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Scanning Electron Microscopy Images and Energy-Dispersive X-Ray Microanalysis of the Stapes in Otosclerosis and Van der Hoeve Syndrome

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Objective/Hypothesis: **The objective of this study was to evaluate the morphological and microchemical changes that affect sclerotic stapes in otospongiosis and van der Hoeve syndrome.** ***Methods:*** **A scanning electron microscope equipped with an energy-dispersive x-ray analyzer was used in the experiments.** ***Results:*** **In otosclerosis, focal lesions are poorly mineralized, with low calcium salt and reduced calcium-to-phosphorus (Ca/P) ratio (1.9:1). This finding correlates with a spongiotic type of lesion and indicates unstable mineralization with possible change from hydroxyapatite to calcium triphosphate. In van der Hoeve syndrome the presence of magnesium in stapes suggests osteoclastic function stimulation. The osteoclasts secrete many protons, causing an acidified microenvironment. Brushite is formed, and Ca/P ratio decreases in comparison with that of control patients (2.0:1 vs. 2.6:1).** ***Key Words:*** **Stapes, otospongiosis, otosclerosis, van der Hoeve syndrome, scanning electron microscopy, Calcium-to-phosphorus ratio, brushite, hydroxyapatite.**

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INTRODUCTION

Bone is one of the strongest biological materials known. It is composed of organic components that give force and stability and inorganic components that provide rigidity and convert it to mineral deposits. The most suitable model for the major solid calcium phosphate mineral phase in mature bone is hydroxyapatite $Ca_5(OH)(PO_4)_3$, containing approximately 5% to 10% CO_3^{2-} and approximately 5% to 10% HPO_4^{2-} groups, the latter in a brushite-like configuration ($CaHPO_4 \cdot 2H_2O$). However, under certain circumstances and in different diseases, composition is altered and partial conversions to other related mineral species such as *dahllite* ($Ca_{8.8}(HPO_4)_{0.7}(PO_4)_{4.5}(CO_3)_{0.7}(OH)_{1.3}$) or *struvite* ($MgNH_4PO_4$) are produced. A way of evidencing the transformations that are produced is through quantitative phosphorus (or phosphates) and calcium or magnesium (or both) determinations, because their relative percentages in each of the cited mineral species are significantly different. The calcium-to-phosphorus (Ca/P) ratio is especially useful. In normal bone, the Ca/P ratio is not always constant over time. Thus McLean and Urist[1] postulated that an initial amorphous calcium phosphate phase gradually and successively converts to octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$), tricalcium phosphate ($Ca_3(PO_4)_2$), and hydroxyapatite. These forms of mineralization exist only temporarily in the immature fetus bone, so in adults, only the mature form of calcification represented by hydroxyapatite is found. In some pathological situations, such as in fractures, it is possible to observe brushite near hydroxyapatite, although in a very reduced proportion.

Otosclerosis is a genetically transmitted osteodystrophy of the labyrinthine capsule that is clinically described in relationship with changes in bone mineral composition. In analytical studies, although both calcium and phosphorus levels were determined to be lower in the bone of patients with otosclerosis than in normal controls, molar Ca/P ratio was believed to be similar to that of sound bone. Thus, although poorly mineralized, otosclerotic bone seemed to maintain a hydroxyapatite type of composition.

Van der Hoeve syndrome (VDHS) is a particular form of osteogenesis imperfecta. As in other forms of osteogenesis imperfecta, VDHS is transmitted genetically and is characterized by the associated presence of blue sclerae, bone brittleness, and progressive conductive hearing loss. Mutations in the genes proαa1(I) and proαa2(I) chains of type I procollagen are thought to cause this syndrome, although a severe form of osteogenesis imperfecta unlinked to

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the collagen genes is also known to exist.[2] In addition, anomalous synthesis of the collagen matrix is associated with the presence of bone with an abnormal mineral composition.[3] Using an animal experimental model, it was shown that the cortical bone of patients with VDHS syndrome had a lower molar Ca/P ratio—measured by x-ray microanalysis—than in normal controls.[4]

Despite etiological differences, the auditive characteristics of VDHS most frequently have been compared with otosclerosis because both disorders have been related to alterations in the protein matrix. Whereas VDHS is caused by an alteration in the type I collagen synthesis,[5] it has been postulated that in otosclerosis autoimmunity to type II collagen would exist.[6] This collagen is the main component of embryonic cartilage and it forms the enchondral shell of the labyrinthine capsule. Both diseases have also been related on occasion[7,8] because, as common characteristics, they present mechanical alterations in the bony labyrinth and in the ossicles of the middle ear.

We have studied morphological appearance and mineralization of stapes from patients with otosclerosis and VDHS. These stapes have been compared with normal stapes in the hope of gaining insight into the disease processes of both diseases.

MATERIALS AND METHODS

This study describes the morphological appearance and mineral composition of eight stapes. Three of the eight stapes came from patients with otosclerosis and two came from patients with VDHS. The remaining three stapes were obtained from patients without disease of the ear middle for whom an endaural labyrinthectomy for Meniere's disease was indicated.

A Philips XL30 scanning electron microscope was used in the experiments. Images were obtained in the secondary electron backscattered modes. X-ray energy-dispersive analysis was performed with EDAX New XL-30 Zaphire equipment. The x-rays were collected at optimum angles. Samples were coated with a thin layer of gold to avoid changing the mineral structure.

Scanning electron microscopy (SEM) is a technique in which samples are bombarded with primary electrons: the signals for the images are the secondary and backscattered electrons that result from the topographical and chemical differences in the sample surface. X-ray energy-dispersive analysis is a technique that gives the microanalysis of the sample from the x-ray radiation issued from the interaction of the electrons with the sample and uses semiconductor detectors for the measures. The methodology that is used combines three fundamental advantages. It presents images with greater resolution and greater field depth than optical microscopy, it provides a quantitative and qualitative microanalysis for each image, and, furthermore, samples are not destroyed when their composition is being studied.

In addition, the potential effectiveness of Fourier-transform infrared (FT-IR) spectroscopy for the study of bone mineral changes in bone disease is shown by analysis of several samples. FT-IR spectra were recorded on a Nicolet 205 infrared spectrometer using the potassium bromide disk method. Spectra were collected as absorbency spectra at four-wavelength resolution with 33 scans performed per specimen.

The Ca/P ratios of normal and pathological samples measured by x-ray microanalysis have been expressed in weight (rather than by molar Ca/P relationship).

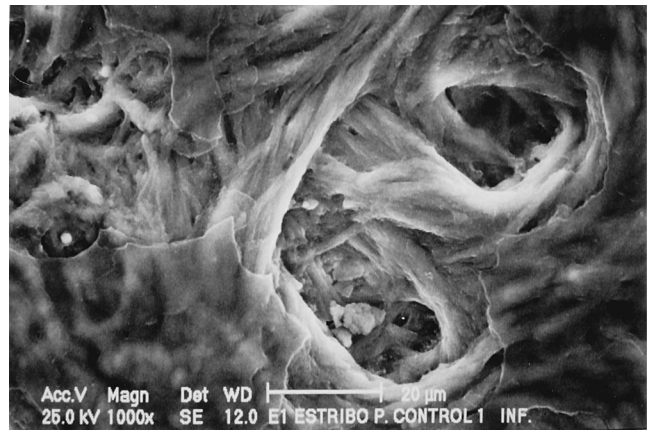

Fig. 1. Scanning electron microscopy (SEM) image (original magnification × 1000) of a normal stapes showing a bone fibrillar structure that resembles thread at great magnification.

RESULTS

Scanning Electron Microscopy

Morphological study of normal stapes at original magnification × 500 shows a fibrillar structure with an irregular trabecular diagram on which rounded expansions are observed. At higher magnification (× 1000) the previously described fibers are observed interlaced with no regular pattern but with an aspect ordered as thread hanks (Fig. 1).

In otosclerotic bone, two clearly differentiated zones corresponding to apparently normal bone (Fig. 2) and otosclerotic foci (Fig. 3) are observed. In disease-free bone at original magnification × 1500, we observed the same fibrillar structure with expansions similar to those described in the previous paragraph for normal stapes. However, otosclerosis plate zone did not have a fibrillar aspect. At original magnification × 1000, multiple spheroid formations (with numerous marks or holes) resembling sea sponges were evident, some of them 10 μm in diameter.

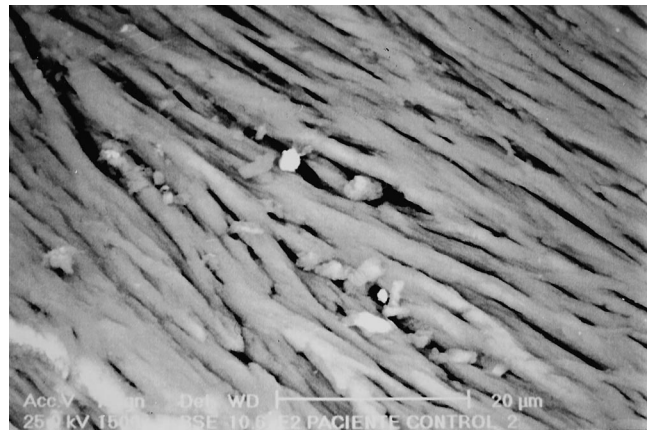

Fig. 2. Scanning electron microscopy image of an otosclerotic stapes (original magnification × 1500) in a corresponding area to normal bone (otosclerosis–free zone). The ultrastructure of the bone adopts a fibrillar morphological appearance, as occurs in the normal bone.

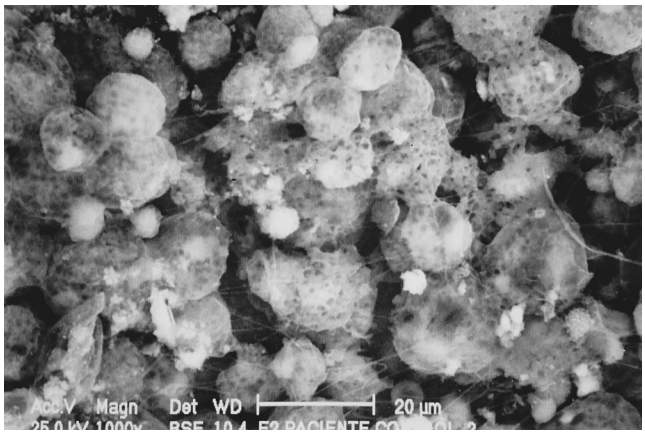

Fig. 3. Scanning electron microscopy image of a otosclerotic stapes (original magnification × 1000) in an otospongiotic area. Multiple spheroid formations ("sea-sponge–like" calcospherites) are evident.

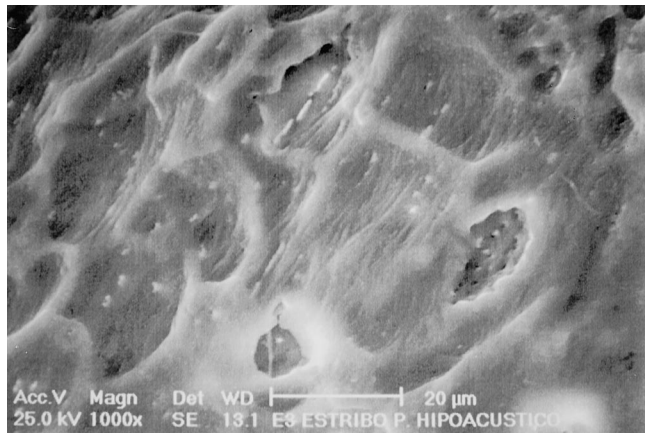

Fig. 5. Higher magnification (original magnification × 1000) of the bone morphological appearance of the stapes in VDHS. The normal bone ultrastructure is replaced with an amorphous structure without any fibrillar pattern.

Stapes corresponding to VDHS presented a pierced, porous, noncompact aspect at original magnification × 500 (Fig. 4). At original magnification × 1000, the fibrillar standard described in normal bone was not observed. There was an amorphous aspect with depressions and abnormally distributed relief (Fig. 5) throughout its extension.

X-Ray Microanalysis

Normal bone mineral composition, as elemental percentage in weight for three different sections, is shown in Table I. The Ca/P ratios for each of the sections were 2.38, 2.61, and 2.73, respectively. Average Ca/P ratio (2.57) shows an apatite type of mineralization.

In agreement with morphological findings for otosclerotic bone, the analytical data shown in Table II reflect a mixed pattern with poorly mineralized lesions and well-mineralized areas. As expected, spongiotic lesions in the foci have lesser mineral components than the apparently disease-free areas (where even components foreign to normal bone can be found). If Ca/P ratio is used as the mineralization criterion, it can be observed that well-mineralized areas show a normal or slightly high (2.87) Ca/P ratio and poorly mineralized lesions are characterized by a low value (1.95).

In the disease-free area of otosclerotic stapes the presence of copper (Cu) is significant. The presence of aluminum (Al) is also noteworthy in otosclerotic foci.

The high level of sulfur (>2%) in otosclerotic bone differentiates it from normal bone. In addition, the quantitatively significant presence of iron (Fe) in nonotosclerotic areas and of silicon (Si) in sclerotic foci helps distinguish them.

Mineral composition of the stirrup in VDHS has been quantified in five different sections. The data are presented in Table III. Ca/P ratios are 2.00, 2.24, 2.04, 1.96, and 1.79 in each section, respectively (mean ratio, 2.0). It is noteworthy that this Ca/P ratio (2.0) is significantly lower for patients with VDHS than for the control subjects (2.6). Another special result is that the presence of magnesium is significantly increased.

Fourier-Transform Infrared Spectroscopy

The observed spectra for all bone samples were in agreement with those from human bone. The spectral range (1240–1030 cm^{-1}) contained the main vibration

Fig. 4. Scanning electron microscopy image of stapes in Van der Hoeve syndrome (VDHS) (original magnification × 500). The porous aspect of their structure is evident.

TABLE I.
Normal Bone Mineral Composition, As Elemental Percentage in Weight for Three Different Sections.

	Section		
Chemical Composition	1	2	3
% Carbon	11.16	16.31	15.41
% Oxygen	19.50	19.33	18.53
% Magnesium	0.52	0.41	—
% Phosphorus	20.35	17.68	17.70
% Calcium	48.47	46.27	48.36
Total	100.00	100.00	100.00

TABLE II.
Mineral Composition of Stapes With Otosclerotic Foci and Apparently Nonlesioned Areas.

Chemical Composition	Nonsclerotic Area	Sclerotic Area
% Carbon	54.71	66.81
% Oxygen	16.85	17.05
% Sodium	0.59	1.03
% Sulfur	4.33	2.20
% Aluminum	0.49	0.50
% Iron	1.04	
% Copper	1.39	
% Phosphorus	5.31	4.04
% Calcium	15.29	7.89
% Silicon		0.48
Total	100.00	100.00

modes: PO_4 bend at 1238 cm^{-1} and asymmetric P-O stretching of PO_4 at 1031 cm^{-1}.

The bone samples from patients with VDHS (Fig. 6) showed a peak value at 871 cm^{-1} caused by P-OH stretching of HPO_4^{2-}. This peak was lower (nearly undetectable) in the spectra of both normal and sclerotic stapes. In contrast, the weak peak value at 1236 cm^{-1}, indicative of P-OH deformation, is also somewhat stronger in VDHS.

DISCUSSION

The morphology of the studied stapes is notably different in control patients, otosclerotic patient, and patients with VDHS.

In the disease-free area of the otosclerotic stapes, the presence of Cu suggests a low bone turnover by suppression of both osteoblastic and osteoclastic functions.[9] This means that mineralization occurs without bone destruction or bone formation (preotosclerotic type according the classification from Lim and Saunders.[10] In the otosclerotic foci the presence of Al suggests only inhibition of osteoblastic function.[9] Because in these foci no presence of Cu was observed, an active otosclerosis was presumed.

In agreement with other authors,[10] we have found poor mineralization in otosclerosis and preserved Ca/P ratio in disease-free areas (the Ca/P is slightly higher than

TABLE III.
Mineral Composition of the Stirrup in van der Hoeve Syndrome.

	Section				
Composition	1	2	3	4	5
% Carbon	17.35	63.44	27.23	23.60	45.33
% Oxygen	30.68	23.14	20.43	23.47	20.39
% Magnesium	0.48			0.86	0.72
% Phosphorus	16.82	3.73	16.58	17.15	11.49
% Calcium	33.67	8.38	33.93	33.63	20.66
% Sodium	1.00	0.61	1.29	1.28	1.41
% Sulfur		0.70	0.54		
Total	100.00	100.00	100.00	100.00	100.00

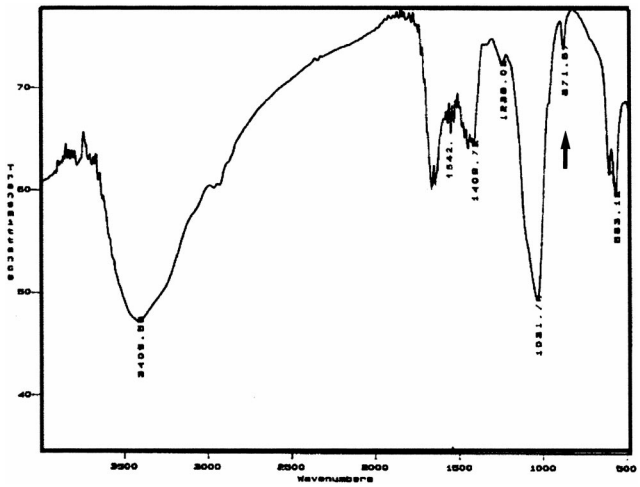

Fig. 6. Fourier-transform infrared spectra of a bone sample from the stapes of a patient with VDHS. A peak value at 871 cm^{-1} (arrow) is caused by P-OH stretching of HPO_4^{2-}.

in the control samples). This suggests a mineralization pattern of apatite type. Nevertheless, Ca/P ratio indicates a changed mineral composition in otosclerotic foci (very probably, transformation of apatite into tricalcium phosphate).

On the other hand, although mineralization was normal in stirrups from patients with VDHS, Ca/P ratio was lower than in normal controls, similar to data communicated previously.[4,11] This Ca/P ratio is compatible with partial substitution of hydroxyapatite by brushite (a change not previously described).

From the Ca/P ratios (Table IV) we estimated that in otosclerotic foci the apatite could be substituted by tricalcium phosphate and other mineral species to three fourths of the overall content. Also, from the data shown in Table IV it can be concluded that in VDHS the substitution by brushite gives rise to one fifth of the apatite lattice (or two fifths of the normal bone mineral composition).

Concerning FT-IR results, our data have shown brushite in stapes of patients with VDHS. Our spectra for VDHS bone produced features similar to those reported by Cassella et al.[4] for osteogenesis imperfecta in a transgenic mouse. In both studies, HPO_4^{2-} was well detected at 872 to 875 cm^{-1} and did not shift to lower wavelengths (as occurs when CO_3-HA contribution is significant). This finding cleared the ambiguity that could be created by the proximity of the bands caused by CO_3^{2-} out-of-plane stretching and by P-OH stretching of HPO_4^{2-} when CO_3^{2-} and HPO_4^{2-} coexist. If, contrary to our results, CO_3^{2-} surpassed HPO_4^{2-} in percentage, the band at 873 cm^{-1} would be shifted to 850 cm^{-1} (Doi et al.,[12] cited by Cassella and Ali[11]). The Ca/P ratios and FT-IR results for VDHS are very encouraging. They correlate well with the result of enhanced magnesium content that always occurs when brushite is detected.[13]

Oligoelement study results of bone from VDHS patients revealed a significant presence of magnesium (Table V). Magnesium is a marker of osteoclastic function stimulation.[9] The osteoclasts secrete a large amount of

TABLE IV.
Calcium-to-Phosphorus Ratios of Mineral Species in Normal and Ill Stapes.

1.3	1.72	1.93	1.95	1.97	2.00	2.15	2.60	2.87	3.0
Brushite	Octocalcium phosphate	Tricalcium phosphate	Otosclerotic foci	B.3HA	Van der Hoeve syndrome	Hydroxyapatite	Control or normal stapes	Normal-like otosclerotic areas	PolyOH or CO_3^-
(B)	(B.2TCP)	(TCP)	(3TCP.HA)		(B.4HA) (2B.3NS)	(HA)	(NS)		-HA

protons[14] causing an acidified microenvironment and HPO_4^{2-} formation. As a result, bone substitutes brushite for apatite in the bone lattice.

From the magnetic resonance imaging line shape, Roufosse et al.[15] deduced that HPO_4^{2-} group fraction was highest in the youngest bone and decreased progressively with increasing specimen age.

Since it has been stated that, from the beginning of the mineralization, octacalcium phosphate is present (not the brushite), and that in some conditions octacalcium phosphate transforms into hydroxyapatite,[16] the usual progressive increase of bone hydroxyapatite with aging is from octacalcium phosphate (but not from brushite). Thus the existence in mature bone of brushite in a significant amount (>10%) must be considered abnormal.

It could be postulated that mineralization in a brushite type of configuration constitutes an immature mineralization form. However, Bonar et al.[17] did not find this configuration in different embryonic bones. Therefore brushite in VDHS stirrups does not in any way constitute an immature persistence of calcification, but rather, a transformation from hydroxyapatite.

Partial transformation of apatite into brushite is favored under either acid or low supersaturation conditions. In fact, with decreasing pH, a preferential dissolution of hydroxyapatite was stated, whereas in weak acid solutions at pH 5 a preferential precipitation of brushite occured.[18] Brushite has never been observed to transform into hydroxyapatite. In contrast, when bone repair is necessary, but local free calcium concentration is scant (e.g., after a fracture), only brushite and amorphous tricalcium phosphate are precipitated.[19]

Different studies have suggested that matrix protean collagen alterations could influence bone mineralization.[3,20,21] This modification in mineralization would be related to the phosphomonoester bindings of O-phosphoserine and O-phosphothreonine of bone protein matrix.[22] Such modification of normal bone mineralization would alter its mechanical properties.

These features lead us to suggest that brushite increase in bones of patients with VDHS can be caused by the following circumstances: 1) osteoclastic function stimulation, 2) acid attack to the bone, 3) apparition of an acidic form of phosphate (which is induced by a changed collagen type), and 4) multiple or abnormally repaired bone microfractures, which require enrichment in this component.

TABLE V.
Analytical Study Results of Bone From Patients With van der Hoeve's Syndrome Compared With That of the Control Subjects and Otosclerotic Patients (Mean Values).

Composition	Van der Hoeve's Syndrome	Control	Otosclerosis
% Carbon	17.3	16.3	54.7
% Oxygen	30.7	19.3	16.9
% Sodium	1.0		0.6
% Magnesium	0.5	0.4	
% Phosphorus	16.8	17.7	5.3
% Calcium	33.7	46.3	15.3
% Iron			1.0
% Sulfur			4.3
% Aluminum			0.5
% Copper			1.4
Total	100.0	100.0	100.0

CONCLUSION

Deposit of tricalcium phosphate and brushite and insufficient mineralization of the collagen matrix may be important features to add to those already known to characterize osteosclerosis and VDHS.

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