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Gentamicin-induced structural damage of human and artificial (biomimetic) otoconia

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Abstract

Conclusions: Gentamicin causes irreversible structural damage of human and artificial otoconia by progressive dissolution of calcite. The inner architecture of otoconia is strongly affected by degradation scenarios during gentamicin exposure. Artificial otoconia can be used as a model system mimicking the chemical attacks for detailed investigations. *Objectives:* To investigate the chemical interactions of gentamicin with natural calcite and human and artificial otoconia under in vivo conditions. *Methods:* Pure calcite crystals and artificial and human otoconia were exposed to gentamicin injection solutions at various concentrations. Morphological changes were observed in time steps by the use of environmental scanning electron microscopy (ESEM). *Results:* Dissolution of pure calcite crystals results in the formation of well oriented nanoshoots indicating an irreversible chemical reaction with gentamicin. Human and artificial otoconia reveal irreversible structural changes of their surface areas as well as of their inner structure, resulting in characteristic changes at different gentamicin concentrations. Minor changes are first observed by surface alterations and dissolution of calcite in the belly region. Major changes result in further reduction of the belly area reaching the center of symmetry. Finally, a complete dissolution of the branches takes place. Artificial otoconia provide detailed insight into surface alterations.

Keywords: *Aminoglycosides, calcium, calcite, calcium carbonate, BPPV, otolith organs, oVEMP, cVEMP, saccule, utricle*

Introduction

Mammalian otoconia are calcite-based biominerals representing an essential part of macular end organs and acting as sensors for linear accelerations and head tilts. Meanwhile, a substantial knowledge of the external and internal morphology of otoconia exists [1–3]. Human otoconia represent nanocomposites of mainly calcite, which is the chemically stable form of calcium carbonate and a minor component of organic material (<5% of their weight), mainly glycoproteins. On the nanoscale, organic fibrils together with calcite show a nanostructured intergrowth arrangement. Because of the slight mismatch between the calcite nanoparticles, otoconia are characterized as highly mosaic-controlled nanocomposites [2]. Otoconia exhibit a typically uniform shape, which is independent of their size. The inner structure shows different volume densities of the calcite nanocomposite, determining a uniform architecture [2,3]. The structure of otoconia can be altered during aging and following chemical attacks due to systemic administration of ototoxic medications such as ethacrynic acid as well as the aminoglycosides streptomycin and gentamicin [1,4–10].

In hair cells, aminoglycosides block mechanotransducer channels, Ca^{2+}-activated K^+ channels, ATP receptors, and nicotinic acetylcholine receptors, respectively. Hair cell death is assumed to be mediated by caspase activation and reactive oxygen

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species. Priuska and Schacht suggest that gentamicin in particular acts as an iron chelator and the iron–gentamicin complex is an effective catalyst of free radical formation [11,12]. However, detailed information about morphological changes of human otoconia is limited at present [1,4].

Morphological changes due to gentamicin, especially in vital human otoconia, have not been studied in detail up to now. Moreover, the underlying chemical mechanisms have not been investigated so far.

Recent studies have shown that it is possible to grow biomimetic (artificial) otoconia such as calcite gelatin composites (CGC) and calcite gelatin/agarose composites (CGAC) [13]. The growth of artificial otoconia and their inner architecture and outer shape, as well as their chemical and structural characterization as nanocomposite material, clearly show that artificial otoconia are closely related to human otoconia in every respect [2,13]. Therefore, it has been proposed only recently that artificial otoconia are suitable model systems for further investigations; for example, the simulation of chemically induced morphological changes [2,3].

The aim of this study was to investigate the effect of gentamicin on structural changes of artificial and vital human otoconia under in vitro conditions in detail.

Material and methods

Dissolution of pure calcite

To clearly show the significant reactivity of gentamicin towards calcite, we exposed the rhombohedral face of a bulk calcite single crystal (1.0 × 0.5 mm in size) to gentamicin injection solution (gentamicin sulfate) (c = 40 mg/ml, 1:2, 1:5, 1:10, and 1:100) at room temperature. Surface changes were monitored in time steps and investigated by an environmental scanning electron microscope (ESEM; FEI Quanta 200 FEGi, Eindhoven, The Netherlands).

Gentamicin exposure of human and artificial otoconia

Vital human otoconia were extracted from patients undergoing transmastoid labyrinthectomy for sporadic vestibular schwannoma as described recently [3]. Human utricles were identified and extracted with the maximum magnification of a surgery microscope (OPMI Vario/S 88; Carl Zeiss, Oberkochen, Germany) after removing the bony structures from the semicircular canals and the vestibule. Specimens were harvested after cutting out endolymphatic tissue with a beaver knife and were immediately fixed in ethanol (96%) for further structural investigation.

Samples lying in the gelatinous matrix were identified by light microscope (Axioplan 2 imaging, Carl-Zeiss) with 300-fold magnification and transferred to conductive (polycarbonate/graphite) foil discs (G3347, FEI/Philips) for investigations by ESEM. Groups of intact human otoconia were identified at higher ESEM magnifications (>1:10 000). For investigation under high vacuum (HV) modes (2×10^{-4} Pa), some samples of human otoconia were coated with gold (Au) to obtain a reliable conductivity of the surface.

Artificial otoconia were produced by double diffusion into a gelatine gel matrix according to methods described previously [2,13]. For CGC a gelatine gel (denatured collagen) and for CGAC agarose together with gelatine (weight-ratio 6:4) was used. The respective gel mixtures were taken as the diffusion matrix, which at the same time was incorporated into the inorganic/organic composite system during growth. Before ESEM investigations the organic net surrounding the composite part of CGC and CGAC was removed by washing with warm water to visualize structural changes in more detail.

Human and artificial otoconia were exposed to sulfate injection solution (c = 40 mg/ml, 1:5, 1:10, and 1:100) for a defined period of time. Structural changes of specimens were studied by ESEM by use of uncoated specimens in low vacuum (LV, 60 Pa). Acceleration voltages varied between 15 and 25 kV in time steps of several minutes. Acceleration voltages varied between 15 and 25 kV.

Ethics

The study was conducted in conformity with the Declaration of Helsinki 1975, revised in 1983, and approved by the ethics committee of the University Medicine Mannheim (2012-612N-MA).

Results

Dissolution of pure calcite

The etching of a pure calcite crystal with gentamicin causes changes in the surface structure. Depending on the concentration, calcite is gradually dissolved by gentamicin, leaving characteristic nanospikes of 500 nm to 1 μm in length. Higher magnifications (up to 10 000-fold) revealed the spikes as etch figures possibly representing trigonal scalenohedra (Figure 1).

Gentamicin-induced structural changes in human and artificial otoconia

Human otoconia show gradual morphological changes depending on concentration and time of

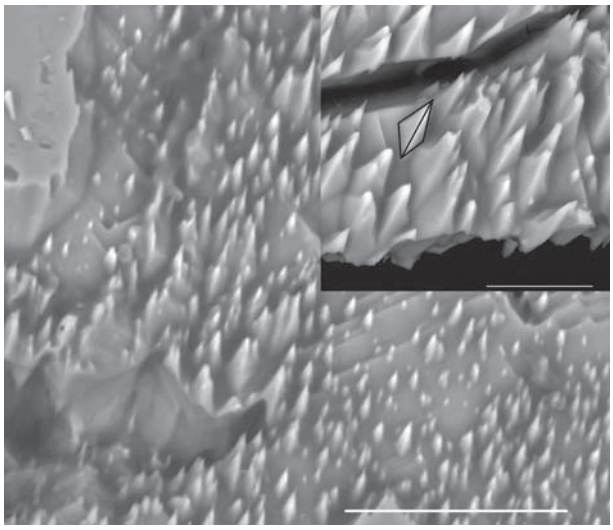

Figure 1. Environmental scanning electron microscope (ESEM) image of a pure calcite crystal (rhombohedral plane) after exposure to gentamicin solution (c = 40 mg/ml, 30 min). The inset shows nanoshoots rooted on the surface of the calcite crystal after the etching procedure. The inset reveals nanoshoots possibly representing trigonal scalenohedra as highlighted by black lines.
Scale bar 20 µm; inset 10 µm, 20 kV; LV, low vacuum.

gentamicin exposure up to the lowest concentrations of gentamicin within a short period of time (Figure 2). The resulting structural damage to the human otoconia in the entire sample reached different stages. Some otoconia located in the lower layers and covered by organic matrix were less affected or were affected at a later stage. However, the structural changes had identical characteristics. Human otoconia lying superficially showed step by step damage of the architecture via different stages up to complete dissolution. Structural changes occurred first superficially in the mid section of the body, which is called the 'belly' region, causing a radial dissolution reaching the center of symmetry of the individual otoconium but leaving trigonal structures in the direction of both ends of the otoconia. The rhombohedral end faces including the trigonal structures ('branches') were affected later. Finally, a complete dissolution of the calcite component of the nanocomposite structure was observed. During the dissolution of human otoconia the outer shape of the individuals remained unaffected because the net of organic fibrils covering the residues remained stable.

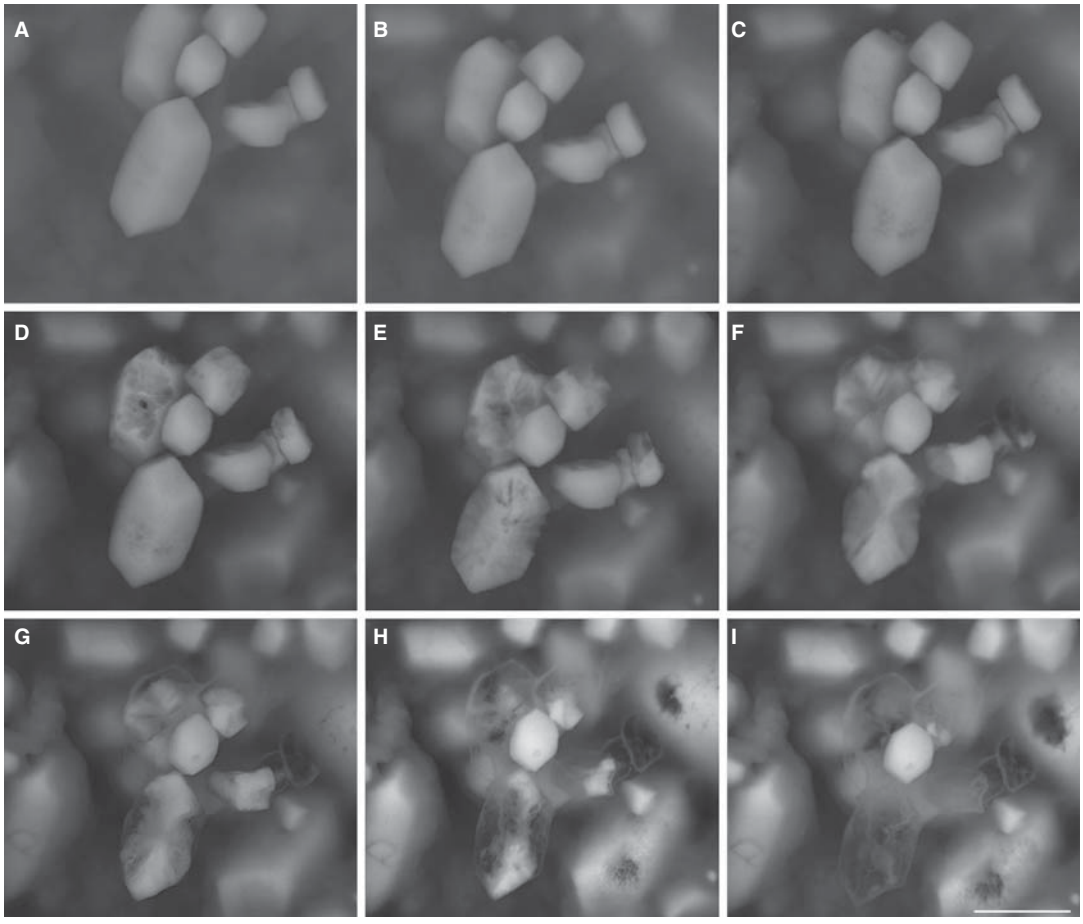

Figure 2. Human otoconia after gentamicin exposure (5 mg/ml) showing gradual changes of irreversible structural damage: (A) before exposure, (B–I) 1 min steps.
ESEM, Environmental scanning electron microscope; LV, low vacuum; scale bar 5 µm, 15 kV.

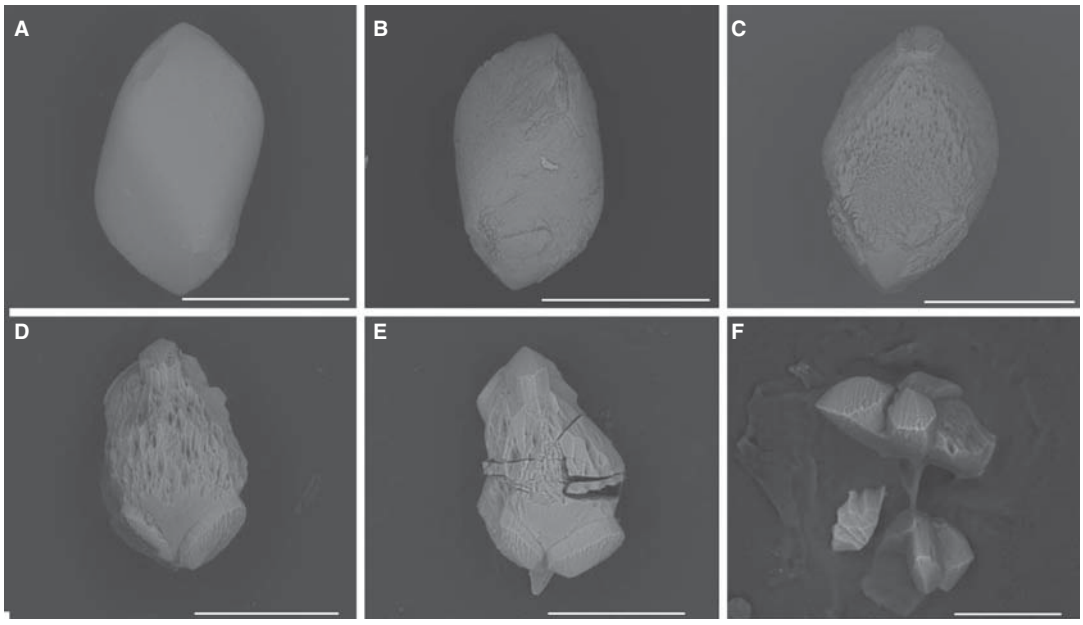

Figure 3. Morphological changes of artificial otoconia (calcite gelatin composites, CGC) after gentamicin exposure (40 mg/ml): (A) before exposure, (B) after 120 min, (C) after 540 min, (D) after 26 h, (E) after 32 h, (F) after 38 h.
ESEM, Environmental scanning electron microscope; LV, low vacuum; scale bars: A and B, 400 μm; C, 300 μm; D, 200 μm; E, 100 μm; F, 50 μm; 15 kV.

Artificial otoconia revealed identical, gradual changes of their architecture down to lowest dilutions comparable to human otoconia (Figure 3). Because of their bigger size, gentamicin-induced dissolution of artificial otoconia takes up to several hours. Structural changes by dissolution of CGC and CGAC revealed the same characteristics. At higher magnifications (up to 10 000-fold) the surface showed detailed changes with an increasing number of fissures and pores dissolving the biomineral structure step by step (Figure 4). The belly region was affected first, while the branches were dissolved at a later stage.

Discussion

The exposure of pure calcite crystals to gentamicin clearly showed that the chemical attack was primarily directed to the inorganic component (calcite) even without nanostructuring of the material as present in the case of otoconia. The formation of trigonal scalenohedra, which is a basic form of calcite, generally supports the irreversible crystal dissolution process, which causes a reduction of material [14]. As human otoconia contain calcite as the majority component, we hypothesized that irreversible structural changes can be observed in human as well as in artificial otoconia when exposed to gentamicin in vitro.

The results of gentamicin exposure of human otoconia clearly revealed gradual structural damage caused by gentamicin at several concentrations and indicated a dissolution reaction with the nanocomposite structure of otoconia. The structural alterations comprised step by step dissolution scenarios beginning with minor alterations and ending with major changes in the sense of complete dissolution of the calcite component. However, although the gentamicin-induced changes occurred in a characteristic manner, the damage of single otoconia differed markedly as a function of time. Some otoconia in upper layers but also in deeper regions of the samples under investigation were observed to remain more or less unaffected. This can be explained by the net of organic fibrils remaining stable around the unaffected inner parts of otoconia and possibly acting as a protective surface layer. Furthermore, the surrounding organic matrix may also cause differences in local gentamicin concentrations.

Our results are comparable with earlier investigations of structural changes of otoconia after systemic administration of aminoglycosides. Johnsson et al. found morphological changes in animals (guinea pigs) caused by streptomycin [4]. Takumida et al. described surface changes in animals (guinea pigs) including a material loss, an aggregation with a formation of giant otoconia, and a loss of contact with the gelatinous layer [15]. In contrast, Campos et al. observed no changes 5 days following daily systemic gentamicin administration in animals (mice); however, the delayed onset of gentamicin ototoxicity in the vestibule might be the reason that otoconia

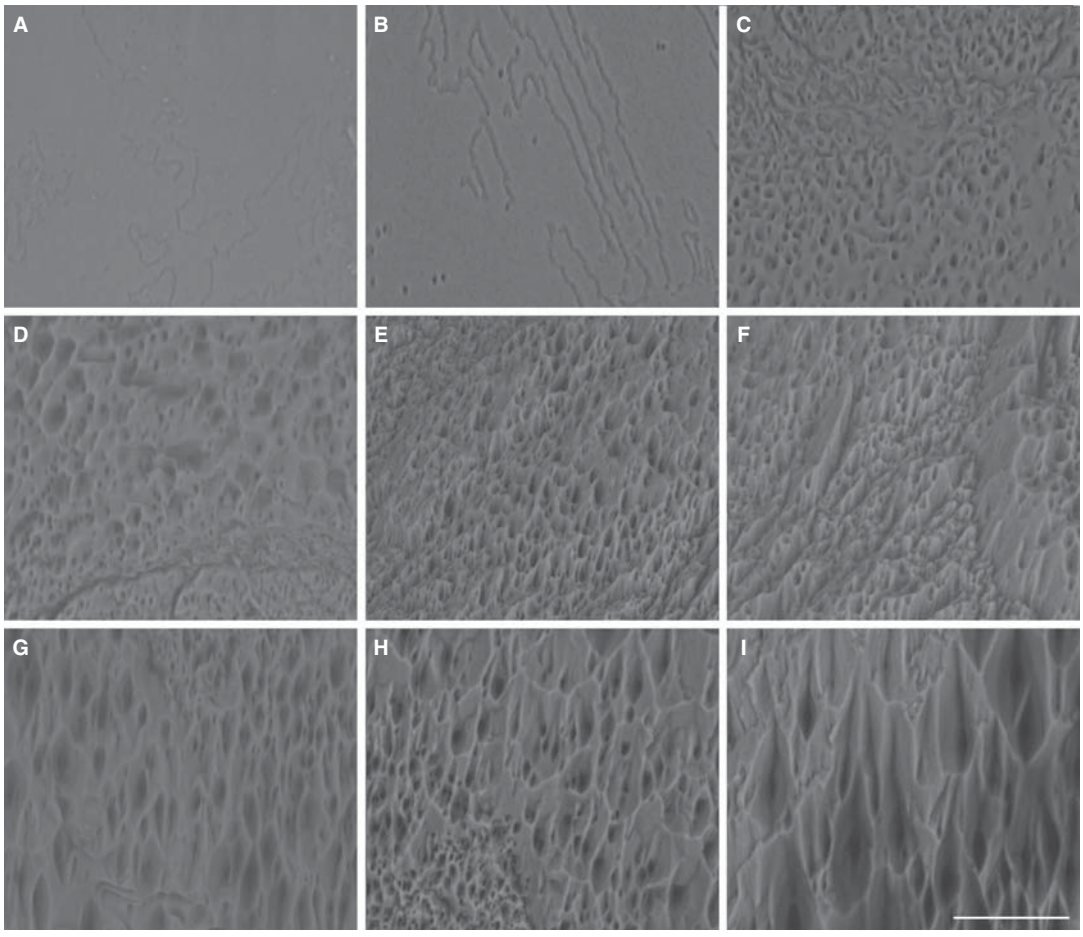

Figure 4. Structural surface changes after gentamicin exposure (40 mg/ml) of artificial otoconia (calcite gelatin composites, CGC): (A) before exposure, (B) 15 min, (C) 30 min, (D) 60 min, (E) 120 min, (F) 180 min, (G) 420 min, (H) 540 min, (I) 720 min.
ESEM, Environmental scanning electron microscope; LV, low vacuum; scale bar, 200 μm, 15 kV.

remained unaffected [16]. Serra and La Mantia found changes of otoconia following systemic gentamicin administration in animals (guinea pigs), which also confirm the observations of the present study [17]. However, a gradual dissolution of human otoconia together with a detailed analysis of the otoconia structure has not been investigated in detail up to now.

The results of a stepwise structural alteration of human and artificial otoconia support recent investigations on their inner structure [2,3,13]. It has been

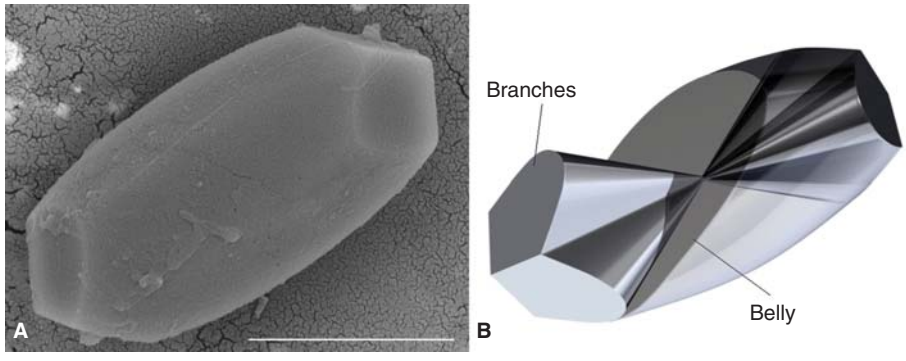

Figure 5. Intact human otoconium (A) and model of the inner structure of otoconia showing the less dense belly (left section partly removed), which is the main target site during gentamicin exposure and the three branches at each side, which include the rhombohedral faces (B). (A) ESEM, Environmental scanning electron microscope; HV, high vacuum; scale bar 5 μm, 15 kV.

shown that otoconia consist of a dumbbell-shaped dense structure (branches), including the six rhombohedral faces, which is surrounded by a less dense nanocomposite structure (belly), which is less structured and more porous (Figure 5). These structural peculiarities of human otoconia are consistent with the fact that the less dense area (belly) of the otoconia nanocomposite structure is more susceptible to gentamicin. Therefore, the belly region represents the predilection site of attacks independent of gentamicin concentration.

The alterations of human and artificial otoconia take place via different stages. At stage 1 the belly region is attacked superficially. The surface is gradually removed by surface reactions and by penetration of gentamicin into the otoconia. By finding its way into the pores of the belly, gentamicin is able to enter even the inner structure of otoconia. By this means, even deeper structures of the belly are gradually dissolved until the center of symmetry, the positions where the branches meet, is reached (stage 2). Then, the more dense branches including the rhombohedral faces are affected (stage 3) until the otoconium is completely dissolved (stage 4). The remaining residue consists of a net of interconnected organic fibrils keeping the shape of the initial otoconium. Similarly, gradual morphological changes were described in earlier studies investigating idiopathic degeneration of animal and human otoconia during lifetime [1]. In general, it can be assumed that the architecture of human otoconia strongly determines degradation processes.

Our results also show that gentamicin-induced alterations of artificial (biomimetic) otoconia are closely related to those obtained with human otoconia. Furthermore, because artificial otoconia are bigger in size (50–500 μm), it was possible to visualize gentamicin-induced surface changes in detail, which is difficult to demonstrate when using human otoconia (size up to 25 μm) (Figure 5). The surface changes of artificial otoconia support the gradual process of the gentamicin attack. In earlier stages of the dissolution process fissures and growing pores indicate structural surface alterations. The dissolution process continues by formation of an increasing number and size of pores and cracks leading to a gradual reduction of the material. The dissolution scenario of artificial otoconia perfectly reflects their inverse growth mechanism, which has been described in detail in recent studies [2,13].

To explain the underlying chemical reaction of aminoglycoside-induced morphological changes of otoconia in the case of streptomycin, Johnsson et al. assumed the responsibility of pH shifts towards the acidic range caused by cell degradation products [4]. In general, calcite can be affected chemically by pH changes into the acidic range as well as by complexation reactions even without pH shifts. In contrast, changes of the pH towards the basic range do not lead to any attacks. Due to its molecular structure, gentamicin contains amino groups in a convenient position, which may act as complexing (chelating) ligands for calcium ions. Therefore it cannot be excluded that a complexation reaction might be the underlying chemical process causing gentamicin-induced structural changes. There is no indication at present that the organic component of otoconia is actively involved in the dissolution process. The fibril net remains stable even after complete decalcification of the nanocomposite.

Apart from systemic administration, studies on intratympanic gentamicin delivery, simulations of clinical drug delivery protocols, and fluorescence experiments after intratympanic administration in animals (guinea pigs, chinchillas) provide evidence that gentamicin enters the vestibular endolymph, leading to a long-lasting effect in the saccule and the utricle [18–20]. Since human otoconia are altered already at low in vitro concentrations, further studies are needed to investigate otoconia damage after intratympanic administration of gentamicin.

In summary, the results of this study clearly demonstrate the destructive effect of gentamicin-induced alterations of the otoconia biomineral structure in both human and artificial otoconia. Artificial (biomimetic) otoconia represent a suitable model system for initial investigations in open biogenic questions. The knowledge of morphological changes in otoconia degeneration and chemical-induced alterations is the basis for further elucidation of destructive processes and provides substantial information for the development of protective mechanisms and possible repair of otoconia damage.

Conclusions

Gentamicin sulfate causes irreversible and gradual structural damage of human otoconia. The main target site of the chemical attack on otoconia is the belly region, whereas the dense branches including the rhombohedral faces are affected in a final stage. Artificial (biomimetic) otoconia can be used as a model mimicking chemical attacks on human otoconia. The peculiar architecture of otoconia strongly determines the degradation processes in case of gentamicin exposure.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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