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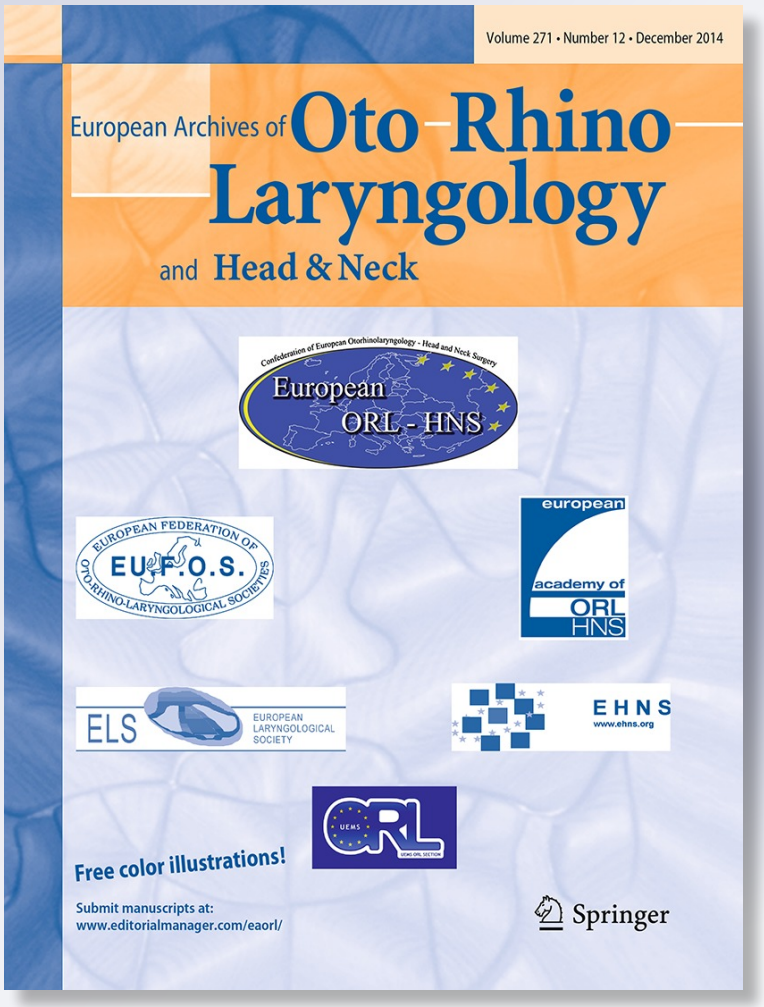

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OTOLOGY

Detection of human utricular otoconia degeneration in vital specimen and implications for benign paroxysmal positional vertigo

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Abstract Otoconia are assumed to be involved in inner ear disorders such as benign paroxysmal positional vertigo (BPPV). Up to now, the distinct structure and morphology of intact and degenerate human utricular otoconia has been only poorly investigated on vital specimen. In this study, human otoconia were obtained from the utricle in five patients undergoing translabyrinthine vestibular schwannoma surgery. Specimens were examined by environmental scanning electron microscopy. Intact and degenerate otoconia as well as fracture particles of otoconia and bone were analyzed by energy dispersive X-ray microanalysis (EDX) and powder X-ray diffraction (XRD). Intact otoconia reveal a uniform size showing characteristic symmetry properties. Degenerative changes can be observed at several stages with gradual minor and major changes in their morphology including fragment formation. EDX analyses reveal the characteristic chemical composition also for otoconia remnants. XRD shows that intact and degenerate otoconia as well as remnants consist of the calcite modification. In conclusion, electron microscopy serves as a standard method for morphological investigations of otoconia. Human utricular otoconia show a uniform outer morphology corresponding to a calcite-based nanocomposite. Morphological changes provide further evidence for degeneration of utricular otoconia in humans, which might be a preconditioning factor causing BPPV. In case of uncertain origin, particles can be clearly assigned to otoconial origin using EDX and XRD analyses.

Keywords Otoconia · Utricle · BPPV · Vertigo · Degeneration · Aging · Ocular VEMP

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Introduction

Human otoconia are calcite-based nanocomposites containing a small amount of organic material (<5 %). Recent studies have investigated the inner structure of human otoconia, consisting of a uniform architecture with a dumbbell-shaped dense structure ("branches"), surrounded by a less dense, more porous structure ("belly") [1, 2].

It is known that otoconia can alter during lifetime [3–7]. A detachment of degenerate utricular otoconia is generally assumed to cause benign paroxysmal positional vertigo (BPPV). There are some observations supporting the scenario that particles gain access to the semicircular canals causing BPPV. Schuknecht and Ruby [8] demonstrated basophilic deposits of the cupula (cupulolithiasis) in patients experiencing BPPV and assumed that they might originate from a degeneration of utricular otoconia. Observations during occlusion surgery of the posterior semicircular canal with free floating endolymph particles [9], particles of proteinaceous and mineral content [10] and degenerate otoconia [11] (canalolithiasis) in patients with BPPV ≥50 years showed further evidence for otoconial origin and degeneration. However, non-otoconia material might also be involved in BPPV. Animal studies and model

experiments [12–14] as well as clinical observations [15–18] confirm the presence of BPPV. The ultrastructural investigation of degeneration of vital utricular otoconia together with analytical methods for identification of particles with uncertain morphology can provide additional evidence for BPPV and its underlying nature in case of otoconial origin.

In the present study, we obtained vital human utricular otoconia from surgical specimens to classify their morphology by electron microscopy in terms of normal and altered shape and to discuss structural changes with regard to degeneration and BPPV at a higher age. Furthermore, we aimed to develop a method for a safe identification of otoconia even in case of a lack of morphological characteristics.

Materials and methods

Surgery and specimens

Patients undergoing transmastoid labyrinthectomy ($n = 5$) for sporadic vestibular schwannoma were included in the study. The mean age at surgery was 56.4 ± 5.4 years (47–63 years). 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 and immediately fixed in ethanol (96 %) for further structural investigation.

Structural investigation

The morphology of vital human otoconia and their size ($n = 1{,}000$, randomly) was studied by environmental scanning electron microscopy (ESEM, FEI Quanta 200 FEGi, Eindhoven, Netherlands) by use of uncoated specimens in low vacuum (LV, 60 Pa) and high vacuum (HV) modes (2×10^{-4} Pa), respectively. Acceleration voltages varied between 15 and 25 kV. For investigation under HV, some samples were coated with gold (Au) for 30 s to obtain a reliable conductivity of the surface.

Chemical and crystallographic analysis

Single otoconia with intact and degenerate morphology, remnants, bone particles and matrix components were investigated by energy dispersive X-ray microanalysis (EDX) to elucidate the element composition. Powder X-ray diffraction (XRD) was used for structure identification by means of the diffraction pattern. EDX analyses were performed by adaptive equipment during ESEM investigations in order to characterize otoconia, matrix and bone. XRD investigations were performed on a Huber image plate Guinier camera G 760 (Cu Kα radiation).

Ethics

The study was conducted in conformity with the declaration of Helsinki 1975, revised in 1983, and approved by the ethic committee of the University Medicine Mannheim, Ruprecht-Karls-University Heidelberg (2012-612 N-[MA]).

Results

Morphological details could be clearly obtained by ESEM at magnifications up to 3,000-fold. The morphology of intact otoconia is characterized by a cylindrical, bulbous-shaped body with tiny pores (Fig. 1a). The 3 + 3 rhombohedral planes on both sides are turned by 60° towards each other and appear less structured than the body region (Fig. 1b). Interconnecting fibrils are observed as filament-like structures between adjacent otoconia (Fig. 1c). A gelatinous matrix surrounding and interconnecting the otoconia is visible (Fig. 1d). A model of an intact otoconium is shown (Fig. 2a, b). The mean size of utricular otoconia is up to 10 μm.

All samples under investigation contained otoconia with surface alterations which we assume to be caused by degeneration effects with a tendency to increase with age. Several types of degeneration were found: (1) otoconia with few fissures and roughening of the belly surface but without morphological changes of the rhombohedral planes. (2) Otoconia with enlarged pores, hole formation and reduction of material in the belly area. The rhombohedral planes also start to be affected. (3) Otoconia with a spongy body due to further material reduction including the rhombohedral planes connected with a successive loss of the typical outer bulbous shape. (4) Single fragments of otoconia which have lost their characteristic outer shape. Identification as fragments of former intact otoconia is possible using EDX and XRD analyses (Fig. 3a–d). The results of EDX analyses show an enhanced content of carbon as well as the absence of calcium in the matrix and a significant amount of phosphorus in bone particles. Powder-XRD diffraction patterns of intact and degenerate otoconia as well as their remnants exclusively correspond to the calcite modification.

Discussion

The results of the present study show that structural details of intact human otoconia can be reliably detected by

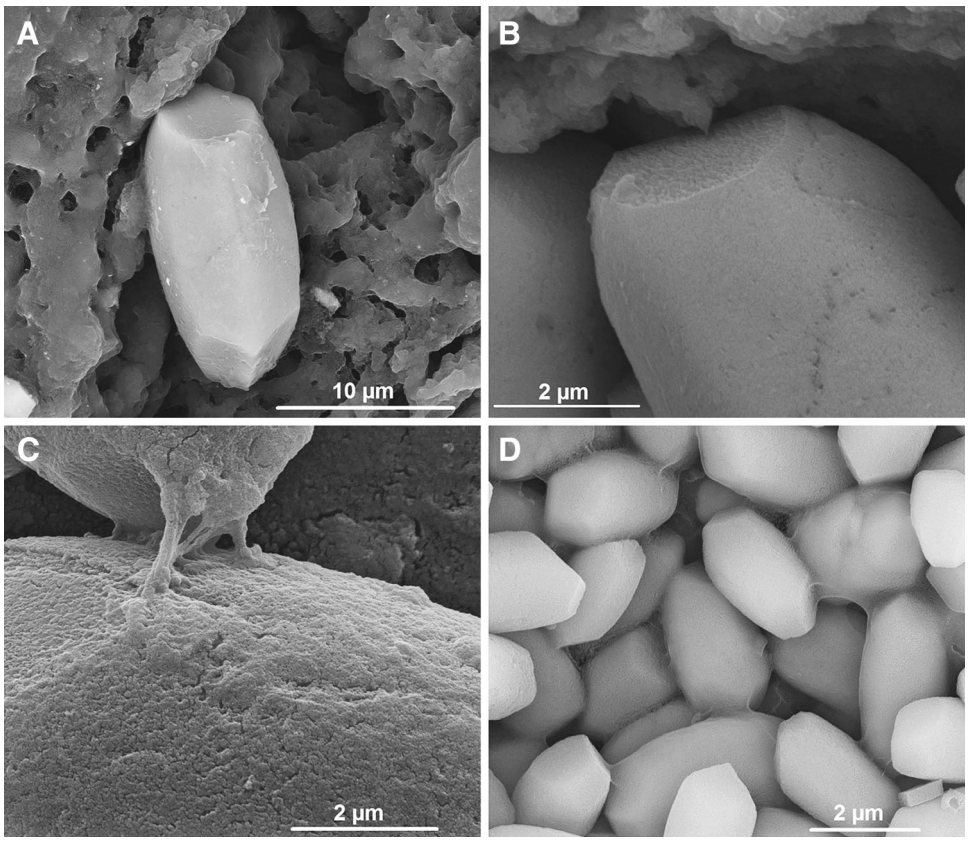

Fig. 1 Intact human otoconia. **a** Intact single human otoconium surrounded by organic matrix. The rhombohedral planes at both ends of the utricular otoconium are turned towards each other by 60°. The center of symmetry of otoconia is located in the center of the body. **b** Compared with the bulbous body, the surface of the rhombohedral planes of intact human otoconia is less structured and more planar. **c** The surface of the bulbous body of intact human utricular otoconia is slightly roughened containing tiny pores. Interconnecting fibrils between otoconia serve as linking filaments. **d** Assembly of human utricular otoconia interconnected by a gelatinous matrix

ultrastructural investigations. The main criterion for the identification of intact human otoconia is their uniform outer shape with a cylindrical bulbous body (belly) and three rhombohedral, terminal planes at both ends which are part of the branches. Intact human utricular otoconia exhibit a roughened surface of the body region due to tiny pores, whereas the rhombohedral planes appear smoother. The results concerning morphology and size are comparable with the observations obtained from animal and post mortem studies showing similar patterns of saccular and utricular otoconia [3–6, 19].

The results also demonstrate that in vital human utricular specimens, degeneration of otoconia can be detected in detail. Intact architectures can be clearly distinguished from degenerate morphologies caused by surface alterations.

The degenerate otoconia morphology at varying degrees indicates that the degeneration processes in the human utricle take place gradually. Minor changes (low grade of degeneration) reveal mild structural alterations such as fissures and surface roughening of the less dense belly area with modest reduction of material. Major changes (high grade degeneration) are characterized by profound morphological alterations (fractures and disintegration) showing a successive loss of otoconia material. The rhombohedral planes as part of the more dense branches are dissolved later. Human otoconia finally become completely dissolved in their belly area leading to fragment formation which hinders a clear morphological identification.

Degenerative changes as shown by the results of our study on vital human utricular otoconia have been demonstrated earlier in animal and human post mortem studies [3–6]. However, alteration sequences in the latter do not always fit to the stage-dependent morphological changes in vital human otoconia. In animals, for example, some otoconia crumble to single fragments by fissuring without any obvious reduction of otoconia material [6].

The presence of degeneration in various stages also shows that morphological alteration leads to a stepwise reduction of material, i.e., a reduction in the volume and the number of otoconia in the utricle which is confirmed by investigations of Igarashi et al. [20] in a post mortem human temporal bone study.

Since degenerative changes have been detected in the present study in all vital specimens showing a tendency to increase with age, it can be assumed that degenerative morphologies in the utricle are developed gradually and start already earlier in lifetime reaching a maximum at higher ages. A mild degeneration of human utricular otoconia at a younger age has been shown in an earlier study [21]. Furthermore, basophilic cupula deposits in younger

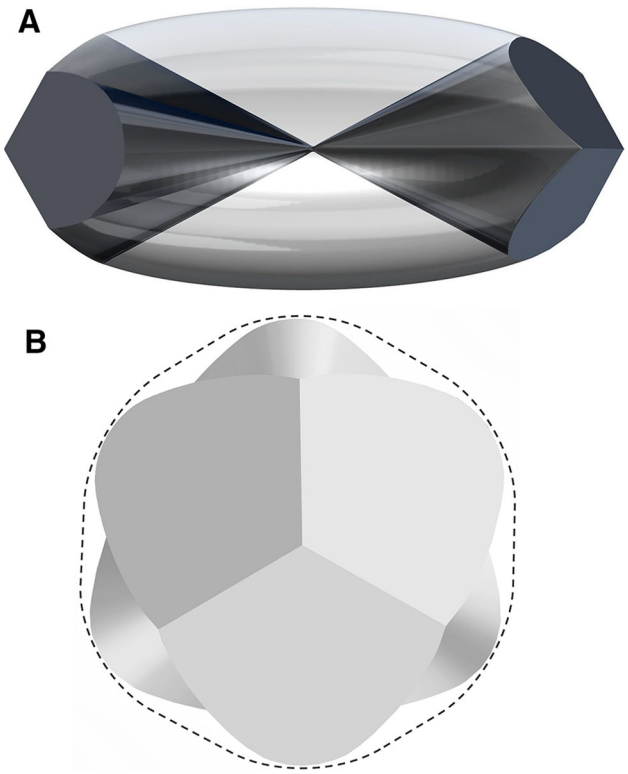

Fig. 2 Model of an intact human otoconium. **a** Model indicating the inner architecture. The *bright area* corresponds to the belly region surrounding the symmetry center in the middle of the otoconium. The *darker areas* correspond to the more dense 3 + 3 branches extending from the center of symmetry of the otoconium [1]. **b** The rhombohedral planes represent the end-faces of the 3 + 3 branches, which are turned by 60° towards each other. Besides the rhombohedral faces, the shape of an intact human otoconium is nearly cylindrical with a slight tendency to a hexagonal equatorial contour

patients suggest degenerative processes which might arise from the utricle [22]. Moreover, epidemiological studies provide evidence for a rare occurrence of BPPV also in younger age groups [23].

A successive reduction of saccular and utricular function was investigated in a recent study on otolith function as measured with vestibular evoked myogenic potentials (VEMP) [24]. This provides evidence for a gradual, age-related decline of otolith function which might be related to hair cell loss but also to otoconia material reduction. Furthermore, it has been shown that utricular dysfunction in patients with BPPV from middle to advanced ages can be measured by ocular VEMP [25–27]. In one of these studies, the authors assumed that pathologic ocular VEMPs in BPPV patients are caused by a detachment of degenerate otoconia leading to a hypermobility of the stereocilia overlying vestibular hair cells type 1 [27].

However, the mechanisms causing otoconia degeneration in humans are unknown up to now, but might play a crucial role in clarifying the etiology of degeneration underlying BPPV. Some medications including aminoglycosides have been shown to alter otoconia morphology [4, 28]. An increased prevalence of basophilic deposits on the surface of the cupula was demonstrated following aminoglycoside therapy in human temporal bones [29].

From the chemical point of view, a reduction of otoconia material as observed in this study can be caused by chemical reactions of the main otoconia component (calcite) either due to complexation reactions or to shifts of the endolymph environment into the acidic range. Recent investigations on the inner structure of human otoconia showed an anisotropic solubility of human otoconia after EDTA exposure [1]. The less dense belly region is affected earlier, whereas the branches with their high volume density are affected in later stages and remain as trigonal entities for the moment (see Fig. 3d). Since the less dense belly surrounds the symmetry center, which is the nucleation point of the branches, it can be assumed that in case of chemical attacks caused by complexing agents such as EDTA, fracturing of otoconia mainly takes place at the center of otoconia (predilection site).

Furthermore, it is assumed from animal experiments (gerbils) that organic components such as glucuronic acid-containing glycosaminoglycans also play a role in degenerative processes [30]. Moreover, it has been shown in animals (mice, rats) only recently that a weakening or loss of anchoring of the organic fibrils interconnecting the otoconia can occur due to aging, which is assumed to be a precondition for a detachment of degenerate otoconia in BPPV [31]. Hence, degeneration of otoconia is obviously caused by reduction of both inorganic (calcite) and organic (e.g., fibrils) material. Thus, degenerative processes are associated not only with a mass reduction causing detachment and BPPV but they also obviously might affect the functionality of otoconia by destruction of the inner architecture, causing a loss of mobility and problems in gravity detection.

In case of fragment formation, the particles morphology cannot be used for safe identification of otoconia material. In this case, the particle identification is performed by analytical methods: EDX analysis detects the main chemical element components. X-ray diffraction can be used to identify the crystalline structure of the particles.

The results show that intact as well as degenerate otoconia and their remnants can be clearly distinguished from bone and matrix material by EDX analysis. The main distinguishing criteria are the enhanced content of carbon as well as the absence of calcium in the matrix and the significant amount of phosphorus in bone particles.

The diagnostic certainty in detection of otoconia can be further increased by XRD. Since the complete internal structure of otoconia is composed of nanodomains of calcite which are intergrown with glycoproteins, it becomes

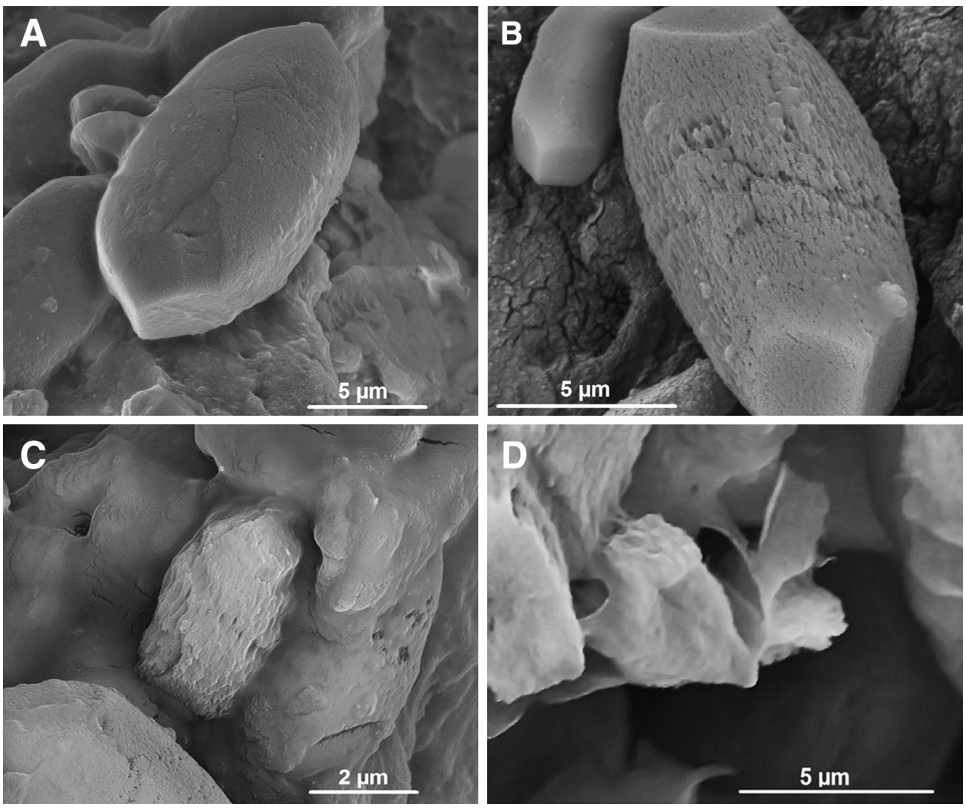

Fig. 3 Degenerate human otoconia. **a** Minor changes on the surface of a single human utricular otoconium showing fissures in the belly. The rhombohedral planes appear to be unaffected (ESEM, stage 1 of degeneration). **b** Structural changes on the surface (belly area) of human utricular otoconia by deepening of pores, a tendency to hole formation and formation of deeper fissures. The terminal planes still remain nearly unaffected (ESEM, major changes on the terminal planes in stage 2 of degeneration). **c** Significant destruction of otoconia by dissolution of the cylindrical body (belly) and affecting the terminal planes (ESEM, major changes, stage 3 of degeneration). **d** Fragment formation caused by heavy material dissolution (ESEM, major changes, stage 4 of degeneration). The fragments probably correspond to parts of the branches. As the otoconia morphology can hardly be identified in **c** and **d** the otoconial origin of the particles was confirmed by EDX and powder XRD analyses

clear that degenerate otoconia and fragments of otoconia show the same characteristics.

EDX and XRD together provide a safe analytical tool for the identification of particles originating from the maculae even without characteristic morphologies. The morphological characterization of intact and degenerate otoconia, the structural details during degeneration scenarios in vital human utricular specimen and the reliable identification of otoconial material by analytical methods provide the basis for further elucidation of destructive processes and their underlying etiology causing BPPV.

Conclusions

Scanning electron microscopy is the standard method to observe morphological changes and destructive phenomena of human otoconia in great detail. The main distinguishing criterion in comparison with intact otoconia is given by changes in the surface structures extending to the interior. Gradual degenerative processes of otoconia with material reduction take place in human utricular otoconia in patients with advanced age providing evidence for BPPV. EDX and XRD techniques provide analytical tools for identification of otoconia in case that morphological criteria cannot be used.

Conflict of interest The authors report no conflict of interest, financial or otherwise.

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