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Intra-tympanic steroid treatments may improve hearing via ion homeostasis alterations and not immune suppression

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Abstract

Hypothesis—The inner ear endothelium is capable of responding to therapeutic steroids by altering local expression of cytokine and ion homeostasis genes that impact inflammation and fluid regulation.

Background—Glucocorticoids are often given trans-tympanically for hearing disorders because of their anti-inflammatory effects, but their direct impact on inner ear ion homeostasis and cytokine gene expression has not been studied.

Methods—The middle ears of Balb/c mice were transtympanically injected with 5 µl of either phosphate-buffered saline (PBS), prednisolone or dexamethasone. Untreated mice were used as controls. Mice were euthanized at 6, 24 and 72 hours, the cochleas harvested and total RNA isolated from the inner ear tissues. Expression of eight cytokine genes and 24 ion homeostasis genes were analyzed with qRT-PCR.

Results—PBS caused upregulation of inflammatory cytokine genes that peaked at 6 hours. Surprisingly, prednisolone and dexamethasone also caused upregulation of most cytokine genes. Interestingly, ion homeostasis genes were predominantly upregulated with dexamethasone and prednisolone, with prednisolone having the larger effect.

Conclusion—In the murine model, intratympanic steroids caused an initial upregulation of inflammatory cytokine genes in the inner ear, as well as predominantly upregulation of ion homeostasis genes. These findings suggest glucocorticoids do not suppress inner ear inflammation, but rather cause an initial inflammatory response in the inner ear. Thus, inflammatory gene suppression is not a likely mechanism for their hearing restorative effects. On the other hand, these steroids have a significant mineralocorticoid function, as demonstrated by increased function of ion homeostasis genes, implicating their ionic and fluid regulatory properties as a mechanism for their therapeutic effects.

Introduction

Local production of cytokines occurs in the inner ear (IE) endothelium in response to infection/inflammation. The IE endothelium is capable of this local response to bacteria as confirmed by immunofluorescence and PCR data^{1–3}. Steroids are used orally or intratympanically (IT) for their anti-inflammatory effect for clinical scenarios such as autoimmune inner ear disease (AIED), Meniere's disease⁴, sudden sensorineural hearing loss (SSNHL)⁵, chronic suppurative otitis media (CSOM) and draining tympanostomy tubes. There also is rat data showing a protective IE effect from IT dexamethasone on endotoxin-induced otitis media (OM)⁶. IT steroids have also been used to preserve hearing during cochlear implantation⁷. Interestingly, steroids can have anti-microbial activity in vitro⁸. In the rodent model, steroids protect hair cell loss from acoustic trauma⁹.

Steroids are widely regarded for their clinical efficacy in controlling inflammation systemically and locally in the ear, presumably by their glucocorticoid (anti-inflammatory) activity. However, it is possible that their mineralocorticoid receptor binding activity is also at work in controlling fluid regulation in the IE via unknown mechanisms¹⁰. While it has been shown that aquaporin 3 mRNA expression is upregulated by intra-endolymphatic sac application of dexamethasone¹¹, little is known of the direct molecular impact on other ion homeostasis genes by therapeutic glucocorticoids delivered trans-tympanically, a common approach to treating hearing disorders. Ion homeostasis genes are active in the inner ear not only in maintaining the ionic microenvironment required for hearing, but also in response to vasopressin¹² and other molecules¹³.

Ion and water transport in the inner ear spaces is essential¹⁴. Several ion homeostasis genes are active in the inner ear to maintain potassium, sodium, chloride and calcium concentrations in the endolymph for optimal functioning of hair cells¹⁵. Defects in many of these genes are also associated with deafness, such as those controlling potassium channels (Kcne1, Kcnj10, Kcnj1), Na⁺, K⁺-ATPase (Atp1a1, Atp1a2), chloride channel (Clnka), gap junctions (Gjb2, Gjb6), and tight junction claudins^{16,17}. Downregulation of ion homeostasis genes is seen in the *middle ear* (ME) both with inflammation and with fluid challenge¹⁸. These results indicate that these genes are downregulated in the early response to both fluid and inflammation, compounding the failure to clear fluid from the middle ear space in the setting of middle ear inflammation. Steroid treatment actually increases ion homeostasis genes to clear middle ear disease¹⁹. However, the local effects of steroids on cytokine and ion homeostasis gene production in IE have not been studied at the gene level. Therefore, to better understand the relationship of ion and water transport genes in response to steroids, the current study investigates the impact of steroids, used frequently in the treatment of clinical otologic conditions, on IE expression of ion homeostasis and cytokine genes. A quantitative RT-PCR study was conducted on a panel of these genes in the mouse in response to two therapeutic steroids, prednisolone (Pred) and dexamethasone (Dex).

Our hypothesis is that steroids will upregulate ion homeostasis gene production in the IE.

Materials and Methods

Balb/c mice (10–12 weeks old) were sedated with a subcutaneous injection of ketamine (100 mg/ml; 0.067 mg/g) and xylazine (20 mg/ml; 0.013 mg/g) and examined under an operating microscope to confirm absence of otitis media (OM). Forty-eight mice were given either Pred (10 mg/kg) or Dex (0.667 mg/kg) trans-tympanically bilaterally and euthanized at three time points post injection (6, 24 and 72 hours). Twenty-four mice were injected with PBS only and euthanized at the three time points to serve as a vehicle control. The no-treatment control group consisted of eight mice.

With microscope assistance, ME tissue was dissected from the IE. IE structures collected for analysis were all components of the periotic bone (membranous and bony labyrinth). This included the cochlear bony wall within the middle ear space. All ME components were thoroughly scraped and washed off this wall to prevent any ME factors from influencing inner ear results. Left and right IE tissues were combined, homogenized, and mRNA extracted for quantitative RT-PCR of key ion homeostasis genes and inflammatory cytokine genes.

RNA Isolation

Inner ears were isolated in RNeasy Lysate (Ambion, Inc., Austin, TX), then blotted to remove excess RNeasy Lysate, flash frozen in liquid nitrogen and stored at 80°C until RNA was extracted. Tissue RNA was extracted with the Qiagen (Valencia, CA) RNeasy Mini Kit. Tissue was transferred to tubes with 600 µl of extraction buffer and homogenized with a PowerGen 125. RNA was quantified using a NanoDrop and all samples were made up to a concentration of at least 62.5 ng/µl.

Quantitative RT-PCR Analyses

Real-time RT-PCR studies used an ABI Step One Plus system (Carlsbad, CA). Total RNA (500 ng) was reverse-transcribed using RT² First Strand Kit (SABiosciences Corp, Frederick, MD) using the manufacturer's instructions. Then samples were prepared for Real-time PCR using the RT² Real-time SYBR Green/Rox PCR master mix. Thermal cycle condition was set as: 95°C 10 min, then 40 cycles: 95°C 15sec, 60°C 1 min followed by a melt curve. Data analysis follows the suggestion of the manufacturer (SABiosciences PCR Array Data Analysis Web Portal). The parameter CT (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline.

Genes Analyzed

The study utilized custom PCR Arrays (SABiosciences Corp, Frederick, MD) already optimized for reaction conditions, primers, and probes (RT² Profiler PCR Array System) for actual quantification of gene expression. Our standard PCR Array plates measure expression of eight key inflammation related cytokines: IL-1α, IL-1β, IL-6, IL-10, CCL3, CXCL2, CXCL11, and TNFα (Table 1). Cytokine genes were also chosen for analysis in this study and include eight key inflammation related cytokines (IL-1α, IL-1β, IL-6, IL-10, CCL3, CXCL2, CXCL11, and TNFα). These genes have been shown to be very active in the mouse model of otitis

media in both the middle and inner ear^{1,2,18,20}. Therefore, they were chosen to measure the inner ear inflammatory response to a middle ear steroid injection. In particular, IL-1 α and TNF α were chosen as they are both acute phase cytokines and act synergistically. While IL-1 β is pro-inflammatory, IL-6 acts both as a pro- and anti-inflammatory cytokine, inhibiting IL-1 and TNF α via activation of IL-10. IL-10 acts as an immunoregulator and anti-inflammatory cytokine. CCL3, CXCL2, CXCL1 are chemokines that participate in the acute phase of inflammation by providing chemotaxis for polymorphonuclear leukocytes and hematopoietic stem cells.

Additional custom PCR array plates were made to analyze 24 ion homeostasis genes (Table 1). These include isoforms of Na⁺, K⁺-ATPase, channels that transport K⁺, Na⁺, Cl⁻, gap junction connexins, water transporting aquaporins, and tight junction claudins. These ion homeostasis genes were selected for their known importance in the function of the inner ear.

Statistical Analysis

Gene expression in the vehicle and steroid-treated inner ears were compared to mice who received no treatment to the middle ear. The statistical significance and fold change were calculated using the C_t method. Individual sample fold change was calculated by subtracting the housekeeping gene of each sample (C_t), then subtracting the C_t average of the control group to formulate C_t from which the fold change is derived. The housekeeping gene used for this method was gluceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was chosen as the housekeeping gene for the standardization of the results since this gene is the most reliable of all proposed standardization genes and additionally, is recommended by the company. We have run our own standardization comparisons with other potential housekeeping genes (Mouse 18S rRNA, actin beta, beta-2 microglobulin, glucuronidase beta, lactate dehydrogenase A-like 6B, ribosomal protein L13a, heat shock protein 90 alpha class B member 1, among others) and find this the most consistent and reliable. Its use also permits comparisons with other qRT-PCR results published by our laboratory^{1,2,18}

ANOVA and post hoc Tukey tests were run on individual fold changes in all groups for each gene at each time point to compare gene expression of the treatment groups (PBS, Dex, Pred) to the untreated group.

All animal procedures in the study were approved by the OHSU Institutional Animal Care and Use Committee protocol #ISO1603-P.I. Dennis R. Trune, PhD.

Results

Cytokine Results

Transtympanic PBS (vehicle) alone caused a significant cytokine response in the inner ear (Table 2). This was particularly evident in the fold change for CXCL1, CXCL2, and IL-6 at 6 hours. This upregulation of inner ear cytokines was still significant at 24 and 72 hours, although it had subsided considerably in magnitude (Table 2). At 72 hours CXCL2 (MIP2) expression was still 7 fold higher than untreated ears and CXCL1 (KC) was 8.61 fold higher. Because of this significant impact of vehicle only on the inner ear, we statistically compared

our steroid induced changes to vehicle-alone (ANOVA) to determine what changes in gene expression were from steroid and not their vehicle. Saline or PBS is the vehicle for these therapeutic glucocorticoids, so it is important to differentiate steroid from vehicle effects.

Interestingly, Pred caused increased expression of many of these same cytokine genes (Table 2), showing even greater upregulation than PBS alone (Cxcl2, IL-6 and Cxcl1 at 6 hours; IL-1 α , Cxcl1 at 24 hours; $p>0.05$, $p<0.05$) In fact, Pred induced 3-4 fold higher expression than vehicle alone for these genes (PBS-Pred posthoc), suggesting this steroid has a dramatic inflammation induction effect rather than inflammation suppression. By 72 hours, Pred showed lower gene expression than vehicle alone, indicating it had overcome the inflammatory reaction it induced and was finally suppressing the inflammatory genes. Thus, it appeared that Pred takes longer than 24 hours to suppress inflammatory genes in the inner ear, requiring at least that long to overcome its own inflammation induction.

Dex showed a similar early upregulation effect on some cytokines (Table 2). For example, it induced IL-6 (7.3 fold), IL-10 (5.39 fold), Cxcl2 (9.3 fold) and Cxcl1 (19.8 fold) at 6 hours. Generally Dex was less inductive than PBS and Pred in its early effects on cytokine genes, still inducing inflammatory gene expression, but less so. IL-10 was the only cytokine gene induced to greater levels than that seen with vehicle or Pred, but IL-10 is an anti-inflammatory cytokine, so Dex in this case was still suppressing inflammation better than vehicle or Pred. These results presumably reflect the ability of Dex to ameliorate the inflammatory effects of the vehicle in as short a period as 6 hours. Analysis of variance (ANOVA) between PBS/Pred/Dex shows that there were statistically significant differences among the treatments at 6 hours in IL-1 β , IL-6, IL-10, Cxcl2, TNF α and Cxcl1 (Table 2). Post hoc Tukey tests for steroids vs. PBS showed significant differences between Pred and Dex activity in most cytokines at 6 and 24 hours (Table 2). Most of these differences drop off at 72 hours, however, where Pred show significant downregulation in IL-6, Ccl3, Cxcl2 at 72 hours and Dex in IL-6, Ccl3 and Cxcl2 at 72 hours ($p < 0.05$; Table 2). Post hoc Tukey tests for Pred vs. Dex also showed that Pred induces more cytokine gene expression than Dex at 6 and 24 hours (Table 2).

When assessing statistically significant fold change by ANOVA, Pred and Dex caused much more upregulation than downregulation (Table 2). In fact, these steroids seldom reduced any gene below its normal expression levels in the vehicle-only ears. Dex only reduced IL-1 β to about half normal expression (0.49) at 6 hours. Otherwise the best Dex accomplished was to overcome the effects of the vehicle and keep expression at normal levels by 24 hours and thereafter. However, neither Pred nor Dex reduced inflammatory gene expression at all in the inner ear except to overcome vehicle effects.

Ion Homeostasis Gene Results

Unlike the cytokine genes, ion homeostasis gene expression change with PBS alone was virtually unseen and limited to only a three-fold increase at 6 hours in Aqp3 (Table 3). This increase was still significant at 24 hours, as well. Aside from this gene, all others were virtually unchanged following vehicle-only inoculation.

In general, the steroids tended to upregulate these genes more than suppress them. Because little or no change was seen with vehicle alone, it appears the steroids had a direct impact on the genes in question. The only exception was Aqp3, which appeared to be upregulated by steroids similar to vehicle, suggesting its expression was due to the fluid alone. The gap junction genes Gja1 and Gjb2 were suppressed by both steroids (Table 3). This suppression was still seen at 72 hours as well. Aside from slight suppression of these gap junction genes, all other steroid-induced gene changes were upregulation. However, these changes due to steroids were small, seldom doubling normal expression. So while they were statistically different from vehicle alone, their impact was moderate.

The most activity in ion homeostasis gene expression change was seen at 24 hours with significant changes in 17 of 24 genes (Table 3). Although both steroids had comparable effects, Pred caused more gene expression changes than Dex. At 72 hours, both Dex and Pred appeared to be less effective, both causing slightly reduced expression compared to PBS alone.

Overall the two steroids had fairly comparable effects. The most changes were seen at 24 hours following treatment. In general, if vehicle alone caused upregulation of expression, the steroids suppressed it. If vehicle alone caused downregulation of gene expression, the steroids restored it. Seldom did the steroids cause expression changes similar to vehicle, so it is concluded their transtympanic delivery had a direct and significant effect on ion homeostasis genes in the inner ear.

Specific Gene Family Changes

Gap Junction Genes: At 6 hours, significant downregulation was seen in both Dex and Pred compared to PBS in Gja1 and Gjb2. *ATPase Genes:* Upregulation was seen at 24 hours in Pred compared to PBS in Atp1b2 and Atp1b1. *Epithelial Sodium Channel Genes:* Significant steroid induced upregulation compared to PBS was seen at 6 hours in all three Scnn isoforms, with Dex causing significantly more upregulation activity than Pred in Scnn1g. This pattern continued for Dex at 24 hours and 72 hours (Scnn1a). *Aquaporin Genes:* Both steroids caused significant alteration of expression in Aquaporins 1,2,3 at 24 hours. The vehicle-only induced overexpression of Aqp3 was suppressed by Dex a little more effectively at 24 hours. *Potassium voltage gated Channel genes:* Most alteration of gene expression was seen at 24 hours in Kcnq1, Kcnq4 and Kcnj10. Vehicle-only caused a slight upregulation of Kcnq1 and Kcnq4 that was reduced back to normal by Pred better than Dex. *Tight Junction Genes:* Both steroids caused downregulation in Gjb2 and Gja1 at 6 hours and Gja1 72 hours.

Osmolality of the three injected substances was measured by osmometer and the mean results of three readings are as follows: PBS 409 mmol/kg, Pred 1016.8 mmol/kg, and Dex 213.7 mmol/kg.

Discussion

In spite of therapeutic glucocorticosteroids being used for decades, little is known of their exact role in hearing recovery¹⁰. While it is presumed their mechanism of action is immune

suppression, restoration of the ion homeostatic processes of the inner ear is also a known action. Furthermore, the intratympanic injection of steroids appears to offer advantages over systemic delivery, yet many questions remain regarding the direct impact of such steroid delivery on inner ear processes. Therefore, in order to better understand these inner ear functions of steroids, the present study was conducted on normal ears.

Surprisingly, middle ear delivery of the PBS vehicle alone had a significant effect of inducing inflammatory gene expression in the inner ear. This actually was unexpected since it (or sterile saline) is the common solution for most therapeutic corticosteroids. This establishes a basis for frequently reported pain following intratympanic delivery as inflammation of the middle ear also occurs. Thus the steroids were evaluated for their impact on inflammation and ion homeostasis genes in comparison to vehicle in order to differentiate their various effects. It appears steroids induce considerable expression of inflammatory genes, which would be detrimental to the ear, while generally causing expression of ion homeostasis genes, which would restore inner ear functions.

Cytokines

In the current study, steroids are shown to cause an early upregulation of cytokine genes in the IE at 6 hours tapering off at 24 and 72 hours in comparison to control ears. While these results are surprising given the known anti-inflammatory properties of glucocorticoids, the early inflammatory response may be partially due to the vehicle alone as shown with the upregulation seen with PBS injections, yet the ANOVA would support that steroids in and of themselves may stimulate the immune system initially. The early inflammatory effect of Pred was stronger than Dex in terms of upregulation, especially at 6 and 24 hours. Pred in and of itself is not enough to overcome the inflammation, seen especially at 6 hours in IL-6 and Cxcl1 and Cxcl2. Interestingly, Dex showed significant inflammatory reaction, but was less than vehicle, suggesting its anti-inflammatory properties were operating to mitigate partially the reaction of tissues to vehicle.

Ion Homeostasis

Steroids are shown to cause inner ear downregulation of several important classes of ion homeostasis genes: aquaporins (Aqp 1,2), Na⁺-K/ATPase, Tmprss3, gap junction (Gjb2, Gja1), and potassium-regulatory genes (Kcnq1, Kcnq4). Other genes were upregulated (in particular Aqp3 and Na⁺ channel families). The differences seen between PBS/Pred/Dex by ANOVA were greatest at 6 and 24 hours, while the differences in cytokine genes were strongest at 6 hours. As seen with the cytokine results, PBS alone stimulates inflammatory gene upregulation, but does not impact ion homeostasis gene expression much, whereas steroids increase ion channel and transporter gene expression in the inner ear. Intratympanic steroid use may cause some IE inflammation in response to the vehicle, then, steroids acting on the IE are able to overcome that inflammation and induce upregulation of ion homeostasis genes. The presence of a strong mineralocorticoid steroid (Pred) impacts this upregulation. Dex, which is reported to have less of a mineralocorticoid effect, appears to affect ion homeostasis gene expression less.

Aquaporin family

Aquaporins are embedded in the cell wall and regulate water flow. Aquaporin (aqp) channels move water and several have been described for the IE. Steroid effects on the IE may in part be in adjusting IE water volume that is altered by inflammation. In particular, Aqp3 appears to be upregulated most strongly and may be the most active in this IE protection. This points out that steroid effect may not be only an anti-inflammatory effect on the IE, but also fluid regulatory.

Na⁺,K⁺ATPase family

Pred and Dex upregulated the ATPases at 24 hours. ATP1b1,2 are both modestly, but significantly, upregulated at 24 hours, then downregulated at 72 hours. Na⁺,K⁺ATPases are integral membrane proteins responsible for controlling cell volume and maintaining the electrochemical gradient of Na⁺ and K⁺ ions across the cell membrane. Altered regulation of expression of these proteins in the IE may increase ion movement and decrease extracellular water.

Claudin family

Claudins are transmembrane proteins that maintain tight junctions and establish the paracellular barrier controlling flow of molecules in the intercellular space between the epithelial cells. At all time points, Pred and Dex cause primarily upregulation of all three Claudin genes.

Gap Junction gene family

Gap junctions are specialized intercellular connections, directly connecting the cytoplasm of cells and allowing various molecules and ions to pass between cells. These hydrophilic channels ensure that molecules passing thru the gap junction do not leak into the intercellular space. Gap junction proteins are involved in the transport of K⁺ in the organ of Corti and lateral wall. Downregulation of these genes by steroids (seen primarily in Gjb2 and Gja1) may facilitate maintenance of IE ion homeostasis, thus supporting the efficacy of steroids in steroid-responsive SNHL and other disorders that are so-called “steroid-responsive”.

K⁺ and Na⁺ channel genes

These genes regulate ion transport in the IE and are here seen to be active in the post-inflammatory response to an IE challenge caused by the PBS. Potassium and sodium regulatory genes were largely upregulated by the steroids.

Limitations of this study are several. Firstly, while the mouse model is widely used for otitis media research, its translatability to the human condition is not necessarily universal. Secondly, the possible effect of the differing osmolalities of the injected medications on the gene change results, is unavoidable, and may play a role in the outcomes. Given that the mammalian extracellular osmolarity is in the range of 290 mOsmol/liter, all of these substances may cause fluid shifts by virtue of being hyper- or hypo-osmolar to extracellular fluid in the middle and inner ear. However, in order to study the human condition where

steroids are injected into the middle ear for inner ear effects, our mouse model mimicked the human scenario, including the osmolality differences. Also, the similarity of our steroid results point to a minimal impact from osmolality differences in the two steroids. Thirdly, the model used in this study is a non-disease mouse model and results may not translate to a diseased state in mice or humans. Lastly, the use of a single housekeeping gene is a potential limitation of the study, but GAPDH has been shown to be the most reliable of standardization genes and is widely used as a housekeeping gene for gene expression studies.

The findings of the current study lend credence to the theory that mixed steroids (Pred) act on the IE via alteration of cytokine *and* via ion homeostasis gene expression. This lends credibility to the fluid regulatory property of steroids on the inner ear, challenging the widely-held belief that steroids act on the IE by anti-inflammatory properties alone¹⁰. Steroids impart a combination of up-regulation and down-regulation on ion homeostasis gene expression at all time points studied, and upregulation of cytokines occurs most strongly at 6 hours²¹. Thus, this two-step effect on the IE appears to be an early inflammatory effect by the instillation of the steroid in its vehicle, while later, fluid regulation occurs.

Ion homeostasis gene expression is significantly altered by Dex and Pred. Understanding the effect of steroids on ion homeostasis genes in the IE may help lead to directed IE therapies with trans-tympanic or systemic agents to control ion homeostasis genes that react to glucocorticoid and mineralocorticoid steroids. Ultimately, control of sensorineural hearing loss from IE or systemic pathology, and understanding the mechanism by which steroids stabilize hearing in conditions such as sudden SNHL is the goal of such translational research. A better, molecular level understanding of how steroids act in the IE could lead to new therapies directed at different mechanisms of hearing loss.

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Table 1**Inflammatory Cytokine and Ion Homeostasis Genes Studied**Inflammatory Genes:

IL-1 α , IL-1 β , IL-6, IL-10 = Interleukins 1 α , 1 β , 6, 10

Cxcl2, chemokine C-X-C motif, ligand 2 [MIP-2],

CCl3, chemokine C-C motif, ligand 3 [MIP-1 α]

Cxcl1 chemokine C-X-C motif, ligand 1 [KC, Keratinocyte derived chemokine]

TNF α = tumor necrosis factor α

Ion Homeostasis Genes:

SLC12A2 = Na-K-2Cl cotransporter

Atp1 β 1, Atp1 β 2 = Na⁺,K⁺-ATPase, Na⁺/K⁺ transporting, beta 1,2, polypeptides

Atp1 α 1 = Na⁺,K⁺-ATPase, Na⁺/K⁺ transporting, alpha 1 polypeptide

Clcnka = chloride channel Ka

Cldn 3, 4, 14 = Claudins 3, 4, 14

Gja1, Gjb2, 3, 6 = gap junction proteins, alpha 1, beta 2,3,6

Aqp1, 2, 3, 5 = aquaporins 1, 2, 3, 5

Kcne1, Kcnq1, Kcnq4 = potassium voltage-gated channel

Kcnj10 = potassium inwardly-rectifying channel

Scnn1 α , 1 β , 1 γ = ENaC (epithelial Na⁺ channels), nonvoltage-gated 1 α , 1 β , 1 γ

Tmprss3 = transmembrane protease, serine 3

Table 2

ANOVA and post-hoc analysis Fold Change in Inner Ear Cytokine Gene Production

	6 hour Fold Change				post-hoc p-values				24 hour Fold Change				post-hoc p-values				72 hour Fold Change				post-hoc p-values			
	PBS	Pred	Dex	ANOVA	PBS-Pred	PBS-Dex	Pred-Dex	PBS	Pred	Dex	ANOVA	PBS-Pred	PBS-Dex	Pred-Dex	PBS	Pred	Dex	ANOVA	PBS-Pred	PBS-Dex	Pred-Dex			
IL1a	0.96	0.76	1.21	0.072				0.85	1.70	0.95	0.011	0.016	0.935	0.034	1.34	0.81	1.15	0.207						
IL1B	2.51	1.04	0.49	0.001	0.019	0.001		0.506	2.25	0.92	0.037	0.444	0.296	0.029	2.38	1.45	2.00	0.243						
IL6	24.71	69.00	7.30	0.001	0.008	0.405		0.001	3.69	1.78	0.059				2.46	1.09	1.37	0.045	0.047	0.133	0.856			
IL10	3.12	4.46	5.39	0.005	0.096	0.004		0.308	1.48	1.43	0.046	0.065	0.089	0.987	0.94	0.78	0.93	0.613						
CCl3	1.07	0.86	0.95	0.547				1.15	1.69	1.05	0.016	0.055	0.879	0.020	1.39	0.90	1.05	0.049	0.045	0.189	0.732			
Cxcl2	32.21	91.38	9.30	0.003	0.030	0.543		0.003	13.55	2.10	0.005	0.087	0.334	0.004	7.06	2.53	2.67	0.022	0.037	0.043	0.996			
TNF	1.62	1.02	0.84	0.001	0.001	0.001		0.388	1.43	1.21	0.280				1.23	1.20	1.25	0.961						
Cxcl1	56.30	183.85	19.82	0.001	0.007	0.595		0.001	17.03	2.59	0.001	0.014	0.021	0.001	8.61	3.08	2.90	0.222						

N = 8 for all groups (Untreated controls, PBS, Dex, Pred)

Note: post-hoc tests were not run where ANOVAs were non-significant.

Bold = p<0.05

Table 3

ANOVA and post-hoc analysis Fold Change Inner Ear Ion Homeostasis Gene Production (Bold = p<0.05; N=8 for all groups).

	6 hour Fold Change				post-hoc p-values				24 hour Fold Change				post-hoc p-values				72 hour Fold Change				post-hoc p-values			
	PBS	Pred	Dex	ANOVA	PBS-ANOVA	PBS-Pred	PBS-Dex	Pred-Dex	PBS	Pred	Dex	ANOVA	PBS-ANOVA	PBS-Pred	PBS-Dex	Pred-Dex	PBS	Pred	Dex	ANOVA	PBS-ANOVA	PBS-Pred	PBS-Dex	Pred-Dex
Sle12a2	0.87	1.17	1.57	0.021	0.418	0.017	0.212	0.73	1.39	0.89	0.001	0.001	0.576	0.001	0.001	0.012	1.11	0.72	1.07	0.141				
Atp1b2	0.79	0.79	0.96	0.235				0.87	1.30	1.02	0.022	0.019	0.565	0.019	0.151	1.05	0.86	1.06	0.311					
Atp1b1	0.85	0.84	0.92	0.771				0.82	1.09	1.04	0.006	0.007	0.035	0.007	0.749	1.03	0.73	0.96	0.001	0.001	0.001	0.001	0.513	0.005
Atp1a1	0.97	1.09	1.18	0.249				0.96	1.22	1.09	0.077				1.15	0.92	1.10	0.056						
Clenka	1.01	0.96	1.87	0.001	0.970	0.001	0.001	0.87	1.87	1.19	0.008	0.007	0.540	0.007	0.073	1.13	0.82	1.20	0.281					
Cldn3	1.15	1.78	1.04	0.032	0.085	0.919	0.038	2.08	0.98	1.69	0.001	0.001	0.201	0.001	0.009	0.96	1.37	0.93	0.126					
Cldn4	0.93	1.47	0.75	0.001	0.004	0.449	0.001	1.13	0.84	1.16	0.025	0.059	0.963	0.059	0.034	1.21	1.07	0.98	0.352					
Cldn14	0.95	1.29	1.06	0.086				1.00	0.84	1.22	0.020	0.426	0.194	0.426	0.015	1.01	1.00	1.06	0.901					
Gjb2	0.70	0.39	0.51	0.001	0.001	0.015	0.145	0.87	1.10	0.75	0.058				1.02	0.84	1.02	0.224						
Gja1	0.96	0.58	0.57	0.001	0.001	0.001	0.955	0.84	0.72	0.70	0.065				1.12	0.79	0.89	0.001	0.001	0.015	0.318			
Gjb3	1.16	1.59	1.53	0.211				1.68	2.30	2.49	0.185				1.85	2.58	1.98	0.085						
Gjb6	0.91	0.78	0.94	0.155				0.82	1.01	0.97	0.113				0.97	0.84	1.04	0.012	0.132	0.435	0.010			
Aqp1	1.00	0.97	0.98	0.954				0.91	0.63	0.77	0.003	0.002	0.144	0.002	0.128	1.06	0.91	0.98	0.195					
Aqp2	0.85	0.63	0.83	0.346				0.92	1.39	1.04	0.044	0.042	0.773	0.042	0.159	1.05	0.79	1.04	0.099					
Aqp3	3.22	3.93	3.01	0.277				3.69	2.49	1.46	0.001	0.059	0.001	0.059	0.001	2.05	1.97	1.55	0.457					
Aqp5	0.59	0.40	0.58	0.077				0.84	1.24	0.73	0.008	0.037	0.787	0.037	0.009	1.28	0.64	0.90	0.001	0.001	0.001	0.014	0.129	
Kenel	0.69	0.55	0.65	0.058				0.93	0.91	0.83	0.696				0.95	0.91	1.04	0.141						
Kenq1	0.91	0.94	0.96	0.886				1.36	0.98	1.26	0.007	0.007	0.655	0.007	0.048	1.08	0.99	1.23	0.043	0.533	0.263	0.035		
Kenq4	1.29	1.29	1.12	0.380				1.42	0.86	1.62	0.001	0.002	0.319	0.002	0.001	1.27	1.15	1.25	0.857					
Kenj10	0.95	0.83	0.93	0.543				0.96	0.91	1.18	0.031	0.882	0.092	0.882	0.035	1.02	0.93	1.07	0.371					
Senn1a	1.44	2.41	2.71	0.001	0.001	0.001	0.179	1.10	1.75	2.49	0.001	0.073	0.001	0.073	0.035	1.17	1.25	1.44	0.030	0.735	0.028	0.128		
Senn1b	0.98	1.26	1.30	0.024	0.060	0.033	0.955	1.06	1.17	1.46	0.005	0.602	0.004	0.602	0.038	1.09	1.04	1.27	0.090					
Senn1g	1.05	1.29	1.76	0.001	0.342	0.001	0.029	0.95	1.32	1.19	0.095				1.12	0.82	1.00	0.272						
Tmprss3	0.95	0.89	0.85	0.686				1.10	0.83	0.88	0.003	0.004	0.016	0.004	0.796	0.89	0.81	0.82	0.504					