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Dexamethasone’s Effect in the Retrocochlear Auditory Centers of a Noise-Induced Hearing Loss Mouse Model

Leon Chen, MD1, Clare Dean, MD1, Michele Gandolfi, MD1, Edmund Nahm, MD1, Linda Mattiace, PhD2, and Ana H. Kim, MD1,2

Abstract

Objective. Examine prophylactic effects of dexamethasone (Dex) in retrocochlear auditory centers in a noise-induced hearing loss (NIHL) mouse model.

Study Design. Prospective animal study.

Setting. Academic research center.

Subjects and Methods. Thirty-two mice were divided into control, untreated, saline (2 and 10 μL), and Dex (2 and 10 μL) groups. Dex was applied intratympanically (IT) prior to 110 to 120 dB noise over 6 hours. Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) were performed at 1 day, 1 week, 1 month, and 2 months. Retrocochlear neuronal cells were labeled with FluoroGold and counted. Hair cells of the organ of Corti were labeled with fluorescein isothiocyanate-conjugated phalloidin and counted.

Results. Auditory brainstem response thresholds of untreated NIHL, 2 and 10 μL IT saline, and 2 and 10 μL IT Dex were 21.7 ± 2.9 dB, 20 ± 0 dB, 20 ± 5 dB, 18.3 ± 2.9 dB, and 18.3 ± 2.9 dB, respectively. At 1-day post NIHL, all groups demonstrated profound hearing loss. At 2 weeks, 2 and 10 μL Dex thresholds improved to 47.5 ± 3.5 dB and 48.8 ± 18.9 dB, respectively, whereas the untreated and saline groups remained unchanged. Mean cell counts in the cochlear nucleus (CN), superior olivary complex (SOC), and lateral lemniscus (LL) of control mice were 1483 ± 190, 2807 ± 67, and 112 ± 20, respectively. After acoustic trauma, the untreated, saline, and 2 μL Dex groups yielded decreased neuronal counts in the SOC. In contrast, the 10 μL Dex group had 1883 ± 186 (CN), 2774 ± 182 (SOC), and 166 ± 18 (LL). There was sporadic hair cell loss for all traumatized groups.

Conclusion. Our NIHL mouse model demonstrated dose-dependent Dex pretreatment otoprotection against NIHL with preservation of retrocochlear auditory neurons.

Keywords
dexamethasone, noise-induced hearing loss, retrocochlear

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Introduction

Noise-induced hearing loss (NIHL) has been a growing public health issue over the past half century. According to the National Institute for Occupational Safety and Health, approximately 10 million people in the United States have NIHL, whereas 30 million workers are exposed to potentially damaging noise on the job.1 Noise-induced hearing loss is also the top disability among military personnel returning from combat.2 Given the number of people knowingly exposed to loud noise, further attention to prevention and treatment of NIHL is needed.

The effects of NIHL on the inner ear have been extensively studied, with necrotic and apoptotic cell death in the organ of Corti being the major focus.3-9 Hence, studies have concentrated on interventions in the peripheral auditory system, ranging from glucocorticoid administration to hair cell regeneration.10-14 The use of dexamethasone (Dex), a potent synthetic glucocorticoid, has been demonstrated to attenuate NIHL by reducing outer hair cell (OHC) death and other glucocorticoid receptor mediated effects in the cochlea.15-19 These studies, however, have not investigated the effects of NIHL and Dex in the central auditory system.

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A few studies have shown cell loss or morphologic changes in the retrocochlear auditory centers following NIHL. The major retrocochlear auditory pathway is collectively known as ECOLI (Eighth nerve, Cochlear nucleus, Olivary complex, Laterallemniscus, and Inferior colliculus). Studies that found reduction in cell density or nuclei volume in these regions attribute the cell death to excitotoxicity from excessive stimulus, and/or secondary degeneration after hair cell loss. There is, however, minimal literature on effects of interventions like Dex on retrocochlear regions.

The purpose of our study was to examine effects of Dex on the retrocochlear auditory centers when used prophylactically against NIHL. First, we established appropriate parameters for a consistent NIHL mouse model and the time course for spontaneous hearing recovery. Using this model, we then investigated the functional and morphological changes in select retrocochlear nuclei when Dex is applied prior to NIHL by using hearing tests and neuronal cell labeling with FluoroGold (FG), as previously described by our group. Outer hair cell counts were also used to verify significance of retrocochlear results independent of peripheral changes.

**Methods**

**Animals**

CBA mice, documented to have normal auditory anatomy, physiology, and behavior, were chosen to study the auditory pathway. Thirty-two CBA male mice (Jackson Labs; Bar Harbor, Maine, USA) 13 to 16 weeks of age were housed at New York Medical College Animal Facility under the care of the Department of Comparative Medicine (IACUC No. 30-2-0512). Prior to any procedure, mice were anesthetized with an intraperitoneal injection of ketamine HCL (Ketathesia: 100 mg/kg; Abbott Labs, North Chicago, Illinois, USA) and xylazine (AnaSed: 10 mg/kg; Ben Venue Labs, Bedford, Ohio, USA).

**NIHL**

Hearing was assessed using auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) tests prior to NIHL and at several time points post trauma. To establish a NIHL mouse model, 3 mice were placed into a wire mesh cage (11 cm × 7 cm × 10 cm) within a sound-isolating acoustic chamber (Industrial Acoustic Corporation, Bronx, New York, USA) and subjected to free field broadband noise (6-32 kHz) from a Selenium speaker (D220TI 8 ohms; Nova Santa Rita, Brazil) and AudioSource amplifier (AMP102; Portland, Oregon, USA) for 6 hours at an intensity of 110 to 120 dB sound pressure level (SPL). During the exposure, mice had access to food and water.

**ABR Testing**

Click and tonal (8, 12, 16, 20, 24, 28, and 32 kHz) ABR thresholds were measured at 1 day prior to NIHL, and then at 1 day, 1 week, 1 month, and 2 months post acoustic trauma to establish the NIHL mouse model. Subsequent ABR testings for intervention mouse groups were done 1 day prior and 1 day, 1 week, and 2 weeks post NIHL. Subcutaneous stainless steel electrodes were placed on the vertex of the head (active), right mastoid (reference), and left hind leg (ground) for monitoring. The stimulus was delivered by ES1 speakers (Tucker-Davis Technologies, Alachua, Florida, USA) for open-field stimulation. Auditory brainstem response waveforms were recorded at intensity levels from 80 to 0 dB SPL in 5 dB SPL increments. The ABR threshold was defined as the lowest noise intensity eliciting a distinct reproducible wave V peak.

**DPOAE Testing**

Distortion product otoacoustic emission thresholds were determined at 12, 16, 20, 24, and 28 kHz immediately following ABR testing. An ear probe containing an ER-10B ear canal microphone (Eytonic Research, Elk Grove Village, Illinois, USA) was placed in the right external auditory canal. The probe provided a conduit for both stimulus, generated by 2 EC2 speakers (Tucker-Davis Technologies), and capture of responses in closed-field stimulation. Frequencies were acquired with f2/f1 ratio of 1.20. L1 = L2 with stimulation levels of 80 to 0 dB SPL. Tones were presented at a rate of 11.92/second. Responses were acquired via 100 sweeps per tone-level combination. Distortion product otoacoustic emission thresholds were defined as the lowest noise intensity eliciting a distinct distortion product frequency (fDP) over baseline noise.

**Intratympanic Dex and Saline Injections**

Six mice were used to establish our NIHL mouse model (3 untreated, 3 control). Eighteen mice were then evenly divided among 6 groups: 1 naïve (control), 1 traumatized (untreated), and 4 pretreated groups. Two different volumes (2 and 10 μL) of either Dex (10 mg/mL; West-Ward Pharmaceuticals, Eatontown, New Jersey, USA) or 0.9% normal saline were bilaterally injected intratympanically (IT) 1 time per ear immediately prior to NIHL induction. An additional 4 mice were added to each of the 10 μL Dex and untreated groups for statistical analysis. Under a Zeiss surgical microscope, the tympanic membrane (TM) was visualized through the external auditory canal. A 10-μL Hamilton syringe with a 2-inch-long 32-gauge blunt needle was passed through the inferior/posterior TM quadrant at a marked depth of 1/100th to 2/100th of an inch to ensure injection close to the round and oval window membrane.

**FluoroGold Cell Labeling and Count**

Two microliters of 5% FG (Sigma, St Louis, Missouri, USA) suspended in 0.9% normal saline was slowly injected IT similar to Dex, except with a 5-μL Hamilton syringe, 9 days after acoustic trauma. Five days post FG injection, mice were overdosed with ketamine (100 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 0.9% normal saline followed by 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS) at pH 7.4. Brains were fixed...
in situ overnight in 4% paraformaldehyde at 4°C and then carefully dissected from the cranium and surrounding tissue and placed in 30% sucrose in 0.1M PBS on a shaker at room temperature until the tissue sank to the bottom. Tissue was then embedded in tissue freezing medium (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) at –20°C. Twenty micron serial sagittal sections were cut on a Leica CM1850 cryostat (Leica Microsystems, Inc, Buffalo Grove, Illinois, USA), mounted 2 per slide, and cover-slipped using Fluoromount (Sigma).

Positive FG staining was visualized with a Zeiss Axioskop2 Plus (Carl Zeiss Inc, Thornwood, New York, USA) microscope outfitted with an ultraviolet filter cube (350 nm). Ipsilateral FG-labeled cells in the cochlear nucleus (CN), superior olivary complex (SOC), and lateral lemniscus (LL) were counted, along with cells of the contralateral SOC. Using a 20× objective lens, cells with only the nucleolus present in the section were considered to prevent double counting. All images were captured using the Zeiss Axioskop2 Plus fitted with a Roper Scientific camera using RS Image version 1.7.3 software (Photometrics, Tucson, Arizona, USA).

**Hair Cell Quantification**

Organ of Corti explants were fixed in 4% paraformaldehyde for 30 minutes at room temperature, treated with 10% PBS/0.1% Triton X-100/PBS for 30 minutes, and stained with fluorescein isothiocyanate-conjugated phalloidin (FITC-phalloidin; Sigma) for 30 minutes at 1:300 dilution. Fluorescein isothiocyanate-conjugated phalloidin-stained explants were observed using a Zeiss Axiohot epifluorescent microscope with a blue excitation filter set at 450 to 490 nm. Basal, mid, and apical segments of each organ of Corti explant were counted per 360 μm of cochlear duct length using a 63× objective lens.

**Statistical Analysis**

Statistical analysis between 10 μL Dex and untreated groups was calculated using a 2-factor analysis of variance (ANOVA) with replication for tonal ABR data, and the Mann-Whitney U test for click ABR, neuronal, and hair cell count data. Statistical significance was set at $P \leq .05$.

**Results**

**ABR and DPOAE Measurements**

For our mouse model, initial mean baseline click ABR threshold was established at 25 ± 0 dB. Following noise exposure, mean thresholds changed to 80 ± 0 dB, 80 ± 0 dB, 70 ± 10 dB, and 73.3 ± 7.6 dB after 1 day, 1 week, 1 month, and 2 months, respectively (Figure 1A). Mean baseline tonal ABR thresholds were 20 ± 0 dB at 8 kHz, 23.3 ± 5.8 dB at 16 kHz, and 30 ± 10 dB at 28 kHz (Figure 1B). Mean tonal ABR thresholds were determined by averaging all thresholds within a group of mice at a specific frequency. At 1 day post NIHL, mean tonal ABR thresholds increased to 80 ± 0 dB at 8 kHz, 80 ± 0 dB at 16 kHz, and 80 ± 0 dB at 28 kHz. These thresholds remained unchanged at 1 week, with a marginal decrease in the lower frequencies by 1 month (71.7 ± 7.6 dB at 8 kHz, 80 ± 0 dB at 16 kHz, 80 ± 0 dB at 28 kHz). At 2 months, mean tonal ABR thresholds showed a marginal decrease in the higher frequencies as well (76.7 ± 5.8 dB at 8 kHz, 71.7 ± 7.6 dB at 16 kHz, and 76.7 ± 5.8 dB at 28 kHz). There was a statistically significant difference in the NIHL group out to 2 months compared to baseline ($P < .05$).
Mean baseline DPOAE thresholds were 20 ± 0 dB at 16 kHz, 26.7 ± 2.9 dB at 20 kHz, and 36.7 ± 2.9 dB at 24 kHz (Figure 1C). At 1 day post NIHL, mean DPOAE thresholds were elevated at 46.7 ± 2.9 dB at 16 kHz, 51.7 ± 11.5 dB at 20 kHz, and 43.3 ± 2.9 dB at 24 kHz and remained at these levels 1 and 2 months post NIHL. There was a statistically significant difference in the NIHL group out to 2 months compared to baseline (2-way ANOVA: \( P < .05 \)).

Baseline average click ABR thresholds for the 5 traumatized groups, consisting of untreated, 2 and 10 \( \mu \)L IT saline, and 2 and 10 \( \mu \)L IT Dex, were 21.7 ± 2.9 dB, 20 ± 0 dB, 20 ± 5 dB, 18.3 ± 2.9 dB, and 18.3 ± 2.9 dB, respectively (Figure 2). At 1 day post NIHL, all 5 groups demonstrated a click ABR threshold of 80 dB. By 1 week post NIHL, only the 2 and 10 \( \mu \)L Dex groups demonstrated threshold decrease at 63.3 ± 14.4 dB and 45.7 ± 11.7 dB, respectively, whereas thresholds for the untreated and 2 \( \mu \)L and 10 \( \mu \)L saline groups remained elevated at 80 ± 0 dB, 80 ± 0 dB, and 71.7 ± 14.4 dB, respectively. At 2 weeks post NIHL, a minimal reduction in threshold was found in these 3 groups at 80 ± 0 dB, 75 ± 8.7 dB, and 70 ± 17.3 dB, respectively, whereas the 2 \( \mu \)L and 10 \( \mu \)L Dex thresholds returned toward baseline at 47.5 ± 3.5 dB and 48.8 ± 18.9 dB, respectively.

Tonal ABR thresholds at 1 day post NIHL were elevated at all frequencies, averaging across all frequencies at 78.6 ± 3.8 dB, 78.3 ± 3.7 dB, 78.6 ± 3.8 dB, 78.6 ± 3.8 dB, and 76.4 ± 3.7 dB for the untreated, 2 and 10 \( \mu \)L saline, and 2 and 10 \( \mu \)L Dex groups, respectively. In contrast, the control group threshold averaged 31.1 ± 11.8 dB (Figure 3A). Although thresholds remained relatively unchanged in untreated and saline groups by 1 week post NIHL, the 2 and 10 \( \mu \)L Dex groups were slightly lower at 71 ± 10.9 dB and 61.2 ± 16 dB, respectively (Figure 3B). At 2 weeks post NIHL, the untreated and 2 \( \mu \)L and 10 \( \mu \)L saline group threshold averages were 76.4 ± 7.1 dB, 75.7 ± 7.1 dB, and 73.8 ± 11.9 dB, respectively, whereas the 2 \( \mu \)L and 10 \( \mu \)L Dex treated threshold averages decreased to 65 ± 13.6 dB and 58 ± 15.5 dB, respectively (Figure 3C).

A 2-way ANOVA showed that the 10 \( \mu \)L Dex group had significantly greater tonal ABR hearing threshold recovery 1 and 2 weeks post trauma (\( P < .05 \)). Further analysis with the Mann-Whitney \( U \) test found thresholds at 8, 12, 16, 20, and 24 kHz to be significantly different (\( P < .05 \)) at 1-week post NIHL, and at 8 and 12 kHz at 2 weeks post NIHL. Similarly, there was significantly greater click ABR threshold recovery 1 and 2 weeks post trauma (\( P < .05 \)).

Neuronal Cell Counts

Mean cell counts in the CN, SOC, and LL of control mice (no acoustic trauma, no pretreatment) were 1483 ± 190, 2807 ± 67, and 112 ± 20, respectively (Figure 4). In contrast, untreated and 2 \( \mu \)L and 10 \( \mu \)L saline groups yielded, respectively, 1390 ± 404, 1293 ± 327, and 1357 ± 204 in the CN; 2083 ± 190, 2061 ± 146, and 2034 ± 164 in the SOC; and 104 ± 21, 147 ± 19, and 143 ± 32 in the LL. Whereas the 2 \( \mu \)L Dex group showed similar cell counts of 1321 ± 28, 2124 ± 165, and 171 ± 32 in the CN, SOC, and LL, respectively, the 10 \( \mu \)L Dex group was closer to the control group with counts of 1883 ± 186 (CN), 2774 ± 182 (SOC), and 166 ± 18 (LL). There was a statistically significant (\( P < .05 \)) difference in cell count in the SOC of the 10 \( \mu \)L Dex group compared with untreated, saline, or 2 \( \mu \)L Dex treated groups. Figure 5 shows a representative picture of FG staining in the SOC for control, 10 \( \mu \)L saline, and 10 \( \mu \)L Dex groups at 10× and 20× magnification.

Hair Cell Counts

Mean number of OHCs in apical, mid, and basal turns of a 360 micron section of cochlear duct were 146.7 ± 1.5,
were 57.7 ± 0.6, 63.3 ± 2.3, and 71.5 ± 6.3, respectively, for controls; 52 ± 3.2, 57.5 ± 2.6, and 61.5 ± 5.6 for the untreated NIHL group; 44 ± 3.1, 48.8 ± 3.9, and 58.6 ± 11.7 for the 10 µL saline group; and 51.2 ± 9.8, 55.1 ± 9.5, and 61.2 ± 12.3 for the 10 µL Dex group. There was no statistically significant difference between 10 µL Dex and untreated groups (P = .668). Only the OHC count of the basal turn of 10 µL Dex compared to the control group was statistically significant (P < .05). Figure 7 shows a representative picture of FITC-phalloidin stained hair cells from the mid-cochlear turn of control, 10 µL saline, and 10 µL Dex groups under 63× magnification oil immersion.

Discussion

Our NIHL mouse model showed no spontaneous recovery in either ABR or DPOAE thresholds up to 2 months post NIHL. Although DPOAE signals were present after NIHL, it was present at elevated threshold, suggesting pathology at the level of the organ of Corti OHCs, consistent with prior reported studies.6-9 We observed sporadic missing OHCs at the basal turn in the NIHL group, regardless of whether they received treatment or not, thereby resulting in a decreased total number of OHC and IHC counts in our NIHL mouse model. Pretreatment with 10 µL Dex prior to NIHL, however, resulted in ABR threshold recovery and neuroprotection in the SOC.

Although the 2 µL Dex pretreatment failed to demonstrate the same level of neuroprotection in the CN and SOC, ABR thresholds improved similar to that of the 10 µL Dex group. This suggests that Dex may therapeutically affect these 2 cellular groups. The ABR improvement appears to be independent of cell number in these areas or, as previously suggested, Dex mechanisms are related to modulation of hearing sensitivity, maintenance of homeostasis, and anti-inflammatory properties.16,19 Our study demonstrates that hearing function can be maintained even with hair cell and neuronal cell death, although the critical cell number below which irreversible hearing loss occurs is currently unknown. It is interesting that intratympanic Dex treatment administered once at either 1 day, 1 week, or 2 weeks post NIHL did not result in hearing recovery in our mouse model (data not shown). This may be due to the significant degree of acoustic trauma sustained in our animal model and may further support the potential need for pharmacotherapy prior to high intensity acoustic exposure, as in the military.

Dexamethasone is a potent synthetic glucocorticoid used to treat several inner ear disorders. Glucocorticoid receptors (GR) present in both neuronal and non-neuronal tissues in the inner ear can be modulated by noise trauma.10-12,15,27 For example, Tahera et al16 demonstrated in CBA mice that GR can directly modulate hearing sensitivity in response to NIHL following intraperitoneal injections of dexamethasone. In a more targeted approach, Takemura et al17 showed that direct inner ear infusion of Dex resulted in higher OHC survival rates and attenuation of NIHL in guinea pigs.

Although the mechanism of central auditory neuronal cell loss through acoustic trauma and Dex otoprotection is
beyond the scope of what this study could prove, theories have been postulated based on previous literature. As with peripheral cell loss, the neurons of auditory centers may degenerate from lack of stimulation. Given the OHC counts in our study, however, we felt that there was more than just degeneration contributing to neuronal cell count changes. Acoustic trauma could induce excitotoxic or apoptotic changes retrocochlearly independent of peripheral changes. Intratympanic Dex may be reaching its therapeutic target in the brainstem by either diffusion through cerebral spinal fluid communicating with the perilymphatic space or neuronal retrograde transport, thus providing protection in neurons in addition to proximal cells. The molecular weights of Dex and that of FG are similar—516.41 and 532.6 Daltons, respectively. Since FG can diffuse across the round window membrane and is retrogradely transported, it is feasible that a similarly sized molecule could do the same. Many other factors (charge, channels, etc) determine such transport, but the possibility warrants more investigation. In addition, increasing evidence suggests that the corticotrophin-releasing factor (CRF) pathway exerts important neuroprotective effects against various insults such as hypoxia, ischemia, neuroinflammation, and oxidative stress, all proposed mechanisms of sensorineural hearing loss. In the brainstem, 2 different CRF receptors (CRF1 and CRF2) have been reported on neurons and glial cells. Further studies about chemical and cell pathway changes could lead to a better understanding of mechanisms affecting the central auditory system and Dex’s effect.

Another consideration is the protective quality of fluid volume as an acoustic buffer. Given that the maximum mouse middle ear space is approximately 9 μL in volume, an intratympanic injection of 10 μL could completely fill the middle ear, potentially eliminating air conduction. Since both saline and Dex treatments were administered prior to NIHL, this fluid buffer could have attenuated the noise
intensity, thereby initiating less damage and allowing for greater recovery. The 2 and 10 μL saline groups, designed as negative volume controls, further illustrate this point, as shown by the differential recovery dependent on volume (Figure 2). Although these results were not statistically significant, this trend is important to consider for future study designs and possible improvements of such a fluid buffer on current hearing protection gear. Consideration of both the reflectivity of fluid and impedance of air conduction may lead to a better design for sound dampening than the standard foam and soft plastics currently used. There is a substantial number of workers and military personnel exposed to environments with high noise levels for which a pretreatment can be considered. Our experiments encourage exploration of IT Dex injection as a preventive measure.

Conclusion

Our NIHL mouse model can serve as an innovative research tool for future studies in understanding NIHL. The combination of functional testing (ABR and DPOAE) and central morphologic examination with FG labeling yielded additional insights into the effects of Dex and its potential mechanisms of protection against NIHL.

Author Contributions

Leon Chen, contributed to NIHL protocol; acquisition and analysis of ABR and DPOAE data; manuscript drafts; Clare Dean, acquisition and analysis of ABR, DPOAE, and FG data; manuscript drafts; Michele Gandolfi, organ of Corti hair cell data and analysis; manuscript revisions for intellectual context; Edmund Nahm, organization of data; manuscript revisions for intellectual context; Linda Mattiace, contributed to study design; critical review and editing manuscript; Ana H. Kim, research conception and design, analysis of data, manuscript revisions for intellectual context, final approval of version to be published.

Disclosures

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