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Calcification Characteristics of Porcine Stented Valves in a Juvenile Sheep Model

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Background. Different antimineralization treatments of stented porcine bioprostheses were evaluated: ethanol (Epic), α-amino-oleic acid (AOA) (Mosaic), and sodium dodecyl sulfate (SDS) (Hancock II). A nontreated, glutaraldehyde-fixed valve (Labcor) served as control.

Methods. For each treatment, six valves were implanted in juvenile sheep in the pulmonary position. Valves were explanted after 3 and 6 months and examined macroscopically, by roentgenogram and light and transmission electron microscopy. Calcium content (μg/mg) was determined by atomic absorption spectrometry.

Results. The Labcor valves revealed small calcium deposits in the cusps, although calcium content remained low (median value 0.4 ± 0.8 μg/mg). SDS did not prevent cusp calcification as assessed by histology and calcium content measurement, which was higher than in all other valves: 1.9 ± 4.6 μg/mg (p < 0.05). Cusp retraction and rupture were occasionally found in the Hancock. The Mosaic and Epic valves showed no cusp calcification and had low calcium contents (0.3 ± 2.4 μg/mg and 0.7 ± 0.6 μg/mg, respectively). Epic showed less pannus formation, but had hematoma or iron staining in the cusps.

Conclusions. SDS is inefficient as an antimineralization treatment, in contrast to ethanol or AOA. Cusp hematoma after ethanol treatment needs further investigation.


Tissue calcification is a common cause of clinical failure of stented glutaraldehyde-fixed porcine aortic bioprosthetic valves [1]. Several antimineralization treatments of glutaraldehyde-fixed valves have been developed and processed in porcine bioprostheses. These strategies include treatment of the fixed tissue with compounds such as sodium dodecyl sulfate (SDS) [2], α-amino-oleic acid (AOA) [3, 4], and ethanol [5, 6]. These substances have different properties: ethanol and SDS extract phospholipids from biological tissue, whereas AOA neutralizes free aldehyde moieties that remain present after glutaraldehyde fixation. All these procedures showed some benefit in terms of reduction of tissue calcification in several experimental models [2, 4, 5].

To compare the efficacy of these antimineralization treatments, we implanted glutaraldehyde-fixed, stented porcine aortic bioprosthetic valves in our juvenile sheep model [7, 8]. These valves were either SDS-, AOA- or ethanol-treated and were compared with a nontreated, glutaraldehyde-fixed valve.

Material and Methods

All animals were cared for by a veterinarian in accordance with the “Guide for the Care and Use of Laboratory Animals” (NIH publication 85-23, revised 1985). The study was approved by the Ethics Committee of the Katholieke Universiteit Leuven. Young sheep bred especially for this purpose were selected.

Valves Studied

Four types of stented porcine aortic valves were selected for this study: the Epic (St. Jude Medical, St. Paul, MN), the Mosaic (Medtronic, Minneapolis, MN), the Hancock II (Medtronic, Minneapolis, MN), and the Labcor (Sulzer Carbomedics, Austin, TX). These valves are all fixed by glutaraldehyde. The Epic (n = 7) is treated with ethanol and has a silver-coated fabric. The Mosaic (n = 6) is treated with AOA. The Hancock II (n = 6) has SDS as anticalcification treatment. The Labcor (n = 6) has no antimineralization treatment.

Implantation

All valves were implanted by previously described methods [7, 8]. Through a left thoracotomy at the second intercostal space, the main pulmonary artery was isolated, and a pneumatic right ventricular assist device (Medos-HIA VAD 54-mL ventricle, Medos-Helmholtz Institute, Aachen, Germany) was installed. After proximal and distal clamping of the pulmonary artery, the artery was opened and the stented valves were implanted using a continuous 4-0 polypropylene suture. After removal of the clamps, the native pulmonary valve was destroyed by tearing two cusps with a clamp introduced through a purse-string suture placed at the si-
nuses. The animals received analgesic, antibiotic, and diuretic agents as necessary. Low molecular weight heparin (enoxaparin sodium, 20 mg twice daily, Clexane, Rhone-Poulenc Rorer, Brussels, Belgium) was administered for 6 days. Afterwards, the sheep returned to the controlled animal facility where the general health of the sheep was checked daily.

**Explantation and Analysis**

Three of the six valves in each series were explanted after 3 months, the remaining three after 6 months. Sheep were premedicated and anesthetized as described previously. The left thoracotomy was reopened and after heparinization and exsanguination, the valve was excised.

**GROSS EXAMINATION.** After gross inspection, the valve was transected longitudinally through the commissures. Each of the three resulting specimens thus included one complete cusp with a small part of porcine aortic wall and a pre- and postvalvular part of the sheep pulmonary artery.

**ROENTGENOGRAM ASSESSMENT.** Roentgenogram examination was performed on every cusp in two directions under mammography conditions. The degree of calcification was scored semiquantitatively with three categories: 0 = no calcification, 1 = slight calcification, and 2 = severe calcification.

**HISTOLOGY.** For histology, paraffin sections (4 μm thick) were routinely stained with hematoxylin and eosin, Masson’s trichrome stain for collagen, an elastic Von Giesson stain, a phosphotungstic-acid-hematoxylin for fibrin, and a Von Kossa calcium staining.

**TRANSMISSION ELECTRON MICROSCOPY.** From each valve, several small samples were taken and embedded in dow exopy resin. One-micrometer thick sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were cut, stained with uranylacetate and lead citrate. Sections were treated with 2% potassium pyroantimonate to demonstrate calcium. Grids were examined in a Philips CM10 electron microscope. Random photographs were taken. Afterwards, every photograph was scored in a similar semiquantitative manner as for the roentgenograms: 0 = no calcification, 1 = slight calcification, and 2 = severe calcification.

**QUANTITATIVE CALCIUM DETERMINATION.** The cusps were divided into three parts: the commissural area, basal part, and free edge. The aortic wall portion was also divided in two parts. This resulted in nine cusp samples and six aortic wall samples in every valve. After lyophilization, the tissue was pulverized and desiccated. Pulverized tissue was diluted in 20% hydrochloric acid at a ratio of 10 mg dried tissue/1 mL HCl. Calcium content was measured by flame atomic absorption spectrometry, and expressed as microgram per milligram of dry weight.

| Table 1. Macro- and Microscopical Findings in Explanted Stented Bioprostheses |
|-----------------|-------|--------|--------|--------|
| Macroscopy      | Epic  | Mosaic | Hancock II | Labcor |
| Vegetation      | –     | –      | –       | –      |
| Thrombosis      | –     | –      | –       | –      |
| Pannus          | –     | +      | ++      | ++     |
| Cusp retraction | –     | +      | +       | +      |
| Cuspal hematoma | +     | –      | –       | –      |
| Cusp rupture    | –     | –      | +       | –      |
| Microscopy      | –     | +      | ++      | ++     |
| Calcification   | –     | +      | ++      | ++     |
| Neointima       | –     | +      | ++      | ++     |
| Foreign body reaction | –    | +  | ++     | +      |
| Iron staining   | +     | +     | –       | –      |

Results

**Gross Examination**

In the Epic group, 1 animal was lost at 2 months after implantation because of severe endocarditis. This animal had to be replaced by a new implantation. In all other valves, no infection occurred and valve thrombosis was never found. One of the Hancock valves showed a small cusp rupture.

Pannus formation was seen predominantly in the Labcor (5 of 6 animals) and the Hancock (4 of 6). It was less frequent in the Mosaic (3 of 6) and rare in the Epic valves (1 of 6). This neointima formation induced cusp retraction mainly in the Hancock (4 of 6). Cusp retraction was found in two of six valves in the Labcor and Mosaic. The results are summarized in Table 1.

**Roentgenogram Examination**

Calcification on roentgenogram was mainly seen in both the Hancock and Labcor valves. The other valves showed only minimal radiographic signs of calcification. The result of the semiquantitative roentgenogram analysis is given in Figure 2.

**Light Microscopy**

Microscopically, calcification of the cusp or the aortic wall portion was demonstrated in all Labcor and Hancock valves. Cuspal calcification in these valves was present in the form of small, diffuse calcific deposits. Large accumu-
lations of calcium in the cusp were never seen. The wall portion showed calcium aggregation in the tunica media. In the Mosaic valves, calcification was found only occasionally. Only two of six Mosaic valves showed a small calcium deposit in the cusps. The Epic showed no calcium deposition at all.

Both Labcor and Hancock showed significant neointima formation, which sometimes completely covered the cusp and caused cusp retraction. Foreign body reaction with accumulation of giant cells near the wall portion was also present in these valve types. Neointima formation was also found in the Mosaic valves, but to a lesser extent.

Fig 1. (A) Perls staining for iron in a cusp of an explanted Epic valve: cuspal hematoma (group of red blood cells) with positive iron staining (black dots) in iron macrophages ($\times$200 before 25% reduction). (B) Hematoxylin-eosin staining of an explanted Mosaic with neointima covering the base of the cusp ($\times$12.5 before 25% reduction); (C) Electron microscopy of an explanted Labcor cusp: visible calcification (black dots) between an ultrastructurally preserved environment ($\times$24,400 before 25% reduction).
The layer of fibrous tissue reached the base of the leaflets, but almost never extended further toward the free edge of the cusp. Neointima formation was practically absent in the Epic valves. These valves, however, frequently showed iron deposits in the leaflets. A fresh hematoma was found in the cusp of one Epic valve. A sample of the histologic image is shown in Figure 1. The iron staining is most likely the result of resorbed hemoglobin, coming from intracusal red blood cells from a small cusp hematoma.

Transmission Electron Microscopy
In all valves, the main ultrastructural tissue pattern was well preserved. In all valves, except for the Epic valve, small dots of calcium deposits were found in the cusps, both extracellularly spread between collagen fibers and intracellularly in cell remnants. These calcifications were variable in degree and incidence. The result of the semiquantitative analysis of cusp calcification is shown in Figure 2. Cusp calcification was highest in the Hancock and Labcor valves, and low in the Mosaic and Epic valves.

Calcium Content
When the results of calcium content were compared between valves explanted at 3 months and those explanted at 6 months, no statistically significant difference was found. Therefore, the data of the 3- and 6-month explants were pooled in every group. The results are listed in Table 2. The calcium content in the cusps of all studied valves were low, except for the Hancock valve. In this valve, calcium content in the cusps was $1.9 \pm 4.6 \mu g/mg$ (median value ± interquartile range), which is significantly higher ($p < 0.05$) in the Hancock valve, compared with all other valve types.

Comment
In stented porcine aortic bioprostheses, a small portion of the aortic wall, contiguous with the cusps, is trimmed and incorporated into the prosthesis. This aortic wall portion is known to calcify after implantation of the valve and many antimineralization treatments are obviously unable to prevent this calcification process [9]. However, in contrast to stentless valves, stented valves have only a small portion of aortic wall, located close to the stents, so that stiffening is not expected to change the hemodynamics of the valve. Furthermore, it is known in human explants that calcification of the aortic wall portion of stented porcine aortic bioprostheses is less than that of the corresponding cusps [10]. Therefore, anticalcification approaches need not be effective beyond the cusps of the stented valves. This fact is why we focused our analysis of calcification characteristics of the four stented valves on changes occurring predominantly in the cusps.

In a glutaraldehyde-fixed, nontreated valve (Labcor), we found relatively low calcium contents in the cusps.

![Fig 2. Semiquantitative score for the roentgenogram (A) and the electron microscopy (B) analyses. (□ = proportion of roentgenogram or electron microscopic photographs with no calcification (score 0), □ = proportion with slight calcification (score 1), ■ = proportion with severe calcification (score 2).)](image)

<table>
<thead>
<tr>
<th></th>
<th>Epic</th>
<th>Mosaic</th>
<th>Hancock II</th>
<th>Labcor</th>
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<tbody>
<tr>
<td>Cusp (μg/mg)</td>
<td>0.7 ± 0.6</td>
<td>0.3 ± 2.4</td>
<td>1.9 ± 4.6$^a$</td>
<td>0.4 ± 0.8</td>
</tr>
<tr>
<td>Aortic wall (μg/mg)</td>
<td>3.7 ± 19.0</td>
<td>0.4 ± 12.0</td>
<td>52.5 ± 117.3$^a$</td>
<td>3.3 ± 14.6</td>
</tr>
</tbody>
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Data are median values ± interquartile range.

$^a p < 0.05$ compared with all other valves.
However, cuspal calcification was clearly seen in light microscopy, but only in small deposits. Also, transmission electron microscopy clearly revealed the presence of calcium aggregates in the cusp. This contradiction between histologic findings and calcium content in the Labcor can be related to sampling error because cusp calcification is never homogeneous.

Although roentgenogram examination and light and electron microscopy revealed cusp calcification in the Labcor valve, the extent of calcification was still less than that found in the Hancock. This valve underwent a treatment with SDS to inhibit mineralization. Although this treatment, based on phospholipid extraction, has been shown to inhibit calcification in experimental animals, it was not efficient in our juvenile sheep model. Cusp calcification was clearly demonstrated by roentgenogram, light and electron microscopy, and atomic absorption spectrometry. In our case, also a small cusp rupture was found. In a recent clinical study on explants of the Hancock valve, it was shown that tears of the cusps (possibly caused by collagen disruption) and cusp calcification are the most common causes of valve failure in this stented valve [11].

α-Amino-oleic acid is known to inhibit calcification of glutaraldehyde-pretreated porcine aortic valve cusps, but not the aortic wall portion of bioprosthetic valves [4]. In our study, we found that AOA is much more efficient as an antimineralization agent than SDS. Cusp calcification in the Mosaic valves could be shown only by transmission electron microscopy and was only rarely found in the light microscopic sections of the cusps.

The Labcor, Hancock, and, to a certain extent, the Mosaic valves, showed neointima formation. This finding was always pronounced at the aortic wall portion and at the base of the leaflets, and extended sometimes to the free edges of the cusps. These valves also showed a clear foreign body reaction in the structures surrounding the stent and sewing ring. Both phenomena were rarely found in the ethanol-treated Epic valve. The exact reason for this lack of chronic inflammatory reaction and reduced neointima formation is not known. It cannot be excluded that the incorporation of silver in the polyester fabric plays a certain role.

Cusp calcification was clearly inhibited by the ethanol treatment and confirms results from previous experiments [5]. This inhibition of calcification of valvular tissue is related to selective lipid removal from the tissue [12]. In our study, light and electron microscopy as well as roentgenogram examination and calcium content determination all demonstrated almost optimal prevention of valve mineralization in the Epic. However, the Epic showed consistent iron deposits and small hematomas in the cusps. The iron deposits are most likely the remnants of a previous intracapsular bleeding. Cuspal hematomas were also described after AOA treatment [3, 4] and may result from small tears in the surface related to either unsoluble AOA crystals [4] or lipid extraction [12]. This feature needs further evaluation.

In conclusion, these results show that by using the juvenile sheep model, different antimineralization treatments can be compared and their efficiency determined.

References
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