Effects of combined administration of furosemide and kanamycin on rat auditory nerve

GUO Weiwei¹, YUAN Fen-qian², LIU Hui-zhan¹, YANG Shi-ming¹

¹ Department of Otolaryngology, Head & Neck Surgery, Institute of Otolaryngology, Chinese PLA General Hospital, Beijing 100853, P.R. China
² Department of Head and Neck Surgery, Jiangxi Cancer Hospital, Nanchang 330029, China

Abstract
Objective To determine the effects of combined administration of furosemide and kanamycin on inner ear structures and the auditory nerve in rats.

Methods The rats in the treatment group received intravenous injections of combined furosemide and kanamycin sulfate, and the rats in the normal control group received no treatment. The auditory brainstem response (ABR) test was carried out 7 days after drug administration to determine the effects of drug administration on hearing. Cochlear slice and cochlear wholemount were prepared after 7 days of drug treatment.

Results After 7 days of drug administration, ABR thresholds were significantly higher in the treatment group than in the control group and neurofilaments were significantly reduced, although the number of spiral ganglia showed no decrease and there were no signs of supporting cell injury.

Conclusions Combined administration of furosemide and kanamycin sulfate has an apparent synergistic ototoxic effect. Although spiral ganglion damage may not be apparent within a short time period of drug administration, damage to auditory nerve fibers is obvious.

Key words kanamycin; furosemide; deafness model; hair cell injury

Introduction
Kanamycin is an aminoglycoside antibiotic and its toxicity to the inner ear has been reported in numerous studies. Furosemide is a loop diuretic and its ototoxicity has been shown in studies to involve damage to marginal cells of the stria vascularis. The level of the marginal cell edema is used as an indicator of loop diuretic ototoxicity. The stria vascularis plays an important role to ensure the relative stability of the micro-environment in the inner ear and to maintain normal functioning of hair cells and the auditory nerve.¹ Currently, research on effects of combined ototoxicity mainly focuses on cochlear hair cell damage and subsequent spiral ganglion cell degeneration and rarely on injury to neurofilaments and synapses. In this study, we measured brain stem auditory responses (ABRs) in rats treated with kanamycin and furosemide to better understand how aminoglycoside antibiotics and loop diuretics damage the auditory nervous system and hearing in combination.

Materials and Methods

Animal groups
Care and use of the animals in this study were approved by the Institutional Animal Care and Use Committees of the Chinese PLA General Hospital. Healthy 4-week-old SD rats (100 – 110g, n=20) of either sex were randomly selected to receive kanamycin (100 mg/kg) and furosemide (200 mg/kg) treatment (Group B) or no treatment (Group A).

Drug administration
Animals were anesthetized with 10% chloral hydrate (0.45 ml/100 g) by intraperitoneal injection. The left jugular vein was surgically exposed and furosemide intravenously injected. This was followed by intramuscular injection of kanamycin as detailed in the literature.²

ABR Measurement
Details of ABR measurement were provided else-
For ABR measurement, rats were anaesthetized with xylazine and ketamine. Needle electrodes were inserted at the vertex and pinna. ABRs were evoked with clicks or 5 ms tone pips (0.5 ms rise–fall) at 4, 8, 16, and 32 kHz presented at 30/sec. Responses were amplified, filtered and averaged using the Intelligent Hearing System. The sound level was raised in 20- or 5-dB steps. At each level, 1,024 responses were averaged. Thresholds were determined by visual inspection.

**Immunocytochemistry staining**

Cochleae were perfused with 4% formaldehyde in phosphate buffer and treated with 0.2% Triton X–100/ PBS. Goat serum (10%) was used to block nonspecific binding. The tissue was then incubated with 200 kD Neurofilament Heavy antibody (abcam, diluted 1:200). The samples were washed with phosphate buffered saline (PBS), followed by incubation with secondary antibodies. To stain nuclei, we used the DNA–specific label Propidium iodide (PI) as described previously. The samples were mounted on glass slides with antifade solution (Prolong Antifade Kit, Molecular Probes). The samples were examined using a LSM 510 META confocal scanning system with three lasers mounted on a Zeiss AxioPlan 2IE MOT motorized upright microscope.

**Frozen sections and H–E staining**

The animals from each group were deeply anaesthetized with urethane (1.5 g/kg), and killed by transcardial perfusion of warm physiological saline and 4% paraformaldehyde fixative. The temporal bones were quickly harvested and the stapes removed. The round window membrane and cochlear tip were perforated with a fine needle. The cochlea was gently perfused again with 4% paraformaldehyde fixative in 10 mM PBS (pH 7.4) through the cochlea tip. The temporal bone was immersed in fixative overnight and rinsed in 10 mM PBS twice. Cochleas were decalcified in 10 % EDTA solution overnight in a refrigerator at 4°C for 1.5 days. All cochleae were rinsed in PBS and washed for 30 min in continuous rotation with 20% sucrose, at room temperature and maintained overnight at 4°C in 20% sucrose solution. Cochleae were placed in the cryomold under dissecting microscope and filled halfway with OCT. The cochleae were placed in the OCT and oriented by aligning an imaginary plane through the modiolus parallel with the bottom of the embedding mold. The mold was immediately place in dry ice/ethanol bath. Eight–micron sections were cut with a Leica Cryomicrot 2800 and mounted on Fisherbrand superfrost plus slides. Haematoxylin and Eosin (HE) staining was processed as previously described.

**Spiral ganglion cell count**

As described by Corell 6, the number of spiral ganglion cells was assessed in all 3 cochlear turns on the same side of the modiolus. In each section, all neurons containing a nucleus and having a soma size larger than 20 μm were counted.

**Statistical analysis**

Results were analyzed using student’s t test with STATA7.0 statistical software (STATA Corp, L.P., College Station, TX). Data are presented as mean and standard deviation (SD).

**Results**

**Animals’ general condition**

All animals in the treatment exhibited normal behavior and feeding after drug treatment. Body weight declined in some of the animals following drug administration, which gradually returned to normal. There was no significant difference in body weight between the two groups, and no signs of abnormal vestibular function such as slanting head, limb abduction, forced circular movement and unstable standing. No death occurred.

**ABR threshold after administration**

Seven days after drug administration, no repeatable ABR waveforms were elicited in 10 rats in Group B at 110 dB SPL, while ABR thresholds were normal in Group A (Figure 1).

![Figure 1](image-url)  
Figure 1  ABR thresholds across frequencies on Day 7 after injection of furosemide and kanamycin. A: the treatment group; B: the control group.
**H&E staining and spiral ganglion cell count**

Figure 2 shows that a row of inner hair cells and three rows of outer hair cells from an animal in the control group. In group B, inner and outer hair cells were missing completely on Day 7 after drug administration, although supporting cells remained present and no damage to the spiral ganglion cell body was seen. Average number of spiral ganglion cells was $98.37 \pm 4.56$ ($n = 6$) in the control group and $97.25 \pm 5.73$ ($n = 6$) in the treatment group ($P > 0.05$).

**Immunofluorescence staining**

Neurofilament antibody staining and PI nuclear staining of the whole cochlear preparation showed significantly reduced cochlear nerve fibers in group B 7 days after combined administration of furosemide and kanamycin, compared with the control group. Damage to nerve fibers gradually increased from the apical to the basal turn of the cochlea. In the control group, morphology of the nuclei in inner and outer hair cells was normal with regular nuclear arrangement. However, in the drug treatment group, outer hair cell nuclei had disappeared. Although inner hair cell nuclei were present, their arrangement was disorderly (Figure 3).

**Discussion**
Currently, it is thought that loop diuretics, such as furosemide and ethacrynic acid (EA), mainly cause edema and cystic degeneration of the cochlea stria vascularis, but a single injection of a conventional dose of aminoglycoside antibiotics, such as kanamycin, may not cause inner ear dysfunction or pathologic changes. It usually takes multiple injections for massive necrosis of inner ear hair cells to occur. Studies have confirmed that loop diuretics and aminoglycosides may mutually reinforce their ototoxicity. 7, 8. Yamane et al 9 reported that furosemide could promote the entrance of kanamycin into the inner ear lymphatic space, and speculated that this finding was related to the effects of furosemide enhancing the ototoxicity of kanamycin. They also showed that furosemide and kanamycin had a synergistic effect on ototoxicity. The synergistic effects of kanamycin and furosemide may be because furosemide damages the blood–labyrinth barrier, causing a change in permeability and easier entrance of kanamycin into the inner ear. Furosemide may also damage the excretory function of the cochlear stria vascularis, causing a slower excretion of kanamycin from the inner ear and thus increased accumulation.

In our study, deafness caused by combined furosemide and kanamycin in rats was bilaterally symmetric on Day 7 after drug administration. Studies have shown that after the intramuscular injection of kanamycin, degeneration of supporting cells occurs significantly later than hair cells and degeneration of the spiral ganglion and nerve fibers happens even later. Therefore, it is thought that damage to inner ear sensory cells is the root cause of kanamycin–induced hearing impairment. 10. Our results on the injury of inner ear sensory cells are consistent with these observations, but we find that combined use of furosemide and kanamycin not only causes damage to the inner ear sensory epithelium, but also to auditory nerve fibers. In 2004, Nourski et al. 11 showed that the effects of ethacrynic acid and kanamycin targeted hair cells without causing significant inhibition of responses of the auditory nerve, which is contradictory to our study results. The reason for this difference may be the different time points of observation. The time points of observation were within 10 hours after drug administration in the Nourski study, 12 while our observations were carried out 1 week after drug administration. It is possible that auditory nerve fibers are yet to be damaged within 10 hours after drug administration, but definitely show damage at 1 week.

Furosemide is a loop diuretic. Ototoxicity studies 13 have shown that furosemide can cause pathologic changes in border cells of the cochlear stria vascularis, and that the degree of edema of these cells is a main indicator of loop diuretic caused ototoxicity. The cochlear stria vascularis plays an important role in ensuring the relative stability of the inner ear micro–environment, which is necessary for normal activities of hair cells and the auditory nerve. This is probably an important reason to explain the injury of hair cells and auditory nerve fibers in the current study.

In summary, we find that combined use of kanamycin and furosemide results in severe hearing loss, and that the injury of cochlear outer hair cells is more severe than that of inner hair cells. However, the majority of supporting cells remain undamaged. Spiral ganglion damage is not apparent within a short time period after drug administration, although damage to auditory nerve fibers is present.

Acknowledgments

This work was supported by grants from the Major State Basic Research Development Program of China (973 Program) (#2011CBA01000), National Hi–Tech Research and Development Program of China (863 Program) (#2007AA02Z150) to SMY, the National Natural Science Foundation of China (NSFC) (#30871398, 30730040, 30628030) to SMY, and National Eleventh Scientific Program (2008BAI50B08, 2007 BAI18B12, 2007BAI18B14) to SMY.

References

4  Yang SM, Guo WW, Hu YY, et al. Smad 5 haploinsufficiency leads to hair cell and hearing loss. Dev Neurobiol, 2009, 69:
153–161.

(Received May 30, 2011)