Effects of Prolonged Kanamycin Administration on Cochlear Anatomy and Auditory Brainstem Response Thresholds in Chickens

Ming-Liang Xiang, PhD; Hao Wu, PhD; Qi Huang, MD; Lan Cheng, MD

Objective: To determine whether regenerated hair cells in the basilar papilla of chickens are resistant to kanamycin monosulfate damage.

Design: Randomized controlled trial.

Subjects: Ninety newly hatched Roman chickens.

Intervention: Chickens were injected with kanamycin monosulfate (200 mg/kg/d) for 10, 13, 17, 20, 25, or 30 days.

Results: Scanning electron microscopy revealed that hair cells in the proximal 40% of the basilar papilla degenerated and disappeared after 10 days of kanamycin treatment. Following this, hair cell regeneration and repair was apparent. Regeneration and maturation of hair cells within 20 days in chickens that received treatment for 20 days were similar to those in chickens that were treated for 10 days followed by 10 days of recovery. After 25 days of treatment, many regenerated hair cells of mature appearance were reinjured. Regenerated hair cells of immature appearance were not damaged. The auditory brainstem response assay showed that the loss and recovery thresholds in chickens treated with kanamycin for 20 days were similar to those in chickens treated for 10 days followed by 10 days of recovery. There was a loss of auditory brainstem response thresholds in chickens that were treated with kanamycin for more than 20 days.

Conclusion: The immature regenerated hair cells in the basilar papilla of chickens are resistant to kanamycin ototoxic effects; however, this resistance is not seen in mature hair cells following prolonged kanamycin exposure.


A number of previous studies have demonstrated that immature hair cells are present in the damaged regions of the basilar papilla in chickens at the end of a 10-day course of aminoglycoside treatment.1-4 These findings suggest that immature regenerated hair cells in the basilar papilla of the chickens may be resistant to the ototoxic effects of aminoglycoside injury. Hashino and Salvi3 examined the effects of kanamycin-induced hair cell loss in chickens and found that the hair cells that regenerated after kanamycin treatment displayed no evidence of basilar papilla damage following 25 days exposure to high doses of aminoglycoside antibiotics. Since regenerated hair cells take approximately 25 days to mature,1,3 the findings of Hashino and Salvi strongly suggest that mature regenerated hair cells are resistant to kanamycin-induced ototoxic effects.

In the present study, we further examined the resistance of regenerated hair cells in the basilar papilla of chickens to kanamycin monosulfate. We found that both regenerated hair cells and the auditory brainstem response (ABR) thresholds could be redamaged when kanamycin was administered for more than 20 days. These findings indicate that mature regenerated hair cells in the basilar papilla of the chickens may not be resistant to prolonged (>20 days) kanamycin administration following associated ototoxic effects.

METHODS

ANIMALS AND TREATMENT

The animals used in this study were cared for in strict compliance with the principles of humane treatment approved by the Xinhua Hospital ethics committee. A total of 90 newly hatched Roman chickens (3 days old) were randomly divided into 3 experimental groups. Group 1 chickens (n=36) received daily intramuscular injections of kanamycin monosulfate (200 mg/kg) (Sigma, St Louis, Missouri) for 10 consecutive days and were subsequently killed 0, 3, 7, 10, 15, or 20 days after the last injection (n=6 per subgroup). Group 2 chickens (n=36) received daily intramuscular injections of kanamycin monosulfate (200 mg/kg) for 10, 13, 17, 20, 25, or 30 consecutive days and were killed on the day...
indicate SDs. dB SPL indicates decibel sound pressure level.

Chickens were prepared for ABR testing after anesthesia was induced by celiac injection of 10% chloral hydrate (240 mg/kg). The ABR was recorded using a MEB-3102 physiological response recorder (Nihon Kohden, Tokyo, Japan). Responses were amplified and filtered (50-3000 Hz band pass), and the average of 200 stimulus presentations was determined. The time of analysis was 10 milliseconds, and the sensitivity was 10 µV per division. The pin electrodes were inserted into the bilateral mastoid subcutis as reference and ground electrodes and into the top mid point of the cranium as active electrodes. Since the damage caused by kanamycin is confined to the proximal 40% of the basilar papilla,1,4,5 which corresponds to frequencies over 3000 Hz,6 the stimulus of a half-cycle 4000-Hz sine wave (produced using an SMP-3100 sound stimulation applicator) (Nihon Kohden) was delivered at a rate of 10 times per second by earphone to evoke ABR.

**ELECTRON MICROSCOPY**

Immediately following the ABR test, while still under anesthesia, chickens were decapitated, and each cochlea was perfused via the round window with 2.5% glutaraldehyde in 0.1M phosphate buffered saline (PBS). Following 3 washes with PBS, the temporal bones were removed and the basilar papilla exposed using standard microdissection techniques. The specimens were then placed in 1% osmium tetroxide (in 0.1M PBS) for 2 hours at room temperature and then washed several times in PBS. Thereafter, the specimens were dehydrated in a graded ethanol series and then dissected (this included removing the tegmentum vasculosum and the tectorial membrane). After critical point drying, specimens were sputter-coated with gold palladium and viewed using a XL30ESEM electron microscope (Royal Philips Electronics NV, Amsterdam, the Netherlands).

All ABR data were analyzed by the Mann-Whitney test.

**RESULTS**

**ABR MEASUREMENTS**

**Figure 1** illustrates the ABR threshold results from the control (group 3) chickens (those that were not treated with kanamycin). Hearing function in the control chickens was mature by the 13th day after hatching. No changes were apparent after this time. Thresholds measured at the 33rd day were similar to those measured at the 13th and 23rd days after hatching (P > .05). The ABR threshold results from chickens that received 10 days of kanamycin treatment (group 1) are shown in **Figure 2**. The largest threshold shift occurred at the end of drug administration. The ABR threshold began to recover within a few days and was stable at 10 days following the termination of drug administration.

The average ABR thresholds for animals receiving prolonged kanamycin administration (group 2) are illustrated in **Figure 3**. Threshold values were initially elevated, declined, or recovered to near normal levels, and then increased slightly thereafter. As in group 1 chickens, the largest threshold shift was apparent after 10 days of kanamycin treatment. The threshold recovery in chickens within 20 days of kanamycin treatment was similar to that observed in chickens exposed to kanamycin for 10 days and then allowed to recover for 10 days. Thresholds measured in animals that received kanamycin for 13, 17, or 20 days were similar to those measured in chickens treated with kanamycin for 10 days followed by 3, 7, or 10 days of recovery, respectively. These results are

---

**ABR TESTING**

Chickens were prepared for ABR testing after anesthesia was induced by celiac injection of 10% chloral hydrate (240 mg/kg). The ABR was recorded using a MEB-3102 physiological response recorder (Nihon Kohden, Tokyo, Japan). Responses were amplified and filtered (50-3000 Hz band pass), and the average of 200 stimulus presentations was determined. The time of analysis was 10 milliseconds, and the sensitivity was 10 µV per division. The pin electrodes were inserted into the bilateral mastoid subcutis as reference and ground electrodes and into the top mid point of the cranium as active electrodes. Since the damage caused by kanamycin is confined to the proximal 40% of the basilar papilla,1,4,5 which corresponds to frequencies over 3000 Hz,6 the stimulus of a half-cycle 4000-Hz sine wave (produced using an SMP-3100 sound stimulation applicator) (Nihon Kohden) was delivered at a rate of 10 times per second by earphone to evoke ABR.

**ELECTRON MICROSCOPY**

Immediately following the ABR test, while still under anesthesia, chickens were decapitated, and each cochlea was perfused via the round window with 2.5% glutaraldehyde in 0.1M phosphate buffered saline (PBS). Following 3 washes with PBS, the temporal bones were removed and the basilar papilla exposed using standard microdissection techniques. The specimens were then placed in 1% osmium tetroxide (in 0.1M PBS) for 2 hours at room temperature and then washed several times in PBS. Thereafter, the specimens were dehydrated in a graded ethanol series and then dissected (this included removing the tegmentum vasculosum and the tectorial membrane). After critical point drying, specimens were sputter-coated with gold palladium and viewed using a XL30ESEM electron microscope (Royal Philips Electronics NV, Amsterdam, the Netherlands).

All ABR data were analyzed by the Mann-Whitney test.

**RESULTS**

**ABR MEASUREMENTS**

**Figure 1** illustrates the ABR threshold results from the control (group 3) chickens (those that were not treated with kanamycin). Hearing function in the control chickens was mature by the 13th day after hatching. No changes were apparent after this time. Thresholds measured at the 33rd day were similar to those measured at the 13th and 23rd days after hatching (P > .05). The ABR threshold results from chickens that received 10 days of kanamycin treatment (group 1) are shown in **Figure 2**. The largest threshold shift occurred at the end of drug administration. The ABR threshold began to recover within a few days and was stable at 10 days following the termination of drug administration.

The average ABR thresholds for animals receiving prolonged kanamycin administration (group 2) are illustrated in **Figure 3**. Threshold values were initially elevated, declined, or recovered to near normal levels, and then increased slightly thereafter. As in group 1 chickens, the largest threshold shift was apparent after 10 days of kanamycin treatment. The threshold recovery in chickens within 20 days of kanamycin treatment was similar to that observed in chickens exposed to kanamycin for 10 days and then allowed to recover for 10 days. Thresholds measured in animals that received kanamycin for 13, 17, or 20 days were similar to those measured in chickens treated with kanamycin for 10 days followed by 3, 7, or 10 days of recovery, respectively. These results are
similar to those of Trautwein and colleagues. However, to our surprise, we found that loss of hearing function recurred when kanamycin treatment was continued for more than 20 days. Thresholds measured in chickens treated with kanamycin for 25 and 30 days were higher than in those treated for 10 days followed by 15 and 20 days of recovery, respectively (P < .001 for each) (Figure 4). Threshold shifts were more evident and significantly higher in chickens that were treated with kanamycin for 30 days than in those treated for 20 days (P < .001) (Figure 3).

**SEM OBSERVATIONS**

Figure 5 shows a surface view of the basilar papilla from a control chicken. The oval surface of the hair cells and the thin surface of the supporting cells surrounding each of the hair cells form a mosaic pattern on the surface of the basilar papilla. The stereociliary bundles on the apical surface of the hair cells appear staircaselike and exhibit regular orientation.

In group 1 animals (10 days of kanamycin treatment), there was complete loss of hair cells in the proximal 40% of the basilar papilla following the conclusion of treatment, and new stereociliary bundles were readily identifiable in the damaged region (Figure 6A). Fifteen days after the cessation of treatment, the damaged basilar papilla region had been repaired, demonstrating hair cell regeneration. Most of the regenerated hair cells were mature in appearance and displayed no evidence of damage (Figure 6B).

In group 2, the degrees of hair cell degeneration, regeneration, and maturation in the basilar papilla within 20 days of kanamycin treatment were similar to what was observed in group 1 animals. The most severe basilar papilla injury was also evident 10 days after treatment was initiated. Subsequently, new hair cells appeared in the basilar papilla, repairing the damaged region, despite the fact that the kanamycin treatment was ongoing.

There are 2 possible explanations for the appearance of new hair cells in the basilar papilla of chickens after aminoglycoside ototoxic effects took place. One is regeneration, and the other is recovery. While we have no direct evidence, we believe that the new cells observed in the present study were derived via a regenerative process. Findings from several studies support this assertion. For instance, using transmission electron microscopy, Duckert and Rubel observed that hair cells were completely destroyed in the proximal tip of the basilar papilla in chickens as a result of aminoglycoside ototoxic effects. Lippe and colleagues reported that the restoration of hair cell number following the toxic effects of aminoglycoside administration was due to mitosis. Furthermore, in a previous study using transmission electron microscopy, our research group determined that virtually all hair cells were lost, and many hair cell precursors appeared in the extreme proximal 40% of the basilar papilla after 10 days of kanamycin treatment. The fact that in the present study new cells were observed despite continued kanamycin exposure is also suggestive of a regenerative process. To our knowledge, no reports suggest that new hair cells are simply injured cells that have recovered.

The morphologic characteristics of the basilar papilla in chickens treated with kanamycin for 13 or 17 days were similar to those of chickens treated for 10 days followed by 3 or 7 days of recovery (Figure 7A). We did not find injury or degeneration of these immature regenerating hair cells.

Regenerated hair cells were almost mature by the 20th day of kanamycin injection. The size of the cuticular plate of the regenerated hair cells was nearly normal, and many of the microvilli on the hair cell surface had disappeared. However, at this time point, to our surprise, we found a regenerated hair cell that was mature in appearance and showing signs of degeneration, a bulging cuticular plate (Figure 7B).

When chickens were injected with kanamycin for more than 20 days, there was considerable damage to the regenerated hair cells in the basilar papilla. Unlike the nearly normal appearance of the basilar papilla in the chickens treated for 10 days followed by 10 days of recovery, many degenerated hair cells were evident in the basilar papilla of animals treated with kanamycin for 25 days (Figure 7C). These damaged hair cells had expanded cuticular plates on which no stereociliary bundles were
observed. On occasion, 2 or more separate ciliary bundles were recognized on the apical cell surface of these cells. The number of stereocilia inside these bundles was reduced, or the stereocilia bundles were flattened. In addition, some hair cells appeared to be in the process of being extruded from the epithelium. It is possible that these hair cells were in the process of dying. All injured hair cells were mature in appearance.

Figure 6. Scanning electron microscopy images of the basal portion of basilar papilla from group 1 chickens. A, No original hair cells are evident, and new stereocilia (N) can be seen in the damaged region of the basilar papilla from a chicken treated with kanamycin monosulfate for 10 days followed by 0 days of recovery. Scale bar indicates 15 µm. B, Chicken treated with kanamycin for 10 days followed by 15 days of recovery. Most of the regenerated hair cells are mature in appearance and display no evidence of damage in the basilar papilla. Scale bar indicates 20 µm.

Figure 7. Scanning electron microscopy images of basilar papilla in group 2 chickens. d indicates degenerated hair cell; N, newly regenerated hair cell; S, stereocilia bundle; V, microvilli. Unless otherwise indicated, scale bars indicate 10 µm. A, After 13 days of kanamycin monosulfate treatment, most of the damaged region has been repaired by immature regenerated hair cells. B, Regenerated hair cells further approach maturation. A regenerated hair cell mature in appearance and showing signs of degeneration is observed in the basilar papilla from a chicken treated with kanamycin for 20 days. C, Many basilar papilla–regenerated hair cells were injured after 25 days of kanamycin treatment. D, Massive destruction of regenerated hair cells is evident in the basal region of the basilar papilla from a chicken treated with kanamycin for 30 days. Scale bar indicates 20 µm.
By the end of 30 days of kanamycin treatment, basilar papilla damage was more severe. Most of the original regenerated hair cells were completely destroyed and had disappeared from the epithelium. The remaining cells, as in the basilar papilla of chickens treated for 25 days, were also damaged but remained in the epithelium.

The anatomic changes (as observed by SEM) in the basilar papilla from chickens treated with kanamycin for 30 days were complex (Figure 7D). In addition to differing degrees of damage evident in the original regenerated hair cells, many newly regenerated hair cells were observed in the repeatedly damaged region. Most of these new hair cells were immature in appearance (similar to the regenerated cells in the basilar papilla of chickens after 10 days of kanamycin administration), while a few newly regenerated hair cells were relatively mature. It may be that these new hair cells would have repaired the damaged region completely in the absence of continued kanamycin exposure. None of the regenerated hair cells of immature appearance were injured.

### COMMENT

It is unclear whether regenerated hair cells in the basilar papilla of chickens are resistant to aminoglycoside antibiotics following aminoglycoside administration. Using light microscopy to assess the morphologic changes of regenerated hair cells in the basilar papilla of chickens treated with kanamycin for 20 days, Trautwein et al. observed that the process of hair cell maturation was not suppressed by treatment and that thresholds were elevated again when treatment was continued for more than 20 days. The findings from our electrophysiologic study. Our ABR testings revealed that thresholds were elevated again when treatment was continued for more than 20 days. The activity of regenerated hair cells to kanamycin becomes more apparent with advancing maturation. Many regenerated hair cells were already injured. The sensitivity of regenerated hair cells to kanamycin becomes more apparent with advancing maturation. Many regenerated hair cells in the basilar papilla of chickens treated with kanamycin for 25 days. It was suggested that the hair cells that regenerated after kanamycin-induced hair cell loss could survive for a substantially longer period than their predecessors during prolonged exposure to aminoglycoside antibiotics. These findings indicate that both immature and mature regenerated hair cells in the basilar papilla of chickens are resistant to kanamycin following the initial ototoxic effects.

In the present study, however, we found that many regenerated hair cells were sensitive to kanamycin as soon as they became mature in appearance. Using SEM, we observed that regenerated hair cells were nearly mature after 20 days of kanamycin treatment, and some of these regenerated hair cells were already injured. The sensitivity of regenerated hair cells to kanamycin becomes more apparent with advancing maturation. Many regenerated hair cells in the basilar papilla of chickens treated with kanamycin daily for 25 consecutive days were injured. Degeneration of the basilar papilla in chickens treated with kanamycin for 30 days was more pronounced than in those treated for 25 days, and most of the regenerated hair cells were completely destroyed.

The basilar papilla anatomic changes observed in our morphologic investigation are compatible with the findings from our electrophysiologic study. Our ABR testing revealed that thresholds were elevated again when treatment was continued for more than 20 days. The threshold differences between chickens treated for 30 days and those treated for 20 days were significant. We found that all of the injured regenerated hair cells were completely mature and that there was no evidence of non-injured mature regenerated hair cells. Moreover, no injured immature regenerated hair cells were detected. Therefore, we concluded that immature regenerated hair cells in chickens may be resistant to kanamycin ototoxic effects and that this characteristic is lost on maturation. This finding is in contrast to that from a previous report. The disparity may be explained by the fact that Hashino and Salvi used light microscopy in their analysis, a technique that is less sensitive than SEM.

By the end of 30 days of kanamycin treatment, many newly regenerated hair cells were observed in the repeatedly damaged region. There are 2 likely scenarios regarding the fate of these newly regenerated hair cells in the absence of continued kanamycin exposure. One is that these regenerated hair cells would be destroyed or injured after they become mature in appearance. The other is that these new hair cells would repair the damaged region completely. For several reasons, we consider it likely that mature hair cells destroyed or injured by prolonged kanamycin administration would regenerate and that the ABR thresholds would recover to near normal levels within 15 days after cessation of 30 days of kanamycin administration. First, it is impossible that kanamycin persists in the extracellular environment in the absence of continued kanamycin exposure. Theoretically, chickens with renal failure due to kanamycin nephrotoxic effects would not survive for a substantial period of time, while chickens with normal kidney function would excrete any remaining kanamycin within a short time after cessation of kanamycin administration. Moreover, Marean and colleagues reported that birds had no trace of kanamycin in serum during the recovery period after kanamycin administration.

Second, in our group 1 animals (10 days of kanamycin treatment), we observed that the damaged basilar papilla region was almost completely repaired by regenerated hair cells 15 days after the cessation of 30 days of kanamycin treatment and that no regenerated hair cells (neither immature nor mature) were injured within 20 days of the recovery period.

Third, there was no evidence that mature regenerated hair cells were injured in the absence of continued kanamycin exposure after the end of kanamycin treatment. Further studies are required to explore changes in the basilar papilla after 30 days of daily kanamycin administration followed by a recovery period.

It is not known precisely why immature regenerated hair cells are resistant to the effects of kanamycin. Marean et al. attributed this resistance to systemic factors. However, our finding that regenerated hair cells were damaged only after they attained morphologic maturation during the prolonged period of kanamycin treatment suggests that systemic factors do not play a role. The finding of Hashino and Salvi that regenerated hair cells could be damaged by repeated kanamycin treatment after becoming mature in appearance lends credence to our observations. We suggest that the observed kanamycin resistance occurs at the hair cell level. The finding that...
kanamycin accumulates in original but not in immature regenerated hair cells, suggesting that the resistance of regenerated hair cells to kanamycin may be a consequence of altered and/or dysfunctional drug uptake pathways. Based on the finding that kanamycin resistance in regenerated hair cells was limited to those in the immature stage, and given that hair cells in the basilar papilla of the embryonic chick are also kanamycin insensitive, we are inclined to agree that resistance may be associated with the lack of structural development within immature hair cells.

It is probable that structural development within mature regenerated hair cells is complete, thus enabling kanamycin to enter and exert its cytotoxic effects. The development of cytoskeletal structure may be a very important factor conferring kanamycin sensitivity to mature regenerated hair cells. Very little is known regarding how aminoglycoside molecules are incorporated into hair cells and exert their cytotoxic effects. One possibility is that aminoglycosides block voltage-sensitive calcium $2^+ \text{ ion} (\text{Ca}^{2+})$ channels, which inhibit Ca$^{2+}$ influx into hair cells induced by potassium ions. Although the long-term consequences of such a blockade are unknown, it is conceivable that cellular metabolism or plasma membrane integrity could be disrupted.

Numerous endocytotic vesicles evident on the apical surface of hair cells strongly suggest that the aminoglycoside molecule may be incorporated via endocytosis. Several transmembrane proteins as well as their kinases have been shown to play critical roles in the receptor-mediated endocytosis process. Further studies are warranted to assess if endocytosis is the primary means of kanamycin incorporation into hair cells and, if so, to determine whether expression of the molecules involved in this process are developmentally regulated.

Submitted for Publication: June 2, 2006; final revision received July 4, 2007; accepted September 5, 2007.

Correspondence: Ming-Liang Xiang, PhD, Department of Otolaryngology—Head and Neck Surgery, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, 1665 Kong-Jiang Rd, Shanghai 200092, China (xiangming_liang@yahoo.com.cn).

Author Contributions: Dr Xiang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Xiang. Acquisition of data: Xiang, Wu, and Cheng. Analysis and interpretation of data: Xiang and Huang. Drafting of the manuscript: Xiang, Huang, and Cheng. Critical revision of the manuscript for important intellectual content: Xiang and Wu. Statistical analysis: Xiang and Cheng. Obtained funding: Xiang, Administrative, technical, and material support: Xiang, Wu, and Huang. Study supervision: Xiang.

Financial Disclosure: None reported.

Funding/Support: This study was supported by grant 2007154 from the Shanghai Health Bureau Foundation of China and grant 2007XJ021 from the Shanghai Jiao Tong University School of Medicine Foundation, Shanghai, China (Dr Xiang).

REFERENCES