Ultrastructural morphology of the guinea pig inner ear after systemic gentamicin application.

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Introduction

Aminoglycosides were introduced in the late forties. After several years it was found that these antibiotics could cause ototoxic damage. Wersall (1–4) was one of the first investigators who reported on the morphological changes that took place in the inner ear of guinea pigs, after administration of gentamicin. Aran (5) described altered cochlear and vestibular functions and morphological changes after treatment with gentamicin, dibekacin or tobramycin in guinea pigs. Collins (6) and Twine (7) investigated the influence of the dose of gentamicin on inner ear damage. Collins demonstrated hair cell damage in the cochlea of guinea pigs while Twine described the loss of sensory hair cells in the utricular and saccular maculae after application of various doses of gentamicin.

Over the years, more knowledge was acquired about the mechanism of the effects of gentamicin on the sensory organs at the cellular level. Mitochondrial damage in the inner ear sensory cells has been described by Sjoback (8), while Yoshizawa (9) investigated ribosomal changes after gentamicin treatment. More recently, scientific interest has been focussed on the process of apoptosis. Forge (10) reported the apoptotic death of hair cells in sensory epithelia after gentamicin application in cultured explants of vestibular organs. Different mechanisms of hair cell loss, apoptosis and extrusion were described by Li (11). An important role in the apoptosis of cells was ascribed to membrane blebbing, as explained by Levarrier (12). Takumida reported about the relation between stereociliar fusion and the glycocalyx on the sensory hair cells (13,14) and about the effect of gentamicin treatment on cytoskeletons (15).
This paper reports on an experiment that was performed to investigate the morphological damage in the inner ear organs (utricle, saccule and cochlea) after systemic application of two different doses of gentamicin.

Materials and methods
Eighteen healthy female albino guinea pigs (Harlan, the Netherlands) with a positive Preyer’s reflex and a weight of 400–500 gram were used in this experiment. The experimental Animal Committee of the Groningen University approved animal care and use, protocol number 2325, in accordance with the principles of the Declaration of Helsinki.

Administration of gentamicin
Intra-muscular injections were given daily, during three periods of five days, with a pause of two days between the periods. Gentamicin was given in two doses. One group (n=7) received 50mg/kg, and the other group (n=7) 100mg/kg. One week after the last administration of gentamicin the guinea pigs were sacrificed by means of an intra-cardial injection of sodium pentobarbital (60 mg/kg) for light microscopical (LM), scanning (SEM) and transmission (TEM) electron microscopical investigation of the inner ear organs. Four animals were sacrificed as a control group for TEM and SEM investigation.

Fixation
After termination, the animals were decapitated; the temporal bones were removed and placed in ice-cold HBSS (pH 7.4; 320 Mosm; 0–40C). The vestibulum was opened, and the utricle was located and isolated without damaging the saccule and the cochlea. With a fine pair of tweezers the otolithic membrane was gently removed and then the utricle was fixated in a solution of 2.5% glutaraldehyde in 0.1M Na-cacodylate buffer (pH 7.4; 400 Mosm; 40C) and 2mM calcium chloride. The remaining part of the temporal bone including the cochlea and the saccule, was immersed in fixative of the same composition.
Scanning Electron Microscopy

The TAO–method was performed as post–fixation \(^{(16)}\). This method uses a combination of tannic acid, arginine hydrochloride, glycine, sodium glutamate, sucrose and OsO\(_4\) for optimal preservation and contrast of ultrastructures.

After post–fixation, the specimens (utricle, saccule and cochlea) were rinsed in distilled water and dehydrated in ethanol. All specimens were Critical Point dried with liquid CO\(_2\) and were sputtercoated with Au–Pd (10nm) according to routine procedures. The specimens were studied in a JEOL FEG–SEM, type 6301F, operating at 5kV.

Transmission Electron Microscopy

After fixation, the specimens were decalcified for five days in 10\% EDTA (pH 7.4, RT), post–fixed in 1\% OsO\(_4\) with 1\% K\(_4\)Ru(CN)\(_6\) for three to four hours, gently rinsed in distilled water, dehydrated in a graded ethanol series followed by propylene oxide, and infiltrated using a mixture of 1:1 propylene oxide and Spurr’s low–viscosity resin for one hour and pure resin overnight. Polymerization took place at 700C after exsiccation in vacuum. Sections (\(\mu m\)) of the cochlea (midmodiolar plane), saccule and utricle were cut and stained with toluidine blue, for light microscopic examination. After light microscopic examination, altered areas were selected for further inspection. Parts of the specimens were embedded again and ultra–thin sections of 100nm of the organ of Corti, utricle and saccule were made, contrasted with 2\% uranyl acetate in 100\% methanol and lead citrate according to Reynolds, and examined using a Philips 201 transmission electron microscope (TEM), operating at 40 kV.
Results

Scanning Electron Microscopy (SEM)

**Utricle**
Limited hair cell loss with degeneration was observed on the surface of the utricles of the 50mg group. The number of stereocilia of the hair cells in the peripheral areas near the striola decreased. The hair cell bundles were dissociated. In the striolar region fusion of stereocilia, lifting of the cuticular plate and extrusion of the cytoplasm (blebbing) were found (figure 1a). The striolar area also showed an increased number of microvilli with extended height. The 100mg series displayed severe hair cell loss in the striolar part. This phenomenon was also seen in the peripheral regions next to the striola (figure 1b). The remaining hair bundles in the 100mg series demonstrated a comparable degeneration pattern as described in the 50mg group.

**Saccule**
Both groups showed a minimal loss of hair cells in and around the striola. Fusion of stereocilia and extrusion of whole cells were not seen. In some specimens extrusion of the cytoplasm (blebbing) was found. Hair bundles in and near the striola often showed a dissociated aspect (figure 1c). In some cases the striolar region displayed an increased number of supporting cells in the 100mg group (figure 1d). Overall the damage was less extensive than in the utricle.

**Cochlea**
In the 50mg series the basal turn of the cochlea showed no damage. In the middle turns, the third row of the outer hair cells showed a loss of stereocilia and disintegration of the hair cell bundles (figure 2a). In the apical turn, in some cases, a loss of the third row of the outer hair cells was observed. The second row demonstrated loss of stereocilia and dissociating hair bundles. The inner hair cells sometimes showed stereociliar disarrangement, but severe signs of degeneration were not found (figure 2b).

In the 100mg group most specimens showed severe damage in the basal, middle and apical turns (figure 2c). A total loss of hair cells (inner and outer hair cells) was observed in some specimens (figure 2d). In most specimens disarrangement of the hair bundles in the outer hair cells and fusion of the stereocilia of the inner hair cells were detected. Deteriorated hair bundles
Figure 1  A (50mg/kg) and B (100mg/kg) gentamicin–treated maculae of the utricle. The striolar regions are shown at different magnifications. Fusion (F), dissociation (D), lifting of the cuticular plate and a decreased number of stereocilia are clearly visible. In figure B hair cell loss in and next to the striola (S) is found. The holes (arrows) are extruded hair cells. C and D–show maculae of the saccule treated with gentamicin–treated saccules (C–100mg/kg, D–50mg/kg). C–Disintegration (D) of the stereocilia and a decreased number of stereocilia is visible in the striolar part (S). D–figure D shows no hair cell loss in the saccule (the cracks in the surface are artefacts caused by the critical point drying procedure).
Figure 2. Cochlear damage caused by gentamicin application (A and B 50 mg/kg, C and D 100 mg/kg). A, B and C show disintegration (D) of the hair bundles and a diminishing number of stereocilia in OHC2 and OHC3. The inner hair cell bundles and OHC1 show less damage. In the outer rows the lifting of the cuticular plate is visible (arrow). Figure D shows an inner hair cell with fusion (F) of the remaining stereocilia. Remarkable is the increased number of microvilli (M) with extended height. Scale bar A, B, C and D = 10 µm.
always demonstrated a decreased number of stereocilia and in most cases the hair cells showed a total loss of the shortest and a partial loss of the middle row of stereocilia.

**Control group**

In the control group a normal morphology without pathological alterations was observed in the utricle, saccule and cochlea.

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**Figure 3** Light-microscopic view of a normal (A) and gentamicin-treated (B, 100mg/kg) utricle. In figure B, the damage and loss of sensory hair cells loss is easily seen. The nuclei (arrows) seem to be pyknotic and vacuolization (arrowheads) is clearly visible in the sensory epithelium. NF = nerve fiber, HCB = hair cell bundle. Scale bar = 20µm.
Transmission Electron Microscopy (TEM)

After light microscopic examination (figures 3a and 3b), altered areas were selected for further inspection at ultrastructural level to evaluate the degeneration of the inner ear organ.

Utricle and saccule
Both dose groups showed similar intracellular degenerative forms in the utricle and saccule (figure 4, 5). This degeneration consisted of swollen or leaking mitochondria, vacuolisation, dilatation of the endoplasmic reticulum, in some cases absence of ribosomes and glycogen, extrusion of the cytoplasm, leaking cell membranes, lifting of the cuticular plate and fusion of stereocilia. In some cases the nucleus of the cell was pyknotic, dislocated and irregular shaped in affected hair cells. In most specimens efferent and afferent nerve endings were present, and in some cases mitochondria in these nerve endings showed degeneration. The junctions between the cells remained intact, but when extrusion of the cytoplasm occurred, the cell membrane dissociated from the supporting cells and the junctions were interrupted.

Cochlea
The affected inner and outer hair cells showed degeneration of the mitochondria, intracellular vacuoles (caused by dilatation of the endoplasmic reticulum), vacuoles in the cuticular plate, occasionally absence of stereocilia and multivesicular bodies and Hensen’s bodies. In severely damaged hair cells the number of lysosomes had increased, while the ribosomes and glycogen were dissolved. In some cases the cytoplasm disappeared, and only the cell membrane remained (ghost cells) (figure 6a). The innervation of the hair cells appeared to be normal. Some specimens demonstrated degenerated mitochondria and vacuoles in the nerve endings (figure 6b).

Control group
In the control group a normal morphology without pathological alterations was observed in the utricle, saccule and cochlea.
Figure 4: Transmission electron microscopic (TEM) views of various stages of degenerated utricular sensory epithelium, treated with gentamicin. Figure A shows fusion (F) of stereocilia (S). The actin components are totally disintegrated (inset). In figure B the reduced cytoplasm volume and the increased calyx (C) volume are remarkable. Only remnants (R) of the cytoplasm are left in figure C. Figure D demonstrates vacuolization (V) in the cuticular plate and degenerating mitochondria (arrows). The nucleus (arrowhead) is pyknotic. Some mitochondria (arrows) display dilated and swollen membranes in figure E. Figure F shows protrusion (P) of cell contents. Scale bar A, B, C, F = 5 µm and scale bar A (inset), D, E = 1 µm.
Figure 5  TEM view of saccules treated with gentamicin. Figure A displays vacuolization (V) in the cytoplasm and the cuticular plate. Arrows show lifting of the cuticular plate. The remnants (R) of the cytoplasm and pyknotic nuclei (P) are visible in figure B. Scale bar A, B = 5 µm.
Figure 6  In figure A, the cytoplasmic contents of the outer hair cell (arrow) nearly disappeared. Figure B shows afferent nerve endings (NE) of an outer hair cell with swollen and leaking mitochondria (arrows). Scale bar A = 5µm and scale bar B = 1µµm.
Discussion

This study demonstrates morphological damage to the utricle, saccule and cochlea at light microscopical and ultrastructural level after systemic application of gentamicin in two doses.

In the control group a normal morphology without pathological alterations was observed in the utricle, saccule and cochlea, which excludes artefacts a possible explanation for the described changes. Comparable damage of the cochlea and vestibular system is described after systemic administration of gentamicin, as shown by Wersall (1), Twine (7) and Forge (10). However, topical administration near the round window membrane provides a quicker and more devastating effect on all inner ear organs (10,1).

The utricles of the 50mg group showed limited hair cell loss in and near the striolar region while the 100mg group displayed severe hair cell loss in and next to the striola and the lesions extended to the peripheral areas. The disarrangement of the hair bundles can be explained by the loss of the side and tip links between the stereocilia (13-14). In many cases holes were found in the striolar region and these holes can be interpreted as rejection of complete hair cells, which is in agreement with earlier observations (11). In the saccule, only limited hair cell loss was observed by scanning electron microscopy, indicating less susceptibility of the saccule for gentamicin.

The cochlear results display clear differences between both groups. In the 50mg group the organ of Corti showed no hair cell loss while in the 100mg group the loss of great numbers of sensory cells (inner and outer hair cells) in all turns were observed. In both groups the cochlea demonstrated more extensive damage in the apical and middle turns in comparison to the basal turn, while the most pathological changes were found in the third row of the outer hair cells. The cochlear damage we found in our experiment is in contrast with the findings of Forge (10) and Collins (6). They observed the start of hair cell degeneration in the first row of the outer hair cells in the upper basal and middle turns of the organ of Corti. The use of albino guinea pigs in our experiment might explain the difference in result. Conlee (17-18) reported on differential susceptibility to gentamicin ototoxicity between albino and pigmented guinea pigs. In these papers it was suggested that cochlear melanin might inhibit gentamicin activity explaining the greater outer hair cell loss in albino guinea pigs in comparison to pigmented guinea pigs. However, the difference in location between our observations and other reports remains unclear and needs further investigation.
The severe intracellular degeneration in the utricle, saccule and cochlea as observed with transmission electron microscopy are in agreement with earlier studies (8, 3). However, scanning electron microscopy showed evident differences in degeneration between utricle and saccule in both groups, while an identical pattern in degeneration was observed with transmission electron microscopy. These observations may indicate that the pathological changes initiated in the intracellular part of the sensory cell, ultimately lead to the degradation of the apical located stereociliar complex.

In conclusion, gentamicin application induces extensive morphological changes of the inner ear in a clear dosage response. Scanning and transmission electron microscopy demonstrate a different susceptibility for gentamicin ototoxicity between the saccule and utricle.