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The structure and material composition of ossified aortic valves identified using a set of scientific methods

Antonín Zeman [a], Michal Šmíd [b], Martina Havelcová [c,*], Lucie Coufalová [d], Štěpánka Kučková [d,e], Martina Velčovská [d], Radovan Hynek [d]

[a] Institute of Theoretical and Applied Mechanics, Academy of Sciences of the Czech Republic, Prosecká 76, 190 00 Prague, Czech Republic
[b] University Hospital Pilsen, Cardiology, alej Svobody 80, 304 60 Pilsen, Czech Republic
[c] Institute of Rock Structure and Mechanics, Academy of Sciences of the Czech Republic, V Holešovičkách 41, 182 09 Prague, Czech Republic
[d] Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 166 28 Prague, Czech Republic
[e] Charles University, Department of Chemistry and Chemical Education, M.D. Rettigové 4, 116 39 Prague, Czech Republic

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ABSTRACT

Degenerative aortic stenosis has become a common and dangerous disease in recent decades. This disease leads to the mineralization of aortic valves, their gradual thickening and loss of functionality. We studied the detailed assessment of the proportion and composition of inorganic and organic components in the ossified aortic valve, using a set of analytical methods applied in science: polarized light microscopy, scanning electron microscopy, X-ray fluorescence, X-ray diffraction, gas chromatography/mass spectrometry and liquid chromatography–tandem mass spectrometry. The sample valves showed the occurrence of phosphorus and calcium in the form of phosphate and calcium carbonate, hydroxyapatite, fluorapatite and hydroxy-fluorapatite, with varying content of inorganic components from 65 to 90 wt%, and with phased development of degenerative disability. The outer layers of the plaque contained an organic component with peptide bonds, fatty acids, proteins and cholesterol. The results show a correlation between the formation of fluorapatite in aortic valves and in other parts of the human bodies, associated with the formation of bones.

1. Introduction

Degenerative aortic stenosis (AS) has become a very common and dangerous disease in recent years, and it is the most common indication for surgical valve replacement (Stewart et al., 1997). Histopathologically, the changes associated with AS include valve thickening and the accumulation of irregular fibroclastic masses, the mineralization of aortic valves, their gradual enlargement and progressive loss of functionality (Otto et al., 1994). AS can be considered to be at a significant level when the aortic valve area index reaches <1 cm^2 or 0.6 cm^2/m^2. Degenerative aortic valve stenosis etiology is currently the most frequently operated valvular disease in developed countries, prevalence increases with age and affects about 4% of the population aged over 80 years. The increasing number of patients with this type of valve defect is due to the aging of population, and the improved means of diagnosis. The final phase is characterized by degenerative annulus calcification and aortic valve cusps with their limited separation. The initial presenting symptoms include progressive shortness of breath on exertion, syncope, chest pain, and frank heart failure. AS affects older age groups and treatment failure can have fatal consequences.

In a broader sense, AS is the obstruction of blood flow from the left ventricle. This may be below the aortic valve (subvalvular), in the aortic valve (valvular) or above the aortic valve (supravalvular). Histologically, the aortic valve consists of three principal layers: (a) the fibrosa at the outflow surface containing densely packed collagen fibers, (b) the spongiosa in the center composed of glycosaminoglycans, and (c) the ventricularis at the inflow surface containing collagen and elastin (Schoen and Levy, 1999). The aortic stenosis disease affects trileaflet aortic valves, often in patients with other risk factors for atherosclerotic disease. The AS is an active process, with lipid deposition, inflammation, and mineralization. This form of aortic stenosis progresses slowly, and patients often fall into the age group of between 70 and 90 years (Stewart et al., 1997). Until recently, the concept was accepted that AS is a degenerative and unmodifiable process induced by long-lasting stress, cardiovascular risk factors, genetic factors, and valve biology (Rosenhek et al., 2000).

The pathogenesis of AS is an active process with two distinguishable stages: nonobstructive aortic valve thickening, or sclerosis, and progressive calcification, which causes valve obstruction (Linhartová et al., 2008). This process shares features in common

* Corresponding author. Tel.: +420 266 009 283; fax: +420 284 680 105.
E-mail address: havelcova@irsm.cas.cz (M. Havelcová).

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with skeletal bone formation: cells with osteoblast phenotype have been identified in the stenotic aortic valve, and mature lamellar bone commonly occurs in end-stage AS (Mohler et al., 2001; Rajamannan et al., 2003).

The early lesion is an active inflammatory process with some similarities (lipid deposition, macrophage and T-cell infiltration, and basement membrane disruption) and some dissimilarities (presence of prominent mineralization and small numbers of smooth muscle cells) to atherosclerosis (Otto et al., 1994). Mature laminar bone formation (Mohler et al., 2001) and smooth muscle cells of the osteoblast phenotype (Rajamannan et al., 2003) have been demonstrated in calcified parts of valves. Boström et al. (1995) have defined cardiovascular calcification as being composed of hydroxyapatite deposited on a bone-like matrix of collagen, osteopontin, and other minor bone matrix proteins. This was confirmed histologically with the presence of osteoblast bone formation in calcified aortic valves removed via surgical valve replacement.

The current management of patients with AS comprises monitoring disease progression. However, the majority of patients with AS do not have symptoms or an indication for surgery. Anti-inflammatory and antiproliferative agents are used to alter the natural history of aortic stenosis. Statins, beta blockers and diuretics are commonly used treatments that have proven secondary preventive benefits in cardiovascular disease and exhibit some of these desirable anti-inflammatory and antiproliferative properties.

So far, no studies have convincingly demonstrated a clear positive effect of this drug group, which was confirmed by a large prospective SEAS (Simvastatin and Ezetimibe in Aortic Stenosis) study (Rossebo et al., 2008).

Our findings (Zeman, in preparation; Zeman et al., 2011; Šmíd et al., 2011) concerning the proportion of inorganic and organic components in the ossified aortic valve and its material composition led us to the idea to initiate an action research, using a set of analytical methods applied in the exploration of natural inorganic and organic materials. It turned out that the main inorganic component of "calcified" flap is hydroxyapatite, its content varied in 20 samples ranging from 65.0% to 91.0% by weight. The remaining part to 100% consisted of organic matter (mostly proteins, polyamides, collagen, cholesterol).

2. Materials and methods

2.1. Samples

Aortic valves were obtained from the Surgical Clinic of the University Hospital in Pilsen and the First Medical Faculty of Charles University in Pilsen. Analysis was performed in explanted aortic valves after cardiac surgery during 2009. The mean age of the persons was 68.3 in the range of 60–80 years, and all of them were indicated for cardiac surgery for significant symptomatic degenerative aortic stenosis, which was verified by echocardiography. Twenty samples of aortic valves were explanted from affected patients, washed with distilled water and stored at −20 °C.

2.2. Methods

The studied aortic valves were dried at a constant temperature of 50 °C for 24 h. The dried samples were then processed into petrographic polished thin sections. The thus-prepared samples were used in research utilizing a polarized microscope and electron microprobe.

2.2.1. Polarized light microscopy

The structures of ossified aortic valves were examined using the polarizing microscope Leitz Orthoplan (GmbHWetzlar, Germany). Backscattered electrons (BSE) were used for the detailed valve structure research. Microphotos were taken by a Nikon Coolpix 4500 (Nikon Inc., USA).

2.2.2. Scanning Electron Microscopy (SEM)

Analytical studies on the valves were performed on the electron microprobe Cameca SX 100 (Cameca, France).

Photos in BSE were taken at a magnification of 205–1000×, and this was followed by a quantitative analysis of the inorganic components and the determination of their quantities in relation to organic constituents. Analyses were done at an accelerating voltage of 20 kV. For standardization, the SPI Supplies set of standards was used.

2.2.3. X-ray fluorescence (XRF)

Control analyses of the qualitative and quantitative composition of the investigated materials were carried out using an X-ray fluorescence analysis (XRF) spectrometer ARL 9400 XP (Thermo ARL, Switzerland).

2.2.4. X-ray diffraction (XRD)

Diffraction experiments were performed using a Bruker D8 Discover (Bruker, Germany) equipped with copper radiation, monochromatized with a germanium primary focusing monochromator. The diffracted beam was detected using a LynxEye 1-D detector. The diffraction diagrams were recorded with a step time of 1 s and a step size of 0.02° from 5° up to 70° in a 2θ angle. The diffraction data were analyzed using DIFRAC plus Evaluation Package (EVA) software with PDF-2 Release 2009 databases. The standard diffraction data were identified according to the databases of the International Centre for Diffraction Data (ICDD).

2.2.5. Furier transform infrared spectroscopy (FTIR)

Samples were examined under a stereomicroscope SM XX, viewing the study matter through an infrared objective with 25× magnification, using a Nicolet spectrophotometer and LUCIA image analysis. The samples were analyzed in the 600–4000 cm^{-1} range with a resolution of 4 cm^{-1}, and compared with library spectra of macromolecular and inorganic compounds (Kössler, 1960, 1970; Horák and Papoušek, 1976).

2.2.6. Carbon content analysis

Total carbon content was determined using a microanalyser Flash FA 1112 (ThermoFinnigan Flash FA 1112, Milan, Italy). Noncarbonate C content (NCC) was determined by elemental analysis after elimination of inorganic carbonates with 1 N HCl heated to 80 °C.

2.2.7. Gas chromatography/mass spectrometry (GC/MS)

Sample extraction was carried out with dichloromethane in a Soxhlet apparatus for five hours. The solvent amount was reduced using a vacuum evaporator. The extract was analyzed by gas chromatography/mass spectrometry (GC/MS) using a Thermo Scientific Trace Ultra DSQ II instrument (Austin, USA) equipped with a capillary column with a fixed stationary phase DB 5 (30 m × 0.25 mm × 0.25 μm film). The GC oven was heated from 35 °C to 300 °C at a rate of 10 °C/min. The analysis was carried out in splitless mode. Helium was used as a carrier gas. Mass spectra were recorded at EI 70 eV from 40 to 600 amu. Chromatograms and mass spectra were evaluated using Xcalibur software (ThermoElectron, Manchester, UK).

2.2.8. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

All samples for protein analyses were cleaved in 60 μL of trypsin solution (1 μg of trypsin in 50 μL of 50 mM NH_4HCO_3 buffer).