studies employing impulse noise required animals to be exposed to 100–150 repeated exposures to obtain comparable levels of cochlear hair cell loss or PTS as were obtained in this study with 3 exposures to a 14 psi blast (194 peak dB SPL) due to the lower peak pressure output and thus energy content of the impulse noise. By further comparison, chinchillas exposed to 105 dB SPL octave-band noise centered at 4 kHz for 6 h show a comparable level of missing OHCs at 21 days post exposure (Choi et al., 2008) as found in this study of rats exposed 4 times to 14 psi blasts.

4.3. Effect of blast overpressure on hearing loss

Although there is considerable audiometric data on blast related hearing loss in humans there are only a few reports of similar studies in animal models (Fausti et al., 2009; Hamernik, 1987; Kopke et al., 2005). PTS of 37–43 dB was documented at 31 days post exposure in a study of chinchillas exposed to repeated impulses (100) high intensity noise (139–147 dB SPL) (Hamernik, 1987). Similarly, PTS of 30 dB at 1 kHz to 60 dB at 6 kHz, were obtained by exposure of chinchillas to 150 impulses of 155 dB SPL noise (Kopke et al., 2005).

We observed a dose dependent relationship between the number of blast exposures and the level of hearing loss, as evidenced by ABR threshold shifts (Fig. 2.) and DPOAE level shifts.
By fine-tuning the conditions to minimize physical damage to the TM an average ABR threshold shift at 21 days of 30 dB was obtained with 3 exposures to 14 psi blasts. Four exposures produced an average ABR threshold shift of about 40 dB but also increased the incidence of TM ruptures. This dose-related increase in ABR threshold shifts and DPOAE level shifts may reflect progressive injury to the TM, e.g. stretching and swelling, with each blast. DPOAE data for 3-blast exposures taken at 21 days showed a level shift of about 25 dB between 2.5 and 13.3 kHz indicative of the extensive damage to the cochlea caused by the blast trauma (Fig. 6). These physiological data are consistent with the cochlear hair cell loss of 40% between 5 and 44 kHz (Fig. 7). Also, ABR threshold shifts reached similar levels across 2–16 kHz (Fig. 4), consistent with the distribution of outer hair cell loss.

4.4. Effects of antioxidants

Data from three sources ABR, DPOAE and cochlear hair cell counts support the finding that administration of HPN-07 and NAC after blast exposure significantly reduces the permanent structural

Fig. 7. Percent OHC loss as a function of percent distance (frequency place in kHz) from the OC apex at 3 h, 24 h, 7 d and 21 d after exposure to three blasts of 14 psi in the control and treated groups. The data are plotted as mean ± SEM. n represents the number of cochleae. Vertical arrows at right of figures show comparison of mean values for all control and treated readings for groups where there was a significant difference. Data analysis was performed by one-way ANOVA followed by Tukey HSD post hoc comparisons: *p < 0.05 (F(7, 47) = 2.37–2.67); **p < 0.01 (F(7, 47) = 5.33–6.84); ***p < 0.001 (F(7, 863) = 34.21). OHC loss progressively increased in the control group while the loss remained constant at a low level in the treated group over the 21 days after blast exposure.

Fig. 8. Percent IHC loss as a function of percent distance (frequency place in kHz) from the OC apex at 3 h, 24 h, 7 d and 21 d after exposure to 4 blasts in the control and treated groups. The data are plotted as mean ± SEM. n represents the number of cochleae. Vertical arrows at right of figures show comparison of mean values for all control and treated readings for groups where there was a significant difference. Data analysis was performed by one-way ANOVA followed by Tukey HSD post hoc comparisons: *p < 0.05 (F(7, 47) = 2.56–2.64); ***p < 0.001 (F(7, 863) = 12.16). IHC loss increased with time across a broad range of frequencies in the control group but was nearly absent at 21 days in the treated group.
and functional damage that results from exposure to blast over-pressure. The DPOAE analysis and hair cell counts show that the protection is nearly complete and extends across the range of auditory frequencies (2–16 kHz) (Figs. 6 and 7). ABR data show that at 21 days following blast trauma there was about a 20 dB reduction of average threshold shifts across 2–16 kHz in the antioxidant treated group relative to untreated animals. These results are similar to those obtained in a study of the effects of the antioxidants ALCAR and NAC on impulse noise-induced hearing loss in chinchillas (Kopke et al., 2005). Permanent threshold shifts at 21 day post exposure in chinchillas exposed to repeated noise impulses were reduced by 10–20 dB in the treated groups relative to the untreated group. Thus the level of antioxidant protection against permanent hearing loss produced by blast trauma was similar to that found in studies of impulse noise despite the fact that the energy level of the insult and the number of exposures were quite different.

The level of OHC loss observed in this study was also similar a study of chinchillas exposed to 6 h of octave band steady state noise at 105 dB SPL (Choi et al., 2008). The loss of an average of 43% OHC observed at 21 days after noise exposure was essentially the same observed in rats exposed 3 times to a 14 psi blast in this study. A similar level of protection as seen in this study was observed with administration (3 days) of the antioxidant 4-hydroxy PBN plus NAC and ALCAR, reducing the level of hair cell loss by 95% compared to controls as reported in the Choi et al., study.

Our current study therefore extends the findings of a protective effect of antioxidants against permanent auditory damage caused by steady state noise and impulse noise-induced hearing loss to damage caused by mid-range blast overpressures encountered in current battlefield explosions and artillery and civilian terrorist attacks.

The ABR threshold recovery, indicative of a TTS, shows a parallel improvement for both control and treated groups with most of the recovery occurring by 7 days post exposure. The effects of a single or reduced dose of antioxidants was apparent at 24 h after blast exposure by reduced averaged ABR threshold shifts of 9.3 dB, and average DPOAE level shifts of 6.2 dB compared to that of the untreated ears. Most (88%) of the recovery of hearing response in treated ears compared to controls occurred in the first 24 h after exposure. By contrast DPOAE level shifts progressively decreased over the 21 days in the treated group with little or no change in the control group after blast exposure. These findings suggest that under the treatment regimen used here the antioxidants act within the first 24 h to block the initiation of biological processes that result in permanent hearing loss, underscoring the importance of the timing of the antioxidant administration to achieving maximum impact on TTS and PTS. This treatment clearly reduces both TTS and PTS.

**Fig. 9.** Percent OHC loss as a function of percent distance (frequency place in kHz) from the OC apex at 21 days after exposure to 4 blasts in the control and treated groups. Data analysis was performed using two-way ANOVA for the main effect: ***p < 0.001 (F(1, 84) = 245.4).

**Fig. 10.** Representative photomicrographs of cochlear hair cells at the middle turn from control (A, B) and treated (C, D) animals at 21 days after exposure to three 14 psi blasts. Missing OHCs were replaced with scars (arrows show examples). Higher numbers of missing hair cells are in the outer hair cell (OHC) region than in the inner hair cell (IHC) region, and in controls than in treated ears, corresponding to quantifications in the cytocochleograms (Figs. 7 and 8).
The time history of the changes in ABR threshold shifts and DPOAE level shifts reveal some interesting features of the damage produced to the auditory system. Initial ABR threshold shifts partially recovered by 24 h post blast exposure in control groups, indicative of a temporary threshold shift, however DPOAE level shifts did not appreciably change by the terminal 21 day testing in blast-exposed unattended control animals. This difference may be a function of the effect of the blast on the different components of the sensory organ. DPOAEs test the biomechanical gain of the outer hair cells of the cochlea. ABRs test the neural component, i.e. synchronous synaptic activity of the cochlea along the auditory pathway including the brainstem. Comparing the progression of OHC loss with that of the corresponding DPOAE data indicates that functional impairment the hair cells can be detected much sooner (reaching a maximum by 3 h post exposure) than their physical deterioration which continues to increase over the 21 days. Drug treatment suppresses both the functional and physical damage to the OHCs. The failure to detect a TTS in the control blast-exposed group by DPOAE testing suggests that the damage to the OHC may be so severe that it is not reversible without the therapeutic intervention, whereas, the neural component can partially recover as reflected by the ABR data showing a TTS. Henry and Mulroy (1995) have shown that noise exposure results in reduction of the afferent synapses between the auditory hair cells and auditory nerve fibers contributes to the TTS. Kujawa and Lieberman (2009) have demonstrated that noise overexposure results in acute loss of afferent nerve terminals and delayed degeneration of the cochlear nerve. The ABR data reported herein are consistent with both a temporary and prolonged deterioration of the auditory nerve fibers and show that antioxidant treatment can both enhance TTS recovery and prevent long-term damage to the neural components of the auditory system.

5. Conclusion

The blast simulator developed for these studies produced reproducible blast overpressures and physiological and physical damage to the auditory system of rats, consistent with open-field blast exposures of humans. Results of auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) testing and outer hair cell analysis suggest that blast exposure causes damage to both the synchronous synaptic activity of the auditory nerve and brainstem and the mechanical activity of OHC associated with cochlear amplification. Furthermore this damage can be significantly reduced by treatment with a combination of the antioxidants, HPN-07 and N-acetylcysteine (NAC). The therapeutic regimen using antioxidants proposed in these studies is particularly adaptable to situations where hearing protection is not practical or where blast exposure cannot be predicted, or where the blast trauma exceeds the protective capability of hearing protection devices.

Author disclosure statement

Dr. Kopke and Dr. Floyd have financial interests in Otological Pharmaceuticals Inc. Drs Ewert, Lu, Du, and Li have no conflicts of interest.

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