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An optimized, clinically relevant mouse model of cisplatin-induced ototoxicity

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Abstract

Cisplatin-induced ototoxicity results in significant, permanent hearing loss in pediatric and adult cancer survivors. Elucidating the mechanisms underlying cisplatin-induced hearing loss as well as the development of therapies to reduce and/or reverse cisplatin ototoxicity have been impeded by suboptimal animal models. Clinically, cisplatin is most commonly administered in multi-dose, multi-cycle protocols. However, many animal studies are conducted using single injections of high-dose cisplatin, which is not reflective of clinical cisplatin administration protocols. Significant limitations of both high-dose, single-injection protocols and previous multi-dose protocols in rodent models include high mortality rates and relatively small changes in hearing sensitivity. These limitations restrict assessment of both long-term changes in hearing sensitivity and effects of potential protective therapies. Here, we present a detailed method for an optimized mouse model of cisplatin ototoxicity that utilizes a multi-cycle administration protocol that better approximates the type and degree of hearing loss observed clinically. This protocol results in significant hearing loss with very low mortality. This mouse model of cisplatin ototoxicity provides a platform for examining mechanisms of cisplatin-induced hearing loss as well as developing therapies to protect the hearing of cancer patients receiving cisplatin therapy.

Keywords

Cisplatin; ototoxicity; mouse; distortion product otoacoustic emissions (DPOAE); auditory brainstem response (ABR); vestibular sensory evoked potential (VsEP)

1. INTRODUCTION

Cisplatin is a highly effective cytotoxic drug used in the standard treatment, alone or in combination with radiation therapy, for a variety of cancers in both pediatric and adult patients (Langer et al., 2013). Cisplatin-based chemotherapy can have substantial side effects including neurotoxicity, nephrotoxicity, myelosuppression, and ototoxicity (Rademaker-

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Lakshai et al., 2006, Rybak et al., 2007). While some of these can be effectively managed (Waters et al., 1991), ototoxicity is irreversible, is often substantial, and is currently considered largely unavoidable.

Cisplatin-induced ototoxicity results in bilateral, primarily high-frequency sensorineural hearing loss in approximately 62% of adults and 61% of pediatric patients treated with the drug (Bertolini et al., 2004, Coradini et al., 2007, Frisina et al., 2016, Knight et al., 2017, Marnitz et al., 2018). The severity of ototoxicity can range from mild to severe impairment and is influenced by factors such as age, pre-existing hearing impairment, concurrent cranial irradiation, cumulative cisplatin dose, and dose administration schedules (Rybak, 2007, Rybak et al., 2007, Olgun et al., 2016). There is an unmet clinical need for therapies that reduce the ototoxicity of cisplatin while preserving its therapeutic efficacy (van As et al., 2014). A challenge to the development of these therapies has been a lack of optimized animal models of cisplatin ototoxicity that are consistent with the methods of clinical cisplatin administration and result in comparable degrees of hearing loss.

To date most published studies of cisplatin ototoxicity in animal models have utilized high-dose, single-bolus injection protocols that often result in only small changes in hearing sensitivity. These high-dose protocols sometimes result in high mortality rates that limit assessment of cumulative dose effects or progressive effects of cisplatin. Single-injection protocols are not reflective of clinical cisplatin administration, which typically involves multiple cycles over several weeks, with intervening recovery periods during which cisplatin is not administered (Sockalingam et al., 2000, Hughes et al., 2014). Animal models that utilize systemic, multidose cisplatin regimens have been previously described with variable results (Minami et al., 2004, Hyppolito et al., 2006, Poirrier et al., 2010, Roy et al., 2013, Hughes et al., 2014); however, these protocols often resulted in high mortality rates and/or inconsistent changes in auditory function, making it difficult to assess potential therapeutic interventions.

Here we have modified and optimized a previously described (Roy et al., 2013., Breglio et al., 2017) multi-cycle model of cisplatin administration in mice that results in a consistent, significant hearing loss over multiple frequencies with little overall variability and virtually no mortality. In addition to the characterization of the cisplatin-induced ototoxicity, this report is meant to provide a detailed methodology that will offer guidance on the revised methods and outline the nutritional and hydration support the protocol requires. This clinically relevant model of cisplatin-induced hearing loss is a useful model system for studies of potential therapies aimed at protecting the hearing of cancer patients treated with cisplatin.

2. METHODS

2.1 Animals

Thirty-five adult CBA/CaJ male and female mice from Jackson Laboratories (Bar Harbor, Maine, USA) were used across two separate experiments. Mice were randomly assigned to control and cisplatin-treated groups, maintaining a balance of sexes in each group. All mice were individually housed with free access to food and water in accordance with the NIH

NIDCD/NINDS Animal Care and Use Committee-approved protocol (#1327). Of the 35 animals, 23 were used in a pilot-dose comparison study in which mice received three consecutive cycles of once-daily intraperitoneal (IP) injections at 2.5, 3.0, or 3.5 mg/kg cisplatin (1mg/ml) for four days followed by a 10-day recovery period. Twelve-week old mice began the 42-day protocol (Figure 1A) with an average body weight of $24.8 \text{ g} \pm 3.5$ (range 17.9-31.3 g). All cisplatin-treated mice received daily subcutaneous injections of 1 ml 0.9% NaCl each morning and 1 ml of Normasol (Hospira, Inc., San Clemente, CA, USA) each afternoon. Supplemental nutrition (Figure 1C) was provided to maintain acceptable body weight throughout the course of the study. Mice were given 0.3 ml (per os (PO)) STAT[®] high-calorie liquid supplement (PRN Pharmacal, Pensacola, FL, USA) twice daily. DietGel Recovery[®] cups (ClearH₂O, Portland, ME, USA) and additional pellet chow were placed on the cage floor. A separate cohort of twelve-week old mice were used for a follow-up study using the same 3-cycle protocol at a single cisplatin dose; 8 mice (4 females, 4 males) received 3.0 mg/kg cisplatin (1 mg/ml) IP, and 4 mice (2 females, 2 males) received comparable volumes of vehicle (0.9% NaCl, IP) only. All mice were carefully monitored by investigators and veterinary staff for changes in overall health or activity that may have resulted from cisplatin treatment. Body conditioning scoring (BCS) was used to record the overall condition of each animal daily based on muscular tone, body fat content, coat maintenance and overall energy level (Ullman-Culleré and Foltz, 1999). Our animal protocol required that if a mouse received a BCS<2 indicative of severe malnutrition, it would be promptly euthanized; however, no mouse in either the pilot study or the follow-up study met this criterion.

2.2 Functional Assays

All mice underwent auditory testing using distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) measurements prior to cisplatin administration and again following completion of the third (final) cycle of cisplatin administration. A subset of mice was retested 4 months later to determine the stability of cisplatin-induced hearing loss. For auditory testing, mice were initially anesthetized using ketamine (Pulney Inc., Portland, ME, USA; 100 mg/kg IP) and xylazine (Akorn Inc., Lake Forest, IL, USA; 10 mg/kg IP) with supplemental injections at 1/3-1/2 of the original dose as necessary. Each animal's core body temperature was maintained at 37°C using a temperature-controlled heating pad fitted to a rectal probe (World Precision Instruments ATC-2000, Sarasota, FL, USA). All auditory testing was conducted in a sound-attenuated booth (Acoustic Systems, Austin, TX, USA).

DPOAE and ABR measurements were performed on anesthetized mice using Tucker-Davis Technologies (TDT; Alachua, FL, USA) hardware (RZ6 Processor) and software (BioSigRZ). For DPOAE testing, a single acoustic assembly containing an ER-10B+ microphone (Etymotic, Elk Grove Village, IL, USA) connected to two transducers (TDT, MF-1) was inserted into the ear canal, providing an unobstructed path from port to tympanic membrane and an appropriate acoustic seal. Two primary tones were presented at fixed intensity levels of L1= 65 dB SPL and L2 = 55 dB SPL at 14 f_2 frequencies spanning 4 to 40 kHz ($f_2/f_1=1.2$). The magnitude of the DPOAE at $2f_1-f_2$ and background noise estimates at 6 surrounding spectra were recorded.

ABR testing was conducted using tone-burst stimuli (Blackman window, 3 ms, 1.5 ms rise/fall) with alternating polarity at a presentation rate of 29.9/sec. Averaged waveforms from 1,024 presentations were acquired, amplified (20x), filtered (0.3–3 kHz) and digitized (25 kHz). Signals were delivered via a closed-field TDT MF-1 speaker at 8, 11.2, 16, 22.4, 32 and 40 kHz. Responses were recorded from subdermal needle electrodes (Rhythmlink, Columbia, SC, USA) placed at the vertex (noninverting), under the test ear (inverting), and at the base of the tail (ground). Stimuli at each frequency were presented initially at 90 dB SPL. The stimulus intensity level decreased in 20 dB steps and then in 5 dB steps as threshold approached. Threshold was determined by visual inspection and defined as the lowest stimulus level at which an identifiable and repeatable wave was observed for any of the ABR waveforms..

Immediately following ABR testing and while still under surgical anesthesia, a subset of mice was transferred to a vestibular sensory evoked potential (VsEP) station for testing of vestibular function. Mice were placed supine on a heating pad held at 37°C to maintain body temperature. Subcutaneous electrodes were placed at the nuchal crest (noninverting), at the left ear (inverting) and at the right hip (ground). The head was placed with the nose upward in a noninvasive head clip lined with weather stripping. A mechanical shaker (Labworks Inc. ET 132-2, Costa Mesa, CA, USA) coupled to the head clip via a custom metal plate was used to deliver the stimuli. Stimuli were linear acceleration ramps (2-msec duration) delivered at a rate of 17/sec to a power amplifier (QSC Rmx 850, Costa Mesa, CA, USA) which drove a mechanical shaker. The resultant translational head motion was in the naso-occipital axis, intended to stimulate the saccule and utricle of the mouse (Mock et al., 2011). The stimulus was calibrated and monitored throughout testing in jerk, the first derivative of acceleration (da/dt). The output of a calibrated accelerometer (VibraMetrics Model 1018, Princeton Junction, NJ, USA) mounted on the same custom plate to which the head clip was attached was routed to a custom differentiator to convert it to jerk in units of g/ms (where $1g = 9.8 \text{ m/s}^2$; see also Jones et al., 1999, and Jones et al., 2011). Stimulus level was measured as the mean peak jerk amplitude and was expressed in dB (re: 1 g/msec). VsEP responses were measured from the left ear using Tucker-Davis Technologies (TDT; Alachua, FL, USA) hardware (RZ6 Multi I/O processor, Medusa preamplifier and head stage) and software (BioSigRz, v. 5.1). The raw signal was amplified (20x), filtered (0.3–3 kHz) and digitized (25 kHz). The threshold protocol ranged in intensity from +6 dB to –18 dB re:1g/msec in 3 dB steps. Each final averaged VsEP response was obtained by averaging 256 positive polarity and 256 inverted stimuli. Two averaged responses were recorded for each intensity level. Threshold was designated as the level in dB halfway between the lowest level at which a repeatable response was obtained and the level 3 dB below at which no response was obtained. Upon completion of electrophysiology recordings, mice were moved to a warmed cage devoid of bedding to be monitored until fully recovered from anesthesia.

2.3 Histology

Following auditory and vestibular post-tests, mice were euthanized via CO₂ inhalation followed by decapitation. Inner ears were extracted, immediately perfused with 4% paraformaldehyde (PFA) at 4°C through the cochlear round and oval windows and then post-fixed overnight at 4°C. Fixed tissue was decalcified in 0.5M EDTA at pH 8.0 at room

temperature for 48 hours. Utricles were removed and cochleas were microdissected into 5 pieces (Eaton Peabody Laboratories, 2018a). Utricle and microdissected cochlear tissue was suspended in blocking solution comprised of 5% normal horse serum (NHS; Sigma-Aldrich, St. Louis, MO, USA) and Triton X-100 (Sigma-Aldrich; 1:300) and rinsed in PBS.

Cochlear pieces were immunostained with antibodies to (1) C-terminal binding protein 2 (mouse anti-CtBP2; BD Biosciences, San Jose, CA; used at 1:200) and (2) myosin-VIIa (rabbit anti-myosin-VIIa; Proteus Biosciences, Ramona, CA; used at 1:200) with secondary antibodies coupled to Alexa Fluors 568 (Invitrogen; used at 1:1000) and 647 (Invitrogen; used at 1:200), respectively. A cochlear frequency map (Müller et al., 2005) was generated using a custom plug-in to ImageJ (Eaton Peabody Laboratories, 2018b) to localize cochlear structures to corresponding frequency regions. Confocal z-stacks (step size of 0.2 μm) of the apical, middle and basal regions from each ear were collected using an LSM 780 laser scanning confocal microscope (Carl Zeiss AG, Oberkochen, Germany) in a 1024×1024 pixel raster ($135 \mu\text{m}^2$) using a high-resolution, oil-immersion objective (63x, numerical aperture 1.4). Inner hair cells (IHC) and outer hair cells (OHC) were quantified in 50 μm sections along the basilar membrane based on their CtBP2-stained nuclei in subsets of cisplatin-treated and control cochleas.

Utricles were immunostained with anti-myosin-VIIa (rabbit anti-myosin-VIIa; Proteus Biosciences; used at 1:200) and detected with Alexa Fluor 647 followed by phalloidin conjugated to Alexa Fluor 488 (Invitrogen; used at 1:50). Confocal z-stacks (step size of 0.2 μm) from two separate extrastriolar utricular regions were collected using an LSM 780 scanning confocal microscope in a 1024×1024 pixel raster ($135 \mu\text{m}^2$) using a high resolution, oil-immersion objective (63x, numerical aperture 1.4). Vestibular sensory hair cells (VHCs) from the extrastriolar region were quantified and averaged across 3 different 50 μm^2 sections per image (total 6 areas examined per utricle) in subsets of cisplatin-treated and control utricles.

2.4 Statistical Analyses

Statistical analyses were conducted in Graph Pad Prism 6 software. Significance was set to an alpha of 0.05. One- and two-way ANOVA followed by tests for multiple comparisons (Dunnett or Tukey) or multiple and paired two-tailed t-tests corrected for multiple comparisons were used to determine significance where appropriate.

3. RESULTS

3.1 Cisplatin ototoxicity is dose-dependent

Mice underwent three cycles of cisplatin treatment (Figure 1A); each cycle consisted of a 4-day injection period during which mice received daily IP injections of cisplatin (2.5, 3.0, or 3.5 mg/kg/day) followed by 10 days of recovery (no cisplatin). During the 42-day protocol, mice were weighed each morning and assigned a body condition score. Figure 1B illustrates the weight changes for control mice and each of the three cisplatin-treated groups. Weights were calculated as a percentage of initial (pre-cisplatin) body weight on Day 1 of the protocol. Control mice continued to gain weight slightly throughout the protocol. All

cisplatin-treated mice lost 10-15% of their initial body weight following the first injection period. By the end of the first 10-day recovery period, mice in the 2.5 and 3.0 mg/kg cisplatin groups had recovered to approximately 95% of their initial weight; mice in the 3.5 mg/kg group recovered to approximately 90% of their initial body weights. During the 2nd and 3rd cycles, mice lost and then recovered weight in a dose-dependent manner. By the end of the 3rd cycle, mice treated with 2.5, 3.0, and 3.5 mg/kg cisplatin were at approximately 79%, 73%, and 73% of their initial weights, respectively.

Previous administrations of this three-cycle protocol using higher cisplatin doses resulted in more dramatic weight loss. Mice that exceeded 30% weight loss had a higher risk for mortality when anesthetized for post-test auditory testing. With the above described nutrition and hydration support, at this 3 mg/kg dosing scheme, no animals reached this extreme weight loss and no mice died during auditory testing. All mice maintained a BCS of 3 throughout most of the protocol and remained active and alert. In the previous higher dose (3.5 mg/kg cisplatin) protocol, a few animals received a BCS of 2+ or 2 in the last few days of Cycle 3. Our endpoint criteria for all cisplatin-treated mice include a BCS <2 for more than a 24 hr period, an arched posture that would indicate pain or distress, or a veterinarian recommendation for euthanasia. However, no animals met these exclusion criteria at any point during the 3.0 mg/kg cisplatin protocol.

Throughout the protocol, supplemental hydration and nutritional support were provided daily (Figure 1C). All animals remained active throughout the protocol.

Cisplatin treatment resulted in significant changes in hearing sensitivity. Figure 2A illustrates the DPOAE amplitudes as a function of f_2 frequency, an indirect measure of the function of OHCs. At the end of the protocol, all cisplatin-treated mice showed significantly decreased DPOAE amplitudes relative to controls ($F_{3, 490} = 304.3$, $p < 0.0001$). The decrease in DPOAE amplitudes was greater in mice receiving higher cumulative doses of cisplatin. Absent DPOAEs or significantly lower amplitudes were confined primarily to the high frequencies (> 28.8 kHz) in mice treated with 2.5 mg/kg with no evidence of ototoxic effects in the low- to mid-frequency region of the cochlea relative to the control group. Mice treated with 3.0 mg/kg cisplatin showed absent or substantially lower DPOAE amplitudes in the high-frequency region as well moderate amplitude decreases across the mid-frequencies (> 16 kHz) and no changes in low-frequency DPOAEs compared to the control group. Lastly, mice in the 3.5 mg/kg cisplatin group had robust changes across most frequencies tested (> 9.6 kHz). Collectively, these data indicate that cisplatin resulted in diminished OHC function in a dose-dependent manner.

ABR were recorded as a measure of auditory sensitivity. Mice were tested prior to cisplatin administration and at the end of the treatment protocol. Figure 2B shows threshold shifts, which are reported as the difference in hearing sensitivity between the pre-test and the post-test ABR measurements. Control (saline-injected) mice showed no significant changes in auditory sensitivity relative to baseline ($F_{1, 6} = 0.2727$, $p > 0.05$). Like the patterns observed in DPOAE amplitudes, all cisplatin-treated mice showed significant increases in ABR thresholds relative to baseline ($F_{3, 135} = 252.4$, $p < 0.0001$). These changes in hearing sensitivity were confined to high frequencies (> 22.4 kHz) in the mice treated with 2.5

mg/kg cisplatin. In contrast, mice treated with 3.0 mg/kg cisplatin had significant threshold shifts across the entire frequency range tested, and threshold shifts were more severe in mice treated with 3.5 mg/kg cisplatin. These data confirm that three cycles of cisplatin administration in mice results in significant threshold shifts that are both frequency-specific and dose-dependent, consistent with clinical cisplatin ototoxicity in humans (Kopelman et al., 1988, Kalyanam et al., 2018).

3.2 Cisplatin-induced hearing loss is progressive

Since a cisplatin dose of 3.0 mg/kg/day resulted in a robust hearing loss across all frequencies with the greatest threshold elevations in the high frequencies and less weight loss than 3.5 mg/kg/day, we selected this dose for further characterization of cochleotoxicity and vestibulotoxicity. A separate, second cohort of twelve mice (8 cisplatin, 4 control) were treated with 3.0 mg/kg cisplatin. Following our established 3-cycle administration protocol (Figure 1A) and animal care schedule (Figure 1C), The cisplatin-treated mice showed similar weight loss and recovery and similar changes in BCS assessment to those in the 3.0 mg/kg group in the pilot study shown in Figure 1B. All mice remained on the study with a BCS >2+ for the entire protocol.

Figure 3A shows significant differences in DPOAE amplitudes between control and cisplatin-treated mice similar to those we observed for mice treated with the same dose in the first experiment, confirming the consistency of ototoxicity using this protocol. While we did not examine the timecourse of cisplatin-induced hearing loss in the current study, our previous data using a higher dose (3.5 mg/kg) cisplatin regimen indicated that minimal hearing loss was observed after the second injection cycle of our 3-cycle protocol (Breglio et al., 2017), but significant threshold shifts across frequencies were not observed until after the third cycle of cisplatin administration. Since the revised protocol utilizes a lower dose of cisplatin, we would anticipate that the hearing loss would be minimal until after the third cycle. No significant differences in DPOAE amplitudes were observed between male vs. female mice. In order to determine if cisplatin-induced damage progresses after the end of the cisplatin administration protocol, a subset of these mice was tested again four months later (Fig 3). DPOAE amplitudes continued to decrease significantly relative to amplitudes at the end of cycle 3 in the mid-frequency region for all mice ($F_{1, 266} = 10.47$, $p < 0.01$). These data indicate that OHC function continues to worsen over time after the cessation of cisplatin administration.

Consistent with changes reported in the first cohort, ABR thresholds were significantly elevated in cisplatin-treated mice for frequencies >22kHz at the end of cycle 3 in the second cohort (Figure 3B; red). ABR thresholds were not significantly different between sexes. Mice that were retested 4 months after cycle 3 demonstrated significant progression of cisplatin-induced hearing loss beyond that seen at the end of cycle 3 ($F_{1, 78} = 66.52$, $p < 0.0001$). These data indicate that the 3-cycle cisplatin protocol results in reproducible changes in hearing sensitivity and that sensitivity continues to worsen significantly after the cessation of cisplatin administration. This progression of cisplatin ototoxicity is consistent with clinical reports of progressive hearing loss in humans treated with cisplatin (Knight et al., 2005, Kollinsky et al., 2010, Yasui et al., 2014, Waissbluth et al., 2018).

3.3 Cisplatin results in significant loss of outer hair cells

After the final hearing test, mice were euthanized and their cochleas were examined. Representative maximum intensity projections of confocal stacks of the organ of Corti for control and 3.0 mg/kg cisplatin-treated mice at the end of cycle 3 are shown in Figure 4A. IHCs and OHCs were identified based on their Myosin-VIIa-labeled cell bodies (blue) and CtBP2-labeled nuclei (white). No significant loss of IHCs was apparent (Figure 4B). Cisplatin-treated mice had a significant reduction in OHCs in the mid-cochlear region ($t_{30}=3.978$, $p<0.001$) and in the basal region ($t_{30}=15.85$, $p<0.001$); OHC density was reduced by approximately 22% in the mid-frequency region and nearly 95% in the basal region relative to control mice (Figure 4C). Four months after Cycle 3, OHC density was further reduced by an additional 25% in the mid-frequency region. The decline in low-frequency auditory sensitivity we observed in our ABR measurements cannot be accounted for by loss of apical outer hair cells, since we observed no decrease in apical OHC survival. Cochlear hair cells are not the only anatomical structures underpinning the ABR. Auditory sensitivity requires function of the stria vascularis to maintain the endocochlear potential and the spiral ganglion neurons to relay electrical signals along the auditory nerve. Cisplatin affects these cochlear structures (Sluyter et al., 2003, Breglio et al., 2017 Liu et al., 2018), and decreased stria or SGN function may contribute to threshold shifts we observed in the absence of hair cell loss.

3.4 Cisplatin has no effect on measures of vestibular sensitivity or utricular hair cell survival in mice

Previous reports of single-injection, high-dose cisplatin indicate decreased vestibulo-ocular reflexes in animal models but no major changes in vestibular function at lower doses (Takimoto et al., 2016). In the present study, VsEP were recorded to assess the utricular and saccular function at the end of cycle 3 and again 4 months later (Figure 5). No significant decrease in the function of the otolithic organs, as measured by VsEP threshold sensitivity, was observed at either timepoint (Figure 5C) ($F_{2, 13}=1.087$, $p>0.05$). In addition, no loss of utricular hair cells was observed (Figure 5A, B) ($t_{11}=0.4781$, $p>0.05$).

4. DISCUSSION

Since its debut in clinical cancer treatment in the 1970s, cisplatin has been a mainstay of chemotherapy that is used to treat a variety of solid tumors. However, cisplatin-based chemotherapy has significant side effects that can severely compromise quality of life for cancer survivors. Cisplatin is the most ototoxic drug in clinical use (Hellberg et al., 2009, Waissbluth et al., 2018). Thus, there is a critical need for therapeutic interventions that prevent or reverse cisplatin-induced hearing loss. However, the development of therapies to safeguard the hearing of cisplatin-treated patients requires appropriate animal models to evaluate potential protective strategies.

Many previous animal models of cisplatin ototoxicity consisted of single-, high-dose cisplatin administration that resulted in high mortality rates and/or relatively small changes in hearing sensitivity (Hill et al., 2008, Bielefeld et al., 2013, Hazlitt et al., 2018, Teitz et al., 2018). Consequently, these studies typically restricted analyses to only acute effects of

cisplatin (Sockalingham et al., 2000, Poirrer et al., 2010, Hughes et al., 2014). In some cases, where mortality was not an issue, insufficient damage to hearing was observed. To increase the extent of cisplatin-induced hearing loss, investigators have augmented cisplatin administration protocols with loop diuretics or concurrent noise exposure (Gratton et al., 1990, McAlpine & Johnstone 1990, He et al., 2009, Ding et al., 2012). While these augmented protocols result in increased hearing loss in the rodent models, they also increase the difficulty of interpreting the findings in relation to clinical cisplatin ototoxicity. Multidose models where cisplatin is delivered in smaller doses repeatedly over multiple days have been previously reported (Bowers et al., 2002, So et al., 2008, Takimoto et al., 2016, Wu et al., 2017). However, some of these regimens resulted in high mortality rates or an insufficient cochlear lesion. An alternative approach to systemic delivery has been the use of local cisplatin delivery via transtympanic injection (He et al., 2009), round window application (Korver et al., 2002, Tanaka et al., 2003, Whitworth et al., 2004, He et al., 2009, Xia et al., 2012) or direct infusion to the cochlea (McAlpine et al., 1990). These delivery routes aim to reduce systemic toxicities but are invasive, have high associated mortality rates and are ultimately not reflective of clinical cisplatin administration, which is usually systemic. To overcome these potential obstacles, a systemic multi-cycle administration protocol that incorporates recovery periods between multi-day injection periods results in more robust hearing loss with reduced mortality.

In the present study, our 4-day, 3-cycle cisplatin administration resulted in significant hearing loss as determined by DPOAEs and ABRs. Histological evidence of extensive OHC loss in the basal region and moderate loss in the mid-cochlear region was reinforced by diminished/absent DPOAE amplitudes in mid to high frequencies. Overall auditory sensitivity, as measured by ABR thresholds, was impaired across the entire frequency range, with increasing threshold elevations in the mid and high frequencies. Moreover, we observed an additional 10-15 dB threshold shift 4 months after the end of cisplatin treatment and further loss of cochlear outer hair cells, indicating that cisplatin-induced hearing loss continued to progress after the cessation of cisplatin administration, consistent with what has been reported in humans (Bertolini et al., 2004, Einarsson et al., 2010, Waissbluth et al., 2018). This progression in hearing loss may be related to long-term platinum retention in the inner ear (Schweitzer et al., 1986, Brouwers et al., 2008, Hellberg et al., 2009, Breglio et al., 2018). Unlike previous cisplatin administration animal models that generated initial profound hearing shifts or incurred high mortality rates using high-dose cisplatin injections, the model presented here results in a moderate-to-severe cochlear lesion comparable to clinical reports (Lippman et al., 1973, Helson et al., 1978, Pollera et al., 1988, Frisina et al., 2016) that also progresses similar to humans (Bertolini et al., 2004, Einarsson et al., 2010, Sprauten et al., 2012, Yasui et al., 2014) without encountering mortality complications or stimulus ceiling effects. Previous investigations conducted within our own laboratory using earlier versions of this 3-cycle protocol but with higher cisplatin dosing were used to examine protection (Roy et al., 2013) and possible mechanisms of cisplatin-induced hearing loss (Breglio et al., 2018). These higher-dose protocols resulted in more severe hearing loss than is often observed clinically as well as increased mortality rates. The current revision to optimize cisplatin dosing results in a pattern and severity of damage to the cochlea better

suited for future studies examining hearing loss progression and potential protective therapies.

There are inconsistent reports in the literature regarding vestibulotoxicity in humans treated with cisplatin. Some investigators have reported no cisplatin-induced vestibular toxicity (Reddel et al., 1998, Myers et al., 1993), while others report symptoms consistent with vertigo and dizziness (Langer et al., 2013, Kalyanam et al., 2018, Myers et al., 2018). Our mouse model of cisplatin ototoxicity does not result in decreased survival of utricular hair cells or in increased VsEP thresholds, suggesting that the protocol results in little to no cisplatin-induced vestibulotoxicity. There are, however, animal models that have been reported to induce cisplatin-associated vestibular dysfunction and therefore may serve as better models of cisplatin-induced vestibulotoxicity. For example, Sergi et al. (2003) observed significant functional declines in the guinea pig vestibulo-ocular reflex accompanied by significant losses of Type I and Type II VHCs in cisplatin-treated guinea pigs relative to controls following systemic cisplatin administration (2.5 mg/kg/day for 6 days). Callejo et al. (2017) reported significant dose-dependent vestibular dysfunction using behavioral tasks in rats treated transtympanically with cisplatin. The differences in animal model (guinea pig and rat versus mouse), the delivery route (transtympanic versus systemic), the dosing schedule (6 consecutive days versus 3 cycles of 4 days of drug injection) and the functional assays used to assess different elements of the vestibular system (semicircular canals versus otolith organs) likely to account for the differing degrees of reported vestibulotoxicity. The current study did not include comprehensive evaluation of vestibular function. No functional assessment of the semicircular canals was conducted, nor were other anatomical elements of the vestibular system quantified. Thus, though no changes in VsEP thresholds or vestibular hair cell counts were observed, we cannot rule out cisplatin-induced vestibular changes in this model.

There is an unmet need for a reliable model of cisplatin-induced ototoxicity. The optimal model should 1) utilize a cisplatin administration protocol that incorporates the cycles of chemotherapy that are typical in clinical settings, 2) utilize the same types of functional measures available in clinical settings, and 3) result in hearing loss consistent with the pattern of damage to the inner ear that is observed in clinical settings. Here we present an optimized model of cisplatin ototoxicity in adult mice that accomplishes these objectives with little variability and no mortality.

5. CONCLUSION

Here we characterize an optimized mouse model of cisplatin ototoxicity that minimizes health risks and mortality and offers a cisplatin lesion that is consistent with clinical observations regarding its frequency-specific pattern of damage and severity of toxicity. The resultant mild-to-moderate sensory hearing loss offers threshold shifts that align with clinical hearing losses and are well-suited for assessing progressive, functional changes in hearing. This model provides a platform for development of novel therapeutic strategies aimed at treatment or prevention of cisplatin-induced hearing loss in humans.

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Abbreviations:

DPOAE	Distortion Product Otoacoustic Emissions
ABR	Auditory Brainstem Response
VsEP	Vestibular sensory Evoked Potential
IHC	Inner Hair Cell
OHC	Outer Hair Cell
VHC	Vestibular Hair Cell
dB	Decibel
SPL	Sound Pressure Level
IP	Intraperitoneal

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Highlights

1. There is an unmet need for an animal model of cisplatin-induced ototoxicity that results in a clinically relevant hearing loss and low mortality.
2. A model of cisplatin administration that mimics the course of human clinical administration offers a platform to study potential protective therapies.
3. We present an optimized mouse model of cisplatin ototoxicity that reliably results in significant hearing loss across frequencies with low variability and virtually no mortality.

Figure 1: A multi-cycle model of cisplatin administration.

A: Time course for audiometric evaluations and three cycles of cisplatin administration.

Each cycle consists of a 4-day cisplatin injection period followed by 10 days of recovery. **B:**

Changes in mouse weight are shown across the entire cisplatin protocol. Weight and body

conditioning scores (BCS) were recorded daily. Mean $\pm$ SEM, $n=4-9$ mice per group. Mice

lost weight with each injection period and regained weight during each of the recovery

periods. No animals met the criteria for euthanasia. **C:** Twice-daily nutrition and hydration

support was provided throughout the protocol.

Figure 2: Cisplatin causes dose-dependent loss of cochlear outer hair cell function and hearing sensitivity.

Cochlear OHC function was assessed using distortion product otoacoustic emissions (DPOAEs). **A:** Relative to control mice, all cisplatin-treated mice showed significantly reduced DPOAE amplitudes or absent DPOAEs. As (cumulative) cisplatin dose increases, DPOAE amplitudes decrease. Mice treated with 3.5 mg/kg cisplatin (green) showed a nearly complete loss of DPOAEs across all frequencies, whereas mice treated with 3.0 mg/kg (red) showed near-total loss in the high frequencies, moderate loss in the mid-frequencies, and no change in the low frequencies. Low-dose cisplatin (2.5 mg/kg, blue) resulted in absent or reduced DPOAEs at high frequencies only. Grey shaded region denotes the mean biological noise floor. **B:** Auditory sensitivity was measured using auditory brainstem response (ABR) recordings. ABR threshold is defined as the lowest stimulus intensity that elicits a repeatable response. Control mice did not have changes in their ABR thresholds. Cisplatin resulted in significant threshold elevations in the high frequency region (>32 kHz) for all cisplatin-treated mice. As cisplatin dose increases, the hearing loss becomes more severe and spreads to include lower frequencies. 3.0 mg/kg cisplatin resulted in hearing loss across frequencies that was more severe in the higher frequencies. Mean $\pm$ SEM, $n=4-9$ mice per group. Statistical analysis consisted of a 2-way ANOVA with Dunnett's multiple comparisons, $*p<0.05$, $**p<0.001$.

Figure 3: Cisplatin ototoxicity progresses after the cessation of cisplatin administration.

A second cohort of mice was treated with 3.0 mg/kg (controls received saline). Auditory tests were again conducted before cisplatin and repeated after cycle 3. A subset of animals was re-tested 4 months later to assess progression of cisplatin-induced hearing loss. **A:** Changes in DPOAE amplitudes at the end of cycle 3 (red) were similar to those seen in the first cohort. No significant differences in DPOAEs were observed between male and female mice. Four months after cycle 3 (blue) a significant progressive decrease in DPOAE amplitudes was observed in the mid-frequency region relative to end of cycle 3. This progression of OHC dysfunction is evident in both male and female mice. **B:** ABR threshold shifts at the end of cycle 3 in the second cohort were comparable to those seen in the first cohort. No differences in ABR threshold shifts were observed between sexes. Mice evaluated 4 months after cycle 3 demonstrated a significant, progressive loss in hearing sensitivity, shown here as a greater threshold shift, relative to the end of cycle 3 in both sexes (blue). Mean $\pm$ SEM, $n=4-8$ mice per group. Statistical analysis consisted of a 2-way ANOVA with Dunnett's multiple comparisons, * $p<0.05$, ** $p<0.001$.

Figure 4: Cisplatin results in significant loss of outer hair cells.

Microdissected cochlear turns immunostained for myosin-VIIa (blue) and CtBP2 (white) were imaged to assess IHC and OHC loss across the cochlea. A: IHCs were intact in both control (upper row) and cisplatin-treated mice (lower row). In contrast, cochleas from cisplatin-treated mice showed significant loss of OHCs in the middle and basal turns relative to controls at the end of Cycle 3 with significant subsequent deterioration 4 months later in the mid-frequency region. Panels B and C show quantification of IHC and OHC numbers, respectively, and further illustrate no loss of IHCs and significant loss of OHCs in cisplatin-treated mice in the middle and basal regions of the cochlea. Means $\pm$ SEM, n=4-8 per group. Statistical analysis consisted of Multiple t-tests, *** p <0.001, **** p <0.0001, n.s. = no significance.

Figure 5: Cisplatin did not result in vestibulotoxicity.

Mouse utricles were immunostained for myosin-VIIa (hair cell marker, white). No observable (A) or quantifiable (B) loss of utricular hair cells was present within the extrastriolar region. C: VsEP amplitudes were measured to assess vestibular function at the end of cycle 3 (red) and again 4 months later (blue). No significant differences in VsEP thresholds were observed between control and cisplatin-treated mice at either time point. Means±SEM, n=4-8 mice per group (1 ear per mouse). Statistical analysis consisted of a one-way ANOVA with Tukey's multiple comparisons test (B) and paired t-test (C), n.s. = no significance.