COMBINATIONAL ADMINISTRATION OF AMINOGlyCOSIDES AND LOOP DIURETICS AS AN EFFICIENT STRATEGY TO ESTABLISH DEAFNESS MODELS IN RATS

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Abstract
It is known that aminoglycoside antibiotics can damage the vestibular and auditory sensory epithelia, and the loop diuretics can enhance the ototoxic effect of aminoglycosides. Previous studies on the synergistic effect of these two types of drugs have used mice, guinea pigs and cats, but not rats. The aim of this study was to determine this synergistic effects in rat cochleae. Rats received intravenous injections of different doses of furosemide and/or intramuscular injections of kanamycin sulfate. Auditory brainstem response (ABR), scanning electron microscopy (SEM) and immunocytochemistry were used to determine the effects of drug administration. In the group receiving combined administration of furosemide and kanamycin, the ABR threshold showed significant elevation 3 days after drug administration, greater than single drug administration. The hair cells showed various degrees of injury from the apical turn to the basal turn of the cochlea and from the outer hair cells to the inner hair cells. Neuron fibers of the hair cells showed significant loss 7 days after the drug administration, but the number of spiral ganglia did not decrease and supporting cells showed no signs of injury. Our study suggest that combined administration of furosemide and kanamycin has an synergistic ototoxic effect, and can result in hair cell loss and hearing loss in rats.

Keywords: Kanamycin; Furosemide; Deafness model; Hair cell injury, rat

Introduction
The animal model of deafness is an important tool in studying the pathogenesis of deafness and hearing regeneration. It can be created through noise exposure, ototoxic drug administration and gene manipulation. Drug administration is more frequently used than the other two due to its rather complete elimination of auditory hair cells. To date, numerous methods have been developed using ototoxic drugs to induce deafness, including local application of carboplatin and cisplatin, neomycin infiltration through the round window membrane [1,2], and intramuscular injection of kanamycin, gentamicin and amikacin [3]. Nevertheless, these methods may bring uncontrollable side effects other than deafness, e.g., infection and mechanical damage of the inner ear from local administration of ototoxic drugs; and severe kidney dysfunction with high mortality from systemic administration of aminoglycosides. Therefore, safer and more effective drug administration methods need to be explored. Fortunately, combined application of aminoglycosides and loop diuretics, such as kanamycin and furosemide, has been demonstrated to be able to cause hair cell injury in a faster and safer manner [4-9]. This inspired us to explore a novel method to construct deafness models. In this study, we aimed to testify the synergistic effect of kanamycin and furosemide in rat cochleae and establishing a preliminary
criterion in drug dosages for deafness induction. The reason of choosing rats was that they are more advantageous than other rodent species for deafness model construction. First, rats costs lower in breeding than guinea pigs. Second, rats have a larger body than mice, more suitable for cochlear surgeries. The auditory brainstem response (ABR) was measured to determine the hearing threshold after drug application. Immunocytochemistry and confocal microscopy were performed to evaluate the inner ear morphology. To our knowledge, this is the first report of establishing rat deafness model through combinational application of aminoglycosides and loop diuretics.

Materials and Methods

Drug Administration

Ninety healthy 4-week-old SD rats (100-110 g) were randomly assigned into 6 groups, with 15 animals in each group (Table 1). The animals were anesthetized with intraperitoneal injection of 10 % chloral hydrate (0.45 ml/100 g). The left jugular vein was exposed and injected with furosemide, whereas kanamycin was injected intramuscularly in the thigh as described previously [4]. Group A was the control without any treatments. Groups B and C were injected with either furosemide or kanamycin alone in the doses of 200 mg/kg or 1000 mg/kg, respectively. Groups C, D, E and F received both drugs in different dosage combinations. All the animals exhibited normal behavior and were on normal diet after the drug administration, and there were no signs of abnormal vestibular function. Care and use of animals were approved by the Institutional Animal Care and Use Committee of Chinese PLA General Hospital.

ABR Measurements

Details of ABR measurements were provided elsewhere [3]. Briefly, the animals were anaesthetized with xylazine and ketamine. The recording electrodes were inserted at the vertex and pinna. ABRs were evoked with clicks and/or 5 ms tone pips (0.5 ms rise/fall, at 30/sec) at frequencies of 4, 8, 16, and 32 kHz. The signal was amplified, filtered, and averaged using the Intelligent Hearing System (USA). The sound level was raised in 20- and/or 5-dB steps. At each level, 1024 responses were averaged. Both ears were measured.

Immunocytochemistry and Scanning Electron Microscopy

The cochleae were perfused with 4.0 % formaldehyde and were 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 PBS, followed by incubation with the secondary antibodies. To stain nuclei, the DNA-specific label propidium iodide (PI) was used as described previously [9]. The samples were mounted on the glass slides with antidote solution (Prolong Antifade Kit, Molecular Probes) and examined using confocal scanning system (LSM 510 META, USA) with three lasers mounted on a Zeiss AxioPlan 2IE MOT motorized upright microscope.

For SEM, the cochleae were fixed with 2.5 % glutaraldehyde in the 0.1 M sodium cacodylate buffer (pH 7.4) containing 2 mM C,CI, then was washed in PBS and post-fixed for 15 minutes with 1 % OSO, in the same buffer. The tissues were dehydrated in an ethanol series, critical point dried from CO2 and sputter-coated with gold then examined using a Hitachi S-3700N scanning electron microscope.

Statistical Analysis

All the data were analyzed using the statistical software STATA7.0 (STATA Corp, L.P., College Station, TX, USA). The student’s t test was applied with p value 0.05 being significant. The data were presented as mean ±standard deviation (SD).

Results

ABR Results

We performed ABR measurements at three time points after the drug administration (3 days, 1 week and 2 months, respectively). The purpose of doing so was to test whether the combined administration of furosemide and kanamycin would be able to induce stable hearing loss over a long-term period. Fig. 1 shows the average ABR thresholds of each group measured at four testing frequencies (4, 8, 16 and 32 kHz). Compared with the control (group A) at any time point, the ABR thresholds of the groups receiving kanamycin or furosemide alone (Groups B and C) did not show significant changes at all four frequencies tested (Fig. 1; p> 0.05). In the groups receiving both drugs (Groups D, E and F), however, the ABR thresholds were elevated significantly at all tested frequencies (p <0.01). In Groups D and E, we applied the same dose of furosemide (100 mg/kg), but used different doses of kanamycin (500 mg/kg for Group D and 1000 mg/kg for Group E). We noticed that the higher dose of kanamycin...
cin caused more hearing impairment, manifested by a higher elevation of ABR thresholds in Group E than D. By contrast, Group F received twice the dose of furosemide (200 mg/kg), but a very low dose of kanamycin (100 mg/kg). Surprisingly, the ABR thresholds elevation exceeded maximum stimulus intensity (110 dB SPL) of the test system.

**SEM of the Basilar Membrane**

Scanning electron microscopy (SEM) was used to examine the basilar membrane (BM) after the drug administration to evaluated ototoxic effects of furosemide and kanamycin on the auditory hair cells. In the groups treated with furosemide or kanamycin alone (Group B or C), the stereocilia of inner hair cells (IHCs) and outer hair cells (OHCs) were clearly visible on the cuticular plate in normal shape and organization (Fig. 3B and C) as seen in the control (Fig. 3A). In Group D, however, the stereocilia of OHCs were completely depleted in the basal turn while remaining intact in the apical and middle turns (Fig. 3D). The stereocilia of IHCs remained intact in the entire BM. Similar findings were observed in Group E, where the stereocilia of OHCs were completely missing in the BM, while the stereocilia of IHCs remained intact in the apical turn and sporadically visible in the middle turn, but were completely missing in the basal turn. Different from these findings, complete depletion of the stereocilia of both IHCs and OHCs were observed throughout the entire BM in Group F. Notable scarring of the BM was also observed.

**Immunofluorescence staining**

Neurofilament antibody staining and PI nuclear staining of the whole cochlear preparation showed that in group F, the number of the cochlear nerve fibers was significantly reduced 7 days after combined administration of furosemide and kanamycin compared with the control group. Damage to the nerve fibers was gradually aggravated from the apical turn to the basal turn of the cochlea. In the normal control group, the morphology of the nuclei in the inner and outer hair cells was normal and the nuclear arrangement was regular. However, in the drug administration group F, the nuclei in the outer hair cells disappeared. Although the nuclei of the inner hair cells were still present, the architecture of the organ of Corti was disrupted (Figure 3).

**Figure 1:** ABR thresholds measured from six groups. A: ABR thresholds (means SD, n=30) were measured at 3 days after injection. B: ABR thresholds (means SD, n=30) were measured at 7 days after injection. C: ABR thresholds (means SD, n=30) were measured at 2 months after injection. Tone pips with frequencies of 4, 8, 16, and 32 kHz were used to evoke ABRs.
Figure 2. Scanning electron microscopy of cochlear toxicity of injection of furosemide and/or kanamycin sulfate in rats. A1, A2, A3, B1, B2, B3, C1, C2, and C3 were the basal, middle, and apical turns of the rat cochlea in Groups A, B, and C, respectively, and one row of inner hair cells and three rows of outer hair cells could be seen. D1, D2, and D3 were the basal, middle, and apical turns of the rat cochlea in Group D. In the apical and middle turns, one row of inner hair cells and three rows of outer hair cells could be seen. The inner hair cells were intact in the basal turn, and the outer hair cells were completely missing. E1, E2, and E3 were the basal, middle, and apical turns of the rat cochlea in group E. In the apical turn, the inner hair cells were intact, and the outer hair cells were completely missing. In the middle turn, the inner hair cells were missing extensively, and the outer hair cells were missing completely. In the basal turn, inner and outer hair cells were all missing. F1, F2, and F3 were the basal, middle, and apical turns of the rat cochlea in group F and the inner and outer hair cells in the whole cochlea were missing. OHC: outer hair cells, IHC: inner hair cells. Scale: A1, A2, B1, B2, C1, C2, C3, D1, D2: 12 μm; A3, D3: 6 μm; B3, E1, E2, E3: 10 μm; F1, F2, F3: 15 μm.

Figure 3. Immunofluorescence staining of the whole cochlear basilar membrane preparation in group F and in the normal control group 7 days after drug administration. A is the basilar membrane preparation of the basal, in normal rats.B is the basilar membrane preparation of the basal turns of the cochlea in the rats of group F 7 days after drug administration.

Discussion

Currently, it is thought that loop diuretics, such as furosemide and ethacrynic acid, mainly cause edema and cystic degeneration of the cochlear stria vascularis, decrease in the magnitude of the cochlear action potential, inhibition of K⁺-Na⁺-ATP enzyme, and edema of the outer hair cells of the organ of Corti [11,12]. The primary ototoxicity of aminoglycoside antibiotics, such as kanamycin, affects cochlear hair cells, which results in irreversible hearing loss [13]. In animal experiments, a single injection of a conventional dose of loop diuretics, such as furosemide, may cause reversible hearing loss from transient disorders of the microcirculation of the cochlear stria vascularis, but a single injection of a conventional dose of aminoglycoside antibiotics, such as kanamycin, often does not cause inner ear dysfunction or pathologic changes. Only after multiple injections, massive necrosis of hair cells may occur because of the accumulation of high concentrations of kanamycin in the perilymph fluid and delayed excretion [14]. In this study, we showed that a single injection of kanamycin or furosemide did not cause any elevation in ABR threshold or hair cell loss in rats. We speculated that the reason why we did not see threshold change may be the time points of observation after drug administration. Because the effects of loop diuretics on the cochlear stria vascularis are temporary, hearing may have restored to normal in rats on the third day after drug administration. Other studies have shown that administration of low-dose antibiotics do not cause significant changes in cochlear sensory epithelium [15].

Previous studies have demonstrated that loop diuretics and aminoglycosides may mutually reinforce ototoxicity [16] Yamane et al. [17] reported that furosemide could promote the entrance of kanamycin into the inner ear lymphatic space, and suggested that this was related to the effects of furosemide, enhancing the ototoxicity of kanamycin. Our study also showed that furosemide and kanamycin had a synergistic ototoxicity effect. The synergistic effects of kanamycin and furosemide may be produced because furosemide damages the blood-labyrinth barrier, causing a change in permeability, which results in easier entrance of kanamycin into the inner ear. Furosemide may also damage the excretory function of the cochlear stria vascularis, causing slower excretion and slow accumulation of kanamycin in the inner ear. When furosemide was administered at a low dose (100 mg/kg), elevation of the ABR threshold was positively related to the dose of kanamycin within a certain range. The combined injection of kanamycin at a low dosage (500 mg/kg) caused a slight increase in the ABR threshold. Injection of KM at a high dose (1000 mg/kg) caused a significant increase in the ABR threshold. When a high dose of furosemide was given (200 mg/kg), a very small dose of kanamycin (100 mg/kg) was required to cause ABR thresholds > 110 dB SPL at all frequencies. The SEM results are consistent with the ABR thresholds. When low dose of furosemide (100 mg/kg) was given, the loss of cochlear hair cells was positively correlated with the dose of kanamycin within a certain range. When high dose furosemide was given (200 mg/kg), a very small dose of kanamycin (100 mg/kg) was sufficient to cause loss of all inner and outer hair cells in the entire cochlea. We speculate that the mechanism for this phenomenon may be that the damage by
low-dose furosemide (100 mg/kg) to the cochlear stria vascularis was mild, the blood labyrinth barrier was opened partially temporarily, and kanamycin entered into the lymphatic space slowly. Therefore, injection of large-dose kanamycin (1000 mg/kg) can result in a concentration that may cause severe injury to cochlear hair cells. However, when a large dose of furosemide (200 mg/kg) was administered, furosemide caused greater damage to the cochlear stria vascularis, the blood labyrinth barrier was jeopardized for a longer time, and kanamycin entered the lymphatic space more rapidly. Therefore, injection of low-dose kanamycin (100 mg/kg) resulted in rapid accumulation of kanamycin in the inner ear. Russell et al. concluded that the variable efficacy in different animals may be attributed to different clearance rates of kanamycin and EA in animals, and that the toxic effects of combination therapy primarily depends on the dose of EA. Our study also showed that when the dose of EA was low, even an increased dose of kanamycin could only achieve 53.3% hair cell loss. When the dose of EA was high, low-dose kanamycin caused complete hair cell loss.

Deafness caused by combined furosemide and kanamycin in rats was bilaterally symmetric and hearing loss occurred 3 days after drug administration. Histological studies on the cochlea of rats with deafness have found that cochlear hair cell damage always gradually expands from the cochlear basal turn to the apical turn, and the damage to the outer hair cells always precedes that of inner hair cells. The mechanism of furosemide- and kanamycin-induced hair cell injury may be that the outer hair cells have greater capability to engulf kanamycin, and that this capability is greater in hair cells at the basal turn than hair cells at the apical turn. We speculate that the reason why hair cells at different places have different drug uptake capacities is related to the distribution of drug transporters on the cell membrane. Basal turn hair cells also have more stereocilia and transduction channels. Studies have shown that after intramuscular injection of kanamycin, degeneration of supporting cells occur significantly later than hair cells and that degeneration of the spiral ganglion and nerve fibers takes place even later. Therefore, it is thought that damage to the inner ear sensory cells is the root cause of kanamycin-induced hearing impairment. Our results on the injury of the inner ear sensory cells are consistent with previous studies. However, we found that combined use of furosemide and kanamycin not only caused damage to the inner ear sensory epithelium, but also injury to auditory nerve fibers. Nourski et al. showed that the effects of EA and kanamycin targeted hair cell injury without causing significant inhibition of response of the auditory nerve. 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, compared to 1 week after drug administration in our observation. It is possible that the auditory nerve fibers are not yet damaged within 10 hours after drug administration, as the damage may occur slowly. Furosemide is a loop diuretic, and studies on ototoxicity have shown that furosemide can cause pathologic changes of border cells in the stria vascularis, and the degree of edema of border cells is the main indicator of ototoxicity caused by a loop diuretic. The stria vascularis plays an important role in maintaining inner ear micro-environment, which is necessary for the normal activities of hair cells. The stability of this microenvironment is critical for the functioning and survival of hair cells.

In summary, we have established a rat model of deafness through single administration of furosemide or kanamycin or combined administration or the two. We have found that only combined use of kanamycin and furosemide result in severe hearing impairment, with more severe injury in cochlear outer hair cells than in inner hair cells. However, the majority of supporting cells would remain undamaged. Spiral ganglion damage may not be apparent within a short time period after drug administration, although damage to the auditory nerve fibers has appeared. No hair cell repair was observed in this study. When the dose of furosemide is high, even low doses of kanamycin can cause serious damage to the cochlea. Single, one-time injection of furosemide or kanamycin results in little to no cochlear damage.

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References

[4] West BA, Brummett RE, Himes DL. Interaction of kanamy-


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