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Ultrastructural Changes in Otoconia of Osteoporotic Rats

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Key Words
Otoconia · Osteopenia · Osteoporosis · Idiopathic benign paroxysmal positional vertigo, women · Calcium metabolism

Abstract
The etiology of benign paroxysmal positional vertigo (BPPV) remains obscure in many cases and women are affected more often than men. A recent prospective study, performed in women >50 years of age suffering from recurrent BPPV, showed associated osteopenia or osteoporosis in a large percentage of these patients. These results suggested the possible relationship between recurrent BPPV and a decreased fixation of calcium in bone in women >50 years. To test this hypothesis, an experimental study was performed in adult female rats. Utricular otoconia of female rats in which osteopenia/osteoporosis was induced by bilateral ovariectomy (OVX) were compared to those of sham-operated adult females rats (SHAM), as control group. ***First Study:*** The morphology of the utricles of OVX and SHAM rats was analyzed with scanning electron microscopy. In osteopenic/osteoporotic rats, the density of otoconia (i.e. the number of otoconia per unit area) was decreased ($p = 0.036$) and their size was increased ($p = 0.036$) compared to the control group. ***Second Study:*** To test the role of calcium turnover in such morphological changes, utricular otoconia of 2 other groups of OVX and SHAM rats, previously injected with calcein subcutaneously, were examined by conventional and epifluorescence microscopy. In epifluorescence microscopy, labeling with calcein showed no significant fluorescence in either group. This finding was interpreted as a lack of external calcium turnover into otoconia of adult female rats. The ultrastructural modifications of otoconia in osteopenic/osteoporotic female adult rats as well as the role of estrogenic receptors in the inner ear are discussed. The possible pathophysiological mechanisms which support the relationship between recurrent BPPV in women and the disturbance of the calcium metabolism of osteopenia/osteoporosis are debated.

Introduction

Transient vertigo elicited after quick changes of head position is characteristic of benign paroxysmal positional vertigo (BPPV). At the end of the 1960s, Schuknecht [1969] correlated the clinical description of BPPV with his histopathological observations of basophilic deposits

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on the cupula of the posterior semicircular canal. Some years later, Parnes and McClure [1992] reported observations of free-floating particles in the endolymph during occlusion of the posterior semicircular canal in patients suffering from intractable BPPV. The authors interpreted their findings as displaced otoconia fallen from the utricular macula, either directly on to the cupula [Schuknecht, 1969] or floating in the posterior semicircular canal [Parnes and McClure, 1992]. Therefore, BPPV was attributed to cupulolithiasis and canalolithiasis, respectively. As otoconia are known to be heavier than the endolymph fluid in terms of mass-volume ratio, the term 'free-floating particle' might be rediscussed. Indeed, if dislodged, one can expect that they move as free bulk into the semicircular canal with respect to the kinetics of the head movements or to gravity itself, depending on the positions of the subject.

BPPV represents one of the most common peripheral vestibular diseases that frequently occur after head trauma, whiplash injuries and acute peripheral vestibular deficits [Barber, 1964; Longridge and Barber, 1978; Baloh et al., 1987; Schuknecht, 1993; Vibert and Häusler, 2003]. It is also associated with Menière's disease [Baloh et al., 1987]. However, in many cases, BPPV remains idiopathic in origin and affects women more often than men [Semont et al., 1988; Häusler and Pampurik, 1989].

In a recent clinical study performed in women >50 years of age with recurrent idiopathic BPPV, we found either associated osteopenia or osteoporosis in a large number of these patients [Vibert et al., 2003]. We hypothesized that mechanisms of increased resorption and decreased fixation of calcium, thus generating osteoporosis, might also be implicated in the onset of repeated dislocation of otoconia. Morphometric changes in otoconia might also affect the fine network of covering filaments [Lins et al., 2000], generating failures in their interconnection and attachment to the utricular macula and inducing the repeated symptomatology of BPPV.

To corroborate the possible relationship between the two pathologies, we performed an experimental study in animals. The bone loss was induced by surgical bilateral ovariectomy, which has represented a widely used model for postmenopausal osteoporosis in the rat for many years [Saville, 1969; Wronski et al., 1986].

The goal of this work was to study the possible alterations of the otoconial structure due to osteopenia/osteoporosis. The ultrastructure of utricular otoconia of adult female rats, in which osteopenia/osteoporosis was induced by bilateral ovariectomy, was compared to that of a control group of healthy animals.

Material and Methods

Twenty adult (6–8 months old) female retired breeder Wistar rats (Charles River, l'Arbesle, France), with a body weight of 300–400 g, were kept in standard animal facilities that complied with the Swiss and US National Institutes of Health guidelines for care and use of experimental animals. The experiments performed were approved by the State Committee for the Control of Animal Experimentation.

Three days before starting the baseline experiment, trabecular bone mineral density (BMD) and bone mineral content were measured, and the rats were assigned at random to one of 2 groups. One to two days after assessment of bone parameters, either a bilateral ovariectomy (OVX, n = 11) or a sham operation (SHAM, n = 9) were performed under ketamin/xylazin anesthesia (50 mg mixed with 10 mg, respectively, per kg body weight; Ketasol, Gräub, Bern, Switzerland; Xylapan, Globopharm, Egg/Zürich, Switzerland) injected intraperitoneally.

Bone Mineral Measurements

Bone mineral content and BMD were measured every 3 weeks after surgery in the proximal metaphysis of the left tibia by quantitative computed tomography (XCT Research SA, software version 5.40, Stratec Medizintechnik, Pforzheim, Germany), as previously described [Mühlbauer et al., 1998; Li and Mühlbauer, 1999]. A cross-section starting 5 mm distal to the joint space was evaluated, using a threshold for trabecular bone of 400 mg/cm^3.

Scanning Electron Microscopy

Five OVX and 3 SHAM female rats out of the 20 adult rats were used for this study. Nine weeks after surgery, the animals were euthanized and the inner ears were excised. The temporal bone lying over the labyrinth was carefully removed and the utricle and saccule directly exposed to the fixative. The samples were immersed in 2% glutaraldehyde in 0.1 M cacodylate and 3 mM $CaCl_2$ buffer (pH 7.3) for 1 day [Huss and Dickman, 2003]. They were then postfixed in 2% OsO_4 for 2 h. To expose the surface of the otoconial membrane, the membranous labyrinth was gently removed and the samples were dehydrated in a graded series of ethanol solutions. They were dried using the critical point method in liquid CO_2 and coated with silver. Samples were observed with a JEOL 6300F electron microscope.

Fluorescence Microscopy

Six OVX and 6 SHAM female rats out of the 20 adult rats were used for this study. All specimens were dissected as previously described for scanning electron microscopy (SEM), but glutaraldehyde was exchanged for pure ethanol, then the specimens were transferred to glass slides and covered with a coverslip without embedding medium to minimize nonspecific fluorescence [Kawamata and Igarashi, 1995]. The utricles were observed by conventional and epifluorescence microscopy (microscope inverted 3 Zeiss; Axiovert 200M) as well as laser-scanning confocal microscopy (Bio-Rad; MRC1024).

Measurements of the calcium turnover in otoconia was performed by using a calcein solution injected in the rats subcutaneously 5 times at 3-day intervals, 2 weeks before they were to be sacrificed, (3%; pH 7.2; 20 mg/kg body weight) [Takumida et al., 1997b]. The last injection was 48 h before euthanasia.

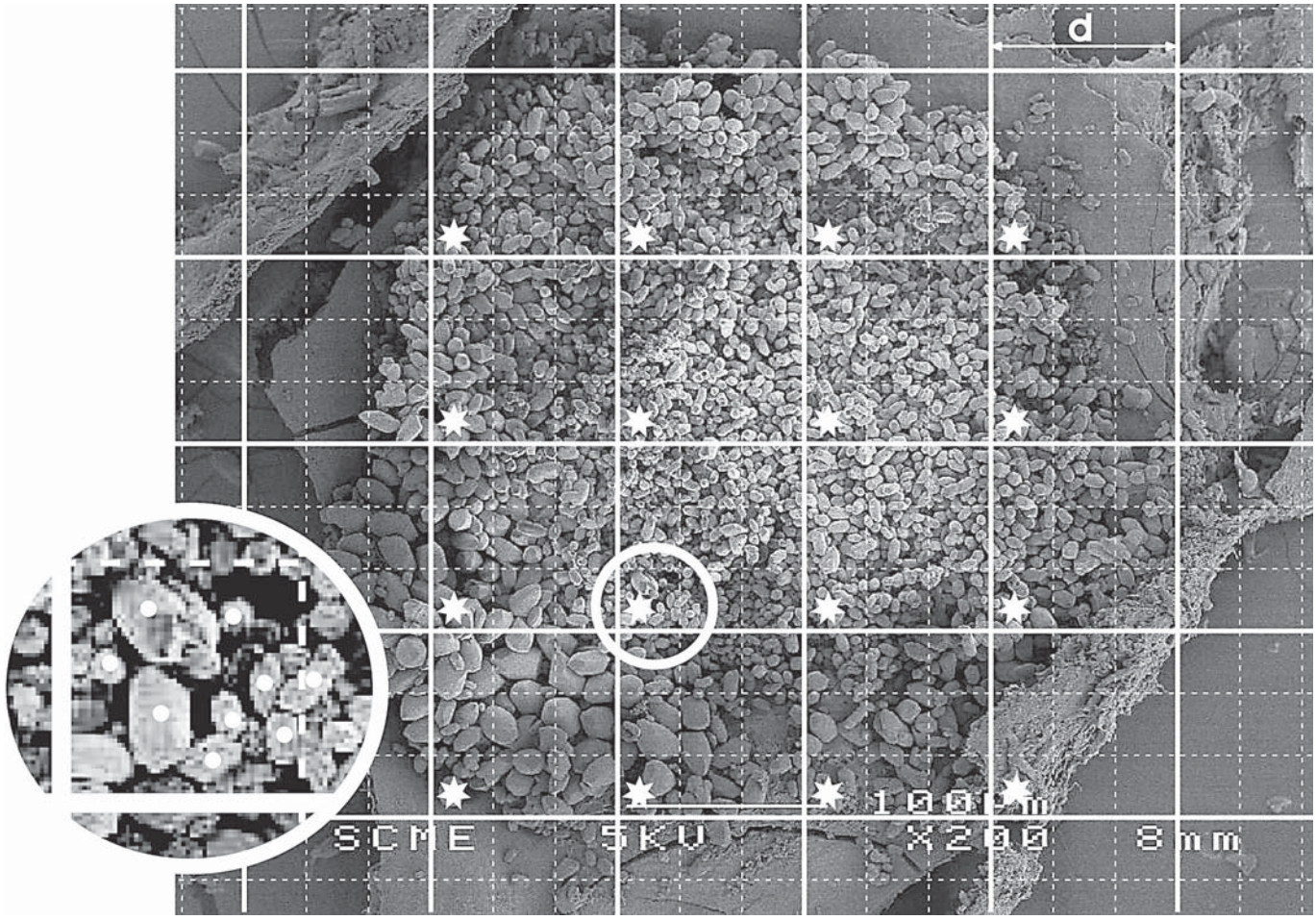

Fig. 1. Quantitative method of otoconial measurement. The square grid was superimposed on the digitalized SEM photographs. Each lower left secondary square marked with a star was analyzed. Sixteen secondary squares were defined on each SEM photograph. d = 93.3 µm; d' = 31.1 × 31.1 µm (967.2-µm^2 area).

As known, calcium is progressively incorporated into otoconia during their development period, i.e. between the 15.5 gestational days and the first postnatal days [Kawamata and Igarashi, 1995]. In contrast, the evidence of a calcium turnover in otoconia of adult animals remains controversial. To be sure that our calcein labeling method was effective in labeling otoconia, we controlled it with a group of 6 young rats, 3–5 days old. They were injected subcutaneously on their backs with the calcein solution during 3 consecutive days. The young rats were sacrificed 4 days after the last injection.

Otoconial Measurement

Measurements of otoconia size and otoconia density were performed on the SEM photographs of the otoconial membrane of all specimens. Throughout this paper, the term 'otoconia density' is used to describe the number of otoconia per unit area. The quantitative morphometry was performed using the coherent double-lattice test systems [Weibel, 1979]. The basic test system is a square lattice of heavy lines whose intersections mark P_T test points. The test point distance d is subdivided q times and finer dotted lines are drawn, whose spacing is evidently d/q. The total number of test points (marked by the intersections of all lines) is $P'_T = q^2 \times P_T$. The test system chosen in this measurement was the C16, where lattice ratio $q^2 = 9$, $P_T = 16$ and $P'_T = 256$. The distance of coarse lines is d = 93.3 µm (8,704.9-µm^2 area). The square grid was superimposed on the digitalized SEM photographs. The utricular maculae were entirely visible on the photograph and the grid was oriented the same way to assure that the same regions were studied under the same conditions in each sample. Each lower left secondary square d' = 31.1 × 31.1 µm (967.2-µm^2 area) was analyzed using digital measurement software. Sixteen secondary squares were defined on each SEM photograph as shown in figure 1. Their analysis consisted of counting all visible otoconia lying either completely within the square or crossing the right or upper dotted line of the square and then measuring the largest visible length of each of these otoconia. Each otoconium measured was then marked to avoid over- and undercounting.

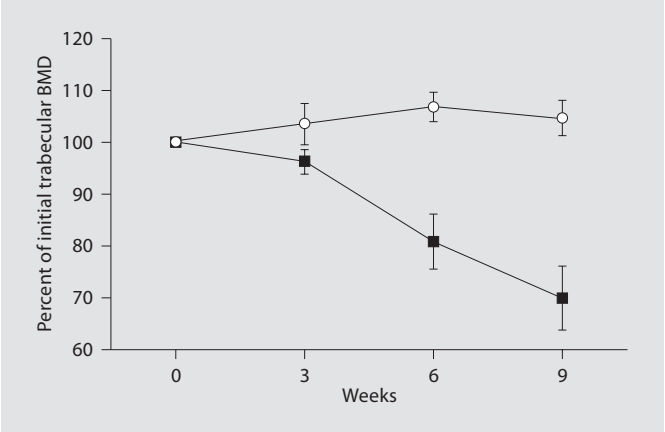

Fig. 2. Measurements of the trabecular BMD of OVX and SHAM rats. The white circles represent the BMD values of the SHAM rats and the black symbols those of the OVX rats, before surgery, as well as 3, 6 and 9 weeks after the operation. The values of BMD (± standard error of the mean) showed the progressive osteopenia/osteoporosis of the OVX rats before euthanasia.

Four to 9 (average = 6.625) secondary squares, representing a total area of 3,868.8–8,704.8 μm^2 (area average = 6,407.7μm^2), were analyzed per rat.

Note that the largest visible or projected length was not necessarily the true largest length due to the oblong shape of the otoconia and different orientations with respect to the direction of viewing. However, the average projected length was proportional to the true average largest diameter in our samples if the distribution of the orientations was assumed to be comparable in all animals analyzed. The validity of this assumption was supported by visual inspection of the photographs performed by SEM.

Data Analyses

We compared the mean projected length and the mean number of otoconia per 1,000 μm^2 of the 5 OVX rats with those of the 3 SHAM controls using 2-tailed Mann-Whitney tests, which does not require normal distribution of the data. The statistical analysis was performed by a certified statistician (M.K.) using the InStat 3.05 (GraphPad Inc., San Diego, Calif., USA) software package.

Results

The density of calcium in bone was measured in all animals using bone mineralometry. Figure 2 shows the trabecular BMD values of the SHAM and OVX rat groups which were measured during the experiment, preoperatively as well as 3, 6 and 9 weeks after surgery. The white circles represent the BMD values of the SHAM group. The values of the trabecular bone volume remained stable during the time of experiment. In contrast, the black symbols show the BMD values for the OVX group. After the bilateral ovariectomy, the values of BMD decreased, showing the progressive reduction of the trabecular bone volume in the OVX rat group at the moment of euthanasia. Histologically, the evidence of osteopenia is confirmed by histomorphometric indices of increased bone turnover in the tibia of OVX rats [Wronski et al., 1986]. The BMD data of the OVX rat group are directly statistically comparable to those of the control SHAM group.

In women, such comparison is not feasible and the results of the BMD are given as a T score. This score is derived by comparing the measured BMD to a normative database of results from 30-year-old women. The difference is expressed as a standard deviation (SD) score. Values between a T score of 0 and –1.0 SD are considered as normal. T scores of 1.1–2 (95% confidence interval = 2 SD) and 2.1–2.5 (95% confidence interval = 2.5 SD) below the normal population are defined as osteopenia and osteoporosis, respectively [Watts, 2000].

SEM: Qualitative Analysis

In the SHAM rats, the otoconia were distributed on the utricular macula according to a specific pattern. Their sizes varied considerably depending on their location on the macula: the otoconia of similarly small sizes were located in the central part of the macula and the large otoconia were found on the lateral part of the utricle (fig. 3a). In the OVX rats, this specific pattern of size distribution was not so clearly defined (fig. 3c).

The detailed view of the central part of the macula of the OVX rats showed large otoconia >30 μm mixed with otoconia of smaller sizes (fig. 3d). In contrast, only small otoconia similar in size were found in SHAM rats (fig. 3b). Furthermore, the lateral part of the macula of both groups showed differences in their otoconial distribution: in SHAM rats, the large otoconia were closely distributed in this area (fig. 3a), while in OVX rats, large otoconia appeared lower in density (fig. 3c). These abnormalities were typically observed in all OVX rats.

Such qualitative observations motivated the following quantitative morphometric analysis to evaluate the differences in size and density of otoconia of the 2 groups of animals.

SEM: Quantitative Analysis

Otoconia Size. The size of 68–228 otoconia (mean = 128) was measured per rat: it represented 68–131 otoconia in each of the 5 OVX rats and 153–228 in each of the SHAM controls.

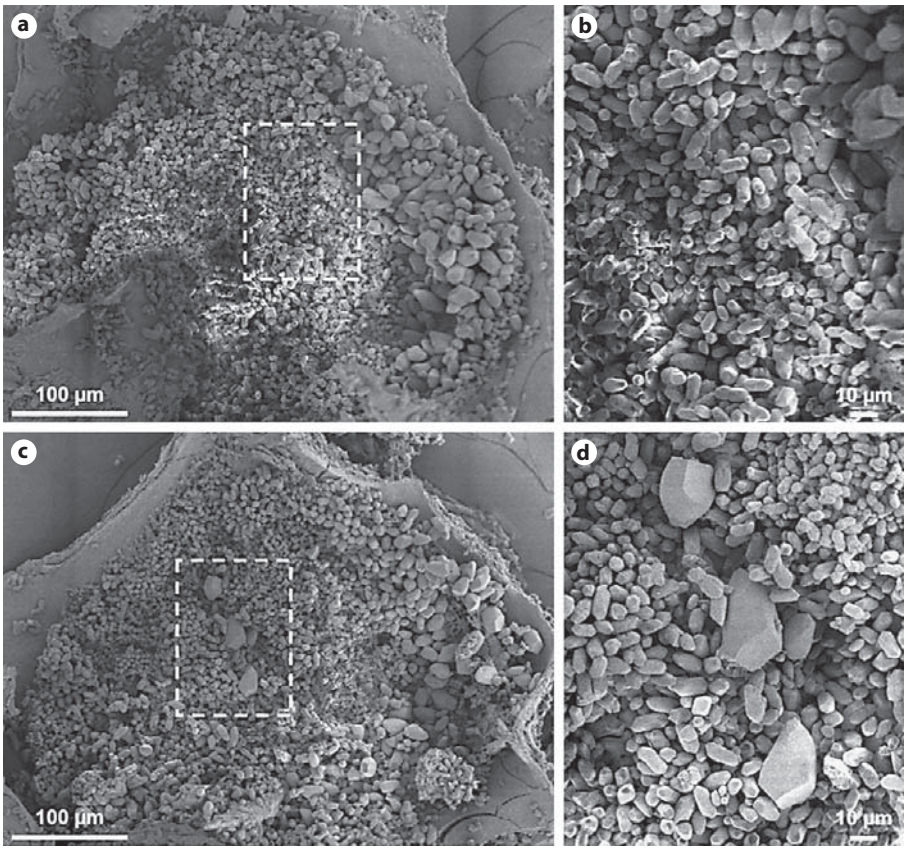

Fig. 3. Otoconia of SHAM and OVX adult female rats by SEM. **a** Survey of utricular macula of SHAM rats. Otoconia are distributed on the macula depending on a specific pattern in size: the smallest are located in the central part and the largest otoconia at the periphery. **b** Detailed view of the marked area in **a**. All otoconia are of small size. **c** Survey of utricular macula of OVX rats. The density of otoconia at the periphery of the macula appears to be lower compared to **a** and large otoconia of >30 µm are located in the central area of the macula. **d** Detailed view of the marked area in **c**. The size of the large otoconia reached 35 µm in length.

Figure 4 shows the measurement results of the largest projected length of the otoconia in each of the 8 rats. The average projected size was 8.8 µm for the 5 OVX rats and 6.2 µm for the 3 SHAM controls. The mean projected length of the otoconia was ≥7.8 for all OVX rats (filled symbols in fig. 4) and ≤6.3 for all SHAM rats (empty symbols in fig. 4). The 95% confidence intervals of the mean length shown in figure 4 do not overlap between any 2 rats from the 2 different groups. The difference in mean projected otoconia lengths is statistically significant between the 2 groups ($p = 0.036$, 2-tailed Mann-Whitney test, $n_{OVX} = 5$ vs. $n_{SHAM} = 3$).

Otoconia Density. Only the squares without significant lumping between otoconia and lying completely on the utricular macula were considered for the analysis. Under this condition, 10–12 (average = 11) squares per rat were considered for the analysis: from 10 to 12 squares per rat in the OVX group and 11 to 12 per rat in the SHAM group.

Figure 5 shows the results of the measurements of otoconia density, i.e. the number of otoconia per area, of the 8 rats. The average density of otoconia was 9.4 per

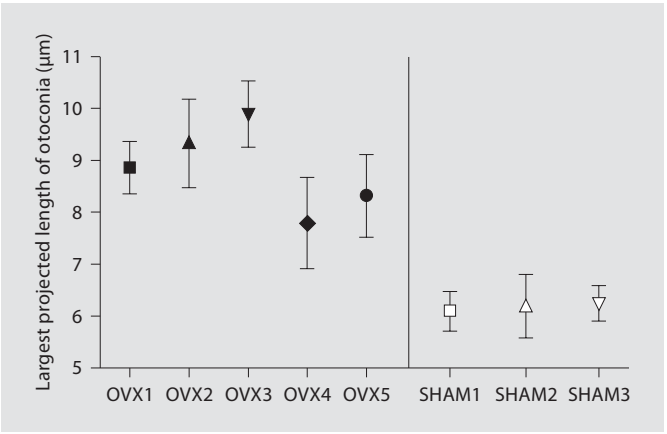

Fig. 4. Otoconia size: comparison between SHAM and OVX rat groups. The distribution of the largest projected length of the otoconia in the OVX rats (OVX1 through OVX5) compared to the SHAM rats (SHAM1 through SHAM3) is illustrated. The plots denote the mean values and 95% confidence intervals. The otoconia size in the OVX rats is statistically higher ($p = 0.036$) than in the SHAM rats.

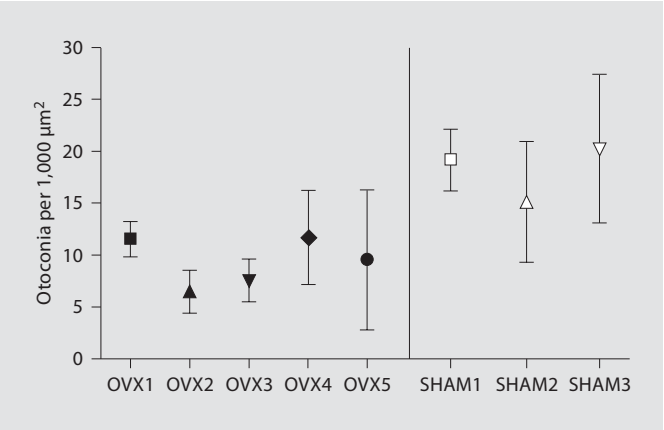

Fig. 5. Otoconia density: comparison between SHAM and OVX rats. Distribution of the number of otoconia per 1,000 μm^2 in the OVX rats (OVX1 through OVX5) compared to the SHAM rats (SHAM1 through SHAM3). The plots denote the mean values and 95% confidence intervals. The otoconia density in the OVX rats is statistically lower (p = 0.036) than in the SHAM rats.

1,000 μm^2 for the 5 OVX rats and 18.2 per 1,000 μm^2 for the 3 SHAM controls. The mean density was $\leq$11.8 per 1,000 μm^2 in all OVX rats and $\geq$15.1 per 1,000 μm^2 for all SHAM controls. As a result of the smaller number of data points, the 95% confidence intervals of the mean density shown in figure 5 overlap between some rats of the OVX group and the SHAM group. However, the difference in mean density is statistically significant between the 2 groups (p = 0.036, 2-tailed Mann-Whitney test).

Otoconia Labeling in Fluorescence Microscopy: Searching for Changes in Calcium Turnover

Based on the morphometric differences observed between both groups in SEM, the calcium turnover in otoconia was evaluated in 2 other groups of adult female rats (6 SHAM rats and 6 OVX rats) under the same experimental conditions.

To control the validity of the fluorescence microscopy technique, the calcium turnover was also measured in the otoconia of young rats using calcein labeling. In the young rats, all otoconia were strongly labeled, showing an intense green fluorescence at their periphery, confirming calcium incorporation into otoconia of young rats during the first week of life (fig. 6a, b).

However, in the OVX and SHAM groups of adult female rats (6–8 months old), the labeling with calcein showed no significant fluorescence into otoconia either with conventional and epifluorescence microscopy (fig. 6c–f) or by laser-scanning confocal microscopy. These findings suggest that in adult female rats there is no detectable calcium turnover into otoconia.

Discussion

In a previous clinical study [Vibert et al., 2003], we pointed out the possible relationship between recurrent idiopathic BPPV in women >50 years of age and the presence of osteopenia or osteoporosis. Indeed, based on previous experimental ultrastructural studies [Anniko, 1980a; Harada and Tagashira, 1981; Usami et al., 1995; Harada et al., 1998; Zucca, 1998; Campos et al., 1999; Karita et al., 1999; Balsamo et al., 2000; Lins et al., 2000; Sans et al., 2001], we hypothesized that, due to decreased estrogen, the disturbances of calcium metabolism, in terms of decreased fixation or/and increased resorption, may induce changes in the otolithic organs. On the one hand, the decreased fixation of calcium may generate failures in the remodeling of the internal structure of the otoconia themselves as well as in their attachment on the otoconial membrane; on the other hand, an increased concentration of free calcium in the endolymph might induce a reduction in its capacity to dissolve the dislodged otoconia.

Our experimental study confirms, in the adult female ovariectomized rats (OVX rats), the presence of ultrastructural modifications of the otoconia in terms of qualitative as well as quantitative changes compared to the SHAM rat group. As reported in previous SEM studies in guinea pigs, the sizes of otoconia ranged from 0.1 to 25 μm and the otoconia were distributed in the utricle according to a specific pattern. Their sizes varied considerably depending on their location on the macula: large otoconia on the lateral part, otoconia of small sizes in the central and medial parts of the utricle [Lim, 1973a, 1973b; Lindeman, 1973]. In our OVX rats, the SEM photographs revealed abnormalities in the distribution of otoconia on the central and lateral parts of the utricle: large otoconia, >30 μm, were also observed on the central area of the utricle, which usually showed only otoconia of small sizes. Furthermore, the morphometric analysis clearly revealed otoconia of larger size and lower in density in the OVX rats compared to the SHAM group (p = 0.036, 2-tailed Mann-Whitney test).

It is interesting to note that Takumida et al. [1997a] have described the formation of giant otoconia (30.7 $\pm$ 5.9 μm) following streptomycin intoxication. They attributed it to the consequences of mechanisms of dissolution-recrystallization of otoconia. Recently, Jang et al.

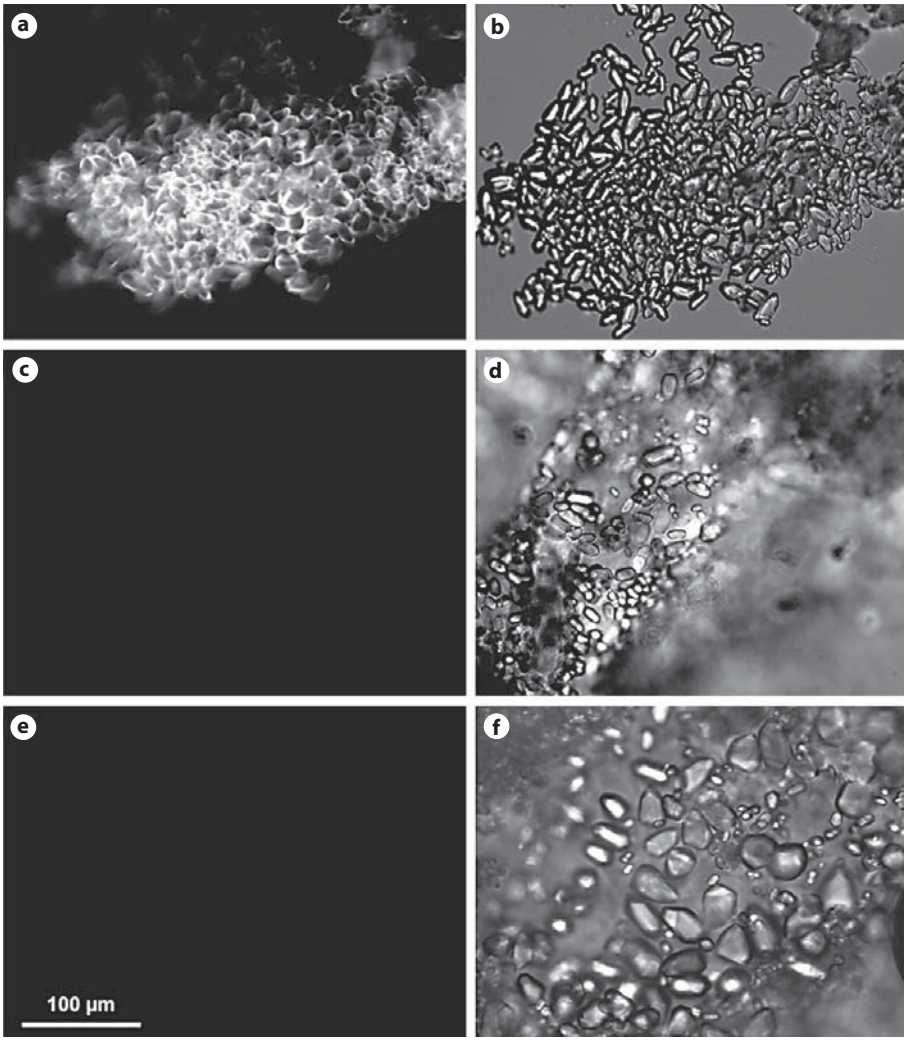

Fig. 6. Otoconia: labeling of calcium using the calcein method. Calcein labeling with conventional and epifluorescence microscopy (**a**, **c**, **e**) and with the laser-scanning confocal microscope (**b**, **d**, **f**) in young (**a**, **b**), OVX (**c**, **d**) and SHAM rats (**e**, **f**). The periphery of otoconia is strongly labeled in the young rats only (**a**). No calcein labeling was detected in otoconia of OVX and SHAM adult rats with epifluorescence microscopy (**c–e**).

[2006] reported that the pattern of otoconial size distribution on the utricular macula was similar in young, middle-aged and aged rats. However, they observed larger otoconia in the peripheral area of the macula compared to those in its central area in middle-aged rats.

In mammals, calcium carbonate as calcite crystals constitutes the crystalline cortex of otoconia. Their organic core is composed of a matrix protein of 90 kDa (otoconin 90) as 90% of its content and other minor proteins [Pote and Ross, 1991; Lundberg et al., 2006]. This matrix protein is directly in contact with the endolymph through pore-like openings located on the crystalline surface [Lins et al., 2000]. These openings may play an important role in the homeostatic control of the chemical environment moving out of and into the core of otoconia [Ross and Williams, 1979; Thalmann et al., 2001]. Thus, changes in the ionic environment of the endolymph, such as modifications of the ions Cl^- [Everett et al., 2001] and HCO_3^- [Royaux et al., 2001] as well as changes of microenvironmental pH near the macular apical region [Stankovic et al., 1997], might generate a demineralization of otoconial crystals.

In rats, the turnover of calcium in otoconia was studied by Kawamata et al. [1995] by labeling them with tetracycline, an antibiotic that precipitates at calcifying fronts and emits fluorescence. They showed that otoconia of newborn rats were strongly labeled at their periphery, which confirmed their high calcium metabolic activity. However, they were not able to demonstrate calcium turnover in the adult rats. In contrast, such calcium turnover was reported by Takumida et al. [1997b] in adult guinea pigs using polychromatic labeling of otoconia. To explain

the morphometric differences that we observed in our osteopenic/osteoporotic rats, we looked for possible changes in calcium turnover of otoconia with a procedure using calcein labeling similar to that described by Takumida et al. [1997b]. Our findings showed no calcein labeling in otoconia of the OVX or the SHAM adult rats. The lack of labeling may be attributed to the fact that the calcium incorporation into otoconia of adult rats is either very low, undetectable using the applied technical methods, or does not exist [Kawamata et al., 1995; Lundberg et al., 2006]. Indeed, the otoconia labeling of neonatal rats might be inherent to their age. On the one hand, calcein might enter the endolymph space of neonatal rats because of the immaturity of their labyrinthine membrane [Anniko, 1980b; Nicolas et al., 2001]. On the other hand, in the first postnatal week, the calcium is massively and very quickly metabolized at the time of otoconia formation and might explain the calcein labeling in comparison to the adult rats.

Finally in our study, the absence of calcium turnover might also indicate that 'exogeneous' calcium ions are not directly implicated in the morphometric changes in size and density of otoconia in that group of rats. The otoconial remodeling might result from slow and progressive mechanisms of mineralization/demineralization involving the 'local' calcium of otoconia themselves. Indeed, these changes might be generated by other biochemical mechanisms of local homeostasis, directly related to the lack of estrogen after bilateral ovariectomy.

In OVX rats, estrogen depletion accelerates the turnover of long bone [Wronski et al., 1985] and also induces a remarkable regression of kidney weight [Hafez et al., 2003] with consecutive alterations in urinary calcium and phosphorus levels [Dick and Prince, 1997]. Because of the similarities between the kidney and the inner ear in terms of fluid regulation and the presence of estrogen receptors in both organs [Kumagami, 1994; Mosselman et al., 1996; Stenberg et al., 1999; Stenberg et al., 2001], one may assume that estrogen depletion in the inner ear, particularly in the utricle, might generate changes in the calcium balance of the endolymph. Playing the role of a calcium ionic reservoir [Ross and Williams, 1979], the otoconia could provide the calcium needed to maintain the biomolecular homeostasis of the endolymph. The direct consequence of such a mechanism should be the progressive demineralization of the otoconia and the alteration of their crystallized shell, generating mechanisms of dissolution-recrystallization of otoconia, with formation of very large otoconia as well as otoconial aggregations [Takumida et al., 1997a]. On one side, such mechanisms of demineralization, dissolution and recrystallization may explain the decreased density of otoconia. The alteration in the otoconial distribution on the utricular macula may be related to faulty adherence of such neo-otoconia due to broken interotoconial linking filaments. On the other side, the observation of an increased size of the otoconia in the osteoporotic rats might be consecutive to a mechanism of control at the molecular level of the organic core of the otoconia by increasing the protein core to compensate the destructured shell. Thus, such morphometric changes of the utricular ultrastructure, by generating altered otoconia, might explain the recurrent symptom of BPPV as well as its frequent resistance to traditional physiotherapeutic maneuvers in women suffering from osteoporosis or osteopenia.

Conclusion

Our experimental study confirms the presence of ultrastructural modifications of the otoconia in terms of changes in their aspect, size and density in ovariectomized osteopenic/osteoporotic female adult rats: the otoconia are increased in size and decreased in their density compared to a control group of rats. These changes are interpreted as the consequence, in the utricle, of the disturbance of the calcium metabolism induced by osteopenia/osteoporosis.

The present results are a further corroboration of the hypothesis that the high frequency of recurrent BPPV in older women suffering from osteopenia and osteoporosis may be related to disturbance of calcium metabolism.

Acknowledgments

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