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Comparison of Gentamicin Distribution in the Inner Ear following Administration via the Endolymphatic Sac or Round Window

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Abstract

Objective/Hypothesis—The distribution of gentamicin in the inner ear via the endolymphatic sac (ES) or round window (RW) routes was investigated.

Study Design—Experimental study.

Methods—A fluorescent gentamicin-Texas Red conjugate (GTTR) was adopted to visualize the gentamicin. Adult guinea pigs were treated with GTTR applied to the ES or RW, the animals were allowed to recover for 3 days, then confocal microscopy was used to observe and quantify GTTR distributions in cochlear and vestibular sensory epithelium.

Results—When GTTR was applied via the ES, strong GTTR labeling was observed in the vestibule while little GTTR was detected in the cochlea ($P < .0001$). However, distinct GTTR fluorescence was observed in the cochlea and vestibule following RW application ($P = .7967$). There was less GTTR labeling in cochlea via ES application than through RW administration ($P < .0001$).

Conclusions—ES drug application may be preferable for the treatment of intractable Meniere's disease.

Keywords

Gentamicin; endolymphatic sac; round window; Meniere's disease

INTRODUCTION

Meniere's disease (MD), characterized by recurrent attacks of vertigo, fluctuating hearing loss (HL), and tinnitus, occurs with an incidence of 15–50 per 100,000 population.¹ Some patients with MD are greatly impacted in their daily activities due to frequent attacks of

vertigo. In 1938, studies of pathologic temporal bones revealed the presence of endolymphatic hydrops during Meniere's disease.² Treatment for MD is usually accomplished by controlling the frequency of vertigo. Control of vertigo using pharmacologic treatment is effective in the majority of cases. If pharmacologic therapy is ineffective, surgical management, including endolymphatic sac (ES) decompression (or shunt surgery), vestibular nerve dissection, or labyrinthectomy, is offered.

Currently, intratympanic (IT) gentamicin administration to treat patients with MD has widespread popularity. Meta-analysis by Chia et al.,³ in 2004, demonstrated that IT gentamicin therapy provided effective vertigo control in 86.8%–96.3% of patients with MD, but 13%–35% of these patients would also experience permanent HL.

Another potential site for drug application is the ES. During ES shunt surgery—which does not disturb auditory function—drugs can be applied within the sac. Morgenstern et al.⁴ injected Thorotrast into the ES; 2 days later aggregated particles were identified in the cochlear endolymph. Lee et al.⁵ observed that gentamicin applied to the ES of guinea pigs produced lesions in the saccule. Yamasoba et al.⁶ injected a replication-deficient adenoviral vector (Ad.RSVntlacZ) into the ES of guinea pigs and observed large numbers of LacZ-positive cells in the vestibule, ampullae, and cochlea. Kitahara et al.⁷ performed ES drainage surgery with steroid instillation into the sac for over 100 patients with intractable MD with regular follow-up for at least 2 years, and reported significantly improved hearing compared to ES drainage without steroids or nonsurgical therapy. Thus, it is generally accepted that drugs administered within the ES are able to diffuse into the vestibule and cochlea.

In this study, gentamicin-conjugated Texas Red (GTTR) was applied into the ES, or to the round window (RW), of guinea pigs. Auditory brainstem response audiometry (ABR) was used to assess cochlear function, and the nystagmus response for vestibular function. Three days after surgery, the distribution of GTTR in cochlear and vestibular epithelia was observed and analyzed using confocal microscopy.

MATERIAL AND METHODS

Conjugation and Purification of GTTR

An excess of gentamicin sulfate (Sigma, St. Louis, MO; MW = 449–477; pH 10) was mixed with succinimidyl esters of Texas Red (Invitrogen, Carlsbad, CA; MW = 816) to ensure a 1:1 ratio of Texas Red conjugated to each molecule of gentamicin.⁸ After conjugation, the reaction mixture was separated by reversed phase chromatography using C-18 columns (Burdick and Jackson, Muskegon, MI) to purify the conjugate from unconjugated gentamicin and potential contamination by unreacted TR.⁹ The purified GTTR conjugate was aliquoted, lyophilized, and stored desiccated in the dark at –20°C until required. The day before the operation, GTTR was dissolved in dimethyl sulfoxide to obtain a 0.2 mg/mL GTTR solution.

Animals and Surgical Procedures

Fourteen albino guinea pigs (weighing 300–350 g) with normal Preyer reflexes and ABR thresholds were used in this study. All the animals were anesthetized using intramuscular xylazine (6 mg/kg) and ketamine (50 mg/kg) and operated under sterile conditions. Under general anesthesia, the animal was put into the sound isolation chamber to detect auditory thresholds. The recording electrode was put on the center of calvaria, the reference electrode was put into subcutaneous post aurem and the ground electrode on the tip of nose. The acoustic stimuli were clicks with pulse length 0.1 ms, sweep duration 10 milliseconds, and superposition 1,024 times. The acoustic stimulus intensity corresponding to the loss of the ABR III wave was adopted as threshold of response.

In the first group of animals ($n = 5$), the ES was exposed through the extradural posterior cranial fossa approach. After local infusion of 1% lidocaine, an oblique incision was made over the region of occipital bone and muscles attached to the suture line were elevated. Drilling began at the lateral supraoccipital ridge until the dura and sigmoid sinus were exposed. The sigmoid sinus was retracted medially and the emissary vein inferiorly to obtain a wide view of the operculum aperture where the ES extended toward the sigmoid sinus. A tiny incision was made in the sac with a fine pin. Gelfoam absorbed GTTR ($5 \mu\text{L}$, 0.2 mg/mL) was placed in the opened sac. The lesion in the occipital bone was filled with gelfoam, and the incision sutured layer by layer.

The same dose of GTTR was placed on the RW membrane in the second group of five animals. All procedures were performed on the right ear, with the left ear serving as a control. In addition, the final four animals received ES or RW surgery to administer $5 \mu\text{L}$ of unconjugated (hydrolyzed) Texas Red as another control.

All the animals in the study were under the guidelines of the Ethical Board of Eye Ear Nose & Throat Hospital, Fudan University, and approved by Committee on Care and Use of Animals.

Tissue Harvesting and Preparation

Under deep anesthesia, all the animals were retested for their ABRs again 3 days later, and then euthanized. Each bulla was rapidly opened and the fluid spaces perfused with 4% paraformaldehyde via the oval window and a fenestra in each semicircular canal. The membranous organs of the inner ear were then carefully dissected out and fixed for 2 hours at 4°C . These included the organ of Corti, the saccule (Sac), and utricle (Utr) as well as the lateral, superior, and posterior semicircular canal (LSC, SSC, and PSC). The otolithic membrane on the Sac and Utr was removed carefully under high magnification.

After washing in phosphate-buffered saline (PBS) (0.01 mol/L), fixed tissues were permeabilized using 0.1% Triton X-100 in PBS for 40 min at 37°C , subsequently labeled with 2% Phalloidin-FITC in PBS (Sigma) for 40 minutes at 37°C to localize filamentous actin, and mounted on glass slides with the ciliary side facing the coverslip.

Confocal Microscopy and Image Analysis

Specimens of the endorgans were scanned using a Leica SP2 confocal microscope (Leica, Germany) from the stereociliary bundle of hair cells to their synaptic region. Eight serial optical slices ($1 \mu\text{m}$) were obtained and merged into one image for each specimen. The laser power settings and pinhole were constant for all specimens. Amplifier offset and photodetector gain were also constant for all serial optical slices in a given tissue. To avoid cross-talk between the fluorescent GTTR and phalloidin-FITC, specimens were excited sequentially with the respective lasers and recorded in $1,024 \times 1,024$ pixel frames.

The fluorescent GTTR intensity was obtained using the image analysis software of Image-Pro Plus v6.0. A region of $2,500 \mu\text{m}^2$ was measured in each quadrant of a sensory epithelium and the sum divided by 4 to obtain the endorgan's average GTTR intensity, including the organ of Corti, Utr, Sac, and semicircular canals. The software reported the intensity as an Optical Density (OD) value.

Comparison and Statistics

The measured outcome variables were the intensity of GTTR in endorgans. Data were treated as spatial repeated measurements with the two different delivery methods as the two levels of the treatment factor and with position for measurements as the repeated factor; then

a Mixed Model with unstructured covariance structure was constructed for the data. Statistical analysis was completed with Proc Mixed Procedure in SAS 9.2 statistical software. *P*-values for effect tests and confidence intervals for means difference were reported. Statistical significance level was set at .05 (simulation based adjustment was made for means comparison to control the family type I error of at a level of .05).

RESULTS

Functional Status

All 14 guinea pigs tolerated the surgical procedures and had normal appetite after anesthesia. The pinna reflex was normal and no head tilt or tottering movement was observed at either 1 day or 3 days after the surgery in the ES, RW, or control animals. No nystagmus was found either. There was no significant deviation in ABR thresholds obtained prior to surgery or 3 days postsurgery in each group.

GTTR Distribution following ES Application

In all five animals that received GTTR via the ES, punctate GTTR fluorescence in the cochlea could only be observed in inner and outer pillar cells (PCs, also called Corti's tunnel), with no obvious GTTR labeling in outer or inner hair cells (Fig. 1). However, strong punctate GTTR fluorescence was observed in saccular hair cells (Fig. 2). The distribution of GTTR labeling was preferentially localized in the central striola zone and with less intensity in the marginal zone ($P < .05$). Further study revealed that GTTR fluorescence was most intense in at the apical region of the hair cell body (Fig. 2). In Utr, the distribution of GTTR fluorescence was similar to that in the Sac, and GTTR fluorescence was mainly located in the apical region of the hair cell body and hair bundle (Fig. 3). However, the intensity of GTTR fluorescence in the Utr was not as strong as in the Sac ($P < .05$). In the cristae of LSC, SSC, and PSC, punctate GTTR fluorescence was localized mainly in the hair cells of the central cristae; however, GTTR intensities were weaker than in the Utr or Sac (Fig. 4). On the control side (the left ear), there was only weak and uniform fluorescence over the background on all slides.

Quantitative analysis of GTTR intensity in the ES group revealed that saccular hair cells exhibited more GTTR fluorescence than hair cells in the other inner ear organs ($P < .01$). Cochlear outer hair cells (OHCs) displayed negligible GTTR fluorescence (Fig. 5).

GTTR Distribution in the RW Group

When GTTR was applied to the intact RW membrane, there was strong punctate GTTR labeling in cochlear OHCs. The intensity of GTTR fluorescence in OHCs decreased from the basal to apical turn. The punctate GTTR was primarily located in the apical cytoplasm of OHCs. In addition, weak diffuse GTTR fluorescence could be observed in the area of inner hair cells and PCs (Fig. 1).

In vestibular organs, the intensity of GTTR fluorescence in the Sac and Utr hair cells differed. Hair cells in the striola zones displayed greater fluorescence than hair cells in marginal areas in the saccular maculae (Fig. 2). GTTR fluorescence was primarily localized in the apical cytoplasm of these hair cells (Figs. 2 and 3). GTTR fluorescence intensity in hair cells of the three cristae following RW application was similar to that when the drug was applied in the ES (Figs. 4 and 5).

No GTTR fluorescence was found on the control side (left ear) when the drug was applied via the RW of the right ear.

Controls of Unconjugated Texas Red

Animals treated with the same dose of unconjugated Texas Red did not reveal any Texas Red fluorescence within the sensory epithelia of inner ear.

Comparison between the Two Routes

Type III tests of fixed effects showed that the *P* value for interaction between delivery methods and measurement positions was .0005, that is, interaction was significant. To investigate the interaction, further, multiple means comparisons were adopted.

In the RW group, there was no statistical difference between the cochlear and vestibular GTTR ($P = .7967$), whereas it presented significant difference in the ES group ($P < .0001$) with a means difference confidence interval $[-1181.72, -554.61]$.

The confidence interval for means difference of GTTR between the RW method and the ES method in the cochlea was $[1095.59, 1667.88]$, with a corresponding *P* value $< .0001$, showing a huge decrease of GTTR labeling in cochlear hair cells via the ES. Also, the ES route yielded less uptake in the vestibular epithelium than the RW application ($P = .0217$) with a confidence interval $[96.1835, 1106.50]$.

DISCUSSION

Silverstein et al.¹⁰ sent a questionnaire to 700 members of the American Otological Society and the American Neurotology Society to discern trends in surgical procedures used to treat MD during the 1990s, and found that the use of office-administered IT gentamicin therapy increased rapidly through the decade, and by 1999 it had become the most frequently used invasive treatment for MD. This intervention aimed to control vertigo by decreasing vestibular function, while preserving hearing capability. However, current literature revealed that 13.1%–34.7% of these patients suffer from hearing loss after IT gentamicin injection.³ Therefore, although IT gentamicin can provide effective control of vertigo in patients with MD, it was considered suitable only for patients whose hearing has already been substantially affected by the disease, because significant hearing loss may occur immediately after IT application.¹¹

ES surgery is another common procedure for patients with intractable MD due to its low risk of deteriorating auditory function.¹² Despite some early studies suggesting that ES surgery was no more effective than a placebo,¹³ ES surgery is thought to be efficacious for inner ear decompression against endolymphatic hydrops, and several modifications of this surgery have been reported. Gianoli et al.¹⁴ reported 2-year results of their modified ES surgery as follows: complete control of vertigo in 60.0% of patients and significant hearing improvement in 60.0% as well. Ostrowski et al.,¹⁵ however, followed up Gianoli's results for 4 to 5 years, which resulted in 47.0% having complete control of vertigo and 18.0% having significant hearing improvement. Goin et al.¹⁶ suggested that ES surgery did not modify the natural course of MD with respect to hearing, and Sun et al.¹⁷ demonstrated that ES surgery did not confer an increased likelihood of stabilizing or improving hearing compared with medical therapy. Kitahara et al.⁷ examined intra-ES application of steroids as a new therapeutic strategy for intractable MD for a period of 7 years, and reported in 2008 that steroids instilled into ES significantly improved hearing but made no difference in vertigo control, in comparison with ES drainage without steroids and nonsurgery therapy. Thus, ES surgery seems valid to some extent on MD, but still needs further improvement.

Considering both gentamicin's contribution to vertigo control and ES surgery's nondestruction to auditory function, we hypothesized the combination would do better for MD. First, we wanted to establish the distribution of gentamicin in the inner ear after ES

drainage. Therefore, we applied low-dose GTTR (5 μ L, 0.2 mg/mL) into the ES in guinea pigs to explore the distribution of GTTR in the inner ear and to evaluate its uptake patterns within the vestibular and cochlear epithelia. GTTR was previously shown to be a valid fluorescent probe to investigate the pharmacokinetics and mechanisms of gentamicin uptake.¹⁸ This concentration of GTTR is nontoxic in the inner ear; thus, the full complement of hair cells could be retained. Furthermore, 0.2 mg/mL GTTR allowed us to track GTTR (as a tracer of gentamicin) via the ES or RW route under confocal microscopy using the same parameters. In a previous study, we reported that GTTR reached peak level of intensity in the inner ear 3 days after local injection.¹⁹ Therefore, we chose the third day to collect specimens after drug administration. However, to determine the time series of different endpoints of drug distribution after the ES drainage still requires further investigation.

In the present study, a mixed model with unstructured covariance was introduced for statistical analysis because the data of cochlear and vestibular organs (organ of Corti, Sac, Utr, and semicircular canals) were not independent with each other. They were from the same animal; thus, an analysis of variance (ANOVA) could not be adopted. The interaction was significant ($P = .0005$), so multiple means comparisons was used further. Vestibular epithelia in the ES group possessed greater intensities of GTTR fluorescence than the cochlea ($P < .0001$), whereas the RW group exhibited no difference between the two parts ($P = .7967$). The statistical analysis also revealed that the ES route led to a huge decrease in GTTR uptake in the cochlear hair cells, versus the RW method ($P < .0001$). The ES group also displayed a small decline in GTTR uptake in vestibular epithelia compared to the RW group ($P = .0217$). Further investigations are necessary to establish the concentration of gentamicin to attain the effective dose for control of MD.

Animals receiving GTTR via the RW displayed punctate GTTR fluorescence in cochlear OHCs. Because the method relied on the passage of drugs through the RW,²⁰ gentamicin would be greatest in the base of the cochlea, and is then distributed throughout the entire cochlea by “interscalar communication,”²¹ and decreased as it diffused into the vestibular system. Owing to the concentration gradients, damage in basal turn OHCs and hearing loss in high frequency regions invariably occurred before the vestibular hair cells were involved.

When GTTR is administered to the endolymph of the ES, it would diffuse toward the vestibule along the endolymphatic duct, which subsequently divided into two aqueducts connecting Sac and Utr, respectively. Thus, GTTR might be taken up first by cells in Sac or Utr. However, the Sac's GTTR intensity was much higher than the Utr, which was consistent with the result of Lee and Kimura's⁵ study that the Sac was the most injured when gentamicin was applied via ES. We extrapolated that it might be because of Bast's valve (also called the utriculoendolymphatic valve) regulating endolymph flow between Utr and endolymphatic duct. Schuknecht and Belal²² reported that the Bast's valve was always closed in the normal inner ear based on 170 human temporal bones dissection. Their studies showed that the Bast's valve is ideally suited to preserve the humoral and anatomical features of the pars superior (utricle and canals) from disease, and traumatic susceptibilities of the pars inferior (cochlear duct and saccule). Thus, Bast's valve might partially prevent GTTR in the endolymphatic duct entering the Utr. Alternatively, GTTR in endolymph might diffuse into perilymph, prior to tracking into Utr and semicircular canals. Although there was an endolymphatic sinus between the Sac and endolymphatic duct, this is not occluded when perilymphatic pressure is normal.²³

Our results showed that after ES application there was strong GTTR labeling in Sac and other vestibular epithelia, but negligible GTTR fluorescence in cochlear hair cells. One of the possible explanations is that the Sac is the closest sensory organ to the ES, and drugs diffusing from the ES would be absorbed by hair cells in the Sac first before entering the

cochlea through the ductus reunions. In addition, gentamicin is thought to have relatively greater vestibulotoxicity compared to cochleotoxicity, and a strong correlation between the intensity of gentamicin uptake and the cellular degeneration level was reported.²⁴ As to the GTTR fluorescence in the area of PCs (or the Corti's tunnel) following ES administration, we speculate that GTTR entered the spiral vessels which course through Corti's tunnel. During ES surgery, GTTR could diffuse into the sigmoid sinus because of the connection between ES and sigmoid sinus.

CONCLUSIONS

This study quantitatively examined gentamicin distribution in the cochlear and vestibular organs via the ES or RW, providing strong evidence that the drug exhibited much less accumulation in cochlea than in vestibular epithelium after ES delivery, whereas in the RW route it presented no statistical difference between the the cochlea and vestibule. These data suggested that ES drainage with gentamicin administration may be a preferable delivery route when retention of hearing sensitivity is important.

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Fig. 1.

Negligible GTTR fluorescence was detected in cochlear hair cells in the basal or middle turns by the ES route, although the pillar cells (PCs) displayed some punctate GTTR labeling. Strong punctate GTTR labeling was present in cochlear outer hair cells (OHCs) of the basal and middle turns in the RW group, together with dispersed punctate labeling in pillar cells. The punctate GTTR labeling was primarily located in the apical cytoplasm of OHCs (white lines).

Fig. 2.

Strong punctate GTTR fluorescence was observed in the Sac in both the ES and RW group. The panels in the right column for each group are magnification of the boxed area in the adjacent left column. The magnified panels reveal that GTTR fluorescence was primarily located in the apical region of the hair cell bodies (white arrows).

Fig. 3.

Strong punctate GTTR fluorescence was observed within the Utr in both the ES and RW groups. The right panels in each group were the magnifications of the boxed area in the left panels. The magnified panels reveal that GTTR fluorescence was mainly located in the apical region of the hair cell body (white arrows).

Fig. 4. shows the cristae of the posterior semicircular canal. GTTR fluorescence in the hair cells of the lateral, superior, and posterior cristae were similar in both the ES and RW groups. The punctate GTTR fluorescence was largely distributed in the cell bodies of hair cells of the central cristae.

Fig. 5.

Shows the average intensity of GTTR labeling in each inner ear organ following the ES or RW drug application. 1st, 2nd, and 3rd represent the basal, middle, and apical turn of the Corti, respectively. In the RW group, there was no statistical difference between the cochlear and vestibular GTTR intensities ($P = .7967$); however, there was significant difference in cochlear and vestibular GTTR intensities in the ES group ($P < .0001$). The confidence interval for means difference of GTTR between the RW method and the ES method in the cochlea was [1095.59, 1667.88], with a corresponding $P < .0001$, showing a huge decrease of GTTR fluorescence in cochlear hair cells via the ES route. Also, the ES route produced less uptake in the vestibular epithelium than the RW application ($P = .0217$) with a confidence interval [96.1835, 1106.50].