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Perilymph pharmacokinetics of locally-applied gentamicin in the guinea pig

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Abstract

Intratympanic gentamicin therapy is widely used clinically to suppress the vestibular symptoms of Meniere's disease. Dosing in humans was empirically established and we still know remarkably little about where gentamicin enters the inner ear, where it reaches in the inner ear and what time course it follows after local applications. In this study, gentamicin was applied to the round window niche as a 20 μ L bolus of 40 mg/ml solution. Ten 2 μ L samples of perilymph were collected sequentially from the lateral semi-circular canal (LSCC) at times from 1 – 4 hours after application. Gentamicin concentration was typically highest in samples originating from the vestibule and was lower in samples originating from scala tympani. To interpret these results, perilymph elimination kinetics for gentamicin was quantified by loading the entire perilymph space by injection at the LSCC with a 500 μ g/ml gentamicin solution followed by sequential perilymph sampling from the LSCC after different delay times. This allowed concentration decline in perilymph to be followed with time. Gentamicin was retained well in scala vestibuli and the vestibule but declined rapidly at the base of scala tympani, dominated by interactions of perilymph with CSF, as reported for other substances. Quantitative analysis, taking into account perilymph kinetics for gentamicin, showed that more gentamicin entered at the round window membrane (57%) than at the stapes (35%) but the lower concentrations found in scala tympani were due to greater losses there. The gentamicin levels found in perilymph of the vestibule, which are higher than would be expected from round window entry alone, undoubtedly contribute to the vestibulotoxic effects of the drug. Furthermore, calculations of gentamicin distribution following targeted applications to the RW or stapes are more consistent with cochleotoxicity depending on the gentamicin concentration in scala vestibuli rather than that in scala tympani.

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Keywords

intratympanic; drug delivery; cochleotoxicity; round window; stapes; permeability; distribution; elimination

1. Introduction

For years, it has been widely assumed that intratympanically applied drugs such as gentamicin primarily entered perilymph of the inner ear through the round window (RW) membrane (Smith & Myers, 1979; Okuno & Nomura, 1984; Lundman et al., 1987; Goycoolea et al., 1988, 2001). Some histological studies had shown that substances such as tetracycline or horseradish peroxidase were found in tissues of the vestibule, suggesting they may have entered through the stapes footplate (Maass & Stupp, 1974; Tanaka & Motomura, 1981; Saijo & Kimura, 1984). However, the demonstration that small molecules could readily exchange between perilymph of scala tympani (ST) and scala vestibuli (SV) by local, “radial” fluid pathways (Salt et al., 1991) meant that it was difficult to establish the route by which substances reached the vestibule.

Pharmacokinetic studies of locally-applied gentamicin in chinchillas (Hoffer et al., 1997; Hoffer et al., 1999; Balough et al., 1998) were interpreted quantitatively through a computer model by Plontke et al. (2002). In that analysis, entry at the RW membrane was assumed and the passage of drug from ST to SV and the vestibule within the cochlea was able to account for the measured concentration time course of samples taken from the vestibule. A major finding of this analysis was that large basal-apical concentration gradients along ST were predicted to exist. The subsequent development of the sequential sampling technique for perilymph (in which multiple perilymph samples are collected and analyzed individually) allowed the gradients along ST to be confirmed experimentally (Plontke et al. 2007). The existence of gradients was supported by morphological studies showing greater hair cell losses at the base of ST after local applications (Wagner et al. 2005) and by gradients in gentamicin measured by immunochemical methods that were described as “striking” (Imamura & Adams, 2003). The existence of basal-apical gradients along the cochlea has high clinical relevance as it explains how vestibular function can be ablated in humans while preserving the function of speech frequency regions of the cochlea. In contrast to local RWM delivery, systemic application of gentamicin resulted in highest perilymph levels in the apex of the cochlea with decreasing concentrations towards the basal regions of ST (Hahn et al. 2013).

In a recent study, gentamicin application was targeted to the RW or stapes followed by functional and histological assessment after 7 days (King, et al., 2013). These studies found that vestibular hair cell loss, cochlear hair cell loss and hearing loss (measured by ABR) were all substantially higher when gentamicin was delivered to the stapes rather than to the RW. This result was interpreted as suggesting that gentamicin entered the inner ear more readily through the oval window and stapes than through the RW. Previous studies have demonstrated that other substances, such as gadolinium, enter the inner ear more readily at the stapes than at the round window. In the case of gadolinium applied to guinea pigs, it was

estimated that 90% of the total entry occurred at the stapes (King et al., 2011). A similar, prominent entry into the vestibule was demonstrated in humans (Zou et al. 2005; Zou et al., 2010). These combined studies raise questions about where gentamicin enters the inner ear and how it distributes within the inner ear following intratympanic applications. Even though the drug is widely used to treat humans, this has never been assessed before. In the present study, we have directly measured gentamicin entry into perilymph of the vestibule and into ST following local applications to the RW niche.

Interpretation of measured perilymph concentrations in terms of the relative entry at the RW and stapes is complicated by the existence of homeostatic processes for perilymph which vary with cochlear location. A recent study using fluorescent dextran as a marker showed that marker levels declined in perilymph at the base of ST far faster than in perilymph of the vestibule (Salt et al., 2015). The decline was thought to result partly from a slow (~30 nl/min) influx of CSF through the aqueduct, diluting ST perilymph contents. It was also shown that dextran or fluorescein decline at the base of ST occurred more slowly when cyanoacrylate glue was applied to the RW membrane (Salt et al., 2015, Plontke et al., 2016). As there was no indication that dextran leaks from the normal RW membrane, this result was thought to result from a mechanical stiffening of the normally compliant RW membrane by the cyanoacrylate.

This would reduce fluid oscillations across the cochlear aqueduct driven by respiratory CSF pressure changes. Such an exchange would cause marker loss from ST perilymph in the normal, RW compliant state. A comparable oscillatory fluid exchange across a cannula sealed into ST is used in a device to deliver drugs to perilymph (Fiering et al., 2009). Both of these interactions between perilymph and CSF in the basal turn of ST are incorporated into the computer model of the inner ear fluids that was used to interpret the results of gentamicin applications.

2. Materials and Methods

Animals

The study utilized 26 pigmented, NIH-strain guinea pigs weighing 400 – 600 g. Experiments were conducted in accordance with policies of the United States Department of Agriculture, the National Institutes of Health guidelines for the handling and use of laboratory animals, and under protocols 20101035 and 20130069 approved by the Animal Care Committee of Washington University.

Animals were anesthetized with 100 mg/kg sodium thiobutabarbital (Inactin, Sigma, St Louis, MO) and maintained on 0.8 to 1.2 % isoflurane in oxygen using a mechanical ventilator combined with a tracheal cannula. A 5 % end-tidal CO₂ level was maintained, monitored with a CapnoTrue AMP (Bluepoint Medical, The Netherlands), through adjustment of the ventilator's tidal volume. Heart rate and oxygen saturation were monitored with a (Surgivet, Waukesha, WI) pulse-oximeter. Body temperature was maintained at 38 °C with a thermistor-controlled heating blanket.

Experimental Conditions and Surgical Approach

Gentamicin was applied to the ear either by an injection into the RW niche (n=12), or by direct injection into perilymph at the lateral semi circular canal (LSCC)(n=14). Under both protocols, perilymph was subsequently sampled from the LSCC for analysis. Access to the inner ear was gained by an incision behind the pinna and exposure of the lateral portion of the bulla. The LSCC for was prepared for injection and/or fluid sampling by thinning the bone over the canal with a dental burr. A branch of the facial nerve was removed in those animals where it ran parallel to the LSCC. When the canal lumen was visible through the thinned bone, the bone was dried and a layer of thin cyanoacrylate glue (Permabond 101; Permabond, Pottstown, PA) was applied followed by layers of two-part silicone adhesive (Kwik-Cast, World Precision Instruments, Sarasota, FL). The silicone was applied thinly over the canal wall but multiple layers were built up at the edges to form a hydrophobic cup. A 30 – 40 μm fenestration into the bony canal wall was made through the adhesives and canal wall using a 30° House stapes pick (N1705 80, Bausch and Lomb Inc.). The conical shape of the pick prevented substantial entry of the tip into the canal, resulting in minimal damage to the membranous boundaries of endolymph. In mice, the fluid collected in this manner has been shown to be perilymph (Hirose et al., 2014).

For drug injections into the LSCC, an injection pipette was inserted into the fenestration in the canal wall and a tissue wick used to remove the fluid bolus accumulating on the hydrophobic surface. A droplet of cyanoacrylate glue was applied to seal the pipette in place. Following this procedure there was no visible fluid leakage at the injection site. “Invisible” fluid leakage below the adhesives (i.e. between the adhesives and the bone) was prevented by this method as the cyanoacrylate was applied to a dry bone surface, to which it bonded extremely well.

Application of Gentamicin Solutions

Gentamicin was applied to the RW niche as a 20 μL volume of 40 mg/ml Refobacin (Merck, Darmstadt, Germany). When this volume of liquid was applied it immediately contacted both the RW membrane and the stapes footplate, and over time could spread to other areas of the bulla. The ear was orientated so that most fluid remained in the niche in an attempt to minimize the amount reaching the bulla. In experiments where perilymph was sampled at 2 or 4 hours after application, drug solution was reapplied every hour after wicking away the previously-applied solution, avoiding any contact with the RW membrane or stapes. This was performed as we knew middle ear gentamicin was declining quite rapidly and we needed to maintain perilymph levels in a measurable range. Drug solution remained in the RW niche during perilymph sampling from the LSCC, kept clear of the sampling site by silicone adhesive (see below) thereby avoiding sample contamination. A single sample of the fluid in the RW niche was collected after perilymph sampling was complete.

Direct gentamicin injections into perilymph used a 500 $\mu\text{g}/\text{ml}$ solution of gentamicin, made by diluting the 40 mg/ml solution 1:79 with bicarbonate-buffered artificial perilymph. The artificial perilymph contained (in mM) NaCl (125), KCl (3.5), NaHCO_3 (25), CaCl_2 (1.3), MgCl_2 (1.2), NaH_2PO_4 (0.75), and dextrose (5). This solution becomes of near physiologic pH when in the normal CO_2 environment of the ear. Drug solution was injected from a glass

Sequential Sampling

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Sample handling and analysis

Fluid samples from the ear, of the applied solution, and of fluid collected from the RW niche after sampling were diluted in 150 μ L of phosphate-buffered saline (PBS) with sodium azide preservative. Reference standards were also made by performing 5 serial 10 X dilutions of the applied solution in PBS. Diluted fluid samples and equal volumes of standards were analyzed with a particle enhanced turbidimetric inhibition immunoassay on a Beckman Coulter UniCal DXC 800 analyzer or with a kinetic interaction of microparticles in solution (KIMS) assay on a Roche cobas c501 analyzer.

Perilymph concentrations were calculated based on individual sample volumes and the diluent volume. In some cases, measured sample concentration data were normalized with respect to the average measured injection concentration for that experiment, while in other cases absolute concentrations are shown.

Data interpretation through simulations of the experiments

Data were interpreted using an established simulation model of the cochlear fluids that we make available for others to download and use (Mac or PC) from <http://otocore.wustl.edu/saltlab/>. The model simulates physical processes driving solute movements (diffusion, volume flow, etc) in up to 15 interconnected compartments representing the fluid and tissue spaces of the cochlea and vestibular systems of the guinea pig inner ear. Cross-sectional area versus distance is defined for each compartment derived from 3-D reconstructions of anatomic data. Elimination to blood and exchange with adjacent compartments can be set for each compartment with specific half-times. The program incorporates the volume flows associated with perilymphatic injections and sampling, allowing all aspects of the experiment to be included in the simulation. In this manner, the program was able to replicate specific experimental configurations, predicting the 10 sample concentrations for sequentially-taken samples after gentamicin application.

3. Results

Perilymph kinetics of gentamicin measured by injections into perilymph

The characteristics of elimination of gentamicin from perilymph were determined by loading the perilymphatic spaces with gentamicin and sampling perilymph at varying delay times after application. The results from individual animals sampled with delay times of 0, 1, 2 and 4 hours after loading are shown in Figure 1. Each curve shows the concentrations of 10 analyzed samples collected sequentially from the LSCC. Initial samples originate from perilymph in the LSCC and the vestibule, followed by perilymph from scala vestibuli and scala tympani, as indicated below the curves. Later samples are currently assumed to be primarily CSF that has passed through the perilymphatic spaces of the ear, into which gentamicin from adjacent fluid and tissue compartments has redistributed. For each experiment, concentrations were normalized with respect to the measured applied drug concentration based on samples of the injected solution. Injection solution, diluted and measured in the same way as perilymph samples averaged 693.5 μ g/mL (SD 74.5; n=14) across the LSCC injection experiments. It is notable that the repeatability across animals was good and demonstrated that gentamicin concentrations declined only slowly with time. Two-

way ANOVA testing showed delay time to have a highly significant influence ($p<0.001$) and multiple comparisons (Student-Newman-Keuls Method) showed all delay times to be significantly different from each other ($p<0.001$ in all cases)

The mean data from these experiments are compared in Figure 2A, demonstrating the slow, progressive decline of perilymph gentamicin with time. For samples from vestibular regions (samples 1 to 3), the mean concentration was more than 60% of the applied concentration even after 4 hours. A broad view of the decline is shown in Figure 2B, where each point is the total amount of gentamicin recovered in the first 8 samples from each experiment, normalized to the total amount injected. As the loading procedure injected approximately 4 times the normal volume of perilymph, the maximum amount recovered is approximately 25% of the injected amount, the balance having passed through the perilymphatic spaces and into CSF. The fitted exponential curve (black line, $R^2=0.853$) has a half-time of 4.7 hr, although a straight line with slope of -2.46% decrease/hour fits almost as well ($R^2=0.840$) as the time period studied is less than one half-time. In addition, the decline is not uniform throughout the ear as shown in Figure 2C. Here, the amount of decline as a function of sample number for 1, 2 and 4 hours after gentamicin loading is shown, calculated by subtracting the curve for zero delay from each data set. The decline initially occurs to a larger extent in samples 6 and 7 than in other samples. These samples originate from the basal part of ST.

Each of the mean sample curves in Fig 2A were fitted with the simulation program using the sample volumes and times specific to the data set and adjusting just 3 parameters to obtain a best fit. Examples of how the parameters affected the calculated curves for the 2 hr delay data are shown in Figure 3. Elimination from SV and SCC was varied to influence concentration of samples 1–4 (Figure 3, left). Elimination from ST was varied to influence concentration of samples 4–5 (Figure 3 middle) and a volume correction of SV was varied to influence concentration of samples 6–9 (Figure 3 right). Each parameter was varied to minimize the differences between the samples calculated by the model and the measured samples. This fitting procedure was similar to that used in the analysis of similar data with FITC-dextran (Salt et al., 2015). Importantly, all simulations included the basal turn interactions of ST perilymph and CSF quantified in that study. Specifically, a slow entry of CSF volume into ST at the base (through the cochlear aqueduct) of $0.03\ \mu\text{L}/\text{min}$ and an ongoing exchange of CSF and perilymph due to fluid oscillation across the cochlear aqueduct of $3\ \text{nl}/\text{s}$. Both of these processes make substantial contributions to the decline of gentamicin concentration at the base of ST. The mean parameters derived from fitting the four sets of gentamicin data are summarized in Table 1.

A summary of the output of the simulator based on the mean parameters and using identical sample volumes ($2.03\ \mu\text{L}$) and times ($168\ \text{s}$) (based on their mean values) is shown in Figure 4. Figure 4A shows the calculated longitudinal gradients of gentamicin along the perilymphatic spaces prior to sampling for different delay times. The displayed gradients along the perilymph spaces give rise to the calculated sequential sample curves shown in Fig 4B. The calculation demonstrates the slow decline in the vestibule and SV and the more rapid decline of gentamicin concentration near the base of ST. The faster decline in ST is demonstrated in Figure 4D where locations $0.5\ \text{mm}$ apical to the RW (ST $1.6\ \text{mm}$) and 0.5

mm apical to the stapes (SV 2.9 mm) are compared. In addition, calculated concentration changes relative to the zero-delay condition (Figure 4C) have comparable characteristics to the measured values shown in Figure 2C, showing the model represents many aspects of the measured changes with time. The faster loss of gentamicin from the basal part of ST is especially important for the current study where we are comparing drug entry rates at the RW membrane and stapes. If these pharmacokinetic properties of perilymph were not considered the relative entry of drug at the stapes would be considerably overestimated.

Perilymph kinetics of gentamicin for local applications to the round window niche

Gentamicin solution was applied to the RW niche and perilymph was collected by sequential sampling from the LSCC after 1 hr, 2hrs or 4hrs application times in 12 animals. The data from individual experiments and the means for each group are shown in Figure 5. In most experiments (10 of 12) highest concentration of the 10 samples from each animal was found in samples 1 to 3, originating from perilymph of the vestibule and corresponding to gentamicin entry near the stapes. In 2 curves the highest concentration was found in samples 6 or 7, originating from ST and corresponding to entry at the RW membrane. In some instances, other samples were high, such as sample 4 of one 4 hour experiment, possibly representing higher entry through the thin bone at the cochlear apex in that guinea pig. There was higher variability of sample concentrations with RW niche drug applications than we had seen with canal injections, which is thought to originate from inter-animal variations in RW membrane and stapes permeability. The mean perilymph concentrations increased with longer application times. Two-way ANOVA testing indicated that application duration was highly significant ($p < 0.001$), with 4 hour applications generating significantly higher concentrations than either 1 or 2 hr ($p < 0.001$, Student-Newman-Keuls Method). The perilymph concentrations measured after 1 or 2 hour applications were not significantly different from each other ($p = 0.310$).

The gentamicin concentration in the RW niche measured after a single application (in experiments of 1 hr duration) averaged 20.3 mg/ml (SD 3.9, $n = 5$). The solution originally applied to the niche, measured in diluted samples with identical methodology, was 48.3 mg/ml (SD 3.0, $n = 5$). Thus, over the course of the experiments, gentamicin concentration in the RW niche had fallen to 46.2% of the initially applied concentration. The fluid from the RW niche was taken after perilymph sampling at an average of 87 min after the application. An exponential decline with a half time of 79 min accounts for this rate and was subsequently used to represent the declining middle ear concentration in simulations of the sample data with RW niche applications. Simulations of application times longer than 1 hr included a superimposed hourly restoration of the applied concentration.

Each individual curve was analyzed by our simulation program, adjusting entry at the RW and stapes by manipulation of their permeability. To better fit the data from some animals, we also included a variable amount entry at the apical part of the cochlea, corresponding to gentamicin solution in the bulla potentially having access to areas of thin bone at the cochlear apex and entering there (Mikulec et al., 2009). In each case, samples from the model were compared with measured data, adjusting 3 parameters to minimize their differences. Stapes permeability was adjusted to fit samples 2–3, apical entry was adjusted to

fit samples 4–6 and RW membrane permeability was adjusted to fit samples 7–9. The fit of the model to one experiment and the effects of adjustments of entry at the RW membrane and stapes on the calculated curves are shown in Figure 6. Although there is an influence of entry at each location on later samples (as following samples have to pass through the region of higher or lower concentration), these procedures allow the data to be fitted by the model in a quantitative, objective manner. The model also includes and therefore takes into account the differences in volume between ST and that of the vestibule and SV combined.

The relative entry at the RW membrane, stapes and apex for the 12 experiments, derived from the analysis with the fluids model are summarized in Figure 7. While there was considerable variation across animals, the mean entry at the stapes to account for the sample data was 35.5 % of the total entry, less than the mean entry at the RW membrane which made up 57.3 % of the total entry.

The distribution of gentamicin in the perilymph of the inner ear as a function of distance and time, based on the mean entry and kinetic parameters derived in this study, is shown in Figure 8. The perilymph space is shown “unrolled” with distance measured from the base of the vestibule, apically through SV and basally through ST. The calculation shows a 4 hour period following a single application to the RW niche with concentration in the middle ear declining over time as described above. This differs from the repeated applications used experimentally with collection times at 2 and 4 hours. The calculations show that gentamicin concentration at the base of ST could transiently exceed that in the vestibule but this would not necessarily be reflected in the sample measurements for a number of reasons. First, the region of high concentration only occurs close to the RW and the corresponding volume is quite small. Second, during sampling this volume has to pass through substantial regions of the inner ear with lower gentamicin concentration, during which drug will be lost. Finally, as sampling commences, CSF entering the aqueduct will immediately tend to dilute the basal region of high concentration. Each of these factors was included in the simulations used to fit the measured sample data, in which perilymph distribution is related to sample concentrations. It is apparent that although higher concentrations were typically found in the vestibular perilymph samples, the relationship between drug distribution in perilymph and sample concentrations is complex. The main processes involved in perilymph kinetics are schematized graphically in Figure 9 where entry of drug (orange) from the middle ear is greater through the RW than the stapes (green arrows). However, drug levels in the basal turn of ST are continually diluted by the influence of CSF (cyan arrows) volume entry and fluid oscillation across the aqueduct, even though elimination from ST (half time 231 min) and from SV and the vestibule (half time 220 min) occurs at similar rates. Although for a brief period concentration at the base of ST may exceed that in the vestibule, over a longer period the level in the vestibule will remain higher as drug concentration in ST declines due to dilution with CSF.

4. Discussion

Sequential perilymph sampling from the LSCC shows that when gentamicin is applied to the RW niche of guinea pigs, where it contacts both the RW membrane and the stapes footplate, higher concentrations are measured in perilymph originating from the vestibule and scala

vestibuli than in samples originating from ST. This confirms that gentamicin, like other substances, directly enters perilymph of the vestibule and SV by entering at (or near) the stapes footplate/oval window region, as reported elsewhere for other substances (King et al. 2011; Salt et al., 2012a). Our analysis also demonstrates that the drug level at the base of ST is underestimated by the “raw” LSCC sample data in the absence of detailed simulations of the experiments. Simulations that closely match the sample data suggest that the gentamicin concentration at the base of ST may transiently exceed the concentration in the vestibule. Over the longer term (for times over 1 hour), concentration in the vestibule and SV remains high for longer than that in ST, due to the ongoing dilution of drug in the basal region of ST by interactions with CSF. These findings are the first direct measurements that show the predominant vestibulotoxicity of locally-applied gentamicin may in part arise from the distribution properties of gentamicin in perilymph of the ear, specifically the prolonged retention of gentamicin in perilymph of the vestibule. Subsequently, gentamicin is found primarily in the Type I vestibular hair cells (Lyford-Pike et al., 2007) resulting in suppression of vestibular function.

The apparent ongoing dilution of drugs at the base of ST with CSF could represent a significant problem for treating the cochlear portion of the ear with drugs applied intratympanically, when a patent cochlear aqueduct is present. Although CSF dilution processes, comprising of CSF volume entry and pressure-driven fluid oscillations across the aqueduct (Salt et al., 2015; Plontke et al., 2016), dominate the decline of substances that are eliminated slowly from perilymph (such as FITC-dextran and gentamicin) their influence will be less on substances that are eliminated more rapidly to blood, such as TMPA marker and dexamethasone (Salt et al., 2012b). However, as CSF dilution is a non-specific process and will influence all applied substances, it is very unlikely that any substance will remain in ST perilymph for a prolonged period unless it is retained in some way by binding to adjacent tissues.

The CSF-related dilution of locally-applied substances in ST perilymph is difficult to distinguish from other forms of elimination, unless losses by other processes (such as elimination to blood) are low, as is the case for dextran (Salt et al., 2015) and gentamicin in the present study. Although ST perilymph interactions with CSF have only been documented in guinea pigs, they are likely to similarly influence all species with a patent cochlear aqueduct, such as mice, rats and other rodents. Oscillatory movements driven by respiration-induced CSF pressure changes will depend on aqueduct dimensions and cochlear impedance (Gopen et al., 1997) with exchange of just a few nanoliters per cycle sufficient to cause substantial drug loss from ST (Plontke et al., 2016). However, CSF dilution of drugs in ST perilymph is likely to be of lesser influence in humans as the cochlear aqueduct is narrower (Gopen et al., 1997) so the volume exchanged with CSF will be smaller. In addition, ST volume is substantially larger in humans so the influence of fluid exchange will be less. Nevertheless, differences in CSF-perilymph communication between species are likely to complicate the translation of drug therapy studies from experimental animals to humans.

The decline of gentamicin or dextran concentration near the base of scala tympani was greater than can be accounted for by CSF volume entry or elimination to blood, and the simulations currently include a loss driven by fluid oscillation across the cochlear aqueduct.

As part of these calculations the amount of entry and loss by each route is totaled. Using the mean kinetic parameters for perilymph derived in this study, the simulations calculated that 53% of the gentamicin entering the inner ear for RW niche applications will be lost to CSF over a 24 hour period. This movement of gentamicin into CSF may contribute to the gentamicin demonstrated in tissues of the brain following intratympanic administrations (Zhang et al, 2012), although other, neural pathways may also be implicated. The degree to which intratympanically-applied drugs reach the brain and the pathways involved become of greater clinical importance as the range of drugs applied intratympanically to humans increases.

For gentamicin injections into the lateral SCC, 60 μ L was injected over a 1 hr period to load the fluids and tissues, compared to perilymph volume which is $\sim$ 15 μ L. About 75% of the injected gentamicin is therefore driven into the CSF during the loading procedure. At present, the gentamicin kinetics in CSF with time following this procedure remains unknown. We have assumed that CSF levels generated will be substantially lower than those in perilymph and that CSF flow will rapidly clear gentamicin away from the cochlear aqueduct region. So, in the absence of data to the contrary, we have assumed a negligible contribution of gentamicin in CSF to the concentrations of sequentially-collected samples. The assumption appears justified by the rapid decline in sample concentration in later samples at all delay times. Nevertheless a small proportion of the gentamicin in later samples could originate from that present in CSF.

The time course of gentamicin in ST was previously measured using a microdialysis method (Hibi et al., 2001). With intratympanic administration they found gentamicin concentration at the base of ST peaked at 56 min and declined with a half-time that averaged 117 min. In the curve in Figure 8 for a 34.5 mm distance (corresponding to 1.5 mm from the RW) we found concentration to peak at 50 min and to subsequently decline with a half-time of 103 min (by fitted exponential). This is remarkably similar to the observations of Hibi et al. We now know that the perilymph kinetics at this location depends on numerous factors, including the time course of gentamicin in the middle ear, entry through the RW membrane, diffusion along the cochlea, dilution effects of CSF, and elimination to blood.

Our analysis of perilymph kinetics of gentamicin following local applications to the ear casts new light on prior studies in which a small volume (3 μ L) of concentrated gentamicin solution (330 mg/ml) was applied in a highly targeted manner, specifically to either the stapes or to the RW membrane (King et al., 2013). In that study, it was shown that 7 days after gentamicin was applied to the stapes, there was more vestibular hair cell loss, more cochlear hair cell loss and more hearing loss than when the same volume was applied to the RW membrane. At the time, the findings were interpreted as suggesting that gentamicin more readily entered the inner ear via the stapes than by the RW and that the greater toxicity resulted from higher gentamicin concentrations in the ear after applications to the stapes. In view of the current study, a more quantitative interpretation is now possible. The delivery protocol described by King et al, 2013 was simulated using the perilymph kinetic parameters and entry properties at the RW and stapes quantified in the current study. The calculated concentration time courses at cochlear locations corresponding to the 24 kHz region of the guinea pig are shown in Figure 10. It is first apparent that when gentamicin is applied to the

RW alone, drug concentration in ST perilymph will initially be higher than in SV, although drug is retained better in SV. Similarly, when drug is applied to only the stapes, drug level in SV is substantially higher than in ST. The higher vestibulotoxicity with stapes applications could certainly be accounted for by higher concentrations, as even at this cochlear location SV concentrations are greater for a stapes application ($C_{\max}$ 993 $\mu\text{g/ml}$ for RW application, 1305 $\mu\text{g/ml}$ for stapes application; AUC (area under the curve) 7513 $\mu\text{g}\cdot\text{hr/ml}$ for RW application and 9471 $\mu\text{g}\cdot\text{hr/ml}$ for stapes application). Perhaps of greater importance is the hearing loss and cochlear hair cell loss found in this study, which was also greater for stapes applications. The calculations show that the ST concentration is substantially lower (by a factor of approximately 100) for stapes application of the drug. Therefore, if cochlear toxicity results from gentamicin in ST perilymph, we would certainly not have expected greater hearing loss with the stapes application. On the other hand, the findings could be explained if cochleotoxicity was determined by the gentamicin concentration in SV. As shown above, both $C_{\max}$ and AUC in SV were greater for the stapes application, lending support to a new concept that cochleotoxicity may result from the prevailing gentamicin level generated in SV rather than in ST. This concept has considerable support from studies tracking gentamicin distribution following systemic applications. Studies with texas red labeled gentamicin (GTTR) have found the drug to enter the inner ear in stria vascularis, passing from there to the hair cells via endolymph (Dai & Steyger, 2008). In vitro hair cell studies with organ of Corti explants confirm that aminoglycosides including gentamicin (a cation) enter hair cells through the mechanosensitive transduction channel, which is a non-specific cation channel (Marcotti et al., 2005; Alharazneh et al., 2011; Vu et al., 2013). The route for gentamicin trafficking was therefore thought to be via stria vascularis and endolymph into the hair cells. A pivotal series of experiments in support of this pathway was reported by Li & Steyger (2011). They compared GTTR entry into hair cells when given systemically, but while perfusing ST with drug-free artificial perilymph, with entry when ST was perfused with a GTTR-containing solution. The hair cells showed low levels of GTTR when GTTR was perfused through ST but showed substantially higher levels when GTTR was given systemically, while simultaneously perfusing ST to keep GTTR concentration there low. This provides strong support for the endolymphatic pathway from blood to the hair cells. An endolymphatic pathway was also an explanation the distribution of gentamicin along ST following systemic applications (Hahn et al., 2013). For local gentamicin applications, the pathway of gentamicin from the middle ear to the cochlea was previously assumed to be via the RW membrane and the perilymph in ST. The substantial entry of gentamicin at the stapes documented here and the greater hearing loss and cochlear hair cell loss with gentamicin applied to the stapes now calls into question the route through ST perilymph. Rather, a pathway from SV perilymph to endolymph must be considered. Although we would expect Reissner's membrane to be relatively impermeable to gentamicin and gentamicin entry into endolymph to be opposed by the positive endocochlear potential, the presence of gentamicin in SV would establish an endolymph-directed gradient. A substantial proportion of gentamicin entering endolymph would be driven into the hair cells as part of the cationic transduction current. As gentamicin is retained in SV for a long time it is therefore possible that over a longer time period, even with a low rate of entry into endolymph, toxic levels could accumulate in the hair cells by this route.

If cochleotoxicity from intratympanic injections does result from the gentamicin level in SV, rather than that in ST, then attempts to target gentamicin delivery specifically to the oval window of humans, such as by protecting the RW membrane (Jahnke, 1989; Quaranta et al., 1998) may not be worthwhile. In addition, any change of treatment protocol that increases the efficiency of drug application to the stapes should be considered as increasing the potential risk of hearing loss. This is particularly relevant for changes in protocol that increase the duration of gentamicin exposure. In both animals and humans, hearing loss has been found to be greater when drug delivery is prolonged with the use of a microcatheter (Shoendorf et al., 2001; Hashimoto et al., 2007) or by delivering the gentamicin in a hydrogel (Mackeith et al., 2014). The use of computer simulations to predict the effects of protocol changes on inner ear drug levels could help prevent the unnecessary deafening of patients in future if gentamicin is delivered more effectively.

The gentamicin used in this study (Refobacin: 40 mg/ml gentamicin) is a formulation in common use in Europe, intended for intravenous use and applied to the middle ear “off-label”. We measured the osmolarity to be 247 mOsm/L, which is slightly hypotonic, and to have a pH of 4.5. However, commercially available gentamicin formulations do not have a standardized content and they differ markedly between manufacturers. For example, for the 40 mg/ml gentamicin produced by Ratiopharm (Ulm, Germany) we measured the osmolarity to be 160 mOsm/L and the pH to be 2.8. In Europe the reformulation of drugs to be given clinically is not permitted. In contrast, in the USA, the formulation of gentamicin applied to the ear is commonly adjusted by adding sodium bicarbonate solution to the commercial drug solution to increase pH (Nedzelski et al., 1993; Minor 1999; Nguyen et al., 2009). This makes a solution that is closer to physiologic pH and osmolarity, causing less discomfort to the patient, but of lower gentamicin content (26.7 mg/ml). It remains unknown to what degree these formulation differences influence gentamicin entry into the inner ear, but it has been shown that RW membrane permeability can be significantly influenced by osmolarity (Mikulec et al. 2008) and toxicity can be affected by solution pH (Tanaka et al., 2003). It is therefore important to be aware that formulation differences could be a significant factor in gentamicin entry into the ear and therefore potentially influence patient outcome.

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Highlights

Locally applied gentamicin enters the ear at the round window and stapes.

Gradients for gentamicin exist along the perilymphatic spaces after local applications.

Cochleotoxicity from IT gentamicin is probably dominated by drug from scala vestibuli.

Figure 1.

Each gray curve shows the gentamicin concentrations of ten sequentially-collected samples from a single experiment after perilymph loading with gentamicin solution. Each panel shows data as time between gentamicin loading and fluid sampling was varied (No Delay, 1 hour, 2 hour and 4 hour delays). A progressive decline of concentration is apparent as delay time increases. Concentrations were normalized with respect to the measured concentration of the injected solution for each experiment. Heavy black lines show the group mean curves for each condition. The number of experiments in each group were No Delay:4; 1 hour delay:3; 2 hour delay:3; 4 hour delay: 4.

Figure 2.

A: Comparison of mean gentamicin concentrations for perilymph samples for delay times between loading and sampling of 0, 1, 2 and 4 hr. Perilymph gentamicin concentration falls progressively with time. **B:** Amount of gentamicin recovered in all perilymph samples, calculated by summing gentamicin content for the initial 8 samples from each experiment relative to the amount injected. The solid line is an exponential curve fitted to the data, which declines with a half time of 4.7 hrs. **C:** Decline of mean samples for different delays, calculated by subtracting the zero delay data from each curve. Initially the greatest decline occurs in samples 6 and 7, originating in ST.

Figure 3.

Derivation of model parameters from measured sample curves. Each panel shows the fitting of a single parameter. Measured data are shown as solid black circles and the best fit as open black circles. Samples 1–4 were used to fit SV elimination (left panel), samples 4–5 were used to fit ST elimination (middle panel) and samples 6–10 were used to fit a volume scaling factor of SV and the vestibule (right panel). Parameters were varied to minimize the differences between measured and calculated curves. In each case, the influence of decreasing (triangles) or increasing (squares) the parameter on the calculated sample curve is shown.

Figure 4.

Calculated distribution of gentamicin as a function of location and time following loading by injection at the LSCC. Simulations used the mean parameters derived from fitting the mean perilymph sample data in Figure 3A. **A:** Distribution of gentamicin along the perilymphatic spaces (“unrolled” from the base of SV to the apex and to the base of ST) as a function of time. Concentration falls most rapidly in the basal turn of scala tympani, dominated by interactions with CSF that have previously been documented (Salt et al., 2015). **B:** Calculated sample concentration curves at each time (using the same parameters for all curves) showing the relationship to the concentration profiles in (A). **C:** Decline of concentration for different delay times, calculated by subtracting the zero delay sample curve. The maximum decline initially occurs around sample 7, originating from the base of ST. **D:** Calculated concentration decreases with time for two locations, in SV 0.5 mm apical to the stapes (SV 2.9 mm), and in ST 0.5 mm apical to the RW membrane (ST 1.6 mm). Gentamicin concentration in ST declines far more rapidly than that in SV.

Figure 5. Measured gentamicin concentration in sequential samples of perilymph taken from the LSCC after 1 hr (n=5), 2 hr (n=4) and 4 hr (n=3) applications to the RW niche. Gray curves are measures of the 10 sequentially-collected samples in individual experiments. Black curves are the group means which are shown superimposed in the lower right panel.

Figure 6.

Model fits (black curves) to measured sample data from one animal (gray curves) after application of gentamicin to the RW niche. The best fit (solid black symbols, shown on both panels) was obtained with 45% entry at the RW membrane, 51% entry at the stapes and 4% entry at the apex. Also shown is the influence that doubling and halving RW membrane permeability (upper panel) and stapes permeability (lower panel) has on the calculated curves. In each case the relative gentamicin entry for the condition is shown on the curve. Varying entry at the RW membrane influences samples 6 and later. Varying entry at the stapes affects all samples, but initial samples more than later samples. The curves show how parameter adjustments are readily used to fit the model to the measured data.

Figure 7.

Relative entry rates at the stapes, apex and RW membrane derived by fitting the measured data with the fluids simulator, incorporating kinetic parameters derived from perilymph-loading studies. The mean entry rates were: stapes: 35.5%; Apex: 7.2% and RW membrane: 57.3 %, although variation was high with entry at the stapes varying from 7.2 % of the total to 65.1 % of the total.

Figure 8.

Calculated distribution of gentamicin in perilymph as a function of distance and time following a single application of 20 μL of 40 mg/ml gentamicin to the RW niche of the guinea pig, based on the mean parameters shown in Figure 7. Concentration at the base of ST transiently exceeds that in the vestibule, but as fluid from this region has to pass through regions of the ear with substantially lower concentration during collection, the elevation is not be represented prominently in samples taken from the LSCC. Although partially hidden in this view, the concentration in ST declines more rapidly with time than it does in SV (where the trailing edge of the curve remains visible) due to the influence of interactions with CSF in the basal region of ST. The small amount of entry into SV through the thin bone at the apex of the cochlea (at 16–18 mm on the distance axis) is also represented in this figure.

Figure 9.

Schematic showing the processes affecting perilymph gentamicin concentration in the basal turn of scala tympani (ST) and scala vestibuli (SV) following an application to the middle ear (drug shown orange). Although the rate of elimination to blood is similar for both scalae (red arrows), drug level at the base of ST is influenced by interactions with CSF, indicated by cyan arrows. In the sealed cochlea, there is a slow entry of CSF through the cochlear aqueduct (CA), diluting the drug level at the base of ST. An additional oscillation of CSF across the aqueduct driven by CSF pressure fluctuations intermixes CSF and perilymph, also contributing to drug decline in ST. As a result, even though entry at the RW is greater than the entry at the stapes (green arrows) and the volume of ST is smaller than the combined vestibule and SV, drug concentrations reached in the vestibule and SV may be greater than those in ST. It is apparent that drug pharmacokinetics in the basal turn of ST is extremely complex and must consider numerous processes.

Figure 10.

Calculated concentration time courses at cochlear locations in ST and SV corresponding to the 24 kHz region of the guinea pig (1.3 mm from base of ST, 4.7 mm from the base of the vestibule/SV), when a 3 μ L volume of 337 mg/ml gentamicin is applied to either the RW membrane (left) or to the stapes (right) only, (based on the protocol described by King et al., 2013). The first 12 hours after application is shown. The influence of the two delivery conditions on ST concentration and total dose (AUC, area under the curve) are much greater than on SV. Drug concentration calculated for ST (black lines) would be substantially lower when drug is applied to the stapes (right panel) than when it is applied to the RW (left panel). Conversely, the concentration in SV (gray lines) is higher when drug is applied to the stapes (right panel), compared with application to the RW (left panel).

Table 1

	Elimination half-time from SV + SCC (min)	Elimination half-time from ST (min)	SV Volume correction (%)
1 hr delay	252	63	106
2 hr delay	218.5	275	90.5
4 hr delay	221	322	107
Zero delay	-	-	75.5
Average	231	220	95

Parameters providing best fits between the calculated and mean measured data for each delay time. Elimination was not varied for the zero-delay condition, instead using the mean value from the other conditions where experiment duration was long enough for elimination to have influence.