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What is This?
Kanamycin-Furosemide Ototoxicity in the Mouse Cochlea: A 3-Dimensional Analysis

Heather M. Schmitz¹, Shane B. Johnson¹, and Peter A. Santi, PhD¹

Abstract
Objective. Administration of an aminoglycoside antibiotic and loop diuretic causes damage to hair cells in the organ of Corti, resulting in their death and the death of their corresponding spiral ganglion neurons. While this phenomenon has been studied previously, analysis of its effects in the whole cochlea has not been reported. The authors sought to evaluate the effects of a combination dose of kanamycin and furosemide in mice cochlea using an imaging system and computer analysis that allowed for nondestructive, whole-cochlea visualization.

Study Design. Study using an animal model.

Setting. Cochlear analysis laboratory.

Subjects and Methods. Five mice received kanamycin and furosemide and 3 mice received saline. Cochleas were harvested and imaged with scanning thin-sheet laser imaging microscopy (sTSLIM) to analyze sensory cells and cochlea structures.

Results. The drug-treated animals showed substantial loss of inner hair cells and complete outer hair cell loss. All treated mice showed spiral ganglion neuron loss with fewer neurons than control animals and decreased cell density in the middle turn of the cochlea. The spiral ligament and spiral limbus in the treated animals also showed a decrease in fibrocyte cell density in the middle to apical portion of the cochlea. The stria vascularis appeared normal in all animals.

Conclusion. Imaging methods that allow for whole-cochlea analysis provide insight into changes that occur in the cochlea after ototoxic insult. Trends that may not be apparent in cross-section samples of the cochlea can be observed. Computer analysis of these trends allows them to be assessed accurately.

Keywords
kanamycin, furosemide, hair cell, organ of Corti, spiral ganglion neuron, spiral limbus, spiral ligament, cell count, sTSLIM, TSLIM

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Complex 3-dimensional (3D) tissues like the cochlea can be best analyzed and understood when methods used to image the cochlea preserve their geometry and allow for 3D analysis.¹ Many of the structures of the mouse cochlea change significantly from base to apex, often with a direct correlation to the structure’s function.²⁻³ One such example is the width of the basilar membrane, which is narrow and relatively thick in the base but gradually becomes wider and thinner toward the apex, which relates to the characteristic frequency (CF) at which it vibrates.² Therefore, imaging methods such as orthogonal-plane fluorescence optical sectioning (OPFOS) and scanning thin-sheet laser imaging microscopy (sTSLIM) are good tools for cochlear analysis.⁴⁻⁵ sTSLIM, when combined with 3D reconstruction software such as Amira (Visage Imaging, San Diego, California), allows for high-resolution qualitative and quantitative assessment of cochlear structures in 3D space.⁶⁻⁷ Individual structures can be isolated by segmentation for their visualization, and when combined with the proper staining techniques, the resolution is adequate for quantitative assessment of cells. Counting cells (especially inner hair cells [IHCs] of the organ of Corti) in 3D space using a computer has a significant advantage over counting cells in cross sections; the cells are easier to visualize and the cell counts are more accurate. This is because specialized software can be used to keep track of cell number and location, and there is no tissue loss since the complete cochlea is digitized and not mechanically sectioned.

The ototoxicity of aminoglycoside antibiotics has been well established.⁸⁻¹⁰ Repeated injections of the aminoglycoside antibiotic kanamycin result primarily in outer hair cell (OHC) loss in the organ of Corti.⁸¹¹ Furosemide and other loop diuretics have well-known synergistic effects with aminoglycoside antibiotics when the 2 drugs are administered closely in time and cause profound hearing loss.⁸¹²⁻¹³ Several mechanisms have been proposed for this synergistic effect, including an alteration to phosphoinositides in the hair cell membrane¹⁴ and other mechanisms described in a
review by Hirose and Sato.11 With a single, combined dose of each drug, complete OHC loss with IHC damage has been observed.12,15 Because mammalian hair cells cannot regenerate, loss of sensory hair cells results in irreversible deafness.10 Furthermore, loss of hair cells appears to be followed by spiral ganglion neuron (SGN) death, presumably because SGNs rely on the afferent input from IHCs.16,17 Deafferented SGNs eventually lose the distal processes that project to the organ of Corti and then degenerate.17,18 Given the intricate 3D shape of the cochlea, analysis of the IHCs and SGNs in 3D space could help elucidate this process.

This study seeks to establish a novel method of counting IHCs by using sTSLIM and to better ascertain the relationship of IHC loss to SGN loss. This was accomplished by counting IHCs and SGNs and using computer analysis to make correlations between the remaining IHCs, SGNs, and their positions in 3D space. It also examines spiral ligament, spiral limbus, and stria vascularis for changes as a result of the drug insult to better quantify and analyze damage throughout the cochlea.

Materials and Methods

All animal maintenance, care, and use were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Drug Administration

Five 8-week-old female CBA/JCr mice were injected subcutaneously with kanamycin (1 mg/g of body weight; Sigma-Aldrich, St Louis, Missouri), dissolved in phosphate-buffered saline (PBS), followed by an intraperitoneal injection of furosemide (0.4 mg/g of body weight) 30 minutes later. Due to an injection error, 1 mouse (Treated5) was not given the complete dose of kanamycin. It was estimated that one-third of the dose was lost. Three 8-week-old female CBA/JCr mice were given a subcutaneous injection of saline followed by an intraperitoneal injection of saline 30 minutes later to serve as control animals. All animals survived the drug administration.

Tissue Preparation

After 28 days, the mice were anesthetized with an overdose of ketamine/xylazine (0.350 mg/g of body weight) followed by cardiac vascular perfusion with 2 mL of 4% paraformaldehyde (PFA). The cochleas were harvested from the temporal bone and immersed in PFA overnight at 4°C. After 3 washes with PBS, cochleas were decalcified by immersion in 10% ethylenediaminetetraacetic acid (EDTA) for 4 days with daily changes of the solution. Following decalcification and washing with PBS, the cochleas were dehydrated in an ascending ethanol series (50%, 75%, and 100%). Whole cochleas were immunostained following the protocol in MacDonald and Rubel19 with rabbit anti–myosin VIIa antibody (Abcam, Cambridge, UK) and goat anti–rabbit Alexa Fluor 532 antibody (Invitrogen, Carlsbad, California). The cochleas were cleared to transparency with Spalteholz solution (5:3 methyl salicylate/benzyl benzoate) and glued to a plastic rod for sTSLIM imaging. After the myosin VIIa–stained cochleas were imaged, they were transferred back to 100% ethanol, restained with Rhodamine B isothiocyanate in 100% ethanol, cleared in Spalteholz solution, and imaged with sTSLIM to visualize spiral ganglion neurons.

Imaging

Cochleas were imaged with sTLISIM, which uses a scanned laser light-sheet to produce a fluorescent plane in the cleared tissue. This plane was then collected as an image by a Retiga camera (QImaging, Surrey, Canada) mounted to an Olympus MVX10 microscope (Olympus America, Center Valley, Pennsylvania). High-resolution micropositioners translated the specimen through the illumination plane to collect a serial z-stack of optical sections through the entire cochlea, and a custom LabVIEW program (National Instruments, Austin, Texas) controlled every step of the stack collection process. A complete z-stack of optical sections containing the entire inner ear was obtained in approximately 75 minutes and resulted in ~300 well-registered images for each cochlea. Two stacks of images were obtained for each cochlea, one after myosin VIIa staining and another after Rhodamine B isothiocyanate staining. Image voxel size was 1.5 × 1.5 × 5 μm. Images were adjusted for brightness and contrast and saved as .tif files. To map damage along the length of the basilar membrane, a B-spline curve was fit to control points and manually placed from the base to the apex along the center of the basilar membrane. When damage in the spiral ligament and limbus was observed in orthogonal cross sections, its beginning and end were marked so that its extent and location could be mapped in 3D space.

Cell Counting and Data Collection

The sTSLIM z-stacks of myosin VIIa–stained cochleas were loaded into Amira (version 5.2; Visage Imaging), and the organ of Corti was segmented by outlining its borders in each section. Segmentation was semi-automated through Amira by manually segmenting every other section and then automatically segmenting the skipped section with linear interpolation. The organ of Corti was defined as a region above the basilar membrane that included the IHCs, inner supporting cells, cuticular plate, tunnel of Corti, pillar cells, OHCs, and Deiters cells. Even though the IHCs and OHCs were missing in the treated specimens, supporting cells of the organ of Corti remained, and it was segmented using the previous criteria. The reliability of segmentation was determined by having 2 observers segment the same cross section of the cochlea in 5 different animals. The results of their segmentations showed a strong correlation (r = 0.999) in the areas of the segmented structures. The observers also counted SGNs in each cross section of Rosenthal’s canal with a strong correlation (r = 0.995) between their results.

A 3D volume rendering of the organ of Corti was created from the segmentation using the Arithmetic module tool in Amira and a simple masking operation. This allowed for visualization of only the organ of Corti and its immunostained hair cells in 3D space. A 3D isosurface volume
rendering was generated from the segmentation label fields to provide both an estimation of the volume of the organ of Corti based on voxel size and a 3D structure upon which to place landmarks. The isosurface volume rendering was made translucent so the volume-rendered organ of Corti could be seen and landmarks were placed over every observed IHC.

A smooth B-spline curve, composed of equidistant points representing percent distance along the centroid of the organ of Corti, was produced from the CenterlineTree module in Amira. The percent distance location of each IHC was determined and cell number vs percent distance histograms were created for the entire data set. The sTSLIM z-stack that was collected after Rhodamine B isothiocyanate staining was then loaded into Amira. The organ of Corti, basilar membrane, and Rosenthal’s canal were segmented in these stacks, and B-spline curves were created for each structure to determine their length and to allow for comparison between the myosin VIIa and Rhodamine B isothiocyanate data sets.

Since 10 μm is the approximate diameter of the SGN cell body, SGNs were counted in 10-μm z-step 2-dimensional (2D) cross sections by placing a landmark over each cell. To avoid counting a SGN twice, the 2D sections were continuously shifted in the z-dimension to check for previously marked cells. The percent distance location of each SGN was determined using the B-spline centroid of Rosenthal’s canal, and cell number vs percent distance histograms were created. The percent distance histograms of the IHCs and SGNs were compared with each other. Local SGN density was determined by counting the number of cells within a 100-μm radius sphere around each cell using Amira’s ClusterDensity module.

Results

Despite almost complete loss of IHCs and OHCs, the 2D sTSLIM cross sections of the treated mice cochlea showed that the supporting cells make the organ of Corti still recognizable for segmentation. This is demonstrated in Figure 1, which shows a 2D sTSLIM cross section of the middle of the cochlea in a treated and a control mouse. Treated mouse organs of Corti were mostly unreactive with myosin VIIa antibody (Figure 1A), while control cochleas exhibited strong myosin VIIa staining (Figure 1B). This lack of myosin VIIa staining in the treated mice was better visualized when the segmented organs of Corti were masked out and volume rendered. Figure 2 shows a volume-rendered segment of the organ of Corti from the middle of the cochlea with myosin VIIa staining in both a treated mouse (Figure 2A) and control mouse (Figure 2B). In the treated cochlea, few IHCs are present as evidenced by antibody labeling and no OHCs are present. The control shows intense labeling by the antibody for the IHCs and lighter labeling of the OHCs.

Visualization and assessment of the SGNs required more thorough analysis because the loss of SGNs was not as obvious. Figure 3 shows this analysis. Panels A (treated) and B (control) show SGN manual counting in 2D cross sections. Panels C and D show point cloud 3D representations of all of the marked SGNs in a treated and control cochlea, respectively. Comparing the point clouds, it visually appears the treated cochlea contains fewer SGNs than the control cochlea; however, actual counts and calculations such as the ClusterDensity analysis are necessary to demonstrate this. The IHC and SGN data for every cochlea are shown in Figures 4 to 6. Figure 4 shows 3D volume renderings of the organ of Corti, basilar membrane, and Rosenthal’s canal with their corresponding centroids (a line that follows the
center of each volume-rendered structure and was used to calculate lengths. **Figure 5** shows a 3D reconstruction of the organ of Corti centroid and red spheres as labels for the presence of IHCs along the centroid. The paucity of IHCs in treated cochleas is obvious with the exception of Treated5, which received an incomplete dose of antibiotic and has numerous IHCs.

**Figure 6** shows 3D volume renderings of Rosenthal’s canal where cell density is represented by a color map with blue being low density, yellow medium density, and red high density. There are very few high-density areas in the treated cochleas with the exception of Treated5. Control cochleas all show a region of high SGN density in the middle region of the cochlea, which is not as apparent in the treated cochleas. The histogram plots of the presence of IHCs and SGNs in all of the cochleas are displayed in **Figure 7**.

**Table 1** shows the morphometric measurements from the treated and control cochleas. There was very little difference between the lengths and volumes of the organ of Corti, basilar membrane, and Rosenthal’s canal in the treated and control groups. The difference between the numbers of IHCs, however, was substantial when Treated5 was excluded from the treated average due to the incomplete dose of kanamycin. All OHCs were missing in all treated animals, and only 44 (standard error of the mean, 8) were missing in the control animals. The number of SGNs in the treated cochleas was also greatly decreased compared with the controls.

**Figure 8** shows the pathology of the spiral ligament and limbus (**Figure 8A,B**) in treated animals that was not observed in any of the control animals (**Figure 8C**). Decreased cell density within these 2 structures was observed, mapped according to its extent and location along the basilar membrane in orthogonal cross sections, and correlated across the treated animals (**Figure 8D**). The error bars seen in **Figure 8D** represent the standard error of the pathology start and stop points. Spiral ligament damage occurred in the middle of the cochlea and appeared to be more variable than spiral limbus damage, which was observed in the apical part of the cochlea. The stria vascularis appeared normal in all experimental and control animals.

**Discussion**

Otoxic damage to cochlear structures after administration of an aminoglycoside antibiotic followed by a loop diuretic has been well established, and sTSLIM optical sections have allowed us to investigate this damage in detail. While sTSLIM does an excellent job of imaging whole cochleas, a limitation of this imaging method is the inability to resolve stereocilia reliably. Therefore, only the presence or absence of IHCs can be determined. Perhaps a future study can resolve these issues, and partially damaged IHCs can be accurately assessed as well.

Outer hair cell loss was complete in all treated cochleas, even in the 1 animal that did not receive the full dose of kanamycin. The difference in hair cell susceptibility has been reported previously, but its mechanism has not yet been established. A similar study by Taylor et al20 showed almost complete OHC loss 48 hours postinjection in a basal to apical pattern. Inner hair cell loss did not occur until about 2 to 4 weeks postinjection and appeared almost total along the entire length of the organ of Corti. The authors observed that scattered IHC loss followed a basal to apical pattern after shorter survival times than the present study.
Figure 5. Organ of Corti centroids (white lines) shown next to spheres representing counted inner hair cells were used to calculate the percent distance of cells along the organ of Corti’s length. Bar = 200 μm.

Figure 6. Local spiral ganglion neuron density calculated with Amira’s ClusterDensity module shows areas of high (red), medium (yellow-green), and low (blue) cell density. Bar = 200 μm.

Figure 7. Histograms of present spiral ganglion neurons (SGNs) and inner hair cells (IHCs) in treated (red bars) and control (blue bars) specimens along 10% distance increments in the organ of Corti and Rosenthal’s canal.
The loss of SGNs, although not as extensive as the IHC loss, was still apparent. Our results agree with a study by Alam et al., who injected rats with kanamycin alone for 8 days to observe the effect on SGNs. They reported a decrease in SGN density 20 days after the first injection. In the present study, it was observed that SGN cell density was lost in the middle of the cochlea, at approximately a 50% to 75% distance from the base (see Figure 6). In a previous study, there was an increase in that region of Rosenthal’s canal of SGN cell density, which appears to be related to the lowest hearing threshold frequency of the organ of Corti.1 It is possible, since there were no observable OHCs in the treated cochleas, that SGN loss represents the loss of type II SGNs, which innervate these cells.9,21,22 However, it is more reasonable to assume that SGN loss is an incomplete, ongoing process and that with a longer survival time, more SGNs would degenerate.17

While the IHC, OHC, and SGN losses were obvious in the treated specimens, the sample size was not large enough to perform the appropriate statistical tests on the 2 different experimental groups. Since the aim of this study was to establish a whole-cochlear approach to the analysis of sensory cell damage in the mouse cochlea, we did not use a larger sample size for a statistical analysis. Virtual orthogonal sectioning of the cochlea, along a centroid generated along the length of the basilar membrane, produced “true” cross sections along its length. Since the z-sections were 5 μm apart, some small pixel interpolation artifact is present, but cell morphology is still well recognizable. Comparative examination of cochlear structures in treated vs control cochleas revealed which structures had changed, and their pathology could be assessed morphometrically. For example, damage to the spiral ligament, limbus, and hair cells was subjectively detectable in the treated animals. However, spiral ganglion cell damage was less obvious and was detected only after counting all cells and expressing them as total number and cell density. Subjective observations of the thickness of the stria vascularis in “true” cross sections suggested that there were no differences between treated and control animals, which eliminated the need to perform a time-consuming morphometric analysis.

Damage to fibrocytes of the spiral ligament and limbus was observed in all treated animals. The function of these fibrocytes appears to involve ion transport, particularly hair cell K+ recycling.23,24 Fibrocyte damage within these connective tissues has been reported after noise trauma,25,26 Taxol,27 and combination doses of an aminoglycoside antibiotic and a loop diuretic.11 Previous studies have described this pathology as decreased histopathologic density, due to a loss of fibrocytes. While this interpretation appears accurate, cell nuclei were not specifically labeled, so a quantitative estimate was not performed. Although damage to the stria vascularis was not observed, it has been reported previously after Taxol27 and combination aminoglycoside antibiotic and loop diuretic treatment.11,20 Perhaps the lack of damage in our treated animals was related to drug dose, survival time, or the difficulty in assessing stria thickness in less-than-true cross sections.

**Table 1. Measurements and cell counts.**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Treated</th>
<th>Control</th>
<th>Treated</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>OC length, mm</td>
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<td>OC volume, μL</td>
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<tr>
<td>No. of IHCs</td>
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<td>27</td>
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<td>Missing OHCs</td>
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<td>All</td>
<td>All</td>
<td>All</td>
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<tr>
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<tr>
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<tr>
<td>RC volume, μL</td>
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<td>0.040</td>
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<tr>
<td>No. of SGNs</td>
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<td>6689</td>
<td>6930</td>
<td>7557</td>
</tr>
</tbody>
</table>

Abbreviations: BM, basilar membrane; IHC, inner hair cell; OC, organ of Corti; OHC, outer hair cell; RC, Rosenthal’s canal; SEM, standard error of the mean; SGN, spiral ganglion neuron.

*Volumes were obtained from segmentation data, and linear measurements were performed with B-spline curves. Treated5 was not included in the treated mean due to the incomplete kanamycin dose.*
The sTSLIM imaging technology is nondestructive, and the specimen can be imaged multiple times with multiple stains. This is advantageous when counting cells such as IHCs and SGNs, which can be best visualized with different stains. Image analysis methods that would semi-automate the detection and quantitative assessment of damage in treated vs control cochleas are a current object of study now that sTSLIM is able to completely digitize a whole cochlea.

Author Contributions

Heather M. Schmitz, proposed/designed study, collected data, compiled figures, wrote manuscript; Shane B. Johnson, collected data, compiled figures, edited manuscript; Peter A. Santi, designed study, handled animals, interpreted data, wrote manuscript.

Disclosures

Competing interests: None.

Funding sources: None.

References