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Time Dependent Changes in the Crista Neuroepithelium Resulting From Gentamicin Ototoxicity in the Chinchilla*

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Abstract: This experiment was conducted to investigate the time-dependent effects of gentamicin (GM) on the neuroepithelial morphology of the posterior crista ampullares (PCA) of the adult chinchilla. The time course of GM-induced HC pathology was documented through measurements of HC density as well as through HC morphologic evaluation.

HC morphology was examined under light microscopy and HC density measurements were documented as a function of the time course of GM-induced ototoxicity. Six groups of animals (n=12) were treated with daily injections of 30 mg/kg of GM subcutaneously (SC) for six days and were sacrificed 1, 2, 3, 4, 6 and 8 weeks post-treatment. An untreated control group (n=3) was employed. Morphologically, in the first two weeks post-treatment (PT), there was pronounced GM-induced pathology in the crista neuroepithelia (NE), with clear fusion of ciliary bundles, blebs, cytoplasmic

vacuolization and shrinkage of the cytoplasm. The NE recovered from the induced pathology at three weeks post-treatment, displaying a normal morphology which remained unchanged up to 8 weeks post-treatment. Quantitatively, there was a significant decrease ($p < 0.05$) in HC density during the first two weeks post-treatment. The densities returned to normal levels at three weeks PT and were comparable to control values up to 8 weeks PT. These results demonstrate that the chinchilla NE exhibits ototoxicity up to two weeks PT and recovers from the induced pathology at three weeks PT; this recovery remained comparable to control morphology and quantity up to 8 weeks after the treatment demonstrating the capability of self repair in the chinchilla's crista NE.

Key Words: Hair cells, Gentamicin ototoxicity, Recovery, Crista ampullaris, Chinchilla

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Introduction

Hair cells (HC) of the cochleo-vestibular system have been a prime focus for investigation in attempts to elucidate a cure for sensorineural hearing loss (SNHL) and associated balance disorders. Recent studies have demonstrated HC regeneration after aminoglycoside ototoxicity in the cochleo-vestibular system of lower species (1-8). Furthermore, newly-formed hair cells have also been detected in the auditory and vestibular systems of mammals following both aminoglycoside treatment and acoustic trauma (9-14). The sources of such cells were attributed to regeneration, formation through transformation/transdifferentiation or recovery from insult through mechanisms of self repair (8, 10, 15-17). In these studies, however, regeneration was assessed

from a base state of complete HC loss (see below) and in many of these studies, the time course of GM-induced ototoxicity was not rigorously tracked.

In the above studies concerning regeneration, regrowth and self-repair, the goal was to sequentially analyze the NE after complete ablation of the HCs. The underlying assumption was that complete ablation of NE HCs while leaving an intact supporting cell layer would portray a clearer picture of HC recovery (9, 18). However, in clinical practice, GM is not used at doses high enough to produce complete ablation of HCs. More conservative experimental doses of GM that still produce ototoxicity can reveal novel information about the changes in the crista NE due to existence of several factors in the media. Indeed, it has been demonstrated that

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a host of diffusible (trophic) factors are involved in the triggering of HC regeneration (19). In light of this, it would seem more fruitful to investigate HC regeneration and/or the HC self repair process resulting from GM-induced ototoxicity within the limits of a lesser GM-induced damage. This would provide more availability of the HC-derived trophic factors necessary for triggering recovery than a HC-deprived state of the NE would allow. The following study was designed through this line of thinking and addresses at the light microscopy level the independent morphoquantitative changes from GM-induced ototoxicity within the vestibular NE. The goal of this study was aimed at addressing the next question: What is the time course of the GM-induced pathology in the crista NE?

Material and Methods

Adult chinchillas (*Chinchilla Laniger*), 8 to 12 months of age, weighing 450-550 grams were utilized. 30 mg/kg of GM SC was administered to six groups of three animals (each group, n=3; n total=18) daily for six days. The animals were allowed to survive 1, 2, 3, 4, 6 and 8 weeks post treatment and were subsequently sacrificed upon completion of their respective survival times. Two animals (n=2) from each group and three untreated control (n=3) animals were studied both morphologically and quantitatively.

Morphological Analysis

Chinchillas were deeply anesthetized (intramuscular administration of a ketamine/xylazine cocktail: 20 mg/kg and 3 mg/kg respectively) and perfused transcardially with 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M sodium phosphate buffered saline (PBS), pH 7.4, after the infusion of 0.9% NaCl solution. Immediately thereafter the auditory bulla was removed from the skull and the otic capsule opened. The PCA was dissected under the microscope and immersed in the same fixative for three hours. The tissue was post fixed with 1% OsO₄ in 0.12M sodium phosphate buffer for one hour at room temperature, dehydrated through a graded ethanol series and embedded in plastic (araldite). Half of the posterior crista from each animal was cut vertically into 1 thick sections from the planum semilunatum (PS) to the center of the crista (C). At intervals of 20 μm five sections were stained for toluidine blue for light microscopy examination.

Quantitative analysis-Hair cells counting

The HCs in a 1 μm section (representative of a 25 micron length of the cristae whereby the subsequent 24

sections weren't counted) were counted from the PS to the C. The position of the cell body within the NE and presence of a cuticular plate was used to define a HC; the presence or absence of a nerve calyx differentiated Type I from Type II HCs (20). A HC was counted if the border of the nucleus was clearly delineated. The cross-sectional NE area in each quantified section was measured via computer-assisted video microscopy and image analysis (NIH Image software operating on a Macintosh computer). HC densities were calculated as the number of HCs per each 104 square microns.

Statistical analysis

Type I and Type II HC densities were analyzed with respect to post-treatment time and crista location. Comparisons were made using multifactor repeated measures analysis of variance (SuperAnova) and the Tukey multiple comparison test. Hair cell densities were further compared to values obtained from untreated control animals using Tukey multiple comparison tests.

Results

Morphology of the Chinchilla Vestibular Epithelium

The vestibular sensory epithelium of the chinchilla consists of two types of sensory cells (Types I and II) and a complement of supporting cells (Figure 1). Type I HCs are easily recognized because they are surrounded by a nerve calyx. In some instances one afferent provides calyces to more than one HC (21, 22). Type II HCs intermingle with type I HCs and can be identified by their more superficially located nuclei. The nuclei of the supporting cells appear as an irregular row of monolayer cells over the basal membrane in the lower third of the sensory epithelium (22).

Gentamicin Ototoxicity: Morphoquantitative Analysis

Figure 2 reveals the morphological changes in the crista NE in the first two weeks after GM treatment. In the first week after GM treatment, the HCs exhibit damage regardless of type. Extensive vacuolization of the cytoplasm of the HCs can be seen, as well as blebs, empty nerve calyces, pyknosis, and forced retraction of cytoplasm due to displacement of cytoplasm from surrounding cells (Figure 2A). These effects were more prominent in the upper aspects of the cristae (the summit), especially in the more central areas. The supporting cells showed a disrupted mono-layer basal lining in the areas where HCs were lost. It appeared as if the supporting cells migrated to the surface of the NE,

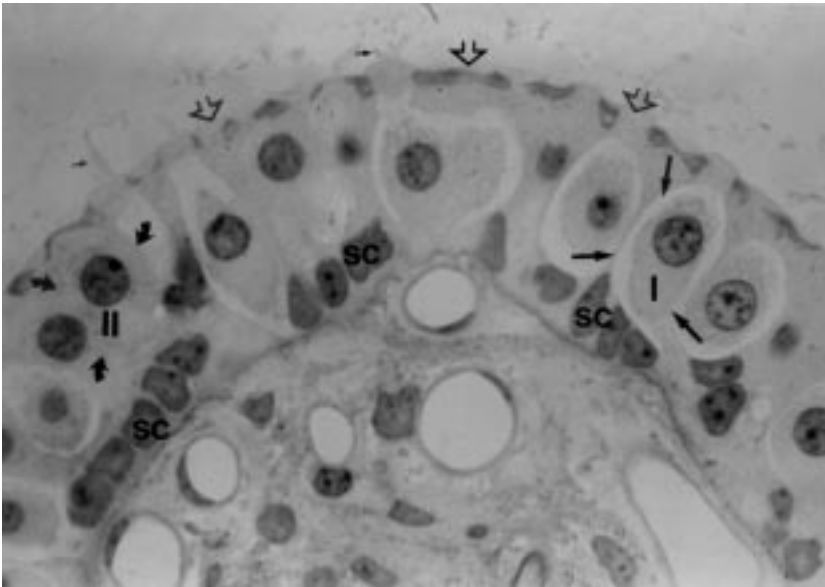


Figure 1. Photomicrograph of the PCA from a control animal showing the normal appearance of the sensory NE.

A circular shaped Type I (I) HC surrounded with a nerve calyx (straight arrow) and a type II (II) HC cylindrical in shape (curved arrow). The basal lining of supporting cells (sc) exhibits a monolayer between this HC layer and stroma. Cuticular plates of the HCs facing the endolymphatic space are continuous (open arrows). Bundles of cilia can be observed across the surface of the crista (small arrows).

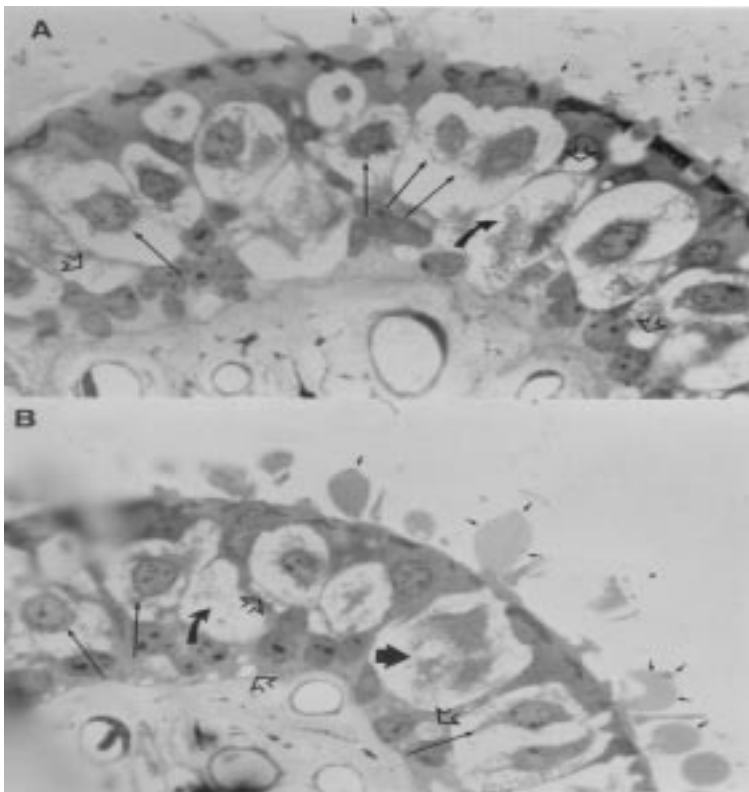


Figure 2. Representative sections of the PCA of animals treated with 30 mg/kg GM at one (A) and two weeks (B) after the treatment.

Blebs are protrusions of the cytoplasm into the endolymphatic space (short arrows). There is shrinkage of the HC cytoplasm (long arrows) and empty nerve calyxes (curved arrows) are shown. A hair cell (thick arrow is shown releasing its cytoplasmic contents into the endolymphatic fluid.

piling on top of each other, particularly in the areas where HCs were lost. The damaged cells did not have ciliary bundles.

In the second week after GM treatment, the morphological changes continued to occur. Blebs, vacuolization of the cytoplasm and empty nerve calyxes were observed along the cristae. Damaged HCs in the

slopes exhibited fusion of the hairs. The hair cells near the top of the cristae show an advanced stage of dissolution (Figure 2B).

At three weeks post treatment, the NE had recovered from the GM-induced pathology exhibiting normal morphology. We did not observe any pathological features of GM ototoxicity in any of the serial sections

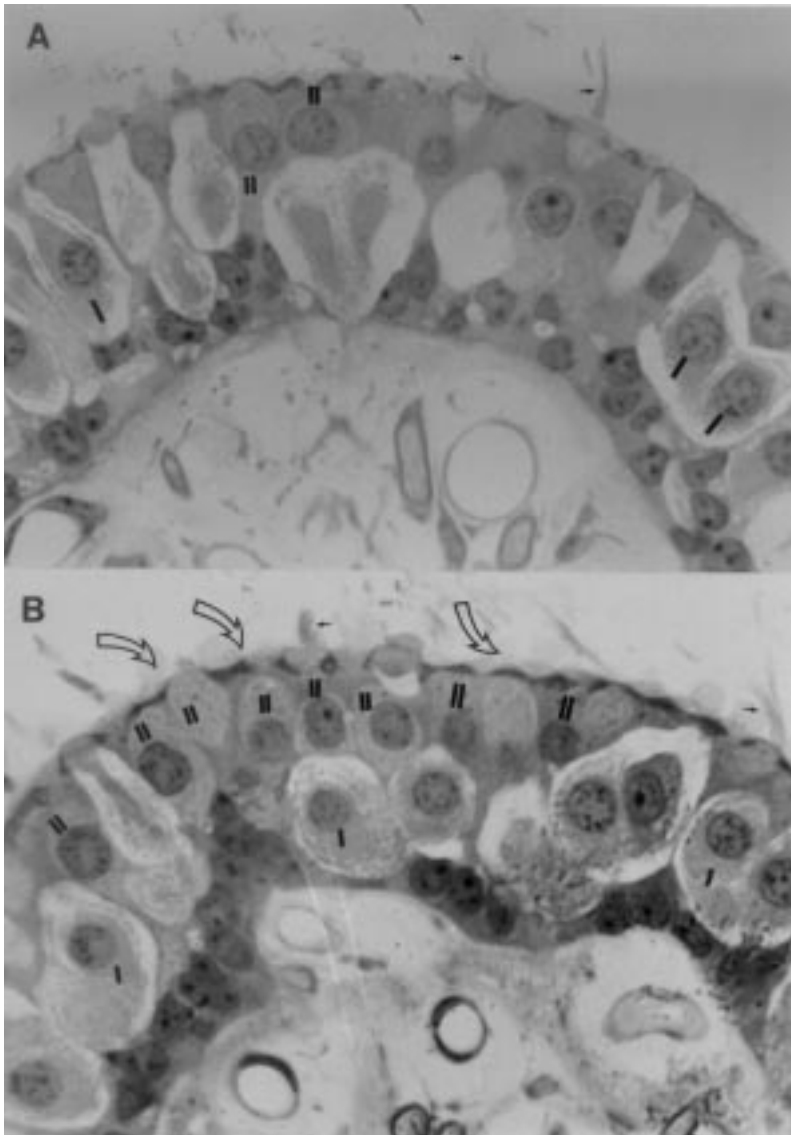


Figure 3. Representative sections of the PCA of animals treated with 30 mg/kg GM at three (A) and eight weeks (B) after treatment. The morphology of the NE resembles normal morphology at 3 weeks post-treatment (A). Ciliary bundles of HCs are seen (short arrows). Type I (I) and Type II (II) HCs and their cytoplasm are shown. The NE continues to show normal appearance at 8 weeks post-treatment (B). There is no HC cytoplasmic vacuolization or shrinkage. The morphological preponderance of the Type II (II) HCs (curved arrows) can be seen.

from both of the animals studied. The HCs were lined up in the upper two thirds of the NE in an ordinary fashion on top of the supporting cell mono-layer (Figure 3). The cuticular plates showed normal continuity and stereocilia were easily detectable. This normal morphology remained up to 8 weeks post-treatment. A morphological preponderance of Type II HCs was observed on top of the crista, particularly in the central regions. Immature-like ciliary bundles arose from the cuticular plate of the hair in some of the sections.

Quantitatively, however, at one week post-treatment, while there was a decrease in the mean densities of both Type I and Type II HCs, there was more of a decrease of

Type I mean density (Figure 4). By three weeks post-treatment and up to week 8, the densities of both Type I and II HCs were not significantly different from control levels (Figure 5).

HC densities decreased in both cellular types regardless of regional sensitivity along the crista (Figure 5). It appeared as though the decrease in HC Type 1 density is greater than that of Type 2 density in the first week. HC density measurements revealed no effect of distance on HC type during the first two weeks post-treatment (Figure 4). Multifactor repeated measures (ANOVA) revealed a significant statistical interaction ($p=0.05$) between HC type and post-treatment time.

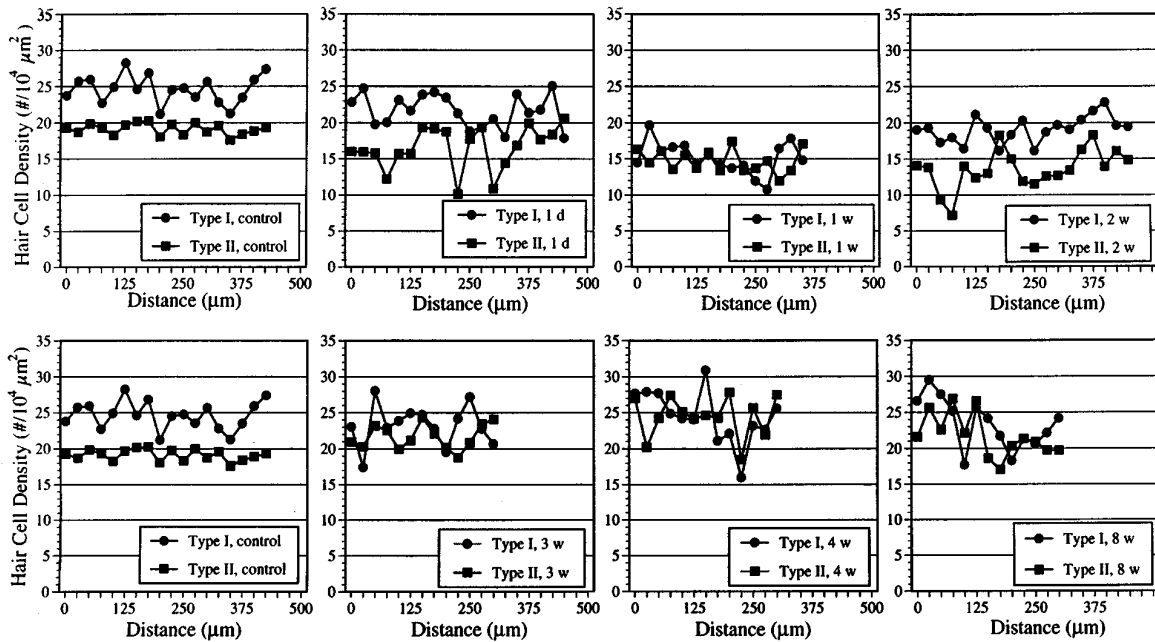


Figure 4. Comparison of control Type I and II HC mean densities a function of length along the cristae from the planum semilunatum (0 μm) to the centre. Standard deviation error bars were omitted to optimize the distinction of Type I and II HC graphs. The upper graphs shown, from left to right, control and post-treatment times of 1 day, 1 week, and 2 weeks. The lower graphs show, from left to right, control and post-treatment times of 3, 4 and 8 weeks.

Discussion

Normal NE

HC Type I to HC Type II ratio was calculated to be 3:1 in the squirrel monkey and 1:1 in the chinchilla (21). Our previous calculations from the posterior semicircular canal of the chinchilla in unoperated controls ($n=3$) revealed a ratio of Type I/Type II of 1.2/1 (22). Our method of HC counting restricted us from making specific commentary about possible regional variability of the Type I/Type II ratio; however, our results do not contradict the literature (21).

GM Ototoxicity

Aminoglycoside-induced HC pathology affects both types of HCs (22-24). We observed damage in both types of HCs in the first two weeks after GM treatment and subsequent morphological recovery at three weeks post treatment. Fusion of the hairs, pyknosis, swollen nerve endings, shrunken cytoplasm and cytoplasmic extrusion into the endolymph are well-documented pathological features of aminoglycoside ototoxicity (18, 24, 25) and our results display similarities to these studies (Figure 2). We observed a well-advanced stage of dissolution of HCs on the top aspects of the cristae. This finding was consistent from the planum semilunatum to the center

regardless of any regional predilection. Other studies have indicated a regional sensitivity pattern predicting a higher Type I HC loss in the central regions of the crista from aminoglycoside treatment (24, 26). It is conceivable that the absence of permanent degenerative changes and subsequent recovery to normal morphology within the crista NE restricted our observation of a regional, ototoxic predilection from GM treatment.

Recovery of Morphology

Aminoglycoside ototoxicity has been reported to establish permanent degenerative changes in the NE of the mammalian vestibular system in experimental animal models (27). In contrast, clinical and experimental models in humans have demonstrated recovery of function after aminoglycoside-induced ototoxicity (28, 29). Our present findings of recovery of morphology lends support to such clinical studies demonstrating recovery of function. Moreover, evidence of regeneration in a mammalian model utricular epithelia has been shown. Forge et al established complete hair cell loss in the striolar regions of the utricular epithelia of the guinea pig two weeks after a ten-day, daily systemic administration of 120 mg/kg GM (9). In this study, the epithelia exhibited immature Type II HCs four weeks after the treatment. Although their microscopy data were not supported with

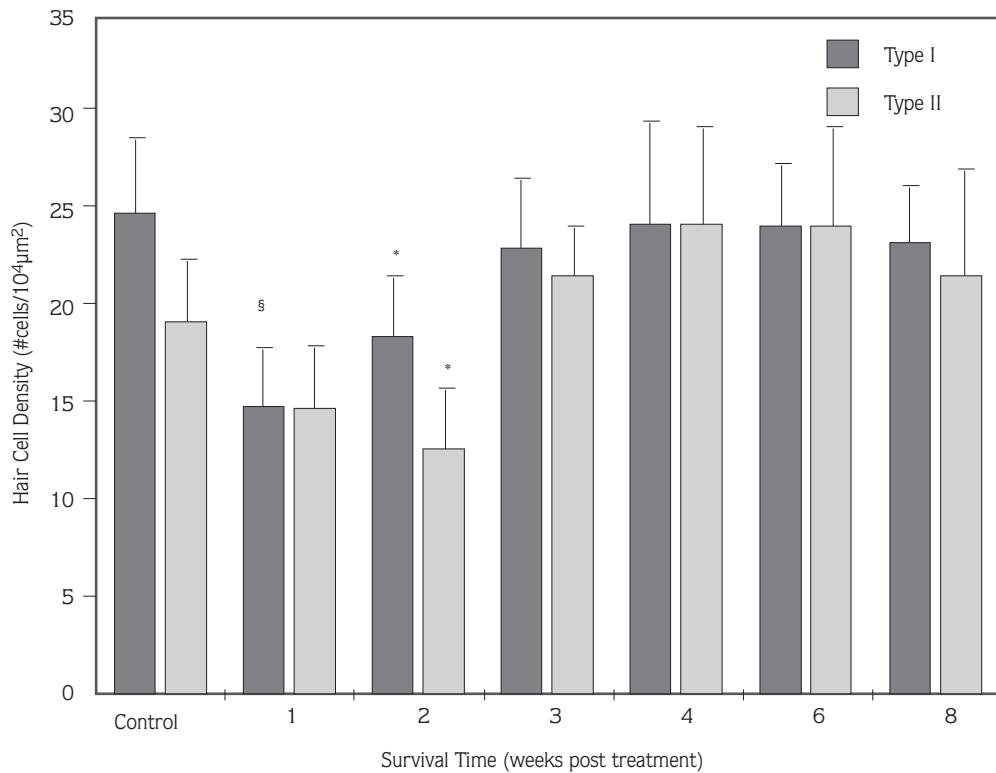


Figure 5. The column bars show the Type I and Type II HC densities (mean ± standard deviation) vs. post-treatment time in experiment II. By one week post-treatment the density of Type I HCs was found to be lower than controls (* $p < 0.005$) and remained significantly lower than control levels at two weeks post-treatment (*, $p < 0.05$). At three weeks post-treatment, however, Type I densities had increased and were not different from the control levels, and remained at comparable values through eight week post-treatment. Type II HC densities exhibited similar differences from controls, whereby the density was significantly lower at the two-week interval (*, $p < 0.05$); the lower density at one week was not statistically different from the control level ($p < 0.2$). As for the Type I HCs, the density of Type II HCs at weeks three through eight were not different from control levels.

electrophysiological assessment, recovery of function after aminoglycoside poisoning has been documented in other experimental models (30, 31). Our results are complimentary to those studies demonstrating morphological recovery of the PCA NE in a mammalian model. In the present study, there was not complete HC loss at any time in the cristae, but significant ($p < 0.05$) HC loss was present in the first two weeks with recovery to normal number and morphology by three weeks post-treatment (Figure 5). This finding was persistent with a morphological preponderance of HC type with marginal significance ($p < 0.02$) from three to eight weeks post-treatment (Figure 5). The NE displayed normal anatomical features comparable to controls without exception during this three to eight weeks post-treatment time.

Regeneration and recovery of function in the bird cochleo-vestibular epithelia is well-accepted (1, 6, 7, 8, 30). In vivo and in vitro evidence of newly-formed HCs in

the mammalian NE has also been shown (9, 12). In the present study, there is recovery of the NE after aminoglycoside-induced ototoxicity. Morphoquantitative evidence supports this finding. There is loss of HCs in the first two weeks after the treatment. Morphological recovery started at three weeks post-treatment and reached control values for up to 8 weeks post-treatment as depicted by HC density measurements. Rubel et al cite a lack of DNA evidence as argument against regeneration explaining newly-formed HCs of the guinea pig utricle post aminoglycoside ototoxicity. These authors postulated that the source of recovery might be either self repair or cellular transformation, but not regeneration (10). Moreover, Kelley et al provided evidence for a HC self repair mechanism in the neonatal mouse organ of corti (32). In our study, there was evidence for morphological HC disruption and death (Figure 2). HCs demonstrated a significant decrease quantitatively in the first two weeks post-treatment (Figure 5). While we cannot discount the possibility of self repair and cellular transformation

mechanisms being operable during the recovery period, a return to control level density of HC number must argue for regeneration in our model.

Tsue et al demonstrated in a culture study involving whole utricular organ explants from the chicken that a soluble factor released from the damaged inner ear epithelium is upregulated to stimulate HC proliferation under such conditions. Furthermore, it was shown that this factor is upregulated as a function of *undamaged* HCs being present in the culture (19). Other studies have demonstrated trophic factors in the mammalian cochleo-vestibular NE that play a role in hair cell regeneration (11, 33). In the present study, we employed a moderate, GM-induced ototoxicity sufficient enough to ensure HC death-but not complete HC ablation- in the crista NE (Figure 2). Our result from a system not totally devoid of HCs are consistent with a HC-derived trophic factor being operable during recovery from ototoxic insult. The damage to the NE might have caused upregulation of some of the trophic factors that are available in the local

media. A crista NE exhibiting complete hair cell loss is more reluctant to generate hair cells than a crista NE that shows partial damage after an ototoxic insult (13).

We have shown both morphologically and quantitatively that the chinchilla's NE is capable of recovering from a GM-induced ototoxic insult. Our results challenge the believed irreversibility of aminoglycoside-induced ototoxicity in the crista NE. Further elucidation of the exact sources and mechanisms operable in such ototoxic recovery of the crista NE should greatly aid in the research of cochleo-vestibular disorders.

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