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Assessing ototoxicity due to chronic lead and cadmium intake with and without noise exposure in the mature mouse

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Abstract

Exposure to heavy metals may lead to hearing impairment. However, experimental studies have not explored this issue with and without noise exposure in mature animals with environmentally relevant doses. The aim of this study was to investigate ototoxicity produced by lead (Pb) and cadmium (Cd) and noise, singly and in combination, in the adult CBA/CaJ mouse. Metals were delivered via drinking water (0.03 mM, 1 mM, and 3 mM Pb; or 30, 100, and 300 μM Cd) for 12 weeks, resulting in environmentally- and occupationally-relevant mean (± standard deviations) blood levels of Pb (2.89 ± 0.44, 38.5 ± 4.9, and 60.1 ± 6.6 μg/dl, respectively) and Cd (1.3 ± 0.23, 6.37 ± 0.87, 27.2 ± 4.1 μg/L, respectively). Metal treatment was also combined with a noise exposure consisting of a 105 dB broadband (2–20 kHz) stimulus for 2 hr or a sham exposure. Auditory performance was determined by comparing auditory brainstem responses (ABR) and otoacoustic emissions (DPOAE) at baseline and after 11 weeks of metal treatment. Metal-exposed animals did not develop significant auditory deficits and did not exhibit morphological damage to cochlear hair cells. In contrast, noise-exposed animals, including those exposed to combinations of metals and noise, demonstrated significant hair cell loss, reduced DPOAE amplitudes, and ABR threshold shifts of 42.2 ± 13 dB at 32 kHz (105 dB noise alone). No significant potentiation or synergistic effects were found in groups exposed to multiple agents. This study establishes a highly reproducible adult mouse model that may be used to evaluate a variety of environmental exposure mixtures.

Keywords

ototoxicity; lead; cadmium; hearing loss; exposure mixtures

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Disclosure

The authors declare no conflicts of interest related to this research.

Introduction

Worldwide, over 360 million people are estimated to suffer from hearing loss (HL) (WHO 2013), and non-age related HL ranks as the fifth cause of years lived with disability globally (Stevens et al. 2013). Hearing loss in almost any form exerts detrimental impact at all stages of life. Even mild losses in children are associated with poorer speech perception and significantly lowered performance on basic skills tests (Tharpe et al. 2009, Rodrigues et al. 2015). In adults, HL may impair a wide variety of adverse social, psychological, educational, clinical and occupational outcomes (Seidman and Standring 2010).

Exposures to high levels of noise produce HL (EPA 1974), and other environmental agents may also cause or contribute to HL (Fechter 2004, Guthrie et al. 2015). Auditory toxicity (ototoxicity) resulting from exposures to certain clinically relevant drugs (Schacht et al. 2012) is well established. Research from the past and present identified another potential source of HL: exposure to non-essential heavy metals, including lead (Pb) and cadmium (Cd) (Schwartz and Otto 1987, Vyskocil et al. 2012), which are common environmental and occupational contaminants in industrialized communities (Guan et al. 2010, Yorita Christensen 2012). Pb is present in paint in US homes built before 1977 and exposure may occur through the water supply in older homes, soil and household dust (Pichery et al. 2011), children's toys (Greenway and Gerstenberger 2010), and even through some food products (Berger et al. 2013). Cd exposure may occur through contact with contaminated soils and dusts, tobacco smoke (Cosselman et al. 2015), and through dietary intake (Tellez-Plaza et al. 2012).

Contradictory information exists on the consequences of prolonged low-level heavy metal exposures in adults. Several reports suggested that Pb may be ototoxic in human adults (Hwang et al. 2009, Park et al. 2010) but not all studies agree (Counter and Buchanan 2002). The potential ototoxicity of Cd in human adults has been addressed in one study (Choi et al. 2012). Epidemiological evidence also suggests that HL associated with Pb and Cd exposures in humans might be synergistically or additively affected by concurrent noise exposure (Farahat et al. 1997; Wu et al. 2000).

Lead has also been implicated as a potential ototoxicant in developing animals, including mice (Jones et al. 2008; Fortune and Lurie 2009; Prins et al. 2010) and monkeys (Rice 1997) and acute Pb exposures may generate auditory dysfunction in guinea pigs (Tuncel et al. 2002). Similarly, Cd in drinking water at 5, 15, or 150 ppm produced ototoxic effects in 30-day exposures in two rat and one mouse study (Ozcaglar et al. 2001; Agirdir et al. 2002; Kim et al. 2008). However, as with Pb, not all studies of Cd demonstrated ototoxicity (Whitworth et al. 1999). These discrepancies largely arise from the lack of an established animal model to guide a consistent approach to potential ototoxic exposures by heavy metals.

While detrimental neurological effects of metals have been established during development in both children (Sharma and Mogra 2014) and animals (Jones et al. 2008), though this is controversial as some studies show no consistent effects (Buchanan et al. 2011; Taylor et al. 2018). Adult animal models using higher occupational exposures to Pb have been exploited

to a lesser extent. Therefore, adult animal models are necessary to address ambiguities in the epidemiological findings and explore potential causal connections between exposure to Pb and/or Cd and HL. In order to close these knowledge gaps, the objective of this study was to evaluate and quantify, in a well-controlled animal model, the relationship between Pb and Cd exposure and ototoxicity as measured by auditory threshold shifts and cochlear hair cell loss. Based upon environmental and occupational exposure parameters, the effects of the heavy metals were evaluated singly, in tandem, and also in combination with noise.

Methods

Animals:

The University of Michigan's University Committee on the Use and Care of Animals approved all animal protocols for this work. Routine care for animals was provided by the University of Michigan's Unit for Laboratory Animal Medicine (ULAM). Mice were housed in a containment facility with unlimited access to both food and treatment water. Treated water bottles were changed twice a week. The facility maintained a 12-hr light-dark cycle. In-cage 72-hr noise exposure measurements were taken monthly using a personal noise dosimeter (Spark 706RC, Larson Davis, Depew, NY). Average ambient sound pressure levels (SPL) were consistently at or below 60 dBA, a level sufficient to eliminate the risk of noise-induced hearing loss in mice (Reynolds et al. 2010, Ohlemiller et al. 2011).

CBA/CaJ mice arrived from the Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age weighing 23 g and were allowed one week for acclimation. All mice were housed 5 to a cage if possible. Baseline auditory brainstem response (ABR) measurements at age 5 weeks determined hearing thresholds before chemical treatments began at 6 weeks of age (Figure 1). A treatment schedule beginning no earlier than age 5 weeks was selected to coincide with mouse reproductive maturity and comparable to the age of young workers in the US. Treatments continued for 11 weeks. Sacrifice by anesthesia and decapitation followed by blood, bone, liver, kidney, and cochlear tissue harvest occurred at 17 weeks of age. Noise exposures were administered during the sixth week of treatment (11 weeks of age). Final hearing thresholds were measured during week 11 of treatment (16 weeks of age).

All animals were visually inspected daily and weighed weekly to verify consistent growth. No cases of overt toxicity from Pb or Cd were observed. Animals were sacrificed after final hearing assessments for pathological analysis, cochlear assessment, and tissue Pb and Cd measurements. One control mouse died during our experiment due to acute obstructive uropathy unrelated to the experimental protocols. All other mice completed the full experimental treatment.

Treatments:

Mouse treatment groups and total group size throughout this study are shown in Table 1. This study was run in three stages. The first stage used various levels of Pb, Cd, and noise treatments to determine the level of treatment that caused damage. During the second stage, doses of 3 mM Pb, 300 uM Cd, and 105 dB noise were selected to investigate effects of these treatments under concurrent dosing regimens. The third stage exclusively investigated

the DPOAE outcome. Controls were included in each period thus larger numbers of control animals are reflected in Table 1. Each treatment group included at least 6 mice to allow for health outcomes to show through individual variability. Only male mice were used due to documented estrus-related hearing fluctuations in female mice (Willott et al. 2008).

Pb exposure—An aqueous 2% w/v lead acetate solution (Fisher Scientific, Waltham, MA; #429132) was diluted into highly purified and filtered water (Merck Millipore, Billerica, MA Milli-Q System). Final concentrations of Pb in drinking water were 0.03 mM, 1 mM, and 3 mM; concentrations were selected to obtain serum levels representing those seen in US workers from average environmental, high environmental and legal limits of occupational exposures, respectively.

Cd exposure—Cd in the aqueous form of $CdCl_2$ (VWR, Radnor, PA; # 101443–260) was diluted into Milli-Q water to final concentrations of 30 μM, 100 μM, or 300 μM, again selected to achieve serum levels to represent US exposures ranging from environmental to occupational.

Combined exposure to Pb and Cd consisted of treated water containing the highest doses of Pb (3 mM) and Cd (300 μM). Random samples from each concentration were taken from all water bottles at monthly intervals to verify consistency in concentrations over the study.

Noise exposure—A single two-hr noise exposure was administered in week 5 of the chemical treatment period. Using a method previously described (Vicente-Torres and Schacht 2006), mice were housed in individual wire cages in a ventilated chamber with a loudspeaker mounted at the top of the chamber. Two or three awake animals were simultaneously exposed to 2 hours of broadband white noise (2 to 20 kHz) at intensities of 102, 105, or 108 dB SPL. Noise levels were confirmed within the wire cage with sound level meters before and during exposure (B&K sound level meter model 2231, with type 4155 1/2” microphone).

Pathophysiology:

Auditory Brainstem Response (ABR)—For ABR testing, animals were anesthetized with intraperitoneal injections of ketamine (65 mg/kg), xylazine (7 mg/kg), and acepromazine (2 mg/kg) to insure relaxation and immobilization as described in previous literature (Sha et al. 2008). Additional injections of ketamine and xylazine were administered as necessary to maintain anesthesia for the duration of the examination; an injection of glycopyrrolate (0.2 mg/kg) was administered to aid recovery. The ear canals and tympanic membranes of all animals were evaluated for signs of obstruction or infection prior to hearing assessments. No obstructions or infections were observed in any of the mice.

Needle electrodes were inserted subdermally at the vertex of each mouse’s skull equidistant to each external auditory meatus, a reference electrode was inserted below the pinna of the left ear, and a ground electrode was inserted contralaterally (Wu et al. 2001; Sha et al. 2008). Sound stimuli were carried in a closed acoustic system to the left external auditory meatus and then transmitted through an ear bar connected to a Beyer DT-48 transducer (Beyer Dynamic, Farmingdale, NY, USA). The test output was transmitted to an amplifier, viewed

via oscilloscope, and recorded using SigGen software (Tucker-Davis Technologies, Gainsville, FL USA). Thresholds were determined at low, mid-range, and high frequencies (8, 16, and 32 kHz, respectively) by progressive reductions in sound intensity by 10 dB SPL steps initially, and 5 dB SPL steps near threshold. Thresholds were defined as the lowest stimulus at which a positive waveform was seen (Hurd et al. 2011) and threshold shifts were calculated for individual animals as the difference between measurement at baseline and the conclusion of the experiment (chemical treatment week 11). Pilot work included preliminary analyses for groups suspected to show the largest changes of final ABR peak I through V amplitude and latency data as well as inter-peak latencies (I-II, II-III, III-IV, IV-V, and I-V) were calculated using MATLAB (Mathworks, Natick, MA) at 80 dB for frequencies of 8, 16, and 32 kHz for control mice, and those in the highest Pb and Cd treatment groups. Due to inconsistencies in interpreting ABR waveforms of noise-exposed animals, we excluded any animal with noise treatment from these analyses.

Distortion Product Otoacoustic Emissions (DPOAEs)—DPAOEs were collected at the same time points as ABR (i.e., baseline and eleventh week of treatment). DPOAE tests were run as described previously (Karolyi et al. 2007, Hurd et al. 2011) at 32 kHz following administration of anesthetics as described above for ABR. The ratio of the intensity of the primary tones f1 and f2, remained constant at f2/f1 = 1.2. F1 intensity was adjusted in 5–10 dB SPL increments, while f2 intensity was held 10 dB SPL below f1. Tucker-Davis Technologies System II (Gainsville, FL) hardware and SigGen/BioSig software captured responses and presented stimuli tones. Amplitude shifts were calculated by subtracting the final amplitude from the initial.

General Pathology:

Immediately following euthanasia via deep anesthesia with ketamine (100 mg/kg) all animals were exsanguinated via cardiac puncture to collect up to 1 ml blood. Upon exsanguination, 0.5 ml blood was placed in a trace metals analysis tube (Becton Dickinson, Franklin Lakes, NJ #368381). Any remaining blood was placed in to a serum separator tube, spun for two min and frozen in preparation for blood chemistry analysis. Blood serum was analyzed for markers of kidney and liver function: creatinine, aspartate amino transferase (AST), albumin, alanine amino transferase (ALT), total bilirubin, blood urea nitrogen (BUN), and alkaline phosphatase (ALP). Mice were necropsied and gross visual inspection was made for systemic damage. Liver and kidneys were removed during necropsy on all mice and placed into formalin fixative for at least 24 hr before they were trimmed and stained with eosin and hemotoxylin. Right femur and right tibia bones were collected, scraped of extraneous tissues with a ceramic blade, weighed and stored under refrigeration. Cochleae were collected and placed on ice in phosphate buffered saline (PBS) for dissection.

Tissue analysis—A University of Michigan Unit for Laboratory Animal Medicine (ULAM) veterinary pathologist blinded to treatment status evaluated liver and kidney slides for organ-specific lesions. ULAM also completed blood chemistry panel analyses. Blood samples were analyzed for Cd and Pb levels using inductively coupled plasma mass spectrometry (ICP-MS) at the Michigan Department of Health (Lansing, MI). Cochlear bone Pb and Cd levels were determined from the entire cochlear-vestibular apparatus along with a

portion of the temporal bone. Right and left cochlea were weighed together and digested in OPTIMA grade nitric acid. Tibia and Femur bones were similarly digested Pb and Cd content was determined using ICP-MS by the Michigan Department of Health. Detection limits were 0.05 mg/kg Pb and 0.05 mg/kg Cd for the bone and cochlear metal analyses.

Hair cell counts—Surface preparations of cochleae were examined to determine numbers of all cochlear inner and outer hair cells. Both left and right auditory systems were examined using a light microscope for abnormalities and signs of infection. The round window, oval window, and apex were opened to perfuse the entire cochlea with 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Cochleae were then decalcified with 4% EDTA in 10 mM PBS, stained with rhodamine phalloidin to label actin and hair cells were counted along the entire length of the cochlea (Matt et al. 2012; Wu et al. 2001). Average outer hair cell losses were tallied in the apex, middle section, and base.

Statistics:

Data were compiled, and descriptive statistics computed, in Excel (Microsoft, Redmond WA). Further analyses were run using R 3.3.1 (Vienna, Austria) and figures were generated in GraphPad Prism 6 (San Diego, CA). Counts of mice with ABR threshold shifts >20 dB, a level that has been used as a threshold for human hearing impairment (Vos et al. 2015), were also computed. Means across exposure groups were compared using Student's t-test and one way ANOVA using a Bonferroni correction for multiple comparisons; distributional comparisons were conducted via χ^2. Results were considered significant at $\alpha = 0.05$. Blood serum above or below established normal values (Charles River Laboratories International 2011) were counted were pooled from similar treatment groups using Pearson's Chi-squared test with Yates' continuity.

Results

Animals:

Pilot work in CBA/J male mice revealed a number of ear infections not identifiable during visual inspection before performing ABR and observed after cochlear dissections. The CBA/CaJ strain of mouse was therefore selected for this investigation due to their low incidence of ear infections compared to other mouse strains (McGinn et al. 1992) and stable hearing thresholds into advanced age (12–18 months) (Sha et al. 2008, Ohlemiller et al. 2011). Our study noted only one mouse with an ear infection on the right (non-ABR) side out of a total 150 mice undergoing ABR and cochlear dissection. Body weight measures were maintained throughout the study and no overt changes of health were noted. As reported in pathology results, hepatotoxicity and systemic toxicity were not found at this dose.

Treatments:

All control water samples tested below the limit of detection (0.2 mg/L) for both Pb and Cd. All Pb treatment water tested below limits of detection (LOD) for Cd and all Cd water tested below LOD for Pb. Water samples showed that mean treatment concentrations of Pb and Cd were within 25% error below the intended concentrations, and no individual samples tested

above the intended concentrations of Pb or Cd. Mean water sample concentrations (all results reported to two significant figures) of Pb were 0.023, 0.91, and 2.7 mM (4.8, 190, and 550 mg/L) for the 0.03, 1, and 3 mM treatment groups, respectively. Mean Cd water sample concentrations were 27, 93, and 240 μM (3.0, 11, and 27 mg/L) for the 30, 100, and 300 μM treatment groups, respectively. Mean water sample concentrations for the combined Pb and Cd treatment groups were 2.4 mM (510 mg/L) and 280 μM (31 mg/L) for Pb and Cd, respectively.

Noise—Pilot study results indicated that noise exposures of 102 dB SPL produced no hearing damage, while 105 dB or 108 dB SPL exposures averaged similar threshold shifts (approximately 35–40 dB). Therefore, the 105 dB SPL exposure, which produced a robust threshold shift without a full elimination of auditory responses, was selected for evaluation of exposures in combination with metals in order to optimize our ability to assess possible potentiation effects of Pb and Cd on hearing thresholds.

Pathophysiology

ABR Baseline Levels—Baseline thresholds decreased with increasing test frequency, as is typical for CBA/CaJ mice. Variability in baseline thresholds was low for the 147 mice that underwent ABR. The highest threshold mean (± SD) of 26 ± 3 dB was observed at 8 kHz, compared to 17 ± 3 dB at 16 kHz and 17 ± 3 dB at 32 kHz.

ABR Threshold Shifts—No significant changes from baseline thresholds were observed in the control animals at 8 and 32 kHz (Table 2 also shown in Figures 2–4). Threshold shift variability in the metal treatment groups was similar at all frequencies to that of the control group. No Pb or Cd treatment groups (including single and combination metal exposures) displayed threshold shifts at any frequency that differed significantly from control, though changes from baseline thresholds among the Pb and Cd single treatment groups were higher on average at 8 and 32 kHz than those of controls. Only 1 of 47 mice (2.1%) in a combination of the control, all Cd single treatment, and all Pb single treatment groups experienced a 20-dB or greater threshold shift at 32 kHz, and only 2 of 42 (4.7%) experienced such a shift at 8 kHz.

Noise-exposed mice showed threshold shifts much larger and more consistent than those from Cd, Pb, or Cd+Pb treatments, and which differed significantly from control (Table 2; results for controls, 105 dB noise exposures, and highest Pb and Cd treatments singly or in combination are shown in Figures 2–4). Mice exposed to 102 dB only exhibited alterations from baseline thresholds 10–11 dB greater than controls at 8 and 16 kHz; however, these differences were not significant. Threshold shifts in the noise-only treatment groups of 105 dB and 108 dB were not significantly different at any frequency. Average threshold shifts were significantly different from controls at 8, 16, and 32 kHz in mice treated with 105 dB noise alone or 105 dB noise in combination with Pb, Cd, or Pb and Cd (data not shown). Threshold shifts in the 105 dB noise plus Pb and/or Cd exposure groups did not differ significantly from the 105 dB noise-only treatment group.

The Cd+Pb+Noise group unexpectedly showed significantly lower changes from baseline thresholds than the Cd+Noise treatment at 16 kHz,. Changes remained lower, though not

significant at 8 kHZ and 32 kHz; differences ranged from 7.3 dB at 8 kHz to 19.8 dB at 16 kHz. The fractions of animals experiencing substantial threshold shifts (greater or equal to 20 dB) were similar among mice treated with mixtures of noise, Pb, and Cd, compared to mice exposed singly to 105 dB noise.

ABR Peak and Latency: ABR waves I, II, III, IV, and V amplitudes and latencies were analyzed as well as inter-peak I-II, IIII, I-IV, and I-V. Preliminary findings did not show significance of any wave peaks or latencies in the highest treatment groups compared with controls, so we did not pursue further deeper analysis. No significant differences from the control group were found among mice treated with 3 mM Pb or 300 μM Cd.

DPOAE Thresholds: DPOAE amplitude shifts at 32 kHz among the Pb, Cd, 105 dB, Pb +Noise, and Cd+Noise groups were similar to those seen in ABR at 32 kHz (Table 3). DPOAE shifts among Pb and Cd treatment groups were not significantly different from zero, and mean differences of amplitude shifts among noise-exposed mice were not significantly different. Mice exposed to noise displayed significantly higher threshold shifts than mice treated with Cd and Pb. One mouse in the noise-only exposure group did not experience a DPOAE threshold shift; this created a larger SD in this group compared to other treatment groups. DPOAE amplitude shift averages for Noise+Pb [28 ± 9.5 dB SPL] were similar to Noise+Cd [26 ± 11.7 dB SPL] and not significantly different.

Tissue Assessment:

Blood levels of metals—Pb blood levels at sacrifice were not altered by addition of noise or Cd treatment. No mice from control or Cd treatment groups had detectable levels of Pb while all mice treated with Pb had detectable levels of Pb in their blood. Blood Pb levels were significantly different among the three Pb-only treatment groups (Table 4). The mean blood Pb levels across the combination exposure groups (using 3 mM Pb) were not significantly different from each other or the single treatment of 3 mM Pb. All mice treated with Cd had detectable levels of blood Cd. Three mice not in a Cd treatment group (one control, two exposed to Pb only) had a detectable level of blood Cd. Blood Cd levels differed significantly among the exposure groups (Table 4 and Figure 5).

In combination treatment, Pb levels were unchanged in mice concurrently exposed to Cd (Figure 6). In contrast, mean levels of blood Cd in mice were altered by addition of Pb treatment, though not among noise-exposed animals (Figure 5). Blood Cd levels for the 300 μM treatment were significantly decreased when combined with Pb exposures. Both the Cd +Pb groups and the Cd+Pb+Noise group exhibited significantly lower blood Cd levels than Cd treatments alone.

Bones and cochleae: All mice had detectable levels of Pb in their tibia and femur, though levels were 1,000-fold higher among the Pb treatment groups than non-Pb-groups. Only mice treated with Cd displayed detectable levels of Cd (Table 5). Among Pb treatment groups, Pb bone levels in the femur were higher than those in the tibia. Mean femur Pb was significantly higher in the Pb-only group than the Cd+Pb group; however this was not the

case for tibia Pb. Bone Cd levels were not significantly different between Cd treatment groups.

As with bones, detectable levels of Pb were found in all cochlear tissue and bone samples, while detectable levels of Cd were observed only in the cochlear tissue and bone of Cd treatment groups (Table 6). Levels of cochlear Pb were not significantly different among non-Pb-treatment groups, and levels of cochlear Cd were not significantly different among non-Cd-treatment groups.

Cochlear Assessments: Significant cochlear hair cell losses were only seen in noise-exposed groups (Table 7). Numerous outer hair cells were missing in the basal area of the cochlea among all noise exposure groups, while no outer hair cells were missing in the apex and mid ranges of all treatment groups. There was a dose-dependent rise in mean loss of basal hair cells with increasing noise exposure; mean levels were 3 ± 2, 6 ± 4, and 14 ± 17 hair cells missing for the 102, 105, and 108 dB exposure groups, respectively. Among combination noise and metal treatment groups, mean basal hair cell loss was highest in the Cd+Noise group [12 ± 10] and lowest in the Pb+Noise group [3 ± 2]; the Cd+Pb+Noise group fell between these [7 ± 5]. Inner hair cells were noted to be intact under all treatment conditions.

General Pathology

Histology: Only mice treated with Pb showed signs of kidney distress, but even the levels noted histologically were not indicative of major systemic health problems. Karyomegaly in the S3 tubular epithelium, which is known to be a characteristic lesion of Pb exposure in mice, was present in 91% of mice treated with 3 mM Pb alone, 86% of mice in the Cd+Pb +Noise group, and in 100% of mice in the Pb+Noise and Cd+Pb groups. Intranuclear inclusions were also present in the highest Pb treatment groups with similar proportions to karyomegaly. One mouse in the 108 dB Noise treatment group displayed both these lesions; that was the only mouse without Pb treatment found to exhibit these lesions. Karyomegaly was absent in the 0.03 mM Pb group and rare (13% of mice) in the 1 mM Pb group. No other lesions were characteristic of any treatment group. Mild (affecting under 10% of tubules in the tissue) tubular hyperplasia occurred in over 50% of animals in all but one treatment group and lesions observed displayed no regeneration or fibrosis. Mild hepatic lesions also presented in a wide range of treatment groups at low levels. Two control animals presented with both centrilobular or random degeneration and necrosis multifocal neutrophilic with mononuclear inflammation. All other groups had 0 to 2 mice with similar lesions; no dose-response patterns were noted. Histology data is shown in Table 8.

Blood serum results—Serum ALP levels at sacrifice tested at biologically relevant low levels in Cd treated mice at higher treatments (4 of 9, or 44 %, in the 300 μM Cd group, 3 of 9, or 33 %, in the Cd+Noise group, and 1 of 12, or 8.3 %, among controls) though these levels were not significantly different from controls.

Most mice showed total bilirubin counts below normal values of 0.12 to 0.58 mg/dl. At sacrifice, blood serum levels of creatinine, AST, ALT, and BUN were not always within the

normal reference ranges but were not significantly different between treatment groups and did not show dose-response changes with increasing treatment levels.

Discussion

Previous studies exploring the ototoxic interaction of chemicals with noise have used rats (Mäkitie et al. 2003). An alternative animal model such as the mouse is increasingly important in modern research making up about 90% of species used today in animal studies and was thus selected. Molecular tools as well as transgenic strains render this model more versatile than any other species for research. In addition, exploration of interactions between genetic and environmental factors in mice offers a much more flexible and yet highly controlled experimental approach than does epidemiological modelling (Mauderly 1993), and the relevance of this research can be increased even further through the use of transgenic mice mimicking human disorders (Vandamme 2014).

In the CBA/CaJ adult mouse model presented here blood levels of Pb and Cd relevant to occupational situations in the US were achieved and simulated environmental exposures that occur in certain areas internationally. Although levels of Pb in the general environment decline in the US, higher levels may still be experienced in certain occupations and remain important to public health. Further, recent research suggests that exposure to noise (both occupational and recreational) may have more severe consequences than previously assumed (Rodrigues et al. 2015). Even low-level exposure that only initiates temporary threshold shifts in young but mature hearing systems may produce earlier and greater age-related hearing losses (Kujawa and Liberman 2006). Globally, adults in low- and middle-income nations have high levels of noise exposures as well as high Pb and Cd exposures in both occupational and community settings (Nelson et al. 2005, Laborde et al. 2015).

With regards to occupational exposures to Pb, the current OSHA Permissible Exposure Limit for Pb exposures among workers is 50 μg/dl; in our study, the exposures achieved in the highest Pb-exposed group was equivalent to 60 μg/dl. The levels of Cd in our highest-exposed mice (27 μg/L) were double the OSHA occupational limit (10 μg/L), and Cd exposures in combined treatment of Pb and Cd still resulted in levels above the same Cd OSHA limit. When environmental exposures are considered, levels in treated mice were above the updated CDC Community Action Limit for Pb (5 μg/dl) (CDC 2012). Even exposures below this CDC limit are coming under increasing scrutiny as research continues to demonstrate the importance of cumulative effects (CDC 2009; Spivey 2007).

Our data appears to be the first study to test for Pb and Cd levels in the mouse cochlea. It was found that levels were detectable and similar to bone levels in the tibia and femur, though these were more similar for Cd than Pb. Mean Pb levels in the cochlea were 36% less than in the femur and 17% less than Pb in tibia. Detectable levels of Pb in non-Pb-treated mice were identified. This Pb exposure may originate from their feed, which contained detectable levels of Pb in our tests. Cd concentrations in bone and cochlea were not detectable in mice that were not treated with Cd. Most animals not exposed to Pb or Cd did not show detectable levels in their blood. However, three mice exposed to the control, or Pb alone showed detectable levels of Cd in their blood. All bone samples, regardless of Pb

treatment status or not, showed detectable levels of Pb. Contamination in animal food has been previously shown as is a likely contributor to the few mice which showed detectable levels of Cd and an ongoing exposure to low levels of Pb (Mesnage et al. 2015, Adamse et al. 2017). For mice treated with a combination of Pb and Cd lowered concentrations of both Pb and Cd were observed in the femur and tibia; this is likely due to competing metal uptake. Cochleae from these mice were not tested for metals.

Mixture-based research such as this is increasingly recognized as a critical area for epidemiological research. Therefore, our study included a thorough analysis of the ototoxicity of Pb and Cd in combination with noise. In agreement with previous investigations (Vicente-Torres and Schacht 2006, Minami et al. 2007), noise exposures produced significant detriments to thresholds in nearly all mice, validating the model. In contrast, our study did not identify any significant differences in hearing loss risk resulting from metal exposure, thus exposing a potential difference between developing and mature animals. It is also possible that Pb or Cd, are more ototoxic in humans, or that longer-term exposures are required in order to produce hearing deficits in older animals.

It is noteworthy that our model explored cochlear histology but did not investigate peripheral or central neurological damage that may disrupt auditory processing. Fortune and Lurie (2009) noted alterations to the superior olivary complex and normal monoaminergic expression albeit in developing mice. These are exciting areas for future studies to examine as Pb treatment may well distort auditory temporal processes. Further, subthreshold damage at a young age might manifest as increased hearing loss as the animals age (Kujawa and Liberman 2009). A long-term follow-up would be an important contribution to our understanding of metal toxicity.

The results of our study appear to disagree with two previously published studies that showed ototoxicity due to Pb exposure. The first used 6 monkeys, treated from birth to age 13, and pure tone thresholds to establish ototoxicity (Rice 1997). Results of this study demonstrated normal thresholds in three monkeys and lowered thresholds at high frequencies in three monkeys (Rice 1997). The second study investigated hearing loss in Wistar rats exposed to 4 mg/kg Pb acetate by gavage for 30 days (Liu et al. 2011). HL was demonstrated as an increased latency in the ABR peaks from I to V (Liu et al. 2011). This was not found in analysis of ABR peaks within our study. Assuming the daily amount of water consumed by one 30 g mouse is about 4 ml (Bachmanov et al. 2002), the Pb dose mice in our study received was about 82.67 mg/kg, a higher level than given to the rats above. However, differences in absorption due to developmental exposure times, species differences, digestion times, and stress levels due to mode of administration may also alter this comparison.

In contrast to Pb, Cd is not as well-studied. Kim et al. (2008; 2009; 2011) demonstrated ototoxic properties attributed to Cd. *In vitro*, over half of hair cells were apoptotic, detected through the use of TUNEL staining, following 24 hr direct exposure of organ of Corti explants from rats to Cd (10 μM) (Kim et al. 2008). This study further explored the ABR threshold shifts in mice following 150 mg/L (1334 μM) Cd in drinking water for 30 days. Thresholds of control mice at 32 kHz were near 30 dB and averaged near 55 dB following

30 days of Cd treatment. A likely reason for the discrepancy to our study is the dosing by Kim et al. (2008) which is over 4-fold higher than the levels administered in our study and should have resulted in comparatively higher serum levels. Increases of ALP in mouse blood due to Cd treatment noted in our highest Cd treatment group were also observed previously by Adefegha *et al.* (2015).

Conclusions

This comprehensive study design with both positive (noise) and negative (water) controls confirms the findings of many previous studies regarding the severity and consistency of noise-related damage to auditory function (Johnson 1993, Barden et al. 2012). For combination treatment, a similar strategy to studies on the interactions of styrene and noise was used (Mäkitie et al. 2003; Venet et al. 2015). To the best of our knowledge, our study is the first of its kind to use mice and had the longest treatment period of experiments of this type identified in a literature search. Therefore, this investigation offers a novel model for examining interactions within the auditory and vestibular (Klimpel et al. 2017) systems.

It is significant that a CBA/CaJ adult mouse model was developed with blood levels of Pb and Cd relevant to human health in real-world mixture exposure settings. Interestingly, these mice did not demonstrate significant ototoxic effects of Pb or Cd singly or in combination. It is also important that throughout the chronic exposure all animals maintained good general health and only showed subtle pathological changes in renal parameters parallel to those seen in rodents (Payne and Saunders 1978) and comparable to observed renal outcomes in humans (Fels et al. 1994). The use of this robust adult model might advance toxicology knowledge and research methods on mixed exposures.

Future studies investigating ototoxicity of metals and possible interactions of chemical agents with noise need to further explore the complex pathways of signals and cell bodies in auditory neurons and ascending pathways for a more complete understanding of health outcomes due to ototoxicant interactions. Metals are known neurotoxicants (Karri *et al.* 2016; Andrade *et al.* 2017); it is not unreasonable that changes in auditory perception may be due to auditory synaptopathy, which may be aggravated by noise exposures (Fernandez et al. 2015), or central effects. One example of a chemical producing adverse effects in the brain stem following noise exposure is 1,3-dinitrobenzene (Ray et al. 1992). Finally, it is possible that the CBA/CaJ mouse may not be sufficiently vulnerable to demonstrate effects potentially seen in more sensitive human populations. Species and strain differences are important and well documented variables in research and deserve further exploration.

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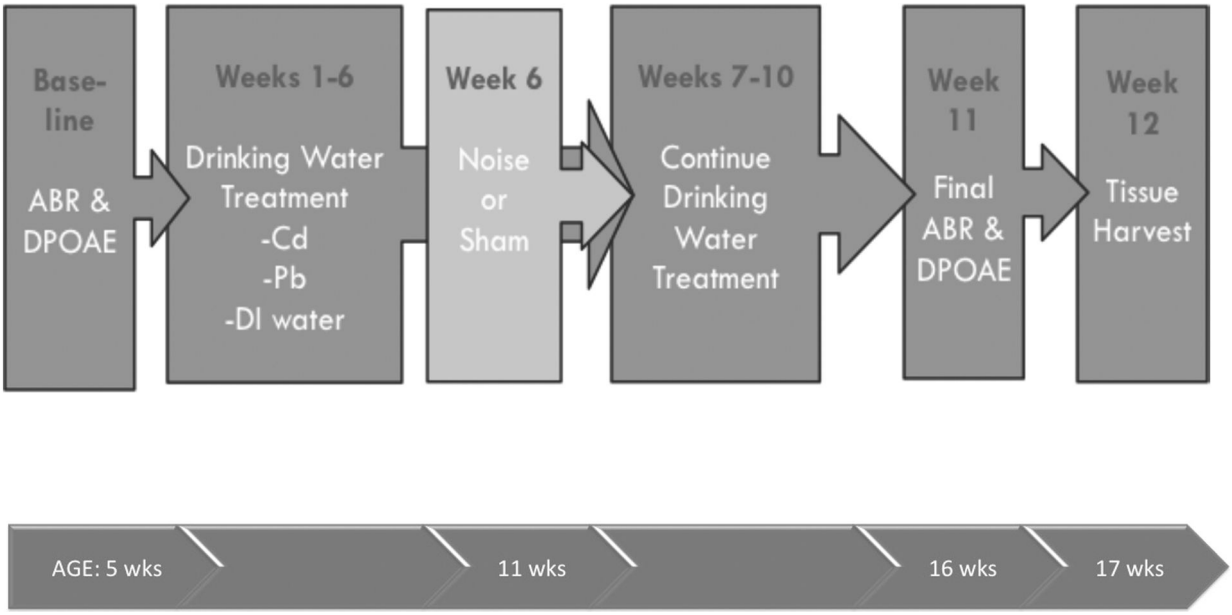

Figure 1.
Timeline of exposures and treatments along with corresponding age of mice.

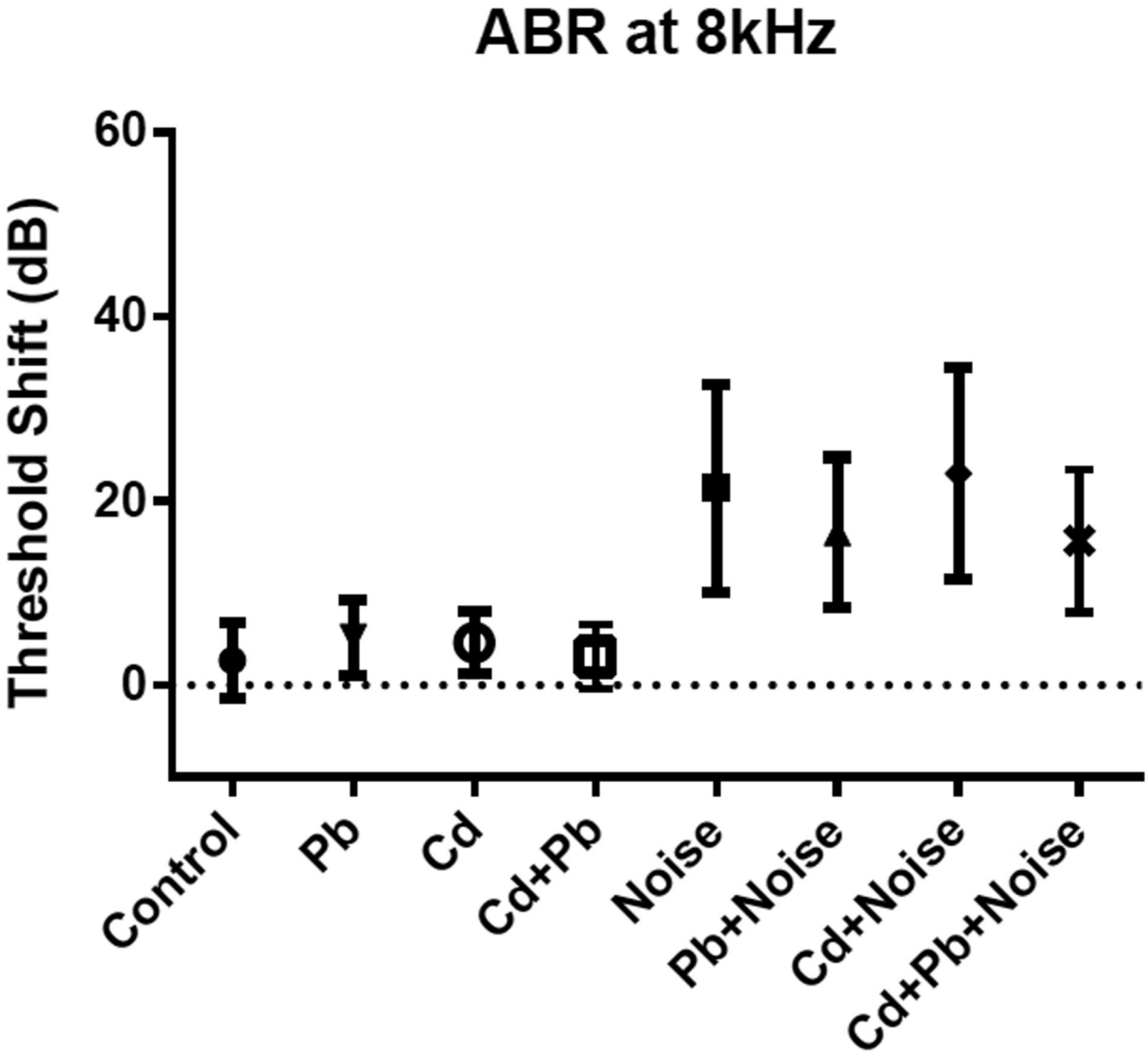

Figure 2.
Mean and standard deviations for ABR threshold shifts at 8 kHz for single treatment and mixture groups with 3 mM Pb, 300 uM Cd, and 105 dB.

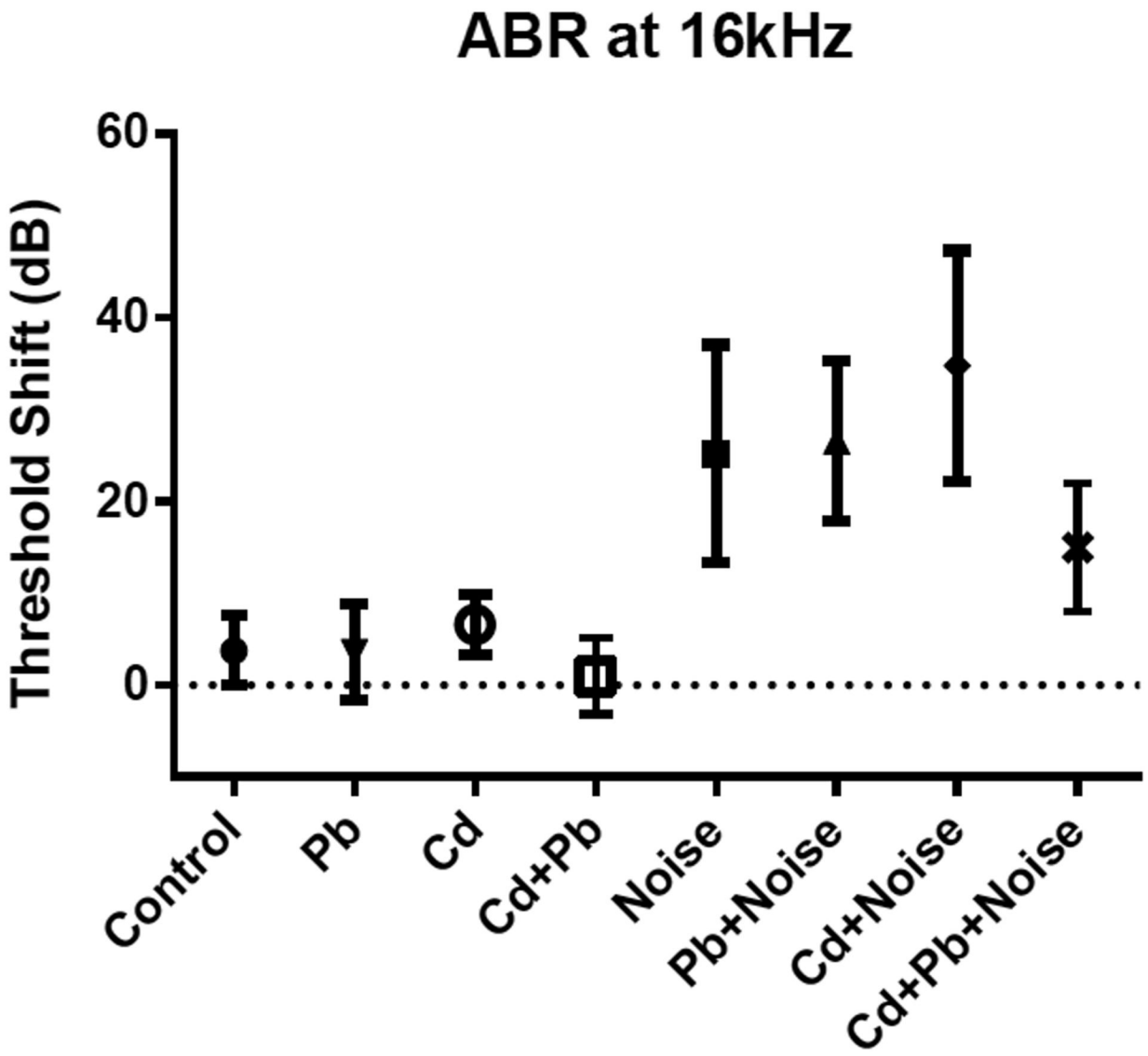

Figure 3.
Mean and standard deviations for ABR Threshold shifts at 16 kHz for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB.

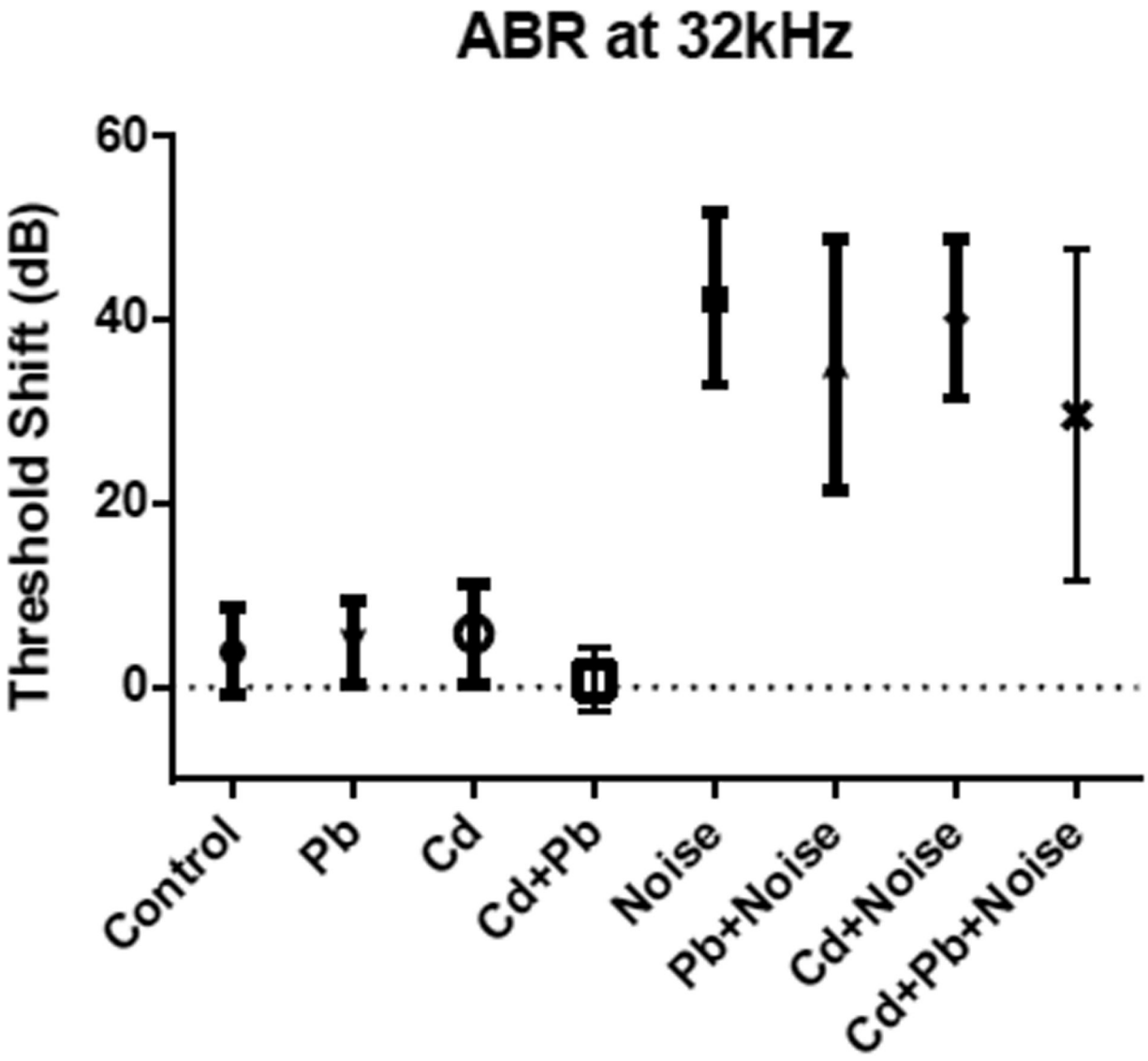

Figure 4.

Mean and standard deviations for ABR Threshold shifts at 32 kHz for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB.

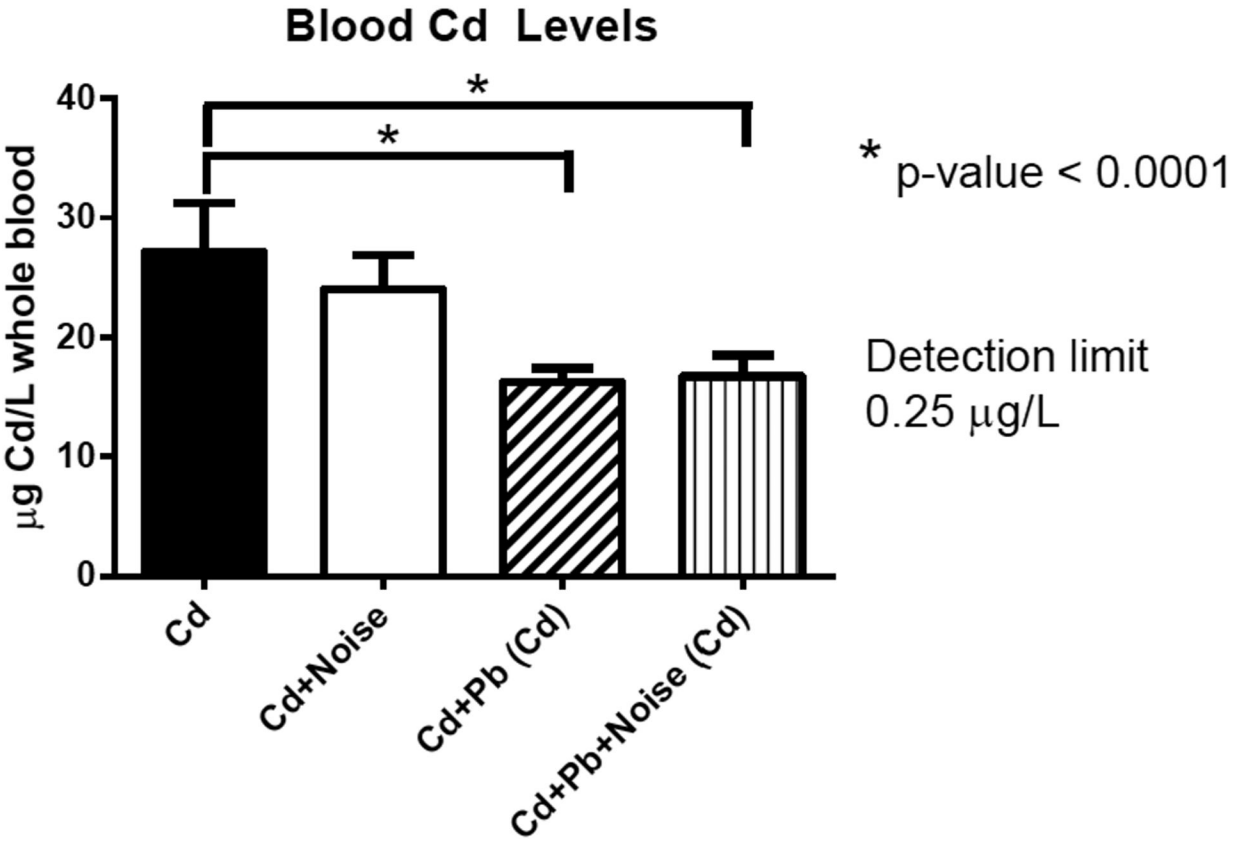

Figure 5.
Blood Cd levels (μg/L) by treatment groups for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB.

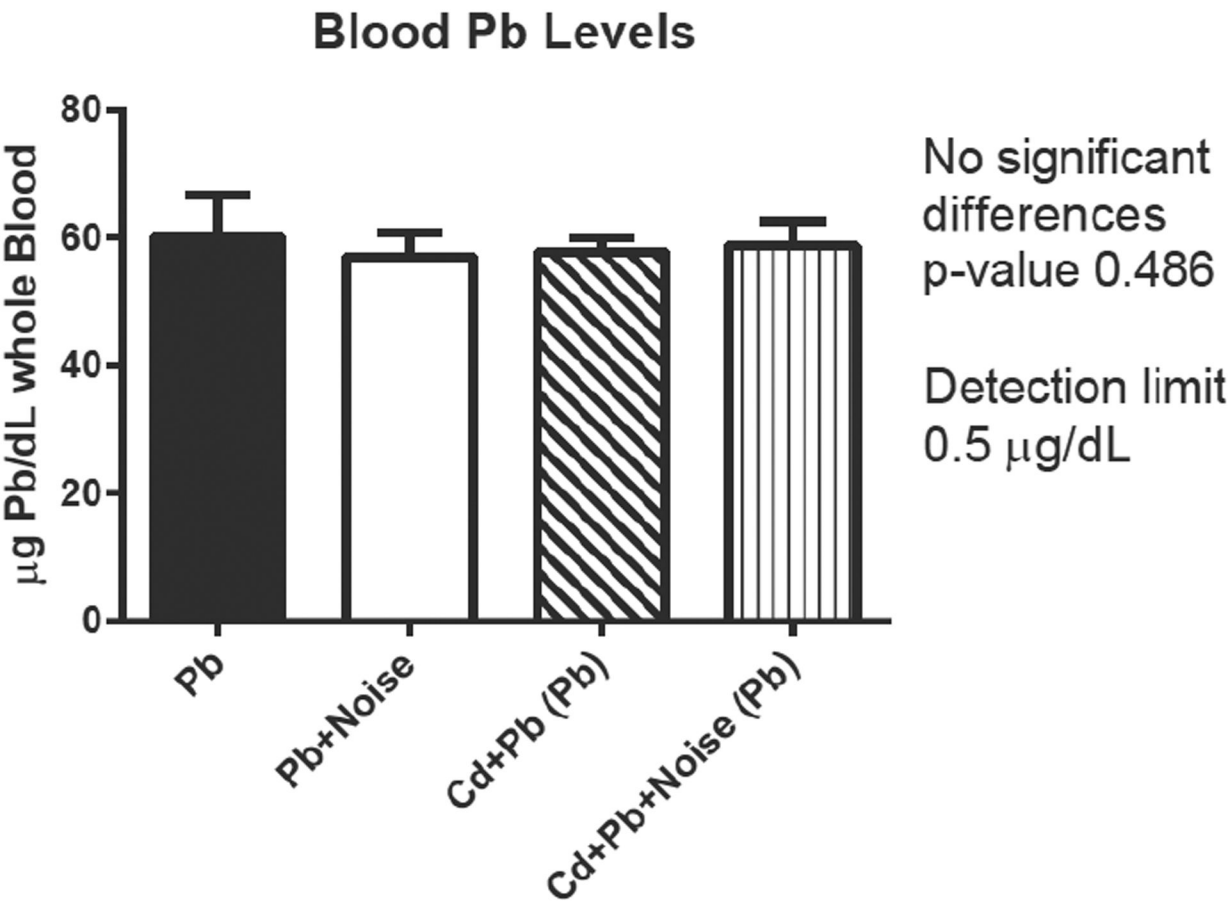

Figure 6.
Blood Pb levels (µg/dl) by treatment groups for single treatment and mixture groups with 3 mM Pb, 300 µM Cd, and 105 dB.

Table 1.

Description of treatment groups.

Group	N
Total	150
Control (no Pb, no Cd, sham noise)	16
Pb (no Cd, sham noise)	
3 mM	16
lmM	8
0.03 mM	8
Cd (no Pb, sham noise)	
300 μM	16
100 μM	7
30 μM	6
Noise (no Pb, no Cd)	
108 dB	7
105 dB	15
102 dB	7
300 μM Cd + 3 mMPb (sham noise)	9
3 mM Pb + 105 dB Noise (no Cd)	14
300 μM Cd + 105 dB Noise (no Pb)	14
300 μM Cd + 3 mM Pb + 105 dB Noise	7

Table 2.

Threshold shift averages (dB) and counts of shifts equal to or over 20 dB by treatment groups.

	32 kHz				8 kHz			
Treatment group	**N**	**Avg Shift**	**SD**	**N Shifts ≥20dB**	**N**	**Avg Shift**	**SD**	**N Shifts ≥20dB**
Control	15	3.9	4.7	0	15	2.6	4.1	0
Pb								
3 mM	16	3.9	4.3	0	11	5.2	4.1	0
l mM	8	6.6	5	0	8	12.75	5.6	2
0.03 mM	8	6.3	8.9	1	8	8.0	4.9	0
Cd								
300 μM	16	4.5	5.3	0	11	4.6	3.5	0
100 μM	6	5.5	3.3	0	5	9.4	5.6	0
30 μM	6	7.8	4.4	0	6	10.7	6.7	0
Noise								
108 dB	6	41.0	18.8	5	6	25.2	16.5	3
105 dB	15	42.2	13.5	14	10	21.4	11.3	5
102 dB	7	15.3	6.5	1	7	13	3.3	0
300 μM Cd + 3 mM Pb	9	0.8	3.5	0	9	3.1	3.4	0
3 mM Pb + 105 dB Noise	14	38.9	12.3	13	9	16.6	8.2	2
300 μM Cd + 105 dB Noise	14	39.3	8	14	9	23	11.5	6
300 μM Cd + 3 mM Pb + 105 dB Noise	7	29.6	18	5	7	15.7	7.8	2

Table 3.

DPOAE Average Amplitude Shifts (dB) and Matched ABR Threshold Shifts (dB) at 32 kHz.

		Threshold Shifts		Amplitude Shifts	
Group	**N**	**ABR**	**SD**	**DPOAE**	**SD**
Pb	5	2	3.1	−1.1	2.6
Cd	4	0.5	2.5	0.6	0.9
Noise	5	42.2	21.0	−21.9	15.7
Pb+Noise	5	45.8	4.3	−27.7	9.5
Cd+Noise	5	37.8	7.3	−26.1	11.7

Table 4.

Concentrations of metals in blood by treatment group.

Treatment group	N	Whole Blood Metals			
		Pb (μg/dL)^		Cd (μg/L)^^	
		Avg	SD	Avg	SD
Control	16	ND	-	ND*	-
Pb					
3mM	11	60.1	6.6	ND	-
lmM	8	38.5	4.9	ND**	-
0.03 mM	8	2.89	0.44	ND***	-
Cd					
300 μM	12	ND	-	27.2	4.1
100 μM	7	ND	-	6.37	0.87
30 μM	6	ND	-	1.3	0.23
Noise					
108 dB	7	ND	-	ND	-
105 dB	10	ND	-	ND	-
102 dB	6	ND	-	ND	-
300 μM Cd + 3 mM Pb	9	57.8	2.2	16.2	1.2
3 mM Pb + 105 dB Noise	9	57.0	3.9	ND	-
300 μM Cd + 105 dB Noise	9	ND	-	24	2.9
300 μM Cd + 3 mM Pb + 105 dB Noise	7	58.9	3.9	16.7	1.8

^ Detection limit is 0.5

^^ Detection limit is 0.25

* One sample above DL: 0.26

** One samples above DL: 0.32

*** One sample above DL: 0.27

Table 5.

Concentrations of metals (mg/kg) in bone.

		Femur				Tibia			
		Pb		Cd		Pb		Cd	
Treatment Group	**N**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Control	5	0.16*	0.14	ND**	-	0.16	0.06	ND	-
3 mM Pb	12	287	45	ND^	-	216	51	ND	-
300 μM Cd	11	0.13^^	0.01	0.27	0.03	0.18	0.04	0.23	0.03
300 μM Cd + 3 mM Pb	9	236	24	0.26	0.02	207	23	0.21	0.02

* All five samples run in control groups showed levels of Pb were detectable and above the limit of detection of 0.05 mg/kg

** All five samples in control groups showed levels of Cd were below the limit of detection of 0.05 mg/kg.

^ Two femur and tibia samples run in Pb treatment groups showed levels of Cd were below the limit of detection of 0.05 mg/kg. Results were similar to control levels.

^^ Two femur and tibia samples run in Cd treatment groups showed levels of Pb were detectable and above the limit of detection of 0.05 mg/kg. Results were similar to control levels.

Table 6.

Concentrations of metals (mg/kg) in cochlea and adjoining tissue

		Cochlea			
		Pb		Cd	
Treatment Group	**N**	**Avg**	**SD**	**Avg**	**SD**
105 dB Noise	5	0.250	0.153	ND*	-
3 mM Pb	5	185.0	34.9	ND*	-
300 μM Cd	4	0.228	0.04	0.287	0.066

*All Pb treatments and Noise treatment groups had levels of Cd that were not detectable at the limit of detection of 0.05 ppm or mg/kg.

Table 7.

Cochlea Cytogram Missing Outer Hair Cell Counts

		Apex		Middle		Base	
Treatment Group	**N**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Control	15	0.2	0.2	0.2	0.3	0.3	0.4
3 mM Pb *	11	0.2	0.2	0.3	0.2	0.1	0.1
300 μM Cd *	12	0.2	0.3	0.2	0.2	0.2	0.2
102 dB Noise	7	0.2	0.2	0.3	0.3	3.1	2.3
105 dB Noise *	10	0.2	0.2	0.4	0.2	5.8	4.4
108 dB Noise	8	0.3	0.1	1.6	2.6	13.8	16.7
Cd+Pb	9	0.1	0.3	0.2	0.2	0.7	1.2
Pb+Noise	9	0.3	0.3	0.3	0.3	2.9	2.3
Cd+Noise	9	0.2	0.1	0.5	0.4	11.6	9.6
Cd+Pb+Noise	6	0.1	0.1	0.3	0.3	7.3	5.0

* Group treatment levels used for mixtures (3 mM Pb, 300 μM Cd, & 105 dB Noise)

Table 8.

Histology data in counts of mice with observed lesions

		Renal Lesions							Hepatic Lesions		
Treatment	**N**	**THP**	**KM**	**TD**	**MII**	**FM**	**INI**	**ANY RENAL LESION**	**DGN**	**MFI**	**ANY HEPATIC LESION**
Control	16	9	0	4	0	0	0	9	2	2	2
102 dB Noise	7	5	0	2	1	0	0	6	1	2	2
105 dB Noise *	10	9	0	5	1	0	0	9	1	1	1
108 dB Noise	7	3	1	4	1	0	1	4	0	0	0
30 μM Cd	6	4	0	2	0	0	0	4	0	0	0
100 μM Cd	7	6	0	4	1	0	0	6	1	1	2
300 μM Cd *	12	9	0	6	1	0	0	9	1	1	1
Cd+Noise	9	5	0	0	0	0	0	5	0	0	0
0.03 mM Pb	8	8	0	3	0	0	0	8	1	1	1
l mM Pb	8	7	1	4	0	0	1	7	2	2	2
3 mM Pb *	11	9	10	6	0	0	10	11	0	0	0
Pb+Noise	9	8	9	1	0	4	8	9	0	1	1
Cd+Pb	9	7	9	1	0	0	9	9	0	1	1
Cd+Pb+Noise	7	6	6	0	0	2	6	7	0	0	0

* Group treatment levels used for mixtures (3 mM Pb, 300 μM Cd, & 105 dB Noise)

DGN - degeneration and necrosis, centrilobular and random

FM - focal mineralization

INI - intranuclear inclusions

KM - karyomegaly, tubular epithelium, S3

MFI - multifocal neutrophilic and mononuclear inflammation

MII - mononuclear interstitial inflammation

TD - tubular degeneration

THP - simple tubular hyperplasia