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An Infrared Spectroscopic Study of Aortic Valve. A Possible Mechanism of Calcification and the Role of Magnesium Salts

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Abstract. *In the present study fourier transform infrared (FT-IR) spectroscopy was used to study the mechanism of pathogenesis of aortic valve calcification. The high intensity bands of vCH_3 and vCH_2 groups of lipids and phospholipids of membranes, in the spectral region 3000-2800 cm^{-1}, show the high concentration of lipids and fatty components in aortic valve, resulting from degradation of the main aliphatic chain of the membranes, with a change of their permeability and fluidity. The presence of bands at 3075 and 1744 cm^{-1}, assigned to olefinic (v=CH) and aldehyde carbonyl groups, respectively, implies that reactive oxygen species are involved in the initiation of peroxidation of the lipids and phospholipids. These latter bands are related to the oxidative stress of the patients. From the shifts of bands to lower frequencies of the characteristic absorption bands of amide I and amide II, it is suggested that the proteins change their secondary structure from α-helix to β-sheets and random coil due to modifications of collagen, associated with the permeability of aortic valve atherosclerosis. From the spectral region 1150-900 cm^{-1}, where the characteristic stretching vibration bands of the phosphate groups (vPO_4^{-3}) absorb, the calcified aortic valve was found to contain biological hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), as well as amorphous hydroxyapatite ($Ca_5(PO_4)_xOH$) and $CaHPO_4$. These findings are in agreement with scanning electron microscopy energy-dispersive X-ray analysis and X-ray diffraction analyses. SEM micrographs show that the valves are rich in fibrils and that the protein-protein cross-linked chemical bonds seem to be the points of initiation of calcification.*

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Infrared (IR) spectroscopy is a physicochemical, non-destructive, sensitive and reproducible method for evaluating the composition and molecular structure of chemical and biological molecules (1-6). The method is based on the interaction between infrared radiation (electromagnetic waves) with matter, which induces changes in the molecular dipole. This means that the strength of absorption increases with increasing polarity of the vibrating bonds, while molecules with no dipole moment change are inactive in infrared (7).

IR spectroscopy is an important tool for the identification and characterization of chemical bonds, materials and biomaterials. As the energy of the IR radiation is absorbed by the molecules their vibrational modes are excited to higher energy levels, which is expressed as the absorption of energy. Each IR absorption band of a chemical bond or group is characteristic and always appears at approximately the same energy or wavenumber (cm^{-1}). Thus, the IR spectra are unique and representative of each molecule and are used for identification purposes. IR passes simultaneously through all frequencies of the molecules and each frequency is absorbed, creating a characteristic spectrum of the molecule, which is finally the ʹfingerprintʹ of the molecule. The exact position of the bands also depends on electron-withdrawing or donating effects of the intra- and intermolecular environment in which the molecules are vibrating. This sensitivity of IR spectroscopy gives us the ability to gain information which could be associated with certain diseases (8-11).

The IR spectrum can be obtained with very small amounts of samples, of few micrograms and very rapidly. The attenuated total reflection (ATR) technique enables samples to be used without any other preparation (1, 12). For instance, in histopathology, the samples are colored, or in the

case of bone or carotid tissues, they must firstly be decalcified. In IR spectroscopy, the spectrum is the sum of all the frequencies of the components present and provides information on all components simultaneously (13-18).

Figure 1 illustrates a representative Fourier transform IR (FT-IR) spectrophotometer, coupled with an accessory for ATR.

IR light is emitted from the source through a crystal with an angle usually at 45˚ and by multiplying the reflections, passes simultaneously through the sample, thus ncreasing its strength and finally through the detector to give the spectrum of the sample. The produced spectrum is the sum of a number of co-added spectra (scans). For clinical samples, the interpretation of the spectra depends on the comparison of the spectrum of diseased and healthy tissues or fluids in order to derive the characteristic pattern of the disease compared to healthy tissue (8-12). The advantage of IR spectroscopy is that it requires only a small quantity (micrograms) of *ex vivo* tissue or biofluid.

Degenerative aortic valve stenosis is a common disorder in the aged population of developed countries, leading to the need for heart valve replacement (19, 20). Aortic valve calcification is a slow, progressive disease that can be diagnosed in early stages by echocardiography. Echocardiographic studies suggest that aortic valve sclerosis is present in approximately 2% of the elderly population (aged over 65 years) (21). Otto *et al*. reported that this disease is age-related, since they found that 40% of patients with valve sclerosis have significant coronary artery disease without stenosis (22). There is a growing body of scientific evidence suggesting that the pathogenesis of aortic valve stenosis is a multifactorial process and is associated with age, hypertension, hypercholesterolemia, diabetes, chronic inflammation and other risk factors, but the mechanism of valve calcification remains unknown. In modern clinical applications, quantitative analytical tests play a pivotal role in monitoring and detecting the development of the disease, but existing analytical methods do not provide data in the conformational changes that take place at the molecular level. The purpose of the present work was to investigate the changes in IR spectra of calcified aortic valve tissues, as well as to characterize the mineral phase and composition generated during the process of valve calcification in order to understand the mechanism of degenerative aortic valve stenosis at the molecular level.

Materials and Methods

Twenty calcified aortic valves were obtained from patients who underwent surgical aortic valve replacement procedures. For better-understanding of the valve calcification mechanism, we excluded patients with rheumatoid arthritis from the present protocol.

The FT-IR spectra were recorded with a Nicolet 6700 thermoscientific spectrometer (USA), equipped with an ATR-FT-IR

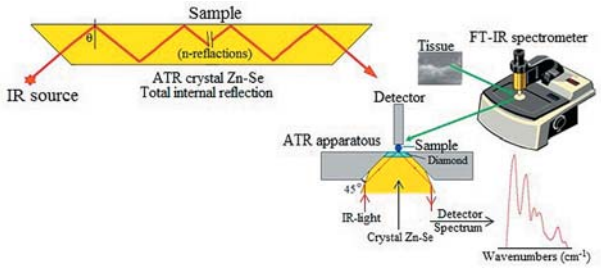


Figure 1. *Schematic presentation of the FT-IR spectroscopic technique coupled with ATR. The IR light passes through the sample by the Zn-Se crystal and after multiplication of the internal reflections the beam is collected by a detector.*

apparatus. Each plot consisted of 120 co-added spectra at a spectral resolution of 4 cm^{-1} and the OMNIC 7.1 software was (from Nicolet 6700) used for data analysis. For fine detection of aortic valve surfaces, a scanning electron microscope (SEM) from Fei Co (the Netherlands) was used. It should be noted that no coating of the samples with carbon or gold. XRD was performed with a Siemens D-500 X-Ray diffractometer (Germany) based on an automatic adjustment and analysis system. The diffraction interval was of 2θ 5-80˚ and scan rate of 0.030˚/s.

Results

FT-IR spectra. The FT-IR spectra of calcified aortic valve tissue in the region 4000-400 cm^{-1} are shown in Figure 2.

The arrows show representative locations of the sample, where the spectra are recorded. Spectrum 1 corresponds to pure organic phase, spectrum 2 is taken from the interface between organic and mineral phase and spectrum 3 is referred to as an individual mineral dot, which was in surrounding mineralized valve tissue. Considerable intensity and shape differences are shown in almost all spectra at the regions between 4000-2600 cm^{-1} and 1800-400 cm^{-1}.

SEM imaging. Scanning electron microscopy is also a non-destructive method, which allows the investigation of the surface of aortic valve tissues, without decalcification or coloring. Under these conditions, there is no change in chemical bonds between mineral and organic face, as there has not been any previous treatment of the sample. On the contrary, in methods like histopathology, the samples are decalcified using EDTA and only the changes of the organic part of the tissues are analyzed. Figure 3 shows the membrane morphology of the aortic valve.

In a higher magnitude of ×400 (Figure 3B) the arrow shows the bone formation, as hydroxyapatite, on the aortic valve. The deposition of minerals on aortic valve tissues is preferential on fibrils of collagen and elastin (arrow Figure 3C). In Figure 3D, the damaged proteins are shown.

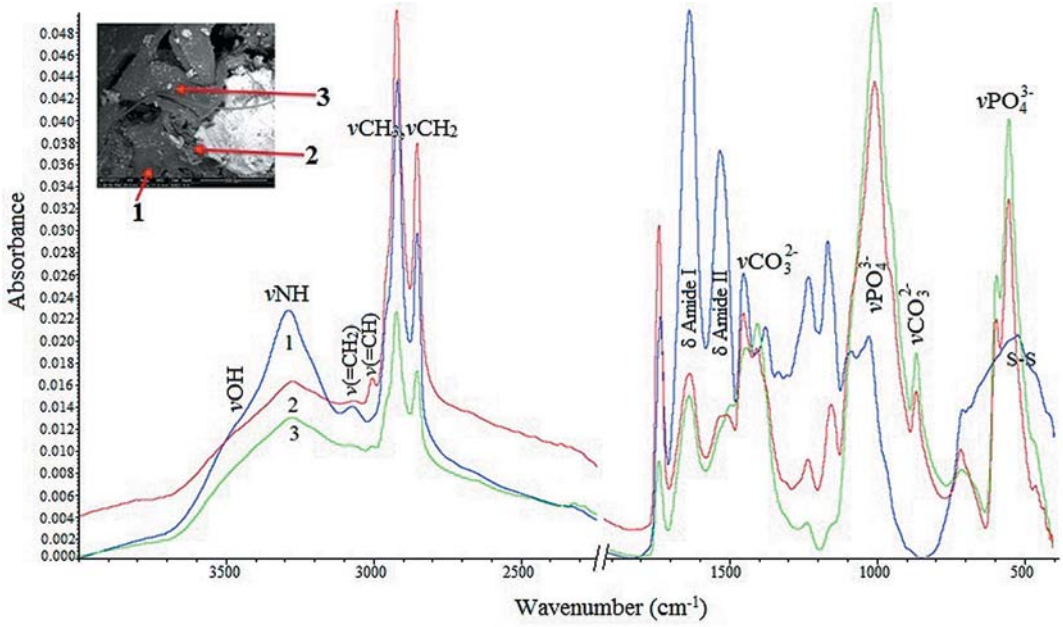


Figure 2. *Representative FT-IR spectra of an aortic valve taken from the spots, 1: organic phase, 2: interface between organic and mineral phase, 3: individual mineral deposit.*

Discussion

In order to understand the mechanism of calcification, a comparison between the IR spectra of the same specimen at different points was performed, which shows considerable changes in the shape and intensity of the bands in several spectral regions, concerning the different components from location to location of the same sample.

The shoulder band at about 3495 cm^{-1} is dominated by absorptions of stretching vibrations of *v*OH functional groups of hydroxyapatite and structural water molecules. The band at 3290 cm^{-1} is assigned to stretching vibrations of *v*NH groups of proteins. This band is sensitive and shows that the NH groups are reduced going from an organic region (Figure 2 spectrum 1) to an individual mineralized spot (Figure 2 spectrum 3). The band at 3075 cm^{-1} is assigned to the stretching vibration mode of *v*(=C-H) groups with olefinic character due to the presence of low-density lipoproteins (LDL). This band in carotid atheromatic plaques was found to be related to serum concentration LDL in patients and could be used as a 'marker band' (6-11). On the contrary, however, in aortic valve the intensity of the band is independent ofthe serum LDL concentration. From this characteristic band it is suggested that in both cases, peroxidation of lipids is taking place during the process of the disease (7-11).

The high-intensity bands in the region between 3000-2850 cm^{-1} are assigned to stretching vibration modes of methyl (νCH_3) and methylene (νCH_2) groups (9-11). Deconvolution of these bands shows that the bands are not simple, suggesting that the aliphatic chains may interact with the lipophilic environment *via* hydrophobic aliphatic tails (hydrophobic regions). It is thus suggested that the length of the aliphatic chains has changed due to free radical disproportionation and degradation reactions, which may explain the changes in permeability and fluidity of the membranes (6, 10, 11). These results are in accordance with SEM data (Figure 2), where the images show the formation of cross-linking bonds.

The strong absorption band near 1744-1734 cm^{-1} corresponds to carbonyl stretching vibrations of phospholipid esters, cholesteric esters and aldehydes (6, 10, 11). It is observed that the intensity of this band increases in the organic region and in the interface, whereas it is decreased in single-spot mineral phase of the sample. These data strongly support the idea that the minerals are produced by lipid and phospholipid peroxidation. Our results are in accordance with the data of Kristal *et al*. who demonstrated that lipid peroxidation leads to production of a variety of aldehydes, such as 4-hydroxy-2-nonenal (HNE) due to arachidonic acid peroxidation (23). In addition, Salaris and Babbs, using Wistar rats as a model, reported the strong dependence of malondialdehyde-like material formation on ischemia/reoxygenation process (24). In order to explain the production of aldehydes, it is suggested that the free radicals which are produced during metabolism or oxidative stress interact with lipids producing lipid peroxides,

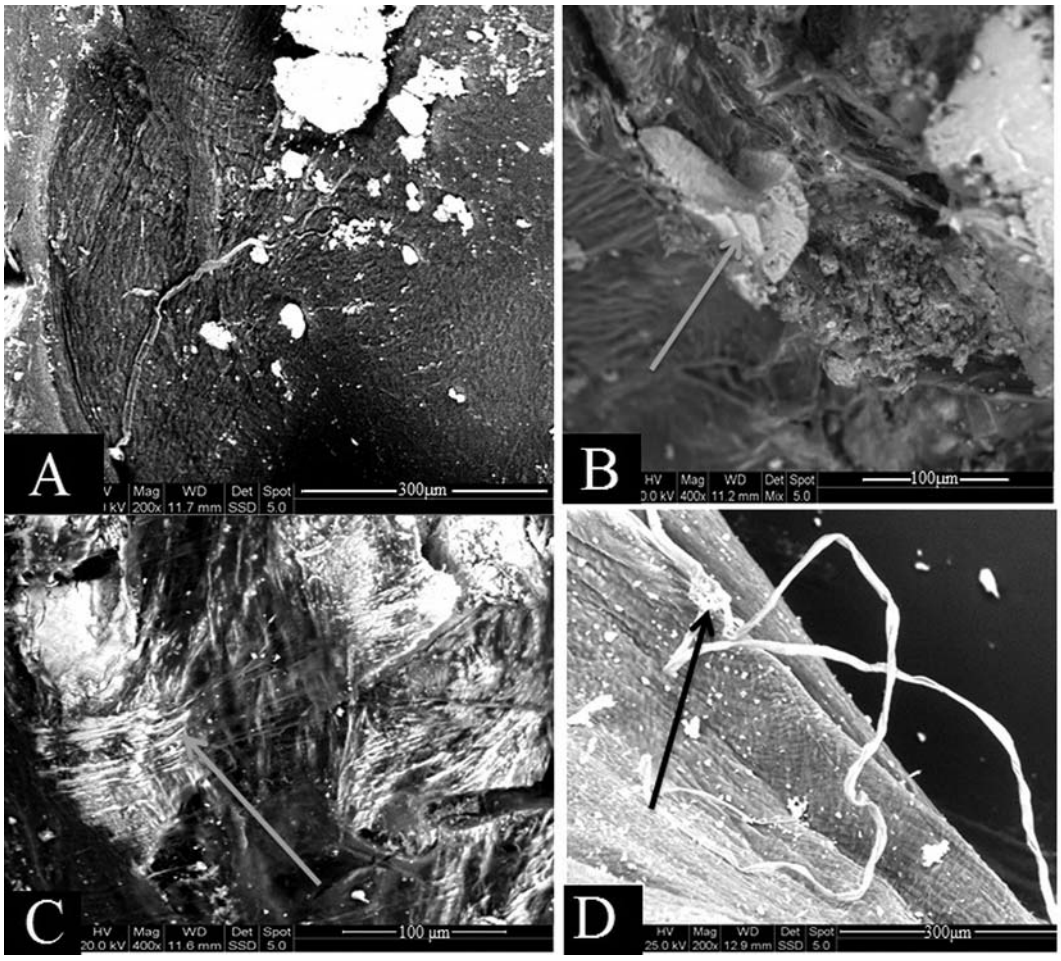
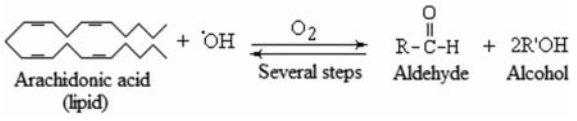


Figure 3. *SEM pictures of calcified aortic valve. A: General architecture of minerals and fibrils (scale 300 µm), B: Bone growth (scale 100 µm), C: Calcified cross-linked proteins (scale 100 µm). D: Clear view of initiation of calcification point on cross-linked bonds (black arrow, scale 300 µm).*

which are not stable and decompose, generating both free and core aldehydes and ketones that covalently modify ε-amino groups of lysine residues of the protein moiety (6, 10, 11) as follows:



(Eq1)

This mechanism is believed to take place during the first phase of oxidation of arachidonic acid from prostaglandine H synthesase. Deconvolution of the complex band near 1744-1734 cm^{-1} indicates that the ester *v*C=O group interacts in different ways with the lipophilic environment. In the case of the individual mineral spot, (Figure 2, spectrum 3), the band at 1714 cm^{-1} indicates that the ester *v*C=O group is located near a polar environment. This band is not observed in the spectra of the organic environment nor in the interface. We have noticed that in this particular case, although the patient had a normal serum total cholesterol and LDL levels, all the characteristic bands of cholesterol peroxidation products were indicated. These findings are in accordance with the observations by Novaro and Griffin, who found that serum lipoprotein levels closely correlate with the presence of aortic sclerosis but they are independent of LDL levels (25). The above discussion leads to the suggestion that oxidative stress activates inflammatory mechanisms following the progression of atherosclerotic lesions.

The absorption bands in the region 1700 to 1500 cm^{-1} are similar to those generated by *v*C=O stretching and *v*NH bending of the amide I (-NHCO-) and amide II modes of vibration in proteins (13-16). The bands at 1629 and 1543 cm^{-1}

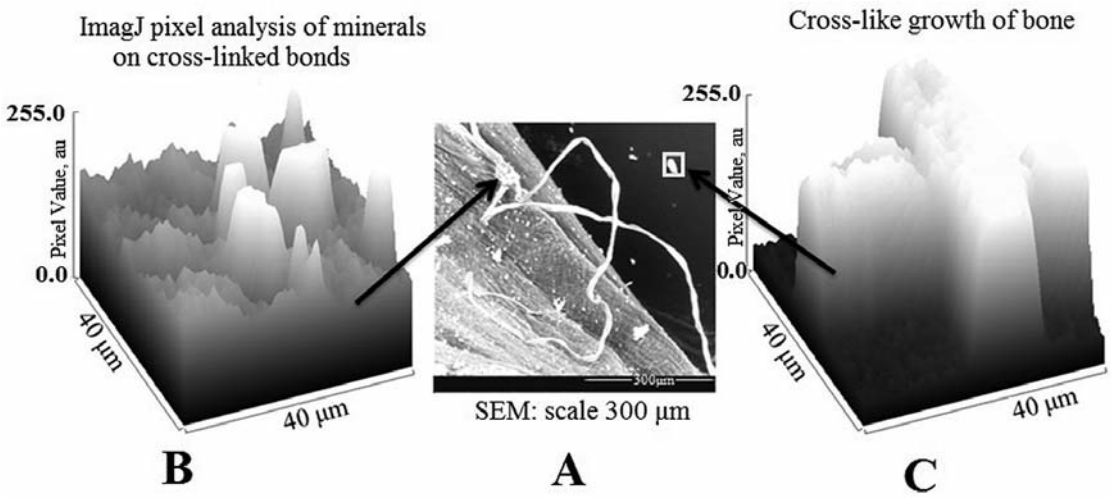


Figure 4. *A: SEM morphology of calcified aortic valve. B: ImagJ analysis of calcified cross-linked proteins. C: The calcium phosphates follow the cross pattern of the chemical bonds.*

are attributed to amide I and amide II vibrations, respectively. These bands are shifted to lower frequencies, comparing them with those of healthy tissues, which are found at 1655 and 1555 cm^{-1}, respectively. These shifts are generally absorptions associated with changes in protein and collagen molecular structure from α-helix to random coil, due to changes of lipophilic environment, as well as due to fragmentation induced by free radical reactions (9-11). In addition, the intensity and shift changes of the bands in the region 1600-1510 cm^{-1} are associated with the decrease of the (APOI)/(APOII) ratio, which dictates the HDL and LDL ratio (9-11).

In the 1500-1000 cm^{-1} fingerprint region, the absorption characteristics at 1454 cm^{-1} and 1370 cm^{-1} were similar to the well-known bending vibration νCH_2 of lipid methylene -CH_2- and the symmetric bending of the lipid methyl νCH_3 groups, respectively (9-11). From the shape, it is suggested that this band is not simple but a combination of bending vibrations of CH_2 and stretching vibrations of the carbonate νCO_3^{2-} anions, which were also present. This band, together with the band at 874 cm^{-1}, suggests that the minerals consist of calcium carbonate (17, 18), with two IR bands of CO_3^{2-}, $\nu_3 CO_3^{2-}$ near 1450 cm^{-1} and $\nu_4 CO_3^{2-}$ near 874 cm^{-1}.

The spectra in the region between 1200-850 cm^{-1} exhibit the characteristic asymmetric and symmetric stretching modes of PO_2^- of DNA, phosphodiester groups of the phospholipids, cholesterol ester and -C-O-C- vibrations of fatty acids (9-11) and hydroxyapatite (17, 18). As shown in spectrum 1 (Figure 2), there are significant changes in the shape and intensity of the bands resulting from the organic phase of the region. On the contrary, the main bands of the other two spectra correspond to the characteristic vibrations of the phosphate groups of hydroxyapatite. The band near 1190-1192 cm^{-1} is attributed to asymmetric stretching vibration of $\nu_{as} HPO_4^{2-}$, in corroboration with XRD analysis data, which shows the presence of $CaHPO_4$. These findings indicate that the initiation of mineral formation requires a polar environment. In the case of the individual mineral spot (Figure 2), there is a high intensity band of phosphate absorption at 1010 cm^{-1}. This particular band indicates the presence of β-$Ca_3(PO_4)_2$ components (26). Deconvolution of this band shows that apart from bone formation as hydroxyapatite, there are also possible crystalline and amorphous calcium salts, resulting from phospholipid fragments due to fragmentation of backbone carbon chain. The presence of $CaHPO_4$ suggests that during salt formation, an acidic environment predominates in the patient. The liberation of phosphates from ATP leads to production of ADP, AMP and hydrogen protons (acidosis). The presence of calcium carbonate anions in the infrared spectra demonstrates that carbon dioxide accumulation takes place, which results in carbonic acid formation, further increasing the acidity of the environment.

Significant changes are observed in the region of 600-400 cm^{-1}. In this region, the absorption bands of groups S-S and C-S of glutathione disulfide and glutathione appear correspondingly, due to protein–protein cross-linking.

Glutathione (γ-glutamyl-cysteinyl-glycine) is the most active endogenous antioxidant protecting against free radical induced cell damage. Glutathione interacts directly with highly reactive hydroxyl radicals, inhibiting damage of lipids. In addition, glutathione acts as a donor of hydrogen atoms, repairing damaged lipids. In both cases, glutathione disulfides, GS-SG, are produced, leading to deficiency of antioxidants as shown in the following equations:

$$GSH + HO^{\cdot} \rightarrow GS^{\cdot} + H_2O \quad \text{(Eq2)}$$

$$2GS^{\cdot} \rightarrow GS - SG \quad \text{(Eq3)}$$

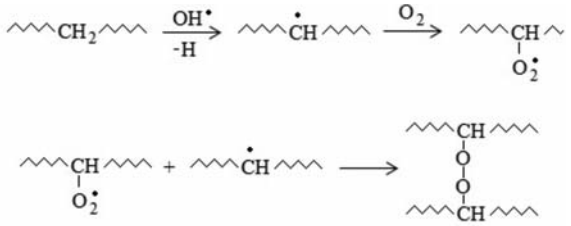
$$GSH + Lip^{\cdot} \rightarrow GS^{\cdot} + LipH \quad \text{(Eq4)}$$

Usually, glutathione reductase in the presence of NADPH reduces glutathione through electron transfer (27, 28):

$$GS - SG + NADPH + H^{+} \rightarrow 2GSH + NADP \quad \text{(Eq5)}$$

In our patients, it seems that NADPH is inactive and does not repair the glutathione disulfide into GSH according to equation 5.

SEM illustrations (Figure 3) show that the pathological alterations and calcification of aortic valve are not homogenous. The fibrils and mineral deposits developed on the aortic valve have different sizes and morphology (Figure 3A). At higher magnification (Figure 3B) bone formation, as hydroxyapatite, can be seen on the aortic valve (arrow). The dispersed smaller mineral deposits maybe the result of other calcium salts. From the morphology of the valve shown in Figure 3D, some of the proteins appear to lose their secondary structure and strands of different proteins or their fragments interact, forming a cross-linked bond (arrow). SEM images revealed that mineralization of aortic valve tissues starts preferentially at cross-linked chemical bonds of proteins, mainly collagenous (arrow, Figure 3C). The simplest mechanism for the peroxidation-induced cross-linked structures could be *via* the following reaction steps of lipids, phospholipids, or proteins:



(Eq 6)

Additionally, ImageJ analysis of the calcified regions confirms that in the early stages, calcium phosphate salts are deposited and organized structurally into the specific pattern of the cross-linked bonds (Figure 4C).

The cross-linking bonds between the insoluble non-collagenous lipoproteins and proteins are the space of calcifiability of aortic valves. We suggest that this reaction takes place at the sites of sulfur atoms of proteins, producing a disulfide bond (29). This *v*S-S frequency was also detected in the IR region between 650-500 cm^{-1} of the FT-IR spectra. Formation of disulfide bonds requires oxidation of cysteine residues, according to the equations (2) and (3). The locations of the cross-linked bonds seem to be the targets of development and progression of the mineral deposition. This is clearly illustrated in Figure 2C, where the cross-linked regions can be seen to be rich in minerals.

XRD analysis demonstrates that calcium deposits in the aortic valve tissues are consistent with the presence of biological hydroxyapatite, ($Ca_{10}(PO_4)_6(OH)_2$), as bone formation (Figure 4, B) and different calcium phosphate salts, such as dicalcium phosphate ($CaHPO_4$), tricalciumphosphate [$Ca_3(PO_4)_2$] and calcium salts formed from the reaction between calcium and phospholipid fragments from membranes. These results are in agreement with IR spectroscopic data, as they result from the shapes and frequencies of the bands. The production of dicalcium phosphate supports the idea that acidosis and anaerobic conditions induce oxidative hydrolysis of ATP affecting calcium homeostasis. It was proposed first by Drury and Szent-Györgyithat ATP is required for myocardial contracture and relaxation, as well as maintenance of normal myocardial impulse conduction (30). Depletion of ATP and 3-fold increase of the production of inorganic phosphate (monophosphates, PO_4^{3-}), at the end of ischemic perfusion was observed (31). Recent research data also indicate that regulation of ATP phosphorylation plays an important role in aortic valve calcification (32).

The molar ratio of Ca/P calculated from SEM-EDEX data analysis shows that this ratio varies from location to location. For a molar ratio Ca/P of 1.625 it is suggested that biological hydroxyapatite is of poor crystallinity (33, 34). As the molar ratio of Ca/P decreases, an increase of magnesium concentration is observed. It seems that magnesium, a calcium antagonist, substitutes the calcium ions on the hydroxyapatite, producing more soluble phosphate salts and thus inhibits bone formation and perhaps the aortic valve stenosis. Holt *et al*. indicated that amorphous calcium phosphate is produced in the presence of magnesium and in alkaline pH (35). Our results show that in order to reduce the progression of calcification of aortic valves a treatment of patients with magnesium salts is needed. Magnesium cations are known to regulate ATP metabolism and are cofactors in more than 300 intracellular enzymes (36, 37). Kruse *et al*. (38), Tufts and Greenberg (39) and most extensively, Seeling (40) have demonstrated that the most prominent feature of magnesium deficiency is the calcification predominantly of arteries. Iseri and French described that Mg^{2+} inhibits the release of Ca^{2+} from the sarcoplasmic reticulum, blocks the influx of Ca^{2+} into the cell by inactivating the Ca^{2+} channels in the cell membrane, and competes with Ca^{2+} at binding sites on troponin C and myosin, thereby inhibiting the ability of Ca^{2+} cations to stimulate myocardial tension (41). Chandy and Sharma using Mg^{2+} reduced both *in vitro* and *in vivo* the calcification of bovine heart valves (42).

Conclusion

The results reported here establish that the spectral changes of the characteristic FT-IR absorption bands of aortic valve are linked to hyperoxidation of membranes due to free radical formation during oxidative stress. The band at 1743 cm^{-1} supportsthe notion there is oxidation of LDL and lipoproteins, and inflammation of aortic valves due to free radical generation. The shape and frequency of the bands in the region 1200-950 cm^{-1} demonstrate that mineral deposits are composed of crystalline, amorphous inorganic calcium phosphates and low crystallinity biological hydroxyapatite. Using FT-IR spectroscopy, the amorphous calcium phosphate salts can be detected quite easily;these cannot be analyzed by X-rays, since this demands a single crystal material for molecular structure resolution. The results strongly suggest that IR spectroscopy has the distinct advantage of characterizing the phosphates in aortic valve. Furthermore, the SEM data show that the protein-protein cross-linking bonds are the starting sites of calcification. In addition, substitution of Ca^{2+} cations by Mg^{2+} cations leads to the formation of amorphous hydroxyapatite, preventing aortic valve stenosis, which suggests that treatment with magnesium salts may reduce stenosis of aortic valves.

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