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Physicochemical and microscopical study of calcific deposits from natural and bioprosthetic heart valves. Comparison and implications for mineralization mechanism

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Natural and bioprosthetic heart valves suffer from calcification, despite their differences in etiology and tissue material. The mechanism of developing calcific deposits in valve tissue is still not elucidated. The calcific deposits developed on human natural and bioprosthetic heart valves have been investigated and compared by physicochemical studies and microscopy investigations and the results were correlated with possible mechanisms of mineral crystal growth. Deposits from 16 surgically excised calcified valves (seven natural aortic and nine bioprosthetic porcine aortic valves) were examined by chemical analysis, FTIR, XRD, and SEM-EDS. The Ca/P molar ratio of the deposits from bioprosthetic valves (1.52 ± 0.06) was significantly lower compared to that of the natural valves (1.83 ± 0.03) ($p = 0.05$, 1-way ANOVA). SEM-EDS examination of the two types of valve deposits revealed the coexistence of large ($> 20\,\mu m$) and medium (5–20 μm) plate-like crystals as well as microcrystalline ($< 5\,\mu m$) calcium phosphate mineral formations. The results confirmed the hypothesis that the mineral salt of calcified valves is a mixture of calcium phosphate phases such as dicalcium phosphate dihydrate (DCPD), octacalcium phosphate (OCP) and hydroxyapatite (HAP). DCPD and OCP are suggested to be precursor phases transformed to HAP by hydrolysis. The lower value of the Ca/P molar ratio found in the bioprostheses, in comparison with that corresponding in natural valves, was ascribed to the higher content in these deposits in precursor phases DCPD and OCP which were subsequently transformed into HAP. On the basis of chemical composition of the deposits and their morphology it is suggested that crystal growth proceeds in both types of valves by the same mechanism (hydrolysis of precursor phases to HAP) in spite of their differences in etiology, material, and possible initiation pathways.

Introduction

Degeneration, mechanical damage, and calcification of the leaflet tissue constitute serious reasons for bioprosthetic valve failure. The formation of calcium phosphates on porcine bioprosthetic heart valves to a percentage of more than 50% is responsible for their dysfunction after 12–15 years due to stenosis or insufficiency [1]. Although intensive research has been done over the past few decades, the mechanism of initiation and development of calcific deposits on tissues in contact with blood is still poorly understood. Biological, chemical, and mechanical factors seem to play a significant role in the kinetics of the process of calcification [2]. As a result, the development and production of biological valves resistant to calcification [2] is still a major challenge.

Natural heart valves also suffer from calcification. However calcification in natural valves is in general associated with certain pathologic etiologies, while this is not a prerequisite for calcification of bioprostheses. Bioprosthetic valve tissue material is quite different from that of natural valves due to the absence of living cells and the presence of dead cell remnants together with the chemical modification of the histologically different [3] natural porcine tissue with glutaraldehyde. These differences give rise to the question if the crystal

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growth in natural and bioprosthetic heart valves proceeds by different calcification pathways or not.

Calcification consists in the formation of sparingly soluble calcium phosphate deposits (CD) in the extracellular matrix of leaflet tissue. From a physicochemical point of view, due to high levels of calcium and phosphate concentration in blood serum a number of calcium phosphate phases may precipitate out in the order of decreasing solubility: Dicalcium phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$, DCPD), octacalcium phosphate ($Ca_8(PO_4)_6H_2 \cdot 5H_2O$, OCP), tricalcium phosphate ($Ca_3(PO_4)_2$, β-TCP) and hydroxyapatite ($Ca_5(PO_4)_3OH$, HAP) [4, 5]. Although valve CDs as end products of biomineralization consist mainly of apatitic calcium phosphate (HAP containing mainly carbonate, fluoride, magnesium, and sodium) [6] *in vitro* and animal studies have proven the formation of transient precursor phases such as DCPD and OCP [7–9]. Studies on explanted calcified natural and bioprosthetic valves suggested that crystal growth proceeded by hydrolysis of the precursor phases to HAP. The presence however of such phases in these CDs has not been proven yet [6, 10].

The aim of this work was to characterize and compare the chemical composition and morphology of CDs from explanted natural human and bioprosthetic porcine aortic heart valves and to look for evidence of possible transformation pathways in the process of mineral phase formation.

Materials and methods

Sixteen calcified valves, explanted from sixteen patients operated for heart valve replacement were investigated. Seven valves were aortic natural ones, which were replaced due to rheumatic $(n = 2)$ or degenerative $(n = 5)$ disease from seven patients (six males, one female), 52–73 years old. Nine valves were porcine aortic bioprostheses, three explanted from the aortic and six from the mitral position. The patients, three males and six females 50–72 years old were reported for valve replacement 8–11 years after the initial implantation. The explanted valves had been stored in neutral phosphate-buffered 10% formalin. This solution does not affect the calcium concentration of calcified tissue [11].

Calcific deposits were first characterized by powder X-ray diffraction (XRD). The solids were powdered in a mortar and the XRD spectra were recorded with a Phillips 1830/1840 powder diffractometer, between 10–70° 2θ at sweep rate of 0.4° 2θ/min. Fourier transformed (FTIR) spectroscopy of CDs (previously dried in 80 °C for 24 h and pulverized) was performed with the method of KBr pellets, with a Perkin Elmer 1600 PC FTIR spectrometer. The morphology of CD crystals formed was studied by SEM. The samples were embedded in paraffin blocks with routine histological techniques. Parallel sections of the blocked tissues were cut perpendicular to the leaflet plane in the circumferential direction with a microtome and placed onto Aluminum sample holders for SEM. The samples were deparafinized very carefully with xylene and the cross-sectional surface was sputtered with gold and examined with a JEOL JSM 6300 SEM with a built-in ability for EDS microanalysis. From the composition, the morphology and the size of the developed CD crystals examined, their nature was determined by comparison with reference calcium phosphate DCPD, OCP, and HAP phases prepared synthetically [12–15]. With this technique it was possible to determine the morphology of CDs developed at the internal sites of the tissue at high magnifications without removing CDs from the associated tissue components, avoiding so possible chemical alterations due to extraction techniques [16].

The chemical analysis of CDs included the determination of the total mineral content (in mg/g dry tissue) and the molar ratio of Ca/P in the CDs examined. For this purpose, the valve leaflets were dried and weighted. Subsequently they were subjected to acid hydrolysis (0.1 N HCl) for 3 days, redried and weighted again. The residual solution was then analyzed for calcium (Ca^{2+}) by atomic absorption spectrometry (Perkin Elmer 305A) and for phosphate (PO_4^{3-}) spectrophotometrically (UV-visible Spectronics Genesys 5) [17].

Results

Fig. 1 shows the XRD spectra of CDs developed on calcified natural human aortic valvular tissue (a) and of reference synthetic HAP (b). The comparison of the peaks of the two spectra at the 20–40° 2θ range of interest suggests that the basic CD is formed as microcrystalline apatitic material. The merging of 211 and 112 peaks at the spectrum of the CD obtained from valves (a) indicate that the apatitic material consisted of carbonated apatite [18].

Fig. 2 shows the FTIR spectra of explanted calcified natural human aortic valves (I) and those of porcine bioprosthetic aortic valves explanted from mitral (IIa) and from aortic (IIb) position. The spectrum from unimplanted bioprosthetic porcine aortic valve (IIc) is presented for comparison. The spectra of the explanted calcified valvular tissue (natural and bioprosthetic) featured the characteristic bands of apatite with significant substitution of phosphate by carbonate. It is known that stoichiometric apatitic materials exhibit a

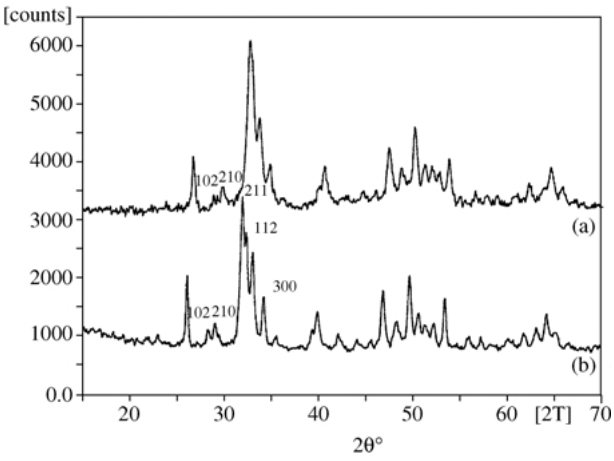

Figure 1 Powder X-ray diffraction spectrum of calcific deposits developed on natural human aortic valvular tissue (a). The spectrum of reference synthetic HAP solid is also shown (b). The merging of 211 and 112 peaks at (a) indicate that the apatitic material consisted of carbonated apatite.

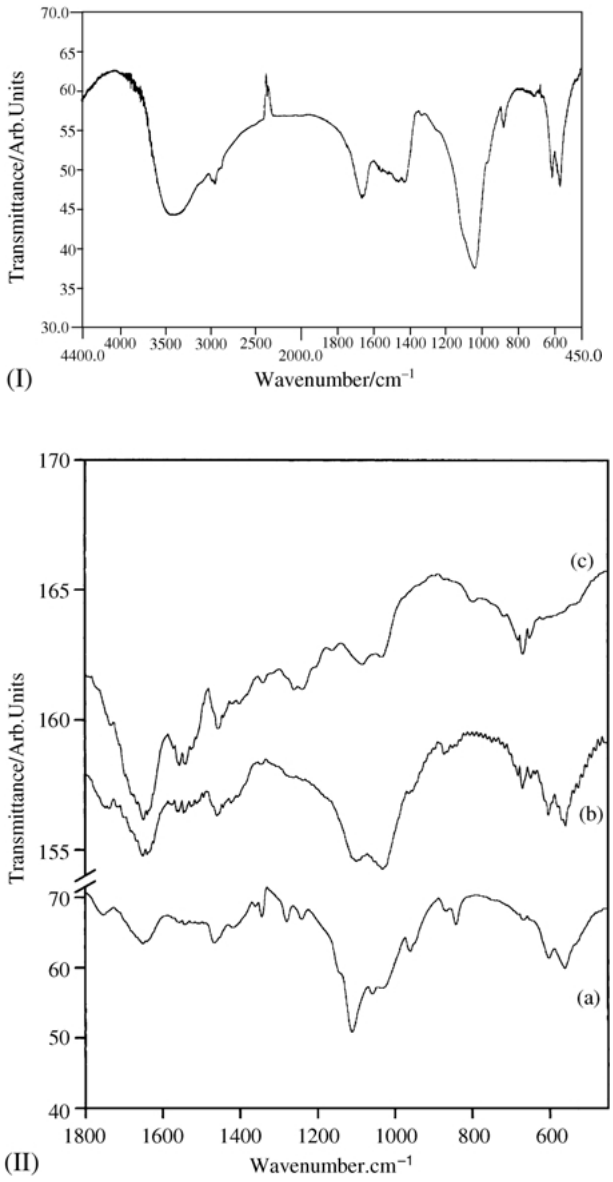

Figure 2 FTIR spectra of calcified valvular tissue explanted: (I) natural human aortic valve and (II) bioprosthetic valves from (a) mitral and (b) aortic position. The spectrum (IIc) belongs to unimplanted bioprosthetic porcine valvular tissue. The presence of carbonated apatite on the deposits appears a double peak at the band 650–500 (I, IIa, and IIb). These peaks are absent at the spectrum IIc.

triple peak band in the phosphate vibration modes (range 650–500 cm^{-1}). In the case of substitution of phosphate ions by carbonate, the triple peak changes to a double peak band [19]. This is the case as may be seen in the spectra of calcified tissues (human natural (I) and porcine bioprosthetic (IIa and IIb)) shown in Fig. 2. These characteristic peak bands at 650–500 cm^{-1} are missing in the spectrum of the unimplanted bioprosthetic porcine valvular tissue (IIc).

The morphology of the calcified deposits is shown in the micrographs presented in Figs. 3–5 in which the results of SEM-EDS examination of porcine bioprosthetic valvular tissue sections may be seen. The characteristic morphology of the solids suggested the coexistence of microcrystalline HAP (size $< 5\,\mu m$) with small and thin plate-like crystallites of OCP (size 5–20 μm). The later phase is believed to be the precursor phase to apatite, while larger and thicker plates (size

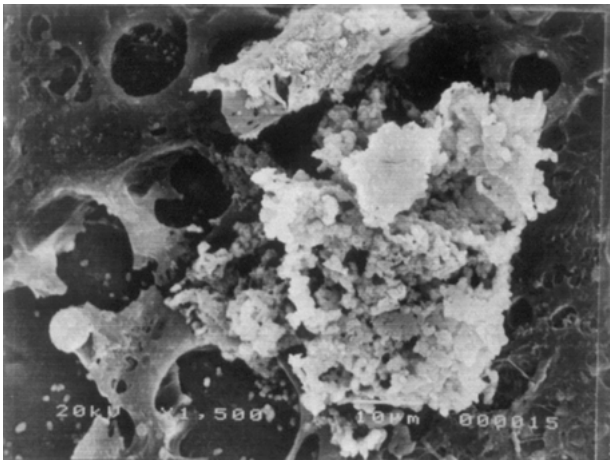

Figure 3 Scanning electron micrograph of section of explanted porcine bioprosthetic heart valve, showing calcific deposits of characteristic fine microcrystalline HAP.

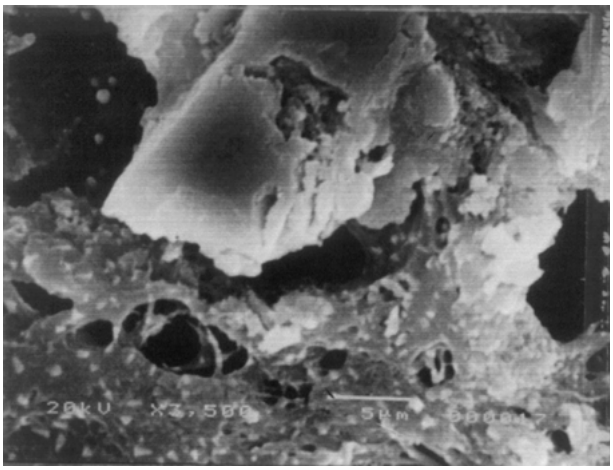

Figure 4 Scanning electron micrograph of section of explanted porcine bioprosthetic heart valve, showing calcific deposits of small and thin plate-like morphology, typical of OCP crystals.

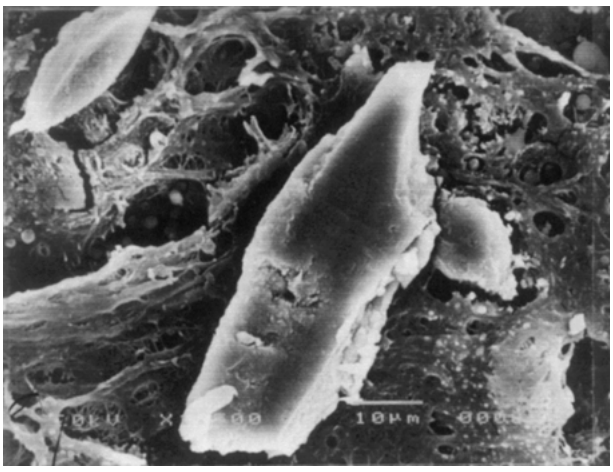

Figure 5 Scanning electron micrograph of section of explanted porcine bioprosthetic heart valve, showing calcific deposits of thick and large platy DCPD formations.

$> 20\,\mu m$), typical of crystal habit of DCPD, the least stable calcium phosphate may also be seen.

The results of the chemical analysis of natural and bioprosthetic heart valve CDs are summarised in Table I. The average Ca/P molar ratio of natural CDs was found

TABLE I Ca/P molar ratio obtained from the chemical analysis of calcific deposits of explanted calcified native human and bioprosthetic porcine aortic heart valves

Sample	Ca/P ratio	
	Native human aortic valves	Porcine bioprosthetic aortic valves
1	1.78	1.41
2	1.84	1.59
3	1.65	1.67
4	2.13	1.33
5	1.62	1.50
6	1.80	1.41
7	1.98	1.62
8		1.15
9		2.01
Average $\pm$ STD	1.83 ± 0.03	1.52 ± 0.06

equal to 1.82 ± 0.03, while the corresponding ratio of the bioprosthetic ones was found equal to 1.52 ± 0.06. This difference was statistically significant ($p = 0.05$, 1-way ANOVA.). It may be suggested that in both cases the CDs consist of mixtures of HAP (stoichiometric Ca/P ratio = 1.67) and OCP (stoichiometric molar ratio Ca/P = 1.33) as well as DCPD (stoichiometric molar ratio Ca/P = 1). A much lower OCP and DCPD content in natural CDs, compared to higher amounts of these phases presented in bioprosthetic ones, could explain the higher Ca/P ratio found in the first, which is close to that found in mature physiological biomineral in bone (Ca/P = 1.75) [20].

Discussion

This study is a contribution to the physicochemical and morphological characterization of the CDs found *in vivo* in natural and bioprosthetic heart valves. The questions we tried to answer are

1. Are the final CDs of natural and bioprosthetic cardiac valves similar
2. Which is the mechanism of CDs crystal creation and maturation

It is obvious that the answer in these questions is very important for the design of an effective strategy for the retardation of the initially forming mineral phases [6, 10].

The nature and physicochemical properties of bioprosthetic valve CDs, although extensively investigated, are poorly understood. From the thermodynamics point of view, stable HAP, usually found on the deposits obtained from explanted valves, may be the final mineral phase developed by the hydrolysis of precursor, less stable phases. Investigations on the mechanism of bone formation provided evidence that DCPD is a precursor phase [19, 21–23]. Moreover, *in vitro* experiments, in which the growth of calcium phosphates on seed crystals was studied, showed that at the physiological pH of 7.40 the initial formation of DCPD is followed by very rapid hydrolysis to OCP and subsequently to HAP [24].

It is very likely that the first nuclei of the mineral deposits in the calcification process are located at transplanted connective tissue cell remnants and also possibly at different stages to the fibers of collagen [2]. The extracellular free calcium concentration is very high (10^{-3} M) and the disruption of cellular calcium regulative mechanism allows for calcium reaction with the phosphate groups of membrane phospholipids [2] which may yield calcium phosphate salts at specific areas in which supersaturation reaches the locally required critical levels.

The XRD patterns of the mineral phase in the specimens examined revealed the presence of microcrystalline apatite material in which OCP and HAP coexisted. The FTIR spectra showed the characteristic bands of apatite with significant substitution of phosphate by carbonate ions. These findings were similar both for the natural and the bioprosthetic heart valve CDs.

The chemical analysis of the natural valve CDs showed increased Ca/P molar ratios compared to the stoichiometric values of the corresponding OCP or HAP pure mineral phases. The higher values may be explained by the presence of substantial amounts of sodium magnesium and carbonate at mature CDs [6]. Bioprosthetic CDs on the other hand showed values of the molar Ca/P ratios in between those corresponding to pure OCP and HAP, in agreement with data reported in the literature [10]. The results support the hypothesis that the final salt, in a long-term mineralization process of valve leaflets *in vivo*, is a mixture of HAP and OCP. The different Ca/P molar ratio of native and bioprosthetic CDs is justified by the fact that the CDs of bioprosthetic heart valves are left in contact with the physiological fluid supersaturated for relatively shorter times in comparison with that of the natural valves. Even if we suppose that mineralization starts upon the implantation of bioprostheses, in the case of natural valves the etiology of dystrophic calcification may have been completed quick earlier prior to explantation, although the initiation time is difficult to be determined. The longer the calcification proceeds, as in the case of natural valves, according to hypothesis presented here, would lead to the development of more mature and stable phases, richer in HAP rather than in the transient OCP phase, which tends to yield increased values of Ca/P molar ratio [6, 10].

The SEM micrographs and the EDS analysis supported this suggestion since the bioprosthetic CDs were found to consist of microcrystalline HAP (Fig. 3) and plate-like OCP crystals (Fig. 4). A more detailed investigation of SEM micrographs (Fig. 5) revealed the presence of DCPD, another precursor phase of HAP in the CDs of bioprosthetic heart valves. The morphological identification of the precursor phases (OCP and DCPD) provided additional evidence for the fact that the initiation of the apatitic mineral formation in the CDs proceeds through the formation of precursor phases.

The formation of amorphous calcium phosphate (ACP) [25–28] cannot be precluded although it was not identified by morphological examination, perhaps because of the long time of equilibration of the minerals with the fluid phase. ACP is an unstable phase, which may be formed spontaneously in highly supersaturated calcium phosphate solutions. It consists of spherullitic crystallites while there is still uncertainty as to whether

this phase is a single stoichiometric unit [29, 30]. X-ray radial distribution measurements on ACP with a Ca/P molar ratio of 1.50 suggested a unit cell corresponding to the stoichiometry $Ca_9(PO_4)_6$ [31].

The results of the present study, concerning the nature of the mineral phase, in conjunction with previous studies on explanted natural and bioprosthetic valves [6, 10, 29] as well with results from studies on bioprosthetic valves *in vitro* and in animal models [7–9] support the idea that HAP is the final stable salt found in CDs of mineralized valve leaflets, which is produced by the formation and subsequent hydrolysis of DCPD and OCP, which are precursor phases.

Conclusion

Detailed examination of the physicochemical and microscopical characteristics of CDs from natural and bioprosthetic heart valves showed that they are similar in composition, texture, and morphology. This finding supports the suggestion that the mineralization mechanism is common in both cases. The plate-like formation of the mineral phase is suggested to be OCP, a precursor phase of the thermodynamically more stable HAP. The Ca/P molar ratio was found to be lower in the bioprosthetic CDs because of higher relative amount OCP : HAP. This difference could be attributed to the fact that the CDs of bioprosthetic valves are mineral phases been in contact with the supersaturated biological fluids for relatively shorter times than the more matured CD phases of the native human valves. Finally, DCPD (another precursor phase of HAP) found in bioprosthetic valve CDs corroborated the suggestion that HAP is the result of a mineralization mechanism involving the formation and subsequent hydrolytic transformation of precursor mineral phases.

The practical implications of these findings are that in the development of bioprosthetic heart valves, the problem of suppression of the precursor mineral phases should be carefully considered and efforts should be done to effectively inhibit the nucleation and crystal growth of DCPD and OCP [6, 10].

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