Original Research—Otology and Neurotology

Prestin as an Otologic Biomarker of Cisplatin Ototoxicity in a Guinea Pig Model

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Abstract

Objective. To evaluate (1) whether changes in serum prestin aid in early detection of cisplatin ototoxicity, (2) the role of diltiazem as an otoprotectant, and (3) whether prestin levels are sensitive to effects of diltiazem.

Study Design. Experimental animal study.

Setting. Translational research laboratory.

Subjects. Twenty female guinea pigs.

Methods. Two groups of 10 guinea pigs were used. The relationship between serum prestin levels and auditory brainstem response (ABR) thresholds was compared between the groups. All animals had baseline blood draws and ABR thresholds recorded prior to cisplatin administration. Intraperitoneal cisplatin bolus (8 mg/kg) was administered followed by 5 consecutive days of intratympanic (IT) diltiazem (2 mg/kg) or sham IT-saline injection. Serum prestin levels and ABR thresholds were measured at days 1, 2, 3, 7, and 14 postcisplatin.

Results. In sham, IT-saline–treated animals, mean prestin levels were elevated above baseline on days 1 to 7. The prestin levels were significantly elevated from baseline on day 1 ($P < .001$), while significant ABR threshold elevations did not occur until day 2 ($P = .028$) for click-evoked ABRs and day 3 ($P = .041$) for tones. In diltiazem-treated animals, prestin levels were not elevated above baseline but ABR thresholds were elevated on days 1 to 3. However, the thresholds returned toward baseline on days 7 and 14.

Conclusion. Changes in serum prestin levels were detectable prior to shifts in ABR thresholds in a guinea pig cisplatin ototoxicity model. These changes did not occur in diltiazem-treated animals. Prestin may serve as a biomarker of cochlear injury that is sensitive to therapeutic interventions in cisplatin ototoxicity.

Keywords

biomarkers, ototoxicity, calcium-channel blockers, otoprotectants

Serum biomarkers are useful indicators of normal and pathological biological processes. They also provide utility in that they indicate response to pharmacological treatments.[1] Biomarkers are easy to collect, sensitive enough to detect the disease of interest, and specific enough to differentiate normal from abnormal. Until recently, biomarkers for otologic diseases were not available. However, several unique proteins specific to the ear offer potential utility as otologic biomarkers. For example, in a proof-of-concept study, we demonstrated that an inner ear protein, otolin-1, increases in circulation in patients with benign paroxysmal positional vertigo (BPPV).[2,3] We extended this concept to sensorineural hearing loss and noise-induced hearing loss through measurement of the outer-hair cell-specific protein, prestin, in circulation.[4,5] In order for the concept of otologic biomarkers to be adopted into clinical practice, validation in other experimental settings, such as ototoxicity, is necessary. In addition, it should be demonstrated that biomarker levels are sensitive to interventions aimed at ameliorating otologic damage. In this study, we propose prestin as an otologic biomarker in the setting of cisplatin ototoxicity.

Cisplatin is a chemotherapeutic agent with known ototoxicity properties.[6,7] It causes irreversible damage to cochlear outer hair cells (OHCs) through calcium-mediated apoptosis.[8-12] Using a murine model of cisplatin ototoxicity, we demonstrated that the calcium-channel blocker (CCB), diltiazem, may have otoprotective properties.[13] Therefore, a

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cisplatin ototoxicity model with and without diltiazem provides a setting for validating prestin as a biomarker and demonstrating its sensitivity to therapeutic interventions. This study also aims to replicate the efficacy of diltiazem as an otoprotectant.

We hypothesize that (1) prestin levels will rise early after cisplatin administration prior to elevations in auditory brainstem response (ABR) thresholds, and (2) diltiazem will prevent elevation of prestin and ABR thresholds. Finally, to support concept of cochlear specificity of prestin,[14] we hypothesize that noncochlear organ levels of prestin will not exceed blood levels.

Methods

This study was approved by the Institutional Animal Care and Use Committee at the UConn Health (IACUC No. 101275-0119).

Animal Subjects

Two groups of 10 female Dunkin Hartley guinea pigs (400-450 g) from Charles River Laboratories (Wilmington, Massachusetts) were used. The animals were handled and evaluated in conjunction with the veterinary staff at UConn Health. They were housed in a 12-hour dark/light cycle, with free access to food and water. No mortality occurred during the study.

Study Design

The animals were assigned to either a sham saline group (n = 10) or treatment diltiazem group (n = 10). Both groups had baseline serum blood draws and click- and tone-evoked ABRs performed. A single dose of cisplatin (8 mg/kg) was administered intraperitoneally (IP), and intratympanic (IT) injections of saline or diltiazem (2 mg/kg) were initiated on the same day for a total of 5 consecutive days. Blood draws and ABRs were then performed at days 1, 2, 3, 7, and 14 postcisplatin administration in both groups. Prestin levels were measured for each sample with enzyme-linked immunosorbent assay (ELISA). All animals were euthanized with CO_2 on day 14. Brain, kidney, skeletal muscle, heart, and liver were harvested from 5 guinea pigs for comparison of prestin levels with blood.

Auditory Brainstem Responses

All ABR testing was performed while the subjects were under inhalational anesthesia in a sound-proof chamber. Both ears were tested during each ABR. The ear not being tested was obstructed to minimize any crossover stimulation. The ABRs were measured with a Tucker-Davis Technologies (TDT, Alachua, Florida) system. A TDT CF1 speaker was placed at the opening of the external canal of the test ear. Electrodes were placed subcutaneously with the active electrode at the vertex, the ground electrode behind the right ear, and the reference electrode behind the left ear.

For tone-evoked ABRs, 5-ms (2-ms rise/fall time) pure-tone bursts of 4, 8, 16, 24, and 32 kHz were delivered at a rate of 21/s. Click-evoked ABRs were recorded in response to 100 μs, alternating polarity, broadband clicks. Stimuli were attenuated in 5-dB steps until no response was present. The 10-ms recorded signal was amplified 100,000 times and filtered between 30 and 3000 Hz. The signals were averaged after 256 presentations. ABR threshold was defined as the highest level that evoked ABR peak III.

Injections/Anesthesia

Prior to any IT injection, inhalation anesthesia was administered. Inhalational route was chosen because it permitted short duration of anesthesia and rapid recovery time. Induction anesthesia of 2.5% to 3.0% isoflurane was administered with concomitant 0.8 to 1.0 L oxygen in a closed chamber. The animal was transferred to a snout mask device for maintenance anesthesia.

IP injections with cisplatin, 8 mg/kg,[15] were performed with a 27-gauge needle. It was followed by a 10-mL bolus of saline for hydration.

While the guinea pig was under anesthesia, an operating Zeiss otomicroscope (Dublin, California) and otic speculum were used to visualize the tympanic membrane (TM). A 27-gauge needle connected to a microsyringe was placed into the middle ear through the TM and the drug administered.

The 2-mg/kg dose of diltiazem was previously determined to provide otoprotection.[16] This was delivered in a 100-μL solution as this is the estimated size of the guinea pig middle ear space.[17] A smaller 10-μL volume was used in the sham IT-saline animals.

Subclavian Blood Draws

With the guinea pigs under anesthesia, the ventral aspect of the neck was shaved and the forelegs retracted. The clavicle was palpated and a 1-mL tuberculin syringe with a 25-gauge needle was inserted below the clavicle just lateral to the midline until venous blood was returned. The volume of blood permissible to obtain at 24-hour intervals in a guinea pig is 1% of total blood volume. The circulating volume in a guinea pig is estimated to be 7.5% of total body weight, which was between 400 and 450 g (33.75 mL). Thus, roughly 0.3 mL was drawn each procedure.

ELISA

Blood samples were collected and centrifuged for 20 minutes at 3000 rpm within 30 minutes of collection. The samples were stored in a –80°C refrigerator until the tissue samples from the harvested organs were weighed, and lysis buffer (Tissue Extraction Reagent I; ThermoFisher, Waltham, Massachusetts), with protease inhibitor (Protease Inhibitor Cocktail Powder; Sigma-Aldrich, St Louis, Missouri), was added at a ratio of 10 mL per 1 g of tissue. Tissue samples from 5 sham-IT saline treated animals were homogenized in an Omni Mixer Homogenizer (Omni International, Kennesaw, Georgia) and centrifuged for 5 minutes to pellet the tissue debris. Supernatants were collected and run on the ELISA.

Prestin concentration was measured using a cavy SLC26A5 ELISA kit (MyBioSource.com) as described in
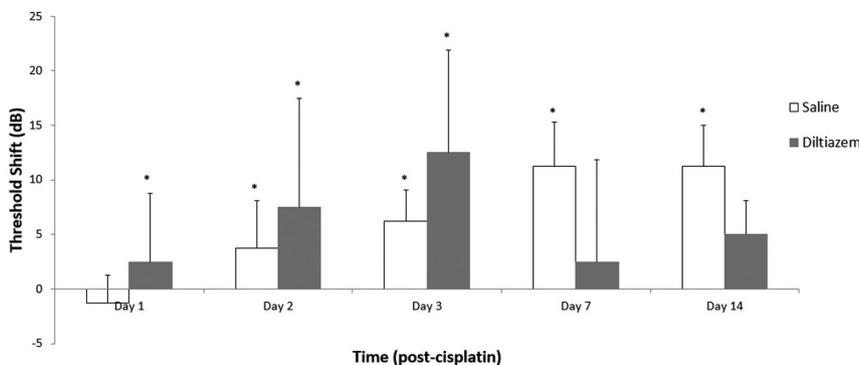
Figure 1. Median click-evoked auditory brainstem response threshold shifts of cisplatin-treated animals who received intratympanic (IT) saline (n = 10) or diltiazem (n = 9). Error bars represent interquartile range. *$P < .05$ compared to baseline threshold.

the manufacturer's instruction manual. The optical density in the wells of the ELISA microplate was measured at 450 nm using a Bio-Tek ELx808 plate reader and data were compiled using the KCJunior software package (Bio-Tek, Winooski, Vermont).

Statistical Analysis

Power analysis, guided by published studies in a guinea pig model of cisplatin,[15,18] determined that a minimum of 8 animals were needed to detect a 10% difference from control with a coefficient of variability of 5% for 95% power at $P < .05$.[19] Left and right ear ABR thresholds were averaged to yield a single threshold value at each stimulus condition per animal. Normality of distributions was tested using the Shapiro-Wilk test. Descriptive statistics included mean and standard error of the mean for normally distributed data and median and interquartile range when distributions departed from normal. Statistical comparisons between each group and its corresponding baseline values were performed. Related samples, Friedman's 2-way analysis of variance (ANOVA) by ranks tests were used to statistically analyze distributions that deviated from normal. Follow-up tests were performed using the Wilcoxon signed rank test. Normally distributed data were analyzed using repeated-measures ANOVA. Statistical significance was set at $P < .05$.

Results

One animal was eliminated from the diltiazem group due to presence of head tilt and cerebral purulence upon postmortem evaluation on day 14.

Click-Evoked ABRs

Distributions of ABR thresholds were significantly different from normal at baseline, day 1, and day 3 postcisplatin. **Figure 1** shows median click-evoked ABR threshold shifts for sham IT-saline and IT-diltiazem groups. Median ABR thresholds progressively increased postcisplatin in the IT-saline group. There was a statistically significant change in threshold shifts as a function of time ($P < .001$). The first statistically significant shift occurred on day 2 ($P = .028$), and thresholds continued to be significantly elevated through day 14 ($P = .005$). In contrast, the IT-diltiazem group showed

threshold shifts that reached statistical significance on days 1 to 3 postcisplatin ($P < .05$). Thresholds returned toward baseline values by days 7 and 14 postcisplatin.
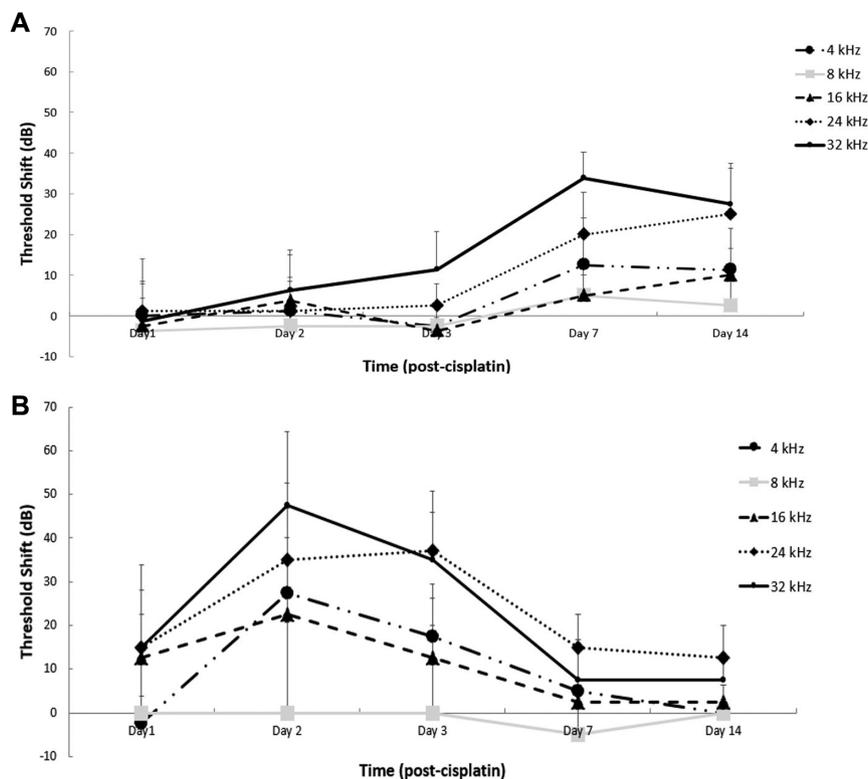
Tone-Evoked ABRs

Distributions of ABR thresholds at each frequency and post-cisplatin day deviated from normal (eg, 32 kHz at days 1 and 3 for the IT-saline group and 24 kHz at baseline for both IT-saline and IT-diltiazem groups). **Figure 2** shows median ABR thresholds for sham IT-saline and IT-diltiazem groups. Significant interaction between day and stimulus frequency was noted. A statistically significant shift in ABR threshold in the sham IT-saline group started at 32 kHz on day 3 ($P = .041$). On days 7 and 14 postcisplatin, there was a statistically significant threshold shift at 4, 16, 24, and 32 kHz ($P < .05$).

For the IT-diltiazem group, significant threshold shifts ($P < .05$) were seen on day 1 at 16 and 24 kHz and on day 2 and day 3 at 4, 16, 24, and 32 kHz. The only significant threshold shift on days 7 and 14 was recorded at 24 kHz on day 14 ($P = .028$).

Prestin. Prestin distributions were normal for both groups. The baseline prestin concentration was 5.90 ± 0.33 ng/mL (mean $\pm$ standard error of the mean). **Figure 3** shows mean percent change in prestin levels change as a function of time postcisplatin administration. In the sham IT-saline group, prestin levels were elevated relative to baseline at all time points postcisplatin administration. The rise from baseline values reached a maximum at day 2 postcisplatin administration (25.6%) and remained elevated at day 3 (20%) before trending back toward baseline at days 7 and 14. The changes in prestin level for days 1 to 3 were statistically significant compared to baseline ($P < .001$, $P < .001$, $P = .022$, respectively). The IT-diltiazem group showed no significant changes in prestin level postcisplatin.

Figure 4 shows prestin levels in major organs as a fraction of median blood concentration in 5 subjects from the IT-saline group. Prestin levels did not significantly exceed circulatory levels in any tissue. In cardiac tissue, brain, skeletal muscle, and kidney, prestin levels were substantially below that of blood, reaching statistical significance in the

Figure 2. Median tone-evoked auditory brainstem response threshold shift in cisplatin-treated animals who received (A) sham intratympanic (IT)–saline (n = 10) (P < .05 compared to baseline at 4, 8, and 24 kHz on days 7 and 14; at 32 kHz on days 3, 7, and 14) and (B) IT-diltiazem (n = 9) (P < .05 compared to baseline at 4 and 32 kHz on days 2 and 3; at 16 kHz on days 1, 2, and 3; 24 kHz on days 1, 2, 3, and 14). Error bars represent interquartile range.

latter 2 organs (P = .009). There was no difference in prestin levels between blood and liver.

Discussion

Currently, there are diagnostic and therapeutic shortcomings in managing cisplatin ototoxicity. Diagnosis is often made after which irreversible damage is present and the window for potential therapies has passed. We hypothesized that an inner ear–specific protein, prestin, would be elevated prior to ABR threshold shifts after cisplatin administration and that diltiazem would prevent prestin elevation and ABR threshold shifts, suggesting otoprotective properties against cisplatin. To test our hypothesis, we undertook a controlled, serial assessment of prestin after cisplatin exposure with and without an otoprotectant.

In the sham IT-saline group, prestin levels increased prior to significant click- and tone-evoked ABR threshold shifts, whereas in the IT-diltiazem group, prestin levels were not elevated above baseline. Both trends provide clinically translatable information. In the IT-saline group, the early rise in prestin before detectable ABR shifts offers early diagnostic information that opens a ''therapeutic widow'' during which potential therapies can be applied. The lack of prestin elevation in the setting of IT-diltiazem suggests that diltiazem may exert its effect at the level of the OHC and that prestin levels are sensitive to treatment interventions. Prestin may also offer the potential to trend therapeutic responses.
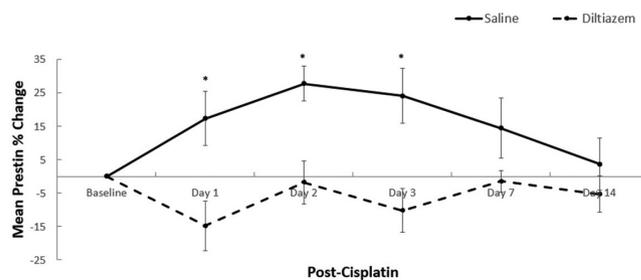
Figure 3. Mean percentage change in prestin levels as a function of time postcisplatin in sham intratympanic (IT)–saline (n = 10) vs IT-diltiazem groups (n = 9). Error bars represent standard error of the mean (SEM). *P < .05 compared to baseline concentrations.

These results suggest that elevated prestin levels early after cisplatin administration are caused by disruption of OHCs followed by release of the protein into circulation.[4] In sham IT-saline–treated animals, it is suspected that after day 3, OHC apoptosis stops and reestablishment of OHC homeostasis occurs, promoting return of prestin levels toward baseline. Similar biomarker trends are seen in cardiac enzymes during myocardial infarction. It is possible that a drop in prestin levels below baseline would have occurred if the study was carried beyond day 14, reflecting a lower number of surviving OHCs. Indeed, 2 weeks after noise trauma when there are missing OHCs in the cochlea,
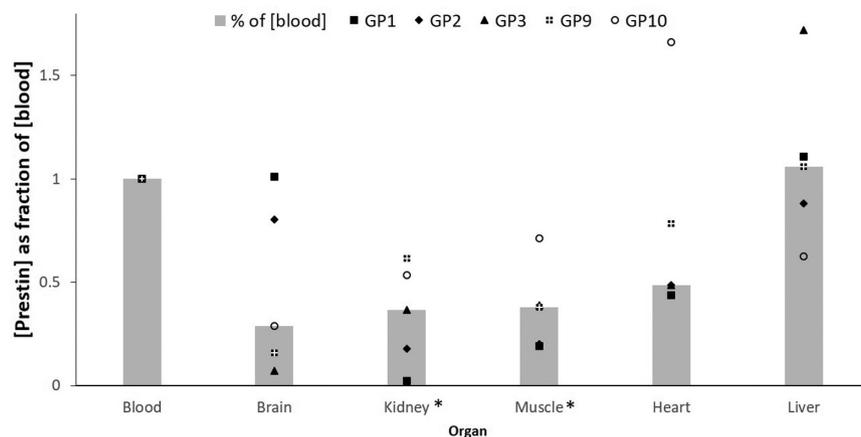
Figure 4. Prestin levels in major organs as a fraction of circulatory serum levels. Bars represent median values. *$P < .05$ in comparison to blood levels.

we have demonstrated decreased prestin levels relative to controls.[5] We only evaluated cisplatin at 8 mg/kg, but varying doses of cisplatin would likely accelerate or decelerate the rate of OHC damage and thus, prestin levels relative to baseline based on the rate of OHC damage. Alternatively, prestin levels may have remained elevated above the baseline due to a temporary elevation of prestin gene expression in the remaining OHCs that maintains circulatory prestin levels near baseline.[20] Future experiments in our cisplatin ototoxicity model could test these notions.

Diltiazem-treated guinea pigs demonstrated temporary ABR threshold elevations during days 1 to 3 postcisplatin administration. Thresholds returned toward baseline at days 7 and 14. A shortcoming of air-conducted ABR is that it is unable to differentiate conductive from sensorineural deficits. The initial rise in ABR thresholds for this group was likely due to the volume (100 μL) of drug placed within the middle ear that created a conductive loss. Persistent diltiazem solution was noted in the middle ear space on days 2 to 5 of IT injections. This volume approximates IT injections in guinea pigs in other models[17] and has been shown to significantly shift ABR thresholds.[21] In our data, ABR threshold shifts were greatest in the high frequencies when middle ear diltiazem solution was present. The shift seen in our model on days 1 to 3 was near the average expected shift due to fluid alone.[21] Nevertheless, we cannot rule out other components contributing to this threshold shift. For example, some of this early shift may represent mild, temporary cisplatin ototoxicity, since threshold shifts in diltiazem-treated animals did not completely return to baseline.

Early ABR threshold shifts were not seen in the sham IT-saline–treated group because a smaller volume (10 μL) was used. The middle ear fluid from 5 daily IT-diltiazem injections cleared by day 7 postcisplatin, and the temporary threshold elevations returned to values near baseline at days 7 and 14. The return toward baseline ABR thresholds in IT-diltiazem–treated animals suggests there may be an otoprotective effect of diltiazem against cisplatin and is consistent with our findings in a mouse model.[13] The fact that prestin levels in the IT-diltiazem group were not elevated above

baseline is also supportive of the concept that the ABR threshold shifts on days 1 to 3 in the IT-diltiazem group were primarily conductive in nature, because if it represented true OHC damage, an elevation in prestin levels would be expected during these threshold shifts.

We found that prestin levels in major solid organs do not exceed circulatory blood levels, suggesting that prestin is likely not produced in these organs. The 5 sham IT-saline–treated animals chosen for organ harvest and analysis had the largest shifts in click ABR thresholds. We suspected that in these animals, tissue concentrations of prestin would be highest if it was produced outside of the cochlea. Given that other organs in these animals did not have elevated levels of prestin, our data support the notion that prestin is likely produced predominantly in the cochlea. Equal levels of prestin in the blood and liver likely are accounted for by the rich vascular supply of hepatic tissue and possibly related to its role in protein metabolism and elimination. These findings further support previous reports that suggest prestin is an inner ear–specific protein, without evaluating prestin levels from tissue other than the cochlea.[22,23] It should be noted that we did not examine organs from untreated animals, so there is room for additional work to confirm our results.

One shortcoming of this study was that different volumes were used in the sham and treatment groups. The purpose of the sham IT-saline group was to control for the effect of potential TM perforation due to IT method of delivery, thus, only a 10-μL volume of saline was used. To adequately administer an adequate concentration of diltiazem, we administered an IT volume of 100 μL, closer to the volume used in a previous guinea pig publication.[17] Given that volume difference may have accounted for early threshold difference between the 2 groups, future work will need to evaluate these IT volumes in different arms of the experiment. Cochlear histology in this experimental model requires future investigation as well. Our group has demonstrated previously in a rat model of noise-induced hearing loss that hair cell counts and distortion product otoacoustic emission levels are reduced in the setting of reduced prestin levels.[5]

In this study, our focus was examining the temporal patterns of change in serum prestin. Therefore, we did not establish the correlation between cochlear histology and prestin at each time point. Finally, we did not evaluate whether diltiazem interferes with chemotherapeutic effects of cisplatin. However, diltiazem is not currently contraindicated in patients undergoing cisplatin chemotherapy, and IT administration should limit systemic distribution of diltiazem.

Cisplatin ototoxicity continues to be a clinical challenge because of limited available therapeutic agents and shortcomings in diagnostic measures. The results of this study extend previous findings[13] to a guinea pig model, suggesting IT-diltiazem has otoprotective properties against cisplatin ototoxicity. In addition, new findings include (1) an early rise in prestin levels after exposure to cisplatin that precedes onset of significant ABR changes, and (2) prestin levels are sensitive to a therapeutic intervention. These findings highlight the promise of prestin as an otologic biomarker that may translate to the clinical setting. This research provides a framework for continued investigations in biomarkers and new otoprotective agents. These concepts may be generalizable to other otologic conditions such as Ménière's disease or sudden sensorineural hearing loss and should be the subject of future clinical studies. Additional work is needed to determine if the methods employed here are specific and reliable enough to use prestin in the clinical setting as a biomarker for otologic disease.

Author Contributions

James Naples, conception hypothesis and design of work; acquisition, analysis, and interpretation of data; drafting and approval of manuscript; presentation of work; **Robert Cox**, conception hypothesis and design of work; acquisition, analysis, and interpretation of data; drafting and approval of manuscript; **Gregory Bonaiuto**, conception hypothesis and design of work; acquisition, analysis, and interpretation of data; drafting and approval of manuscript; presentation of work; **Kourosh Parham**, conception hypothesis and design of work; acquisition, analysis, and interpretation of data; drafting and approval of manuscript; presentation of work.

Disclosures

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