Semiquantitative Vestibulotoxicity to Subablative and Ablative Treated Gerbil Cristae Using Gentamicin or Streptomycin

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Semiquantitative histological evaluation was performed on inner ears from Mongolian gerbils to study vestibular damages using ototoxic drugs. Comparisons were made between animals receiving five daily injections of gentamicin/gelfoam slurry, streptomycin/gelfoam slurry, saline/gelfoam slurry and noninjected controls. For gentamicin treated ears, when percent decrease of nuclei in sensory cell layer was compared, the damage to the sensory cells was significantly more severe in the posterior crista than in the superior and lateral crista from two of three gerbils. For streptomycin treated ears, when percent decrease of nuclei in sensory cell layer was compared, the damage to the posterior crista was similar to superior and lateral crista. These results indicate that our models are useful for detecting different vestibulotoxicity among three cristae.

Key words: vestibule, ototoxicity, crista, gentamicin, streptomycin

I. Introduction

Aminoglycoside antibiotics have been in clinical use for more than 50 years. They are still widely used because of their high efficacy and low cost. Shortly after their discovery the ototoxic potential was identified. Fowler [4] reported streptomycin (SM) treatment of vertigo in 1948. Early attempts to make use of these effects to treat inner ear disorders such as Meniere’s disease were made. Schuknecht [18] improved protocols to administer SM both systemically and transtympanically. To prevent both bilateral and complete vestibular ablation, the subablative titration administration was invented. Recently in unilateral Meniere’s disease local application of gentamicin (GM), such as intratympanic injection [8, 13, 25] and round window membrane application [19], was identified as a potentially beneficial and less invasive treatment. Although many treatment protocols have been reported, efficacy has been identical, and no single technique of GM injection has demonstrated a significant medical advantage over the others [2]. Our understanding in this regard, especially histological evaluation following aminoglycoside administration has largely been limited to the animal models to clarify the dose-related hair cell loss. Although many reports on the effects of aminoglycosides on the vestibular system have been published, few authors have focused on the relative sensitivity between the three semicircular canal cristae. Some have found the posterior canal crista to be more sensitive than the lateral and superior following administration of SM in pigmented guinea pigs [17]. Others showed no difference among the three cristae in guinea pig [9] and in human [21]. The results obtained so far are controversial. This discrepancy may be partly due to different species, drugs, treatments and histological evaluations. Our previous study demonstrated dose-related vestibular posterior crista ototoxicity using GM and SM in the Mongolian gerbil [16, 22, 23]. We characterized linear density throughout normal and drug damaged posterior cristae. We showed a decrease in linear density of nuclei in the sensory cell layer following transtympanic treatment with ototoxic drug. To determine if a differential sensitivity among vestibular endorgans is present in the ears treated with transtympanically injected GM or SM, we additionally performed quantitative evaluation of the superior and lateral cristae, using the same protocol as in our previous study. Percent decreases in sensory, supporting and total cell among three cristae were compared between subablative and ablative treated gerbils in order to examine whether the degree of hair cell loss changes differential sensitivity among vestibular endorgans in the same animal.
II. Materials and Methods

Mongolian gerbils (31-day-old, weighing more than 30 g) were divided into four groups of three animals each. After anesthesia with a mixture of 50 mg/kg ketamine and 8 mg/kg xylazine injected subcutaneously, a single injection of 30 μl of a 50 mg/ml solution of GM with gelfoam powder was performed into the right ears each day for five days in the 5×GM group as a subablative model (n=3). For 5×SM group as an ablative model (n=3), 350 mg of SM was dissolved in 1 ml sterile saline mixed with gelfoam powder and 30 μl was injected once daily for five days into the right ears. Total dosages received by each animal were 7.5 mg of GM and 52.5 mg of SM. For animals in the control groups, either sterile saline was mixed with gelfoam and injected five daily into the right ears, or animals were age matched uninjected controls. Concentration and dosage for each drug were selected based on preliminary studies to determine a protocol that would reliably damage the vestibular system.

The gerbils underwent carbon dioxide inhalation and then were decapitated 2 weeks after the last injection. Each bulla was removed and opened to access the cochlea and vestibular system. Small holes were made in the apex, base, and round window of the cochlea and in each semicircular canal. A fixative solution (3% freshly made paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2) was gently perfused through the holes. The organs were left in the fixative overnight on a rotator. A 5% EDTA solution in phosphate buffer was used to decalcify the bone over 1 week. The cochlea was separated from the vestibular system, and each vestibular endorgan was dissected free from the surrounding bone and fibrous tissues. All specimens were dehydrated in increasing concentrations of ethanol, transferred to propylene oxide, infiltrated and embedded in epoxy resin, and oriented and hardened in a 60°C oven for 1 week.

Each right semicircular canal crista was cut into 1-μm serial cross sections. Every 6th and 7th sections throughout the crista were placed on Fisherbrand Superfrost/Plus microscope slides, stained with methylene blue azure II and coverslipped. The length of each crista was determined based on the total number of sections obtained from each specimen.

Microscopic images of sections to be analyzed from specific points within the crista were captured and analyzed using NIH Image software.

Quantification methods

To quantify the drug induced damages to semicircular canals cristae, a decrease in cell nuclei/μm of sensory epithelium was assumed to reflect cell loss. The total number of nuclei and the number of sensory cell nuclei were counted and the number/μm epithelium length was calculated at eight specific locations along the length of the crista as previously described [16, 22, 23]. The epithelium length along the basement membrane was measured in each reference point from the beginning of sensory cell layer in the lateral of cristae to the end of it.

A method was designed to determine objectively the location of the sections from each crista to be sampled. This was complicated not only because of the different lengths but also because of the presence of the septum cruciatum, an area in the superior and posterior semicircular canals naturally devoid of sensory cells (Fig. 1A, B). Because the septum cruciatum in the gerbil is off-center, graphs plotted according to length of the crista were difficult to interpret. Therefore, we normalized our data to this region. The midpoint of septum cruciatum was set as the zero point; sections were then analyzed at positions corresponding to 10% increments (relative to the entire length of the crista) on either side of the septum cruciatum (Fig. 2, 1st method).

The septum cruciatum is not present in the lateral semicircular canal (Fig. 1C). Therefore, the data was normalized among the three semicircular canals cristae. The septum cruciatum comprised 63% of the total length of epithelium in the uninjected control gerbils. The total length of remaining epithelium in the crista lacking in the septum cruciatum was divided into 10% increments. The beginning of crista was set as the zero point; sections were then analyzed at positions corresponding to 10% increments (relative to the entire length of the crista) from the beginning to the end of crista (Fig. 2, 2nd method). Sensory and supporting cells were identified based on the morphologic structure of the cells (when visible) and the location of nuclei within the epithelium. Sensory cell nuclei were located within the apical half

Table 1. Outline of methods

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Description</th>
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<tr>
<td>Five daily transtympanic injection of gentamicin, streptomycin or saline with gelfoam.</td>
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<td>Sacrificed two weeks after the last injection and tissue preparation of cristae.</td>
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<td>Fixation, dehydration and embedding in epoxy resin.</td>
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<td>One micron serial cross sections and saved every sixth and seventh sections.</td>
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<tr>
<td>Stained with methylene blue azure II.</td>
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<td>Captured and analyzed using NIH imaging.</td>
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<td>Sampled the specific reference points along each crista.</td>
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<td>Used 1st method for posterior or superior crista and 2nd method for lateral crista.</td>
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<td>Counted the number of sensory and supporting cell nuclei.</td>
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<tr>
<td>Measured length of sensory epithelium at the basal lamina.</td>
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<td>Calculated linear density of sensory, supporting and total cell.</td>
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<td>Averaged and compared percent decreases of linear density with each crista.</td>
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of the sensory epithelium, and supporting cell nuclei formed a layer close to the basal lamina. A nucleus was counted if it had a diameter >4.5 μm, a visible nucleolus, round or elliptical shape, and smooth and continuous edges. At each of the eight locations along the length of crista, the results of cell nuclei counts from two sections six microns apart were averaged. The number of sensory cell nuclei/μm the basal epithelial length (linear density of sensory cells) and total cell/nuclei/μm the basal epithelial length (linear density of total cells) versus the reference points along the length of the crista were plotted for comparison purposes. Linear densities were averaged along the length of crista in each semicircular canal crista from the same animal. Obtained data from treated subjects were compared to average saline vehicle control.

To allow for relative differences in linear density found in control posterior crista compared to superior and lateral cristae, we used percent decrease to normalize and compare the data. This ratio was calculated as followed: decreasing percentage of linear density of sensory cells = (average cell density in treated crista - average cell density in control crista) / average cell density in control crista × 100%.
number of linear density of sensory cells in experimental
animal – average number of linear density of sensory cells
in vehicle control)/average number of linear density of sen-
sory cells in vehicle control. Data from each group were
subjected to analysis of variance and Student t-test.

III. Results

Histological analysis of each semicircular canal crista
from the three gerbils treated with 5×saline/gelfoam (Fig.
3A–C) showed uniformly stained cytoplasm and sensory
and supporting cells aligned in separate rows. The sensory
cell nuclei were larger and rounder than those of the support-
ing cells. The supporting cell nuclei rested in a relatively
orderly layer along the basement membrane. The calyxal
nerve terminals around the type I hair cells were seen even at
the apex as large, light areas enclosing all of the cell except
the apex in a cup-like fashion. Each of the posterior and
superior cristae had an eccentrically located septum cruciat-
tum devoid of sensory epithelial cells as Hunter-Duvar [7]
reported in rats. In the lateral cristae no septum cruciatum
was seen. Each cross section through the crista was divided
into three equal portions along the sensory epithelium.
Histological analysis was made in the apical or lateral por-
tion (Fig. 3A).

Histological analysis of posterior semicircular canal
crista from the 5×GM gerbils (Fig. 4A–C) revealed apparent
damage to the sensory cell layer. The decrease in hair cell

![Fig. 3. Light microscopic pictures of 1 μm cross sections at individual crista from the same animal treated with five daily transtympanic injec-
tions of saline/gelfoam for a vehicle control. No damage to the vestibular endorgans two weeks after injections is seen. In a section taken from
the −20% location along the length of crista, the nuclei of the sensory cells (arrow) are found near the surface region in the bilayer of the epithe-
lium, and supporting cell nuclei (asterisk) are lined up along the basal lamina. A; posterior crista, B; superior crista, C; lateral crista, Bars= 50 μm.]

![Fig. 4. Light microscopic pictures of 1 μm cross sections at individual crista from the same animal treated with five daily transtympanic injec-
tions of gentamicin/gelfoam. In 1 μm sections taken at −20% location from septum cruciatum in posterior crista at 2 weeks after five transtym-
panic injections of GM/gelfoam, the numbers of sensory cell (arrow) are decreasing more obvious in the apical region than lateral side of crista.
The supporting cell nuclei (asterisk) are stained normally but arranged staggered and looked smaller than that from control animal. In superior
and lateral crista from the same animal, sensory cell nuclei in the same reference points of posterior crista are damaged to the same extent of that
from the posterior crista. A; posterior crista, B; superior crista, C; lateral crista.]
nuclei was more obvious in the apical region than in the lateral regions of the crista. This caused disruptions of the orderly two-layer arrangement seen in sections from the control ears. The height of the epithelial layer was slightly thinner, especially in the apical regions, compared to the control animals. The supporting cell nuclei were stained normally but the arrangement of nuclei was staggered in the controls. In superior and lateral cristae from the same animal, the sensory cell nuclei in the same reference points were damaged to almost the same extent as in the corresponding areas from the posterior crista.

Histological analysis of posterior semicircular canal crista from the $5\times$SM (Fig. 5A–C) revealed the sensory cell layer to be the most damaged to $5\times$GM and $5\times$saline groups. No hair cell nuclei were found in the apical region, and the lateral regions had only a few degenerated hair cells. The height of the epithelial layer at the apex was obviously thinner than in the control animals. Some supporting cell nuclei

![Light microscopic pictures of 1 μm cross sections at individual crista from the same animal treated with five daily transtympanic injections of SM/gelfoam.](image)

**Fig. 5.** Light microscopic pictures of 1 μm cross sections at individual crista from the same animal treated with five daily transtympanic injections of SM/gelfoam. In a section taken from ~20% location along the length of crista of posterior semicircular canal ampulla, no hair cell is found in the apical region and lateral side of crista has only a few degenerated hair cells (arrow). Some supporting cell nuclei (asterisk) are stained densely and pyknotic. In superior and lateral cristae from the same animal, sensory cell nuclei in the same reference points of posterior crista are damaged to the same extent of that from the posterior crista. A: posterior crista, B: superior crista, C: lateral crista.

![Graph showing cell nuclei density](image)

**Fig. 6.** Linear density (cell number of vestibular sensory, supporting and total cell per μm length of sensory epithelium, measured at the basilar membrane) from five daily GM treated gerbils ($n=3$) and average number of control vehicle ($n=3$) in the five selected locations (~30%, ~20%, ~10%, 10% and 20% relative distance from the septum cruciatum in posterior and superior crista and 30%, 40%, 50%, 60%, and 70% locations of the distance from the beginning of lateral crista). Sensory cell linear density of the posterior crista remains lower than superior and lateral crista from the same animal in two of the three $5\times$GM gerbils. Pos., posterior crista; Lat., lateral crista; Sup., superior crista.
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were stained densely. In superior and lateral cristae from the same animal, sensory cell nuclei in the same reference points were damaged to the same extent as those from the posterior crista.

Quantification of cells in serial sections taken at five selected locations (−30%, −20%, −10%, 10% and 20% relative distance from the septum cruciatum in posterior and superior crista and 30%, 40%, 50%, 60%, and 70% locations of the distance from the beginning of lateral crista). The damage to the sensory cells is significantly more severe in the posterior crista than in the superior and lateral cristae from two of three gerbils. * p<0.05, ** p<0.05. Pos., posterior crista; Lat., lateral crista; Sup., superior crista.

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**Fig. 7.** Average percent decrease of sensory, supporting and total cell linear density from five daily GM treated gerbils in five selected locations (−30%, −20%, −10%, 10% and 20% relative distance from the septum cruciatum in posterior and superior crista and 30%, 40%, 50%, 60%, and 70% locations of the distance from the beginning of lateral crista). The damage to the sensory cells is significantly more severe in the posterior crista than in the superior and lateral cristae from two of three gerbils. * p<0.05, ** p<0.05. Pos., posterior crista; Lat., lateral crista; Sup., superior crista.

**Fig. 8.** Linear density (cell number of vestibular sensory, supporting and total cell per μm length of sensory epithelium, measured at the basilar membrane) from five daily SM treated gerbils (n=3) and average number of control vehicle (n=3) in the five selected locations (−30%, −20%, −10%, 10% and 20% relative distance from the septum cruciatum in posterior and superior crista and 30%, 40%, 50%, 60%, and 70% locations of the distance from the beginning of lateral crista). Sensory cell linear density of the lateral crista obtained from two gerbils (Gerbil #7 and #8) remains lower than posterior and superior cristae. Pos., posterior crista; Lat., Lateral crista; Sup., superior crista.
tion from each crista) was performed. For subablative treated specimens (Fig. 6), linear density of sensory cells from the three cristae showed consistently lower than average sensory cells linear density compared to transtympanic saline injected controls. Sensory cell linear density of the posterior crista remained lower than superior and lateral crista from the same animal in two of the three 5×GM gerbils. No significant difference was observed among three cristae. Analysis of the linear density of the total cells revealed that all organs in the three gerbils treated with GM except for one superior crista that showed lower density than that from the average of three vehicle control gerbils.

To allow for relative differences in linear density found in the average control posterior crista compared to superior and lateral crista, we used percent decrease in each selected location to normalize and compare the data (Fig. 7). Comparison of the average percent decrease in five selected locations (–30%, –20%, –10%, 10% and 20% relative distance from the septum cruciatum in posterior and superior crista and 30%, 40%, 50%, 60%, and 70% locations of the distance from the beginning of lateral crista). The damage to the posterior crista is similar to superior and lateral crista.

IV. Discussion

Intratympanic gentamicin injection therapy for intractable Meniere’s disease has been used clinically in Europe and the United States to achieve pharmacologic ablation of the vestibular system. The disadvantages of using ototoxic drugs to the inner ear have been a cause of concern since treatment can lead to problems such as sensory neural hearing impairment, and no single treatment protocol has been reported to be unilaterally acceptable because clinical effects varied among patients. Reducing cochlear damage and uniform vestibulotoxicity are important to improve this treatment paradigm.

Wanamaker et al. [22, 23] suggested that, in the gerbil,
tr transtympanic gentamicin or streptomycin is ototoxic but not selectively vestibulotoxic. Polger et al. [16] reported that linear density analysis of sensory epithelium had advantages to avoid trial errors in relation to histological processing such as fixation and dehydration in tissue preparation. In this study we evaluated vestibulotoxicity between three cristae on the assumption that intratympanic gentamicin injection achieved unequal effects to the three cristae in the same animal. In subablative animal model we have proved that the percent decrease of hair cells in the posterior crista was much greater than that of the lateral and superior crista. This result illustrated the inconsistency in the therapeutic effects of intratympanic gentamicin treatment.

A potential weakness of this study is using two drugs to obtain different vestibulotoxic animal models. Our treatment paradigm overcomes the difficulty in producing ablative and subablative animal models. Results obtained in this study indicate that these two models offer promise as a suitable method for examining the degree of hair cell loss in vestibular endorgans.

Many animal studies referring to ototoxicities to aminoglycosides have been reported. Berg [1] reviewed early investigations on aminoglycoside otoxicities from 1946 and examined vestibular functions and histological changes to systemic SM injections in cats. Histological comparisons between kanamycin and SM [10], type I and type II sensory cells [24], cochlea and vestibule endorgans [3, 20, 22, 23] are considered to have different susceptibilities to aminoglycosides, regional vulnerability to sensory cells in a vestibular endorgan but no selective ototoxicity in an ear. Otherwise few authors have focused on the relative sensitivity among the three semicircular canal cristae between ablative and subablative toxic animal models. In 1961, McGee [12] reported quantitative graphic representations of vestibular labyrinth pathology in cats following intramuscularly injection of different dosages of SM. Although they did not figure out methods of quantitative cell counting, they estimated that the hair cell loss among three cristae was almost the same in severely damaged animal and in medium damaged animal. Further notable work by Lindeman [10] demonstrated that hair cell damage occurred with surface preparation technique following transtympanic treatment with large doses of SM in guinea pigs, and observed that all three cristae showed almost the same extent and pattern of degeneration. However, Lindeman did not estimate the difference in sensitivity of the vestibular sensory epithilia to smaller doses of ototoxic antibiotics. Rudnick et al. [17] proposed percent damages per mg dosage of SM to sensory cells of each crista following middle ear injection. A comparison of the relative toxicities with SM between three cristae was done. The posterior cristae ampullares appeared to be more sensitive to SM than the superior cristae ampullares, with the lateral cristae ampullares the least affected of the three. The present results agree with those reported by Rudnick et al. [17]. In fact their mean percent damage at maximum dosage was 50% in cristae. Recently, Chen et al. [3] and Mount et al. [14] reported that the posterior canal crista was affected more than the other two cristae following middle ear instillation of GM, SM and carboplatin in chinchillas using scanning electron microscopy. These reports lead us to surmise that the different vulnerabilities between the three cristae do not depend on the drugs, treatments, species, survival time or the method of histological estimation but depends on the extent the vestibular sensory epithelia are damaged. The present results give rise to the view that the degree of hair cell loss changes sensitivity among vestibular endorgans using ablative and subablative animal models.

No comprehensive explanations could account for this different vulnerability between the three cristae. Since the gerbil posterior crista has a septum cructium devoid of sensory cells, the sensory cell linear densities eliminating this region in posterior cristae are greater than the sensory cell linear densities in lateral crista. These findings suggest that this higher linear density in posterior crista sensory cells might cause the different tolerance to ototoxic drugs. Olds and Lyon [15] concluded that the local metabolic rate of glucose utilization for the rat vestibular endorgans is similar with the utricle and saccule and significantly higher than that of for the superior, posterior, or lateral canal ampullae. Lyon and Jensen [11] asserted that blood flow to all of the vestibular endorgans from rats is similar. They did not find significantly different metabolism and blood flow between three semicircular canal ampullae in the rat. It is speculated that the posterior crista having a higher sensory linear density could not maintain homeostasis and tolerate the stress caused by ototoxic drugs.

Variable interanimal sensitivities to aminoglycoside were also observed in our study. Although the transtympanic route has been shown to be effective in inducing ototoxic damage in many species, there are several limitations in transtympanic injections. We had difficulty in proving how much of the drug was absorbed from the round window membrane following transtympanic administration [6]. All drugs might not exist on the round window membrane during treatment periods because the swallowing or body movements of animals may clear away some of the drug from the eustachian tube. Therefore, we administered the drug consecutively for five days and used slurry to prevent the drug from draining out. Obstruction of the surface area of the round window membrane cannot be detected without perforation of the tympanic membrane. In humans, Silverstein et al. [19] noted that the round window niche was completed obstructed by mucous membranes in 12% and partially obstructed in 17%, which would allow for varying absorption rates, although others have not found such rates of obstruction. Variable thickness of round window membrane might also exist and this might change the penetration volume of the drug into the perilymph. Direct round window membrane application of GM application might not eliminate some of the inconsistent results associated with the transtympanic injections. Nor can different absorption routes such as ovarian window or micro fissures between round window niche and posterior crista be ruled out entirely [5].

In conclusion, this study provides important informa-
tion on the different vestibulotoxicity among three cristae in the subablative damaged animals. To answer how this different vulnerability in three semicircular canal cristae affects equilibrium function, attempts to perform vestibular testing have been unsuccessful because of technical difficulties inherent in recording nystagmus in such a small animal that has a completely dark sclera. Research to test the function of individual semicircular canal is proceeding in human subjects. This animal study attempts to establish the fundamental histological evidence. Further study is necessary to investigate optimal inner ear drug application, such as direct inner ear perfusion with cochleostomy via a mini-osmotic pump. We have achieved in establishing a subablative vestibular damage animal model with this method and studies are in progress to clarify time-dependent hair cell loss among three cristae in the same animal. This drug delivery paradigm would not only reduce inter-animal validity but also contribute to solving the problem of how to apply concentrated therapeutic agent to prevent hair cell damage. The present study takes into consideration basic issues of sensory cell kinetics in vestibular endorgans in order to differentiate between subablative and ablative treatment for intractable Meniere’s disease.

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VI. References