Mitigation of Blast-Induced Hearing Damage Using Liraglutide in Animal Model of Chinchilla with Hearing Protection Devices

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Abstract

Introduction: Hearing impairment induced by repetitive exposure to high-intensity sound and blast overpressure (BOP) is a critical health issue in Service members. A recent study observed mitigation of blast-induced hearing damage using liraglutide in chinchillas, but the effect of the drug in ears with hearing protection devices (HPDs) remained unclear. This paper reports the study on hearing function changes in chinchillas with HPDs and liraglutide treatment after multiple exposures to BOP.

Methods: Chinchillas were divided into three groups: pre-blast treatment (N=10), post-blast treatment (N=12), and blast control (N=5). All animals with their ears inserted by foam earplugs were exposed to six consecutive blasts at the level of 3–5 psi (21-35 kPa) on Day 1. The auditory brainstem response (ABR) and middle latency response (MLR) were measured on Days 1 (pre- and post-blast) and Days 4, 7, and 14 after blast exposure. Immunofluorescence studies were performed on the inferior colliculus (IC).

Results: Temporary damage to the chinchilla hearing was reflected by increased ABR threshold and decreased ABR wave I amplitude which was mostly recovered after 14 days. Reduced MLR amplitudes were observed from Day 4 to Day 14 in all chinchillas. Different ABRs and IC histology were observed in the drug-treated animals comparing to blast control chinchillas.

Conclusion: This study demonstrates the protective function of HPDs against repeated low-level BOPs and provides possible novel strategies to treat blast-induced hearing damage using the combination of HPD-based damage prevention and the liraglutide-based function amelioration.

ABBREVIATIONS

BOP: Blast Overpressure; HPD: Hearing Protection Device; PAS: Peripheral Auditory System; CAS: Central Auditory System; TM: Tympanic Membrane; TBI: Traumatic Brain Injury; IC: Inferior Colliculus; GLP-1R: Glucagon-Like Peptide-1 Receptor; ABR: Auditory Brainstem Response; MLR: Middle Latency Response

INTRODUCTION

Repetitive exposure to high-intensity sound and blast overpressure during combat and occupational training, such as breaching exercises and grenade courses, are inherent situations faced by Service members [1-3]. Even with the use of hearing protection devices (HPDs, e.g. earplugs), blast-induced hearing loss and tinnitus are the top-two most prevalent service-connected disabilities among Service members with long-term ramifications for the Veterans’ health care system [4]. However, the detailed mechanisms of the formation, prevention, and restoration of blast-induced auditory injuries were not thoroughly investigated, especially when the HPDs were used.

When exposed to blast overpressure (BOP), both peripheral and central auditory systems (PAS & CAS) are susceptible to structural damage. A literature search indicates that investigation on blast-induced auditory damage was initiated on injuries in the PAS, such as the rupture of the tympanic membrane (TM), disarticulation of the ossicular chain, loss of hair cells, and tearing of the basilar membrane [2,5-9]. With the widespread use of HPDs, recent epidemiologic studies reported the prevalence of the post-blast auditory complaints with insignificant damage in PAS [10-12]. This raised a concern of blast-induced damage to the CAS, which shared a similar mechanism with traumatic brain injury (TBI): abnormally intense shearing and stretching of the auditory-related areas in the brain, such as inferior colliculus (IC) in the brainstem and auditory cortex in the temporal lobe [11,13-15]. Animal studies have been performed to investigate the damage in different regions of the auditory system separately.
and the accumulative effect of repeated low-intensity blasts [16-19]. One of the reasons for the increasing concern on the repeated low-intensity blasts was that the use of HPDs prevented severe auditory injuries such as TM ruptures and middle ear disruptions, while long-term hearing deficits resulted from repeated exposures to low-level BOP were not eliminated and persisted as a chronic health issue in military personnel [1,3,12,20]. However, studies on blast-induced hearing damage in ears with HPDs, which could simulate the actual situations faced by Service members, were rarely performed.

Smith et al. (2020) [21], and Chen et al. (2019) [22], have reported a chinchilla animal model of repeated-blast-induced auditory injuries and investigated the function of HPDs at high (15-20 psi) and low (3-5 psi) BOP levels, respectively. Different levels of acutely and permanently impaired hearing functions were observed in chinchilla ears with and without the HPDs. To ameliorate the hearing function after blast exposure, Jiang et al. (2021, under review) [23] conducted experiments to investigate the therapeutic function of liraglutide in mitigating the progression of auditory injury after multiple blast exposures in chinchillas. Liraglutide is a glucagon-like peptide-1 receptor (GLP-1R) agonist primarily used for the treatment of diabetes, whose neurotrophic and neuroprotective functions are recently discovered [24,25]. More recent studies have assessed the GLP-1R agonists as potential treatment strategies for memory and cognitive deficits in cellular and animal models of stroke and blast-induced TBI [26-29]. Based on the similar mechanism between the TBI and blast-induced hearing damage, Jiang et al. [23], observed significant improvement in the hearing function after blast exposures in chinchillas with the liraglutide treatment, but the severe conductive hearing loss observed in the animal model could not accurately simulate the actual situations faced by Service members with hearing complaints. Therefore, the therapeutic function of the liraglutide treatment needs to be investigated under the protection of HPDs.

This paper reports our recent study to characterize the change of hearing function in chinchillas under the protection of HPDs and liraglutide treatment after multiple exposures to low-intensity BOPs. The protective function of HPD, the therapeutic function of liraglutide treatment, and their interactions were investigated through comparison between the results obtained from present and previous studies.

**METHODS**

**Animal Protocol and Liraglutide (Drug) Administration**

Young, healthy chinchillas (Chinchilla laniger) with mixed gender provided by Ryerson Chinchilla Ranch (Plymouth, OH) were used in this study. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma following the guidelines of the National Institutes of Health and the US Department of Agriculture. Chinchillas were randomly separated into three groups: pre-blast drug treatment (N=10), post-blast drug treatment (N=12), and blast control (N = 5). The time frame and experimental procedures are plotted in Figure 1, similar to the previous liraglutide study on open ears [23]. Blast exposures, hearing function tests, and euthanasia are highlighted by arrows along the time axis. Liraglutide (Victoza, Novo Nordisk Inc., Plainsboro, NJ) was administered to chinchillas via subcutaneous injections with a dose of 246.7 μg/kg/day for consecutive 7 days. The dose used in this study was equivalent to the human dose (20μg/kg/day) normalized to body surface area across species which was determined after considering the dose used for murine animal models in similar studies [27,30]. The 7-day-long liraglutide treatment started 2 days before the blast exposure and continued in consecutive 7 days as shown in Figure 1. Similarly, the liraglutide treatment started 2 hours after the blast on day 1 and continued in consecutive 7 days in the post-blast treatment group. The hearing function tests were performed before and after the blast on Day 1 and on Days 4, 7, and 14. Upon the completion of the experiment on Day 14, all chinchillas were euthanized, and the brains were harvested for histological studies.

**Experimental Setup for Blast Exposure**

Each chinchilla was anesthetized with an intramuscular injection of 35 mg/kg Ketamine (Henry Schein Animal Health, Dublin, OH) and 3 mg/kg Xylazine (Akorn Inc., Lake Forest, IL) during blast exposures and hearing function tests on Day 1. The experimental protocols of blast exposure and earplug insertion followed those reported by Smith et al. (2020) [21] and Chen et al. (2019) [22]. Briefly, the chinchilla was fixed to a specifically designed L-shape animal holder using straps to position the top of its head facing the center of the pressure reservoir and its nose pointed to the front (Figure 2A). Standard polyurethane foam earplugs (3M, Inc. St. Paul, MN) were inserted into both chinchilla ears to provide protection against BOP. The bottom 1/3 of the

An earplug was removed to fit the length of the chinchilla ear canal, and the earplug was deeply inserted to ensure the earplug fitting stably inside the ear canal (Figure 2B). A pressure sensor (Model 102B16, Piezotronics, Depew, NY) was fixed to the animal holder with the measuring plane within 2 cm away from the chinchilla ear canal in the direction facing the front of the blast wave to monitor the BOP level at the entrance of the ear canal. The BOP was generated by a well-controlled compressed nitrogen-driven blast apparatus located inside an anechoic chamber (Figure 2A) [21-23]. BOP at a peak pressure level of 3-5 psi or 21-35 kPa measured at the entrance of the ear canal was generated by rupturing Polycarbonate films (McMaster-Carr, Atlanta, GA) of 0.25 mm, and the typical 10-ms-long BOP waveform was plotted in Figure 2C. Chinchillas experienced 6 consecutive blasts at time intervals of approximately 5 minutes between blasts. The pressure signals from the sensor were processed by a cDAQ 7194 and A/D converter 9215 (National Instruments Inc., Austin, TX) at a sampling rate of 100k/s (10 ms dwell time) and the LabVIEW software package ((National Instruments Inc., Austin, TX) was used for data acquisition.

Hearing Function Measurements

The hearing function tests for each chinchilla were conducted pre- and immediately post-blast on Day 1 and Days 4, 7, and 14, respectively as shown in Figure 1. On Day 1, chinchillas remained sedated under the ketamine and xylazine during the pre-blast hearing function tests, blast exposures, and post-blast hearing function tests. For hearing tests performed on Days 4, 7, and 14, the chinchillas were sedated by isoflurane (covetrus, Dublin, OH) at a concentration of 1%-3% in the oxygen flow of 1L/minute. The reason for using isoflurane was that the chinchillas could wake up faster from the sedation than the ketamine xylazine, but the equipment used to provide isoflurane sedation could not work in the blast chamber to maintain sedation during blast exposure. A 3.9 mm digital otoscope (ScopeAround) was used to examine the conditions of the ear canal and TM of the chinchilla each time before the hearing function tests were performed. The middle ear status was checked by the wide-band tympanometry (Titan, Interacoustics, Denmark) following the otoscopic exam. Because of the protection provided by the HPDs, all of the chinchilla TMs remained intact through the entire experiment and no middle ear abnormalities were observed from the wideband tympanometry during the entire experiment. With the normal outer and middle ear, the results presented in this paper should not be affected by any conductive hearing loss. Auditory function measurements including auditory brainstem response (ABR) and middle latency response (MLR) were recorded pre- and post-blast on Day 1 and on Days 4, 7, and 14.

Auditory Brainstem Response Measurements

ABR comprises the early portion of the auditory evoked potential which was widely employed to test auditory function and used for diagnosis and localization of pathologies affecting brainstem pathways [31,32]. In this study, the ABRs were recorded bilaterally using a TDT system III (Tucker-Davis Technologies, Alachua, FL) following the protocol established in our previous studies on the chinchilla animal model of blast-induced hearing damage [9,21-23]. Under anesthesia, needle electrodes were placed subcutaneously at the vertex of the skull, ventrolateral surfaces of the ear, and in the rear leg which served as the ground. The length of the ABR signal was set to be 10 ms, averaged 150 times, and filtered by a band-pass filter of 100-3000 Hz. The pure tone stimuli used in this study were defined with 0.5-ms rise-fall time, 4-ms duration, alternating polarity at frequencies of 1, 2, 4, 6, and 8 kHz and delivered 21 times per second [31,33]. The stimuli generated was amplified by a power amplifier TYPE 2718 (BRU EL & KJAER, Nærum, Denmark), generated by an MF1 multi-field magnetic speaker (Tucker-Davis Technologies, Alachua, FL), and monitored by a probe microphone inside the chinchilla ear canal (ER-7C, Etymotic Research, Inc.).
Research, Elk Grove Village, IL). The sound pressure level of the stimuli decreased from 100 dB SPL to 20 dB SPL at a step size of 5 dB. ABR thresholds were determined by visually examining the typical ABR peaks to determine the lowest sound level at which reproducible waveforms were observed. If an ABR response was not detected at the maximum acoustic stimulation, the threshold was arbitrarily set to be 100 dB. If the ABR signal continued to appear at 20 dB, the threshold was arbitrarily set to be 20 dB. The ABR threshold shifts on days 1, 4, 7, and 14 were calculated by subtracting the threshold measured before the blast from the threshold measured at the following time points, respectively.

The ABR wave I was generated by the auditory nerve, and the suprathreshold amplitude of ABR wave I could serve as indicators of damaged auditory nerves, inner hair cells, or cochlear ribbon synapses [31,34,35]. However, decreased ABR wave I amplitude could also be induced by the reduced input to the auditory nerve induced by conductive hearing loss, or other lesions located upstream of the ascending auditory pathway. In this study, the ABR wave I amplitudes at the stimulus levels between 80 to 100 dB at 8 kHz were recorded to characterize the injuries in the ascending auditory pathway at the locations up to the auditory nerve.

Middle Latency Response Measurement

MLR is the part of the auditory evoked potentials that occurs approximately 10 ms after the stimulus onset, following the ABR and preceding the late auditory evoked potentials [36-38]. Assessment on MLR signal provides a window into blast-induced alterations in thalamic, thalamocortical, and cortical regions of the auditory neuroaxis, which are more central than those reflected by the ABR [18,36]. The amplitudes of the first negative peak (Na) and the first positive peak (Pa) are supposed to reflect the neural conduction in the thalamocortical regions of the auditory system. Therefore, MLR was used as an indicator of damage to the central auditory nerve pathway after blast exposure.

The tone stimuli of 0.5 ms rise-fall time and 4 ms duration with alternating polarity were used for MLR measurement and delivered 4 times per sec. MLR signals with a length of 100 ms were averaged 100 times and the equipment and electrode placement were the same as the ABR measurement (TD7 System III). Thus, early components (< 10 ms) of the waveform collected under the MLR acquisition setting were the responses from ABR generator regions, while later responses corresponded to the more central generators in the thalamus and cortex [39]. The Na-Pa amplitude measured at the frequencies 0.5 kHz at 80 dB SPL was recorded to characterize the MLR waveform [18,22].

Immunofluorescence Study

Upon the completion of hearing function measurements on Day 14, the animals were euthanized and went through cardiac perfusion using saline solution followed by 4% paraformaldehyde in 9.6g/L phosphate-buffered saline (PBS). The brains were then harvested and fixed in 4% paraformaldehyde in 9.6g/L PBS for 72 hours at 4°C. Following a standard histology protocol, the brains were dehydrated in gradient ethanol solutions, decolored in xylene, and embedded in paraffin. The central axis of the brain along the front-posterior direction was normal to the plane of sectioning and sections at a thickness of 10 μm were obtained using a rotatory microtome (Leitz 1512, Leitz, German) [21,40]. Since the damage in IC could be reflected by both of the hearing function tests used in this study (ABR and MLR), the activity of caspase-3, a direct biomarker of caspase-dependent apoptosis in IC was chosen for immunofluorescence (IF) study to characterize the blast-induced damage in the CAS [27]. The location of IC was determined based on the anatomy of the chinchilla cerebral hemispheres and brainstem [41-43].

After antigen retrieval in 95-100 °C 0.1 M sodium citrate buffer for 30 minutes, the tissues were processed by 3% H2O2 solution, blocked with 10% goat serum (Sigma-Aldrich, Saint Louis, MO) containing 0.03% Triton X-100, and incubated overnight at 4°C with the rabbit-source cleaved caspase-3 primary antibody (1:200, #9661, Cell Signaling Technology Inc., Danvers, MA). The IF was performed using goat anti-mouse IgG secondary antibody (1:1000, Alexafluor 594, Thermo Fisher Scientific, Rockford, IL) and nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, Saint Louis, MO). The immunofluorescence images were collected by an inverted microscope (IX73, OLYMPUS Center Valley, PA). Fluorescence from the chinchilla 20-3-13L in Figure 3A) and the post-blast treatment group (chinchilla 20-4-3L in Figure 3B) ABR waveforms at lengths of 10 ms measured at 8 kHz tone stimuli were plotted from top to bottom with the stimulus level decreased from 10 to 20 dB at a step size of 5 dB. Results measured pre-blast and post-blast on Day 1 and Day 14 were included in Figure 3 The pre-blast threshold was 40 dB in the blast control and 35 dB in the post-blast treatment group. ABR waveforms from both blast control and post-blast treatment ears clearly showed typical ABR waveform with decreasing amplitudes and increasing latency when the acoustic stimuli decreased from 100 to 20 dB.

The pre-blast threshold was 40 dB in the blast control and 35 dB in the post-blast treatment ears, indicating the hearing sensitivity of the ears before the blast was similar. With the protection of the earplug, the temporary damage induced by 6 consecutive blasts was limited in both ears as observed from the post-blast results. The post-blast threshold was 75 dB in the blast control ear and 50 dB in the post-blast treatment ear. The waveform and threshold recovered to the pre-blast level on Day 14 in both ears. The wave V amplitudes in both ears were decreased compared to the pre-blast level, and the wave V amplitude in the post-blast treatment ear seemed diminished. ABR waveforms in Figure 3 indicated the blast-induced acute hearing damage was limited in protected ears and the damage could not be observed on Day 14. Therefore, the effect of drug treatment on ABR was not obvious after 14 days except for minor changes on the waveform, and further investigation is needed.

The mean and SEM of the ABR threshold shifts measured...
Figure 3 Representative ABR waveforms at 8 kHz measured pre- and post-blast and on Day 14 from protected ears: (A) Blast control group (20-3-13L); (B) Post-blast treatment group (20-4-3L) at the stimulus level ranged from 100 to 20 dB with a step size of 5 dB.

from protected ears in pre-blast treatment (n=20 ears), post-blast treatment (n=24 ears), and blast control (n=10 ears) groups were plotted in Figures 4A, 4B, and 4C, respectively. The threshold shifts on Days 1, 4, 7, and 14 were plotted in each figure and represented by different colors. On Day 1, the mean threshold shift ranged between 15 and 25 dB in two drug-treated groups, while ranged between 30 dB and 40 dB in the blast control group. The greater threshold shift in the blast control group could be potentially caused by the relatively smaller sample size than the drug-treated groups. In all three groups, the threshold shifts gradually decreased with time from Day 1 to 14 and the amount of recovery between adjacent time points decreased with time. On Day 14, the mean threshold shifts in drug-treated groups decreased to a level below 5 dB, particularly in the post-blast treated group, while the mean threshold shifts in the blast control group reached approximately 10 dB at 2 and 4 kHz. The ABR threshold shift indicated the blast-induced acute damage on Day 1 was temporary and the hearing function recovered to its original level on Day 14. The effect of liraglutide treatment on reduction of ABR threshold shift was observed after 14 days in comparison with the shift reduction in blast control ears.

ABR Wave I Amplitude

The ABR wave I amplitudes (peak-to-peak) measured from protected ears of pre-blast treatment, post-blast treatment, and blast control groups at 8 kHz are shown in Figure 5. The mean and SEM values were plotted against the level of acoustic stimulus ranged from 80 to 100 dB SPL. The results measured at different time points were represented by different colors as shown in the legend of Figure 5C.

In all three groups, the mean of the pre-blast wave I amplitudes ranged between 1 to 2 μV and the value increased with the stimulus level. A minor reduction (less than 20%) of the wave I amplitude could be observed from pre- to post-blast in all chinchillas and the reduction was uniform across the stimulus level. The amplitudes recovered to their pre-blast level in all three groups on Day 4 and remained unchanged until Day 14. The effect of liraglutide was difficult to be observed by the wave I amplitudes because of the limited amount of damage under the protection of earplugs.

MLR Na-Pa Amplitude

The representative waveform of the MLR signal is shown in Fig. 6A. MLR traces were recorded 100 ms after the acoustic stimulus at a level of 80 dB SPL and a frequency of 0.5 kHz. The waveform (Figure 6A) is comprised of a negative peak (Na peak) at 14-18 ms and a positive peak (Pa peak) at 19-22 ms. In general, reduced Na-Pa amplitude reflects that possible reduced activities at locations at or below the level of the thalamocortical regions along the ascending auditory pathway.

The Na-Pa amplitudes (mean ± SEM) measured from protected ears in pre-blast treatment, post-blast treatment, and blast control groups are plotted against the time points of measurement (pre-blast and post-blast on Day 1, Day 4, Day 7, and Day 14) in Figures 6B, 6C, and 6D, respectively. The mean MLR amplitudes measured before and after blasts on Day 1 were approximately at 3 μV in all three groups. The Na-Pa amplitudes decreased for more than 1 μV from post to D4 which was the greatest change observed in the 14-day-long experiment in all three groups. From Day 4 to Day 14, the amplitudes stayed relatively stable. The mean MLR amplitude from Day 4 to Day 14 was approximately 1.5 μV in pre- and post-blast treatment groups while was approximately 2 μV in the blast control group, which might be caused by individual difference. Overall, the MLR results from three groups of chinchillas showed a similar trend throughout the 14-day-long experiment and the reason for the reduction from post to D4 requires further investigation.

Immunofluorescence Imaging Results

Representative IF images of IC obtained from the chinchillas with protected ears are shown in Figure 7. The IC sections from the post-blast treatment and blast control chinchilla brains are

Figure 4 ABR threshold shifts (mean ± SEM) measured on Days 1, 4, 7 and 14 from protected ears: (A) Pre-blast treatment group (n=20 ears); (B) Post-blast treatment group (n=24 ears); (C) Blast control group (n=10 ears).

Figure 5 ABR wave I amplitudes (mean ± SEM) measured on Days 1, 4, 7 and 14 from protected ears: (A) Pre-blast treatment group (N=20 ears); (B) Post-blast treatment group (N=24 ears); (C) Blast control group (N=10 ears).

Figure 6 MLR signal measured at a stimulus level of 80 dB SPL and 500 Hz. (A) A representative waveform measured traces over 100 ms recorded from a blast control ear before the blast exposure. MLR Na-Pa amplitude measured pre- and post- blast and on Days 4, 7, and 14 (mean ± SEM): (B) Pre-blast treatment group (N=20 ears); (C) Post-blast treatment group (N=24 ears); (D) Blast control group (N=10 ears).

shown in Figures 7A and 7B, respectively. The caspase-3 was highlighted in red and the cell nuclei were highlighted in blue by DAPI.

The expression of caspase-3 could be observed in both groups of chinchillas, but the density of the caspase-3 signal was higher in the blast control group (Figure 7B) with the distribution and shape of the cell nuclei similar in both groups. IF results suggest the apoptosis activities existed in chinchilla IC on Day 14 even with the protection of earplugs, and the liraglutide administration possibility reduces the level of caspase-3 dependent apoptosis in the IC.

Therapeutic function of liraglutide in ears protected by hearing protection devices

The present study is a continuation of previous work on investigating the damage mitigation function of liraglutide in chinchilla ears exposed to repeated BOPs reported by Jiang et al. [23]. Substantial hearing damage was observed in chinchilla ears 14 days following the exposures to 6 blasts of 3-5 psi (or 21-35 kPa), and the liraglutide treatment successfully ameliorate the hearing function as reflected by lower ABR threshold shift, greater wave I amplitude, less reduction of the MLR amplitude, and the lower expression level of caspase-3 in the IC observed.
in the drug-treated chinchillas comparing to blast control chinchillas. However, the involvement of severe conductive hearing loss in Jiang et al. (2021)’s study could potentially shadow the sensorineural hearing loss or damage in the CAS which was believed to cause chronic, and persistent hearing deficits as reported by epidemiology studies on Service members and veterans [1,3,20]. Therefore, the therapeutic function of liraglutide was investigated in chinchilla ears protected by HPDs following the same protocols of blast exposures, drug administration, and hearing function assessment as reported by Jiang et al. [23], to estimate the potential of liraglutide to serve as a treatment strategy of blast-induced chronic hearing complaints in military personnel.

The ABR waveform reflects the function of different regions along the ascending auditory pathway up to the IC while the ABR thresholds are indicators of the general hearing sensitivity [18,31] Figure 8 shows the comparison of ABR waveforms recorded from post-blast treatment chinchillas with protected ears (20-4-3L in Figure 8A) and open ears (20-3-8L in Figure 8B). ABR waveforms at lengths of 10 ms measured at 8 kHz tone stimuli with the stimulus level ranged from 100 to 20 dB were plotted in Figure 8 in the same manner as Figure 3. The open ear results were obtained from Jiang et al. [23], Both ears of animals experienced the same blast exposures and drug treatment, except that one animal was protected by earplugs during the blast exposures while another animal was exposed to blasts directly without earplugs. The pre-blast ABR threshold was 35 dB in both open and protected ears, indicating the hearing sensitivity of the ears before the blast was the same. After the blast exposure, the ABR amplitudes decreased in both ears, but the reduction in the open ear was greater than the protected ear. The post-blast threshold was 85 dB in the open ears and 50 dB in the protected ears, indicating the use of earplugs greatly reduced the acute hearing damage induced by the repeated blasts. On Day 14, recovery was observed in both ears, but the amplitude of the protected ear was greater than the open ears. The Day 14 threshold was 35 dB in the protected ear while 65 dB in the open ear, indicating that the use of earplug and liraglutide treatment could eliminate the permanent hearing damage induced by repeated blast exposures while the liraglutide treatment itself was not sufficient to completely recover the hearing function after 14 days. In addition to the threshold and amplitude, the liraglutide treatment and the use of an earplug also altered the ABR waveform especially those later peaks (waves III to V).

The mean and SEM of the ABR thresholds measured from protected (N=4 ears) and open (N=14) ears in post-blast treatment groups were shown in Figures 9A and 9B, respectively. The open ear results were obtained from Jiang et al. [23], The threshold shifts on Days 1, 4, 7, and 14 were plotted in each figure and represented by different colors. The protective function of the earplugs reduced the ABR threshold shift at all frequencies from Day 1 to Day 14, indicating the use of HPDs provided prevention against blast-induced hearing damage. The results demonstrate that the liraglutide treatment more effectively ameliorates the hearing function after repeated blast exposures for ears without protection.

Comparing to the open ear results reported by Jiang et al. [23], the effect of liraglutide treatment was not clearly observed in protected ears based on the same protocols of blast exposures and hearing function measurements, with only a little evidence observed in histological studies. The major reason was the protection provided by the earplugs which reduced the severity and durability of the damage induced by 6 consecutive blasts as reflected by the ABR threshold and wave 1 result. The post-blast changes on ABR wave V, MLR amplitudes, and the IC histology indicated that the drug might still play certain roles, such as reduced apoptosis level in the CNS, but the analyses on the results from the currently performed hearing function tests were unlikely able to reflect the hearing function improvement induced by the liraglutide treatment. More histological evidence needs to be provided with a more detailed analysis of the ABR and MLR waveforms in the next step of the study.

Effects of Number and Intensity of BOP on Auditory Damage

Two similar studies have been reported to investigate the protective function of HPDs in chinchilla ears: a 7-day-long study on the progressive hearing damage induced by 3 blasts at the BOP level of 3-5 psi (21-35 kPa) by Chen et al. (2019) [22], and a 14-day study using 3 blasts at 15-20 psi (103-238 kPa) by Smith et al. (2020) [21]. The experimental setup and hearing function
assessments were similar to the present study except for the liraglutide treatment. A comparison of the results from the blast control group in this study with the results reported by Chen et al. and Smith et al. can provide insight into the effects of the number and intensity of the blasts on blast-induced damage in ears protected by HPDs. The mean ABR threshold shift in protected ears reported by Chen et al. [22], ranged between 20 to 40 dB on Day 1 and below 5 dB on Day 7 at different frequencies, which was slightly lower than the level reported in Fig. 4C. However, the mean threshold shift on Day 14 shown in Fig. 4C was further decreased from Day 7 to a level below 10 dB. This suggests the increased number of the blast may introduce more severe acute reduction to the hearing sensitivity and the severity decreases over time. The wave I result reported by Chen et al. [22], was consistent with the results shown in Fig. 5, indicating the function of the structures in the auditory pathway below the level of the auditory nerve were well-protected by HPDs. However, results reported by Smith et al. [21], showed more severe damage could be induced by 3 high-intensity BOPs (15-20 psi) than 6 low-intensity (3-5 psi) BOPs as reflected by the ABR threshold shift, indicating the intensity of the blast was a dominating factor than the number of blasts to the damage severity induced in the auditory system even under the protection of HPDs. The investigation on how the parameters of BOP affecting the damage in ears protected by HPDs is new to the field, which reveals that insufficient protection is provided by traditional HPDs against high-intensity BOP.

CONCLUSION

The goal of the present study was to investigate the potential therapeutic function of liraglutide treatment in ears exposed to repeated low-level BOPs under the protection of HPDs. The ABR

![Figure 8](image_url) Figure 8 Comparison of ABR waveforms at 8 kHz measured from pre- and post-blast treatment animals. (A) Protected post-blast treatment group (20-4-3L); (B) Open post-blast treatment group (20-3-8L) (Jiang et al., 2021) at the stimulus level ranged from 100 to 20 dB with a step size of 5 dB.

![Figure 9](image_url) Figure 9 ABR threshold shifts (mean ± SEM) measured on Days 1, 4, 7, and 14 from Post-blast treatment groups: (A) Protected ears (N=24); (B) Open ears (N= 14) as reported by Jiang et al.
and MLRs were measured in blast control and pre- and post-blast treated chinchillas on Day 1 and over a time of 14 days, and the brain tissues (IC) were used for the immunofluorescence study. Results indicated that 6 blasts of 3-5 psi (or 21-35 kPa) only induced temporary damage in the auditory system of chinchillas and most of which were recovered 14 days after the blast exposure when the HPD was used. Reduced MLR amplitudes from Day 4 to Day 14 were observed similarly in all three groups of chinchillas. Different ABR waveforms, ABR thresholds, and expression levels of caspase-3 in the IC were observed in the drug-treated animals comparing to the blast controls, but the detailed mechanisms and their implications for hearing functions required further investigation. Overall, the effect of liraglutide treatment was not clearly observed in protected ears because of the protection provided by the earplugs for low-level blast exposures. The damage in these ears may not be significant enough to detect ABR and MLR signals or histological examinations with liraglutide treatment over 14 days. However, this study demonstrates the protective function of HPDs against low-intensity BOP exposures and provides possible novel strategies to treat blast-induced hearing damage using the combination of HPD-based damage prevention and the liraglutide-based function amelioration.

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