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Natural and Prosthetic Heart Valve Calcification: Morphology and Chemical Composition Characterization

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Abstract: Calcification is the most common cause of damage and subsequent failure of heart valves. Although it is a common phenomenon, little is known about it, and less about the inorganic phase obtained from this type of calcification. This article describes the scanning electron microscopy (SEM)/energy dispersive X-ray spectroscopy and Ca *K*-edge X-ray absorption near edge structure (XANES) characterization performed in natural and bioprosthetic heart valves calcified in vivo (in comparison to in vitro-calcified valves). SEM micrographs indicated the presence of deposits of similar morphology, and XANES results indicate, at a molecular level, that the calcification mechanism of both types of valves are probably similar, resulting in formation of poorly crystalline hydroxyapatite deposits, with Ca/P ratios that increase with time, depending on the maturation state. These findings may contribute to the search for long-term efficient anticalcification treatments. **Key Words:** Calcification—Cardiac valves—Bioprostheses—Hydroxyapatite—X-ray absorption near edge structure.

Calcification is the most frequent cause of failure of natural and bioprosthetic heart valves. Calcification growth can jeopardize leaflet motion of valves and cause tearing, resulting in stenosis and/or regurgitation, bringing about the need for valve replacement (1,2).

The occurrence of calcification requires energy to achieve the phase transformation from solution to solid crystal, and the energy required can be decreased by the presence of nucleation sites, such as surface defects, dead cell residues, collagen fibrils, elastin, lipids, and certain bone regulatory proteins, which serve as a substrate for heterogeneous calcification in heart valves (3).

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In the case of bioprosthetic valves, usually made from porcine and bovine heart tissues, besides the already mentioned factors, calcification is also related to the valve treatment before implantation (4–7). The stabilization of tissues by glutaraldehyde promotes cell death, and it has been demonstrated that its activity persists for many months, slowly liberating active glutaraldehyde monomers, causing the death of host fibrocytes and macrophages that come into contact with the toxic valve material (1).

The calcification of natural and bioprosthetic heart valves in vivo takes place after a period of years, in contrast to the calcification of many in vitro models, which occurs more quickly, indicating that in vivo factors, such as the presence of osteopontin, a regulatory protein, may act as inhibitors of calcification (3). In general, deposits of calcium phosphate are chemically and crystallographically similar to that of bone mineral, and heart valve calcification appears to be regulated by a process very similar to the formation of bone, with apatite crystal nucleation, growth, and association with an extracellular matrix that

regulates tissue mineralization (3,5,8,9). Heart valve calcification deposits consist mainly of poorly crystalline forms of hydroxyapatite (HAP), containing mainly carbonate, fluoride, magnesium, and sodium (9).

Several anticalcification methods have been developed, such as amino oleic acid, surfactants, and bisphosphonates, although none have yet demonstrated proven long-term clinical success (2, 4, 10–12). The distinctions in calcification of natural and bioprosthetic heart valves, due to differences in etiology and tissue material, bring about the question of whether crystal growth in natural and bioprosthetic heart valves follows different calcification pathways, or not (9). A better understanding of these matters may contribute to the development of effective anticalcification methods. However, little is still known about the characteristics of calcification promoted in both kinds of valves.

The present study describes the characterization of natural and bioprosthetic heart valve calcification deposits, by scanning electron microscopy (SEM)/energy dispersive X-ray spectroscopy (EDS) and X-ray absorption near edge structure (XANES) analysis. X-ray absorption spectroscopy (XAS) analysis of calcified heart valves is not present in the literature and, as a further innovation, XANES was applied in this study to refine the investigation of heart valve calcification mechanisms, analyzing at a molecular level possible differences in the calcification of natural and bioprosthetic heart valves. For further comparison, bovine pericardium was subjected to in vitro calcification and examined using the same methods mentioned above.

MATERIALS AND METHODS

Analyzed samples

Three native heart valves and three prosthetic valves that were all calcified in vivo and explanted from different patients were supplied by the Heart Institute (InCor), from the University of São Paulo Medical School (Brazil), and stored in 10% formaldehyde solution. Samples to be studied consisted of fragments of natural valves (NV in vivo 1, 2, and 3) and bioprosthetic, made from bovine pericardium heart valves (BV in vivo 1, 2, and 3). Samples were washed in 0.9% NaCl solution, frozen in liquid nitrogen, and lyophilized for 24 h (Liobras, L101, São Carlos, SP, Brazil).

In order to compare the in vivo calcification to the calcification that occurs in vitro, an in vitro calcification experiment was performed with samples of bovine pericardium (BP in vitro). The samples, of approximately 4 cm^2, were extracted from a commercial patch (Braile Biomédica, São José do Rio Preto, SP, Brazil), with a thickness of 0.3 mm, pretreated with glutaraldehyde solution and stored in a 4% formaldehyde solution.

For in vitro calcification experiments, a simulated body fluid solution (SBF) (13) was prepared by mixing NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, K_2PO_4, and Na_2SO_4 salt solutions, using tris hydroxymethyl aminomethane buffer. After the first 2 days, SBF, at a concentration 1.5-fold higher than that present in human blood plasma, was used to accelerate the calcification process. The pericardium samples were immersed in polyethylene flasks containing SBF, and the calcification experiment was performed over 7 days, in a Dubnoff bath at 36.5 ± 0.5°C. Every 2 days, the solutions in the flasks were exchanged for fresh ones, in order to avoid microbial contamination. By the end of the experiment, the samples were carefully washed with ultrapure water, frozen in liquid nitrogen, and lyophilized (sample referred to as "BP in vitro").

SEM/EDS analysis

Samples were analyzed by SEM and EDS, using a Leica microscope (LEO 440i, Leica, Bannockburn, IL, USA), to observe and characterize the calcification deposits formed. Lyophilized samples were mounted on double-sided carbon tape attached to aluminum stubs and coated with carbon with a sputter coater.

XANES analysis

XANES analyses of in vivo- and in vitro-calcified samples, and of different standards of calcium compounds, were performed at the D04B-XAS beamline, in the Synchrotron Light National Laboratory (LNLS, Campinas, Brazil). Calcium standards were prepared from solutions of calcium oxide, calcium carbonate, and calcium phosphate, dissolved in isopropanol. The solutions were homogenized in ultrasound bath and filtered. After the complete solvent evaporation, the filter papers containing the standards, as well as the calcified samples, were fixed in a holder, made especially for this kind of analysis, using a light-element adhesive tape.

Spectra of standards and samples were collected around the *K*-edge absorption of Ca (4038 eV). The LNLS ring energy was 1.37 GeV, and the current used was up to 175 mA. The X-ray energy incident on the sample was defined using a double-crystal Si(111) monochromator. The instrument was evacuated to ~10^{-8} Pa of pressure in order to reduce X-ray losses due to attenuation in the air (14). Standards were

analyzed in transmission mode, using ion chambers before and after the sample to measure incident and transmitted X-ray intensity, and samples, in fluorescence mode, using a Ge energy-dispersive detector mounted in the horizontal plane perpendicular to the beam, minimizing the contribution of elastic scattering (15). Each sample was analyzed three times.

The raw XANES spectra were treated with the ATHENA software, from the IFFEFIT package (http://cars9.uchicago.edu/~ravel/software/). Spectra from each sample were averaged, subtracted from the pre-edge background, and then normalized. The normalization procedure corrects effects resulting from different sample thickness, and allows comparison of samples with different absorber contents (16).

RESULTS

Figures 1, 2, and 3 present, respectively, the micrographs obtained by SEM from natural and bioprosthetic heart valves calcified in vivo, and bovine pericardium calcified in vitro. It is possible to observe the formation of spherical crystalline deposits. The calcification is also well spread over the surface of all types of tissue. However, in some of the in vivo-calcified samples, the surface microstructure presents a different shape, with lower incidence of spherical deposits. The aspect of these formations is probably due to the fact that, during the calcification process, these samples were located in regions that were susceptible to higher shear stresses caused by the flow of body fluids, and cyclic flexural and compressive stresses during valve opening and closure (1).

In general, the micrographs indicate similar morphology of the calcium-containing phases deposited in all analyzed samples. For in vivo-calcified samples, the Ca/P ratio was calculated by EDS to be approximately 1.7 (about 1.79 for native valve and 1.72 for bovine pericardium), which is very close to HAP Ca/P ratio. Elemental mappings for calcium, phosphorus, and carbon, for natural and bioprosthetic calcified valves are presented in Figs. 4 and 5, respectively, indicating that calcium and phosphorus cover almost all sample surfaces. The white spots indicate low concentration of the analyzed elements.

The XANES spectra of the analyzed patterns and samples are shown in Fig. 6. Clear differences in

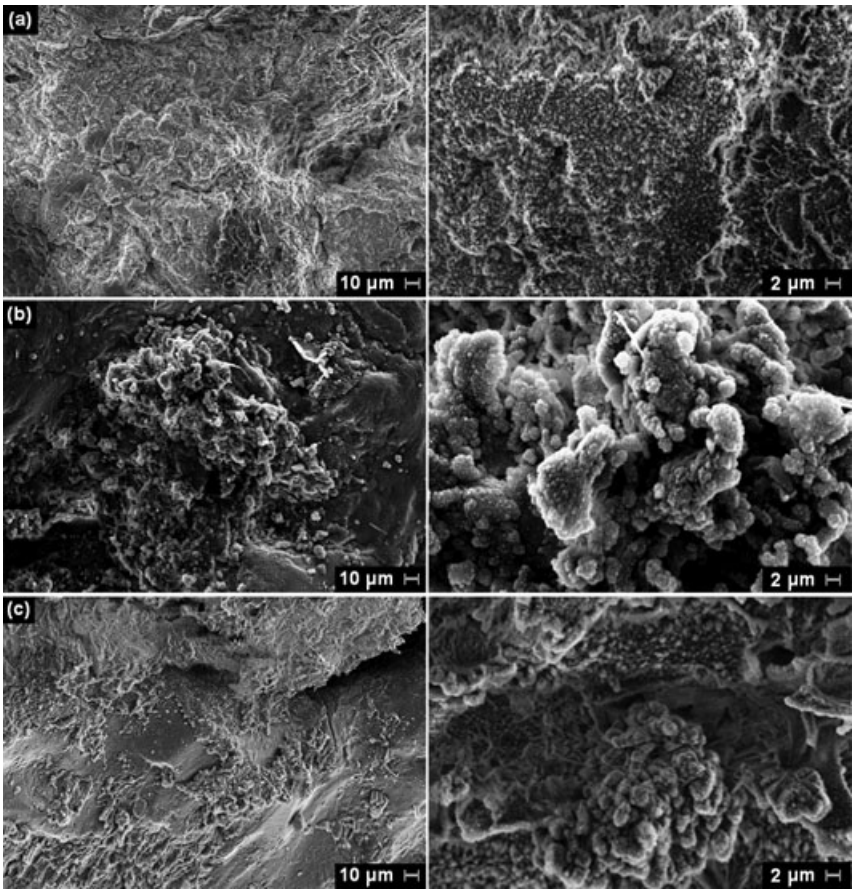

FIG. 1. SEM micrographs of natural heart valve samples that were calcified in vivo, (a) NV in vivo 1, (b) NV in vivo 2, (c) NV in vivo 3.

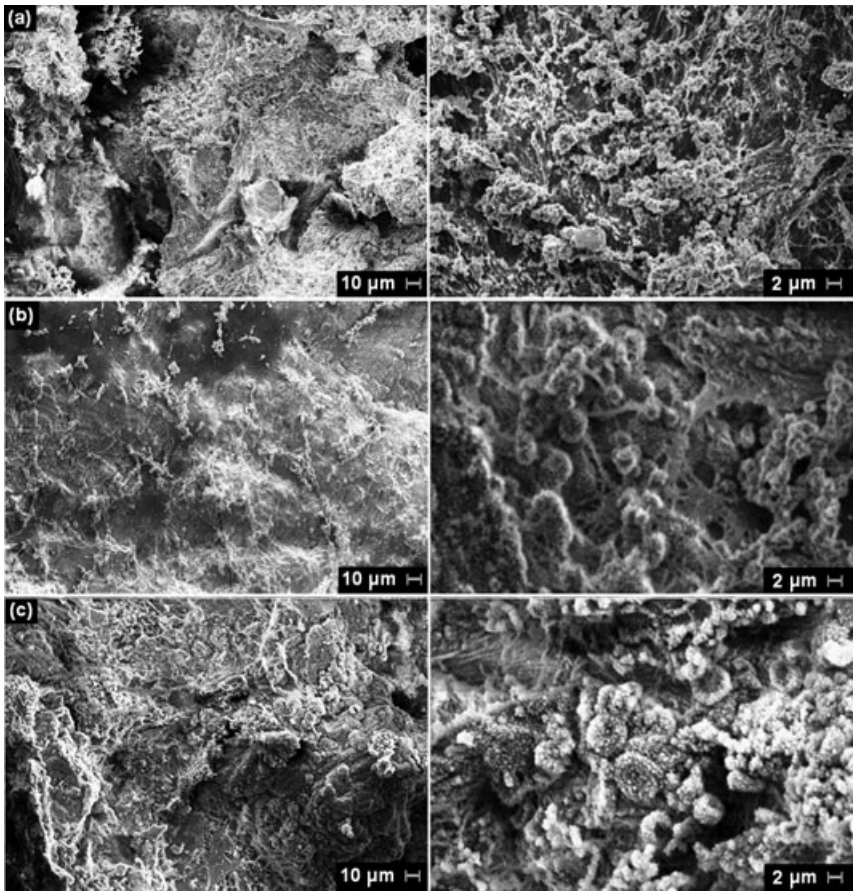

FIG. 2. SEM micrographs of bioprosthetic heart valve samples that were calcified in vivo, (a) BV in vivo 1, (b) BV in vivo 2, (c) BV in vivo 3.

pre- and post-edge features are evident between patterns, and also when compared to the spectra of samples, indicating that XANES can be used to identify different Ca species, as it indicates differences in coordination numbers and environment geometries in the crystal structures of the analyzed compounds (17,18).

In Fig. 7, a magnified area around peak C of the XANES spectra is presented to compare the absorption coefficients of the natural and bioprosthetic calcified heart valve samples. The spectra of natural samples presented relatively higher absorption coefficients, indicating the presence of deposits with higher Ca/P ratios and better organized structures.

DISCUSSION

XANES spectra refers to the secondary peaks and shoulders that modify the appearance of the absorption edge of a specific element, and the near region beyond it, and may serve as a "fingerprint" to identify certain structural aspects of chemical species (19). In Fig. 6, the spectra were labeled moving toward higher energy. The spectra of in vivo- and in vitro-calcified samples are characterized by a small pre-edge

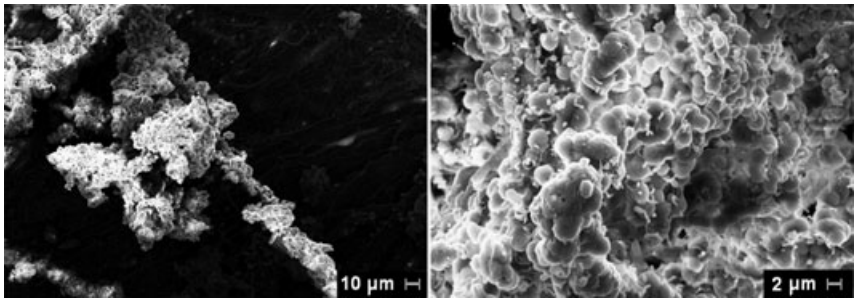

FIG. 3. SEM micrographs of bovine pericardium sample calcified in vitro (BP in vitro).

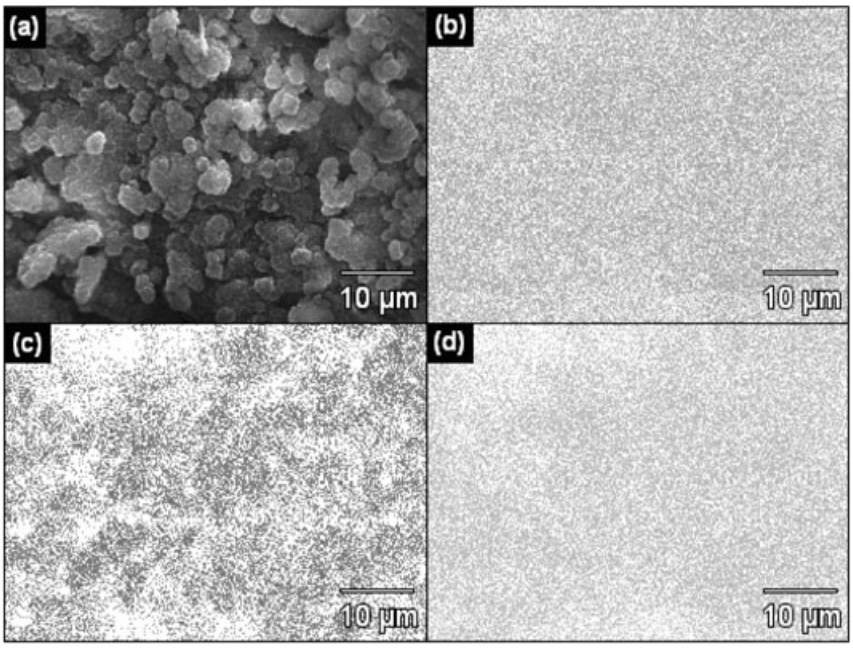

FIG. 4. SEM micrograph of natural heart valve sample calcified in vivo (a), and its elemental mapping using the EDS technique for (b) calcium, (c) carbon, and (d) phosphorus.

(feature A), also observed in lower intensity in the $Ca_3(PO_4)_2$ pattern. This pre-edge may be attributed to the 1s → 3d transition, resulting from the mixing of unoccupied d final states with p-character final states. At higher energy, the shoulder peak B on the rapidly rising absorption curve is assigned to the 1s → 4p transition, and remains unchanged through the series, whereas the intensity ratio of the most prominent peak (C) has a relationship with the Ca content (16,20,21).

The spectra of natural and bioprosthetic heart valves calcified in vivo presented similar features, suggesting that the electronic configuration and the site symmetry of Ca atoms in these samples are similar. The spectra of these samples also presented similar features to those of HAP patterns obtained by Chalmin et al. (22), who analyzed HAP patterns, and also by Wang et al. (23), who analyzed samples of lumbar and femoral bone of rats with different calcium contents, and verified that the

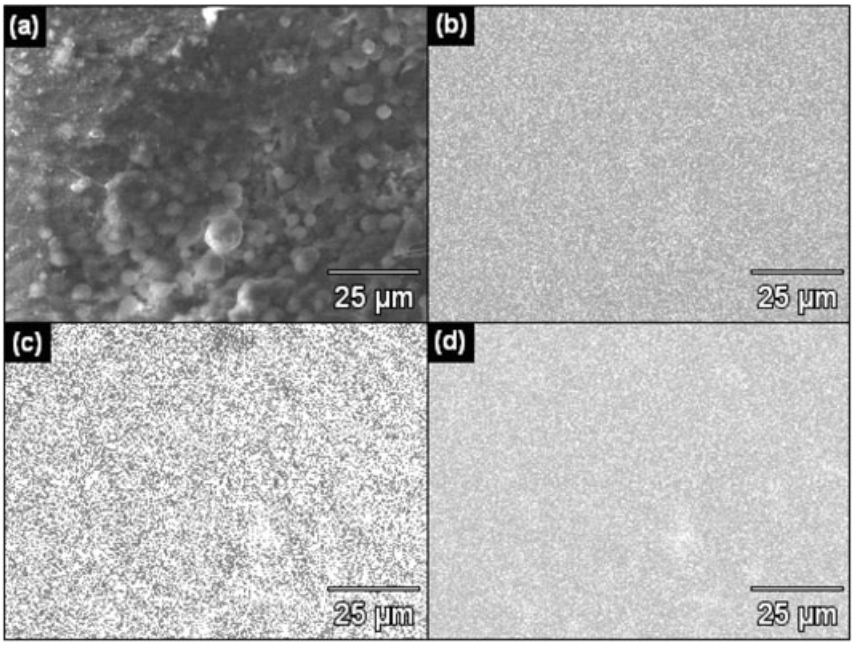

FIG. 5. SEM micrograph of bioprosthetic heart valve sample calcified in vivo (a), and its elemental mapping using the EDS technique for (b) calcium, (c) carbon, and (d) phosphorus.

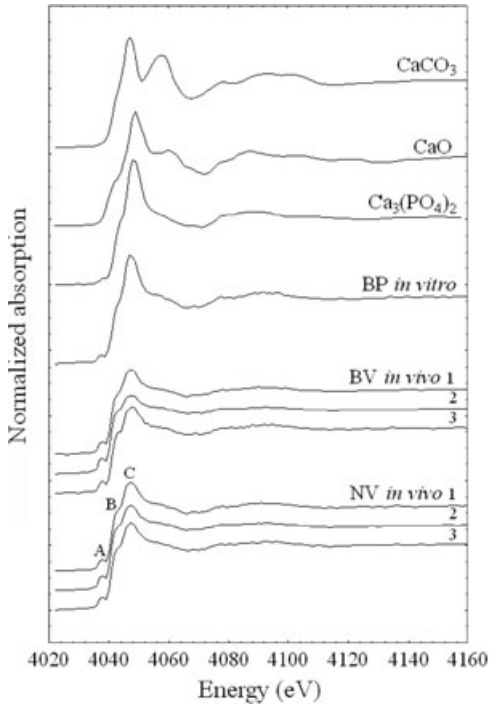

FIG. 6. XANES spectra at the Ca *K*-edge of calcium patterns, natural and bioprosthetic heart valves calcified in vivo, and bovine pericardium calcified in vitro.

XANES spectra of the samples were similar to that of HAP.

Differences in the post-edge region features, when comparing the spectra of patterns to those of calcified samples, may be explained by the difference in coordination number. Calcium carbonate and calcium oxide, for instance, are compounds with well-defined coordination shells, with a lower, six-coordinated environment (14). In stoichiometric HAP, the coordination number may be defined as two distinct Ca environments. Ca_I site is described as 6 (2.43 Å) + 3 (2.79 Å) coordination environment while Ca_{II} is best described as either 4 (2.36 Å) + 2 (2.51 Å) + 1 (2.71 Å) or 5 (2.35 Å) + 2 (2.51 Å) + 1 (2.71 Å) (14,24). The overall shape of the post-edge spectral envelope is evidently indicative of high coordination, and the broad distribution of neighboring distances may explain the features in the spectra of calcified samples, when compared to those of patterns. As it may be seen in Fig. 6, the post-edge features become narrower as the first shell coordination number increases. This is also consistent with the pre-edge feature (A) that becomes more intense as the site is more distorted with a wide range of distances (14,25).

Furthermore, the spectra of the bovine pericardium sample, calcified in vitro, presented similar aspect, when compared with those of in vivo-calcified samples, which may indicate that the growth of calcification clusters may also occur equally, even if nucleation may be caused by several distinct factors found in each in vivo and in vitro system. Hence, the in vitro test, using SBF (26), can be a method to reproduce, to a certain extent, the calcification phenomenon (27,28). From a pragmatic point of view, the in vitro method may be used to exclude biomaterial candidates to be used as heart valves, even if this validation method is not efficient enough to approve them. The trend of developing and studying in vitro tests will be an increasing one, given the practical and social opposition of using living organisms for in vivo tests.

Liou et al. (21) studied the structural environment around Ca of stoichiometric HAP (Ca/P = 1.67), and calcium-deficient hydroxyapatites $[Ca_{10-x}(PO_4)_{6-x}(HPO_4)_x(OH)_{2-x}, 0 \leq x \leq 1]$ with Ca/P ratios of 1.5, 1.55, and 1.6. They concluded from XANES analysis that the adsorption coefficient was dependent on the concentration of the absorbing atom, and that higher concentration corresponds to a higher absorption coefficient. In Fig. 7, the higher absorption coefficients of natural heart valve samples spectra, when compared with those of bioprosthetic samples, may indicate the presence of deposits with higher Ca/P ratios and better organized structures. These results probably arise from the fact that the NV undergo a long-term calcification process, and the mechanism of the initiation of the process is difficult to determine. Although bioprosthetic valves present additional factors that contribute to the calcification process, such as glutaraldehyde-fixative treatment, the implantation period of these valves, resulting in shorter times of contact with biological fluids (9), when compared to NV, probably explains the presence of less-matured calcification deposit phases.

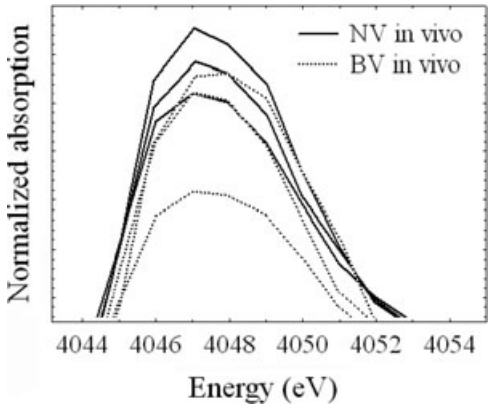

FIG. 7. Magnified area around peak C of XANES at the Ca *K*-edge of the natural and bioprosthetic heart valves calcified in vivo.

These differences in maturation periods have been previously observed by Mikroulis et al. (9), who characterized the calcification of natural and bioprosthetic (porcine) valves, through Fourier transform infrared spectroscopy, X-ray diffraction, SEM, and EDS analysis. Although they concluded that the calcification deposits from both types of valves were similar, probably formed by the same mechanism, they observed that the Ca/P molar ratio of deposits from bioprosthetic valves was significantly lower compared with that of NV. The authors hypothesized that, in both cases, the calcium deposits consisted of mixtures of HAP and precursor phases, transformed to HAP by hydrolysis, such as octacalcium phosphate (Ca/P = 1.33) and dicalcium phosphate dihydrate (Ca/P = 1), and NV presented a lower amount of these precursor phases.

The results from the present study indicate similar structure and morphology of calcification deposits on native and prosthetic valves (in vivo and in vitro). This fact could indicate that the process of calcium growth on native human cardiac valves and on bovine pericardium cardiac valves is similar, and also that microarchitecture of the surface is similar in both kinds of valves and they are able to anchor similar inorganic structures that result from nuclei growth. The nucleation phenomenon, however, should be further studied in order to design new prostheses with a lower tendency to calcify, as the mechanisms may be different in both cases.

CONCLUSIONS

In the analysis of natural and bioprosthetic in vivo-calcified heart valves, SEM micrographs indicated the presence of deposits of similar morphology. XANES results suggest at a molecular level that the calcification mechanism of both types of valves are probably similar, being characterized by the formation of poorly crystalline HAP deposits, with Ca/P ratios that increase with time, depending on the maturation state. These findings may contribute to the search for efficient and lasting anticalcification treatments.

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