ADAPT-Treated Porcine Valve Tissue (Cusp and Wall) versus Medtronic Freestyle and Prima Plus: Crosslink Stability and Calcification Behavior in the Subcutaneous Rat Model

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Background and aim of the study: The study aim was to compare the crosslink stability and calcification behavior of porcine tissue (cusp and wall), treated with a new antimineralization process (ADAPT) with that of commercially prepared Freestyle® and Prima Plus® bioprosthetic tissues.

Methods: Porcine aortic roots were divided into two groups: (i) tissue zero pressure-fixed with 0.625% glutaraldehyde (GA) for seven days and stored in 0.25% GA (as control); and (ii) tissue exposed to the ADAPT process for four days and stored in 0.25% buffered GA. These groups were compared with Freestyle and Prima Plus tissues (cusp and wall). Crosslink stability was assessed by shrinkage temperature and resistance to pronase degradation. Calcification behavior was assessed histologically (Von Kossa staining) and by atomic absorption spectrophotometry of explanted tissue after eight weeks in a subcutaneous rat model.

Results: Crosslink stability and calcification potential of ADAPT-treated porcine valve cusps were comparable to those of Freestyle and Prima Plus cusps (p = NS). ADAPT-treated porcine wall tissue showed improved crosslink stability (p <0.05) and significantly (p <0.001) reduced calcification (-95.95%) compared to control (-0.00%), Freestyle (-47.87%) and Prima Plus (-51.95%) tissues.

Conclusion: The ADAPT process is effective in reducing calcification in both porcine cusp and wall tissues in a subcutaneous rat model, and further suggest that enhanced crosslinking plays an important role in minimizing aortic wall calcification.

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In the present study, the crosslink stability and calci-fication behavior of porcine valve tissue treated with a new multi-step antimineralization process (ADAPT) were compared with those of Freestyle and Prima Plus valve tissues.

Materials and methods

Animals
All animals received humane care in compliance with the Principles of Laboratory Care (National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved by the Animal Ethics committee of the University of Western Australia, Perth.

Valve material
Fresh porcine valves were harvested at the local abattoir (Watsonia, Fremantle) and transported to the laboratory in cold (4°C) phosphate-buffered saline (PBS; 0.1 M, pH 7.4). This was followed by zero-pressure fixation in 0.625% phosphate-buffered GA (BHD, Electron microscopic grade) for seven days at 4°C, or fixation by the ADAPT process for four days. This antimineralization process is based on a multifactorial approach focused on synchronized synergism between enhanced crosslink stability, removal of residual GA, modification of non-bifunctionally reacted GA residues, reduction of the lipid content, and restoration of tissue elasticity. Crosslink stability was enhanced by improved GA penetration and crosslinking of the conformed collagen triple helix, using lipid extraction with a short-chain alcohol. Residual GA was removed and modified by a combination of amine incorporation and carboxyl binding at a high temperature and a low pH. Tissue elasticity was restored by polymerization of the incorporated GA moieties in the tissue by increased temperature. Fixed valve tissue was stored in 0.25% buffered GA at 4°C.

Experimental design
The fixed aortic valve cusps (n = 30) and representative aortic wall samples (n = 30; 10 mm × 15 mm) were removed from the aortic roots and divided into two groups. Group I included valve cusps (n = 15) and representative aortic wall samples (n = 15; 10 mm × 15 mm) stored in 0.25% buffered GA. Group II included valve cusps (n = 15) and representative aortic wall samples (n = 15; 10 mm × 15 mm) exposed to the ADAPT antimineralization process for four days and stored in 0.25% buffered GA. For comparison, a third group (III) consisted of valve cusps (n = 10) and aortic wall samples (n = 10; 10 mm × 15 mm) from Freestyle bioprostheses, while group IV consisted of valve cusps (n = 10) and aortic wall tissue (n = 10; 10 mm × 15 mm) from Prima Plus bioprostheses.

Crosslink stability

Shrinkage temperature
Shrinkage temperature measurement was used to assess the stability of the collagen crosslinks of the tissue (30). Cusp and aortic wall sample tissue strips (5 × 10 mm; n = 10) in each group were attached to an isometric force transducer (MLT0500; AD Instruments, Australia), interfaced with a PowerLab data acquisition system and a desktop personal computer.

Samples were kept in constant extension with a load of 90 ± 5 g and immersed in an open, temperature-controlled water bath filled with 0.9% saline. The temperature of the water bath was gradually increased at approximately 1.5°C/min from 25°C to 95°C. The shrinkage temperature was indicated as a sharp deflection point from constant extension when the collagenous material was denatured.

Enzyme degradation resistance
Resistance to proteolytic enzyme digestion was based upon the method of Girardot and Girardot (28). A pronase solution was prepared by dissolving 100 mg pronase E (type XIV from Streptomyces griseus; Sigma) and 100 mg calcium chloride, in 200 ml HEPES buffer solution (0.01 M, pH 7.4), containing 0.1 M glycine. Fixed tissue samples were rinsed in deionized water for 3 min, blotted, dried overnight at 70°C and weighed. These samples were then incubated in pronase solution at 50°C for 24 h. Remaining tissue samples were rinsed in deionized water, dried overnight at 70°C and weighed. Resistance to pronase digestion was determined by the mass of remaining tissue, expressed as a percentage of the predigested tissue mass.

Rat subcutaneous implants
Surgical implantation was performed at the small animal facility of the Cell Biology Research Unit of Fremantle hospital.

Young, male Wistar rats (bodyweight 150-200g) were divided into two groups; one group (n = 10) received cusp implants and the second group (n = 10) received aortic wall implants. Each animal received one sample of each of the four groups of tissues, making a total of 80 implants.

Rats were anesthetized with pentobarbital (Nembutal®; 45 mg/kg, intraperitoneal). The dorsal muscle area was shaved and disinfected with 15% diluted chlorhexidine gluconate (ICI Pharmaceuticals, Perth, WA) and ethanol (Merck Chemicals, Perth, WA). Implants were thoroughly rinsed in deionized water...
for 2 min to eliminate residual fixative, and then implanted into subcutaneous pouches through an incision of 2.5 cm into the back muscle wall. The incision was closed with 5-0 Prolene sutures.

Rats were sacrificed after eight weeks with an overdose of barbiturates (Euthenase®), and the dorsal muscle wall, containing the subcutaneous implants, was removed for quantitative and qualitative tissue calcium analysis. Each retrieved sample was divided into two anatomically symmetrical halves. One half was used for atomic absorption spectrophotometry, and the other half was fixed in 10% buffered formaldehyde and processed for histology.

Histology

Fixed samples were embedded in paraffin wax, sectioned at 3 µm, and treated with Von Kossa stain for qualitative calcium analysis. Histological examinations were performed using an Olympus BHS light microscope.

Quantitative calcium analysis

Explanted tissue samples from all groups were dissected free of surrounding host tissue and dried in a Biotherm incubator (Selby Scientific, Perth, WA) at 90°C for 48 h. The dried samples were weighed, and the calcium content extracted in 5.0 ml 6 N ultrapure hydrochloric acid (Merck, Perth, WA) at 75°C for 24 h. The extractable calcium content was measured using an atomic absorption spectrophotometer (Varian AA1275) and expressed as µg Ca per mg tissue (dry weight).

Statistical analysis

All numerical results were interpreted according to standard descriptive statistics such as mean, SD, SEM and ranges. The statistical analysis included a one-way variance check (ANOVA), unpaired t-test (Mann-Whitney Rank Sum Test) and pairwise comparison of 95% confidence intervals. A p-value <0.05 was considered to be statistically significant. All statistical analyses were performed using SigmaStat, Version 2.0 software (SPSS. Inc., Chicago, Illinois, USA).

Results

Crosslink stability

The shrinkage temperatures for valve cusps and aortic wall tissues are listed in Table I. No significant differences were identified between the control, ADAPT, Freestyle and Prima Plus cusps. ADAPT-treated aortic wall tissue showed a significantly (p <0.05) higher shrinkage temperature compared to control, Freestyle and Prima Plus wall tissues.

Resistance to enzymatic degradation is illustrated in Figure 1. ADAPT, Freestyle and Prima Plus cusp tissues showed significant (p <0.0001) increases in resistance to proteolytic digestion compared to control (Fig. 1A). No significant difference was seen between ADAPT, Freestyle and Prima Plus cusp tissues.

ADAPT-treated aortic wall tissue showed equal resistance (p = NS) to proteolytic digestion as the control tissue, and a significantly (p <0.01) higher resistance compared to the Freestyle and Prima Plus wall tissues (Fig. 1B).

Table I: Shrinkage temperatures (ºC) of valve cusp and aortic wall tissue (n = 10 per group).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cusp</th>
<th>Aortic wall</th>
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<tbody>
<tr>
<td>Control</td>
<td>84.6 ± 1.40</td>
<td>86.73 ± 0.26</td>
</tr>
<tr>
<td>ADAPT</td>
<td>85.5 ± 0.24</td>
<td>89.34 ± 0.19*</td>
</tr>
<tr>
<td>Freestyle</td>
<td>85.7 ± 0.35</td>
<td>86.16 ± 0.40</td>
</tr>
<tr>
<td>Prima Plus</td>
<td>84.3 ± 0.18</td>
<td>86.37 ± 0.34</td>
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Values are mean ± SE.

*p <0.05 (ADAPT versus Control, Freestyle, Prima Plus).
Histology

Histological examination indicated the presence of severe, intrinsic calcification in the explanted control cusp samples (Fig. 2A). No visible calcification was noted in either the ADAPT, Freestyle or Prima Plus valve cusps (Fig. 2B-D).

Explanted aortic wall tissue revealed severe calcification of the media (Fig. 3A) in control samples. No visible calcification was noted in the explanted ADAPT aortic wall tissue (Fig. 3B). Moderate calcification of the media was noted in the explanted Freestyle (Fig. 3C) and Prima Plus (Fig. 3D) aortic wall tissues.

Tissue calcium analysis

The quantitative tissue calcium levels for explanted cusp samples are illustrated in Figure 4A. Control samples, fixed in GA only, showed the highest level of calcium in this model (92.37 ± 7.9 µg/mg). The calcium levels of ADAPT (2.09 ± 0.22 µg/mg tissue), Freestyle (2.03 ± 0.29 µg/mg) and Prima Plus (1.54 ± 0.17 µg/mg) treated cusps were significantly (p <0.001) reduced compared to control samples. No significant difference was seen between the tissue calcium levels of ADAPT, Freestyle or Prima Plus cusps.

The quantitative tissue calcium levels for explanted aortic wall samples are illustrated in Figure 4B. The calcium content of ADAPT-treated aortic wall samples (4.86 ± 0.12 µg/mg tissue) was significantly (p <0.001) lower (by 95.9%) than that of control samples (120.11 ± 7.48 µg/mg tissue). Freestyle and Prima Plus aortic wall samples showed reductions in calcification of 47.8% and 51.9%, respectively.

Discussion

The long-term durability of GA-fixed heart valve
bioprostheses is mainly dependent on the calcification potential of the valvular tissue (1-4). At present, it is generally accepted that the process of tissue fixation plays a major role in tissue calcification (29). Fixation of heart valve tissue with GA has been shown to be one of the more important factors responsible for the occurrence and extent of tissue mineralization (30,31).

Because of the unique crosslinking and sterilizing properties of GA, some manufacturers have employed additional anticalcification measures such as trivalent cations (13), surface binding of fatty acids (14) and ethanol preincubations (15).

Crosslinking of bioprosthetic tissue with alternative non-GA agents such as epoxide (32), carbodiimide (32) and acyl azide (33) have shown significant reductions in calcification in small animal models. However, none of these additional anticalcification strategies has resulted in complete mitigation of bioprosthetic valve calcification, particularly of the aortic wall. Aortic wall tissue is substantially different in structure from valve leaflet tissue, and is much more susceptible to calcification after GA fixation (34).

In the present study, the crosslink stability and calcification behavior of GA-fixed porcine valvular tissues, treated with a new antimineralization process called ADAPT, was compared with that of commercially prepared Freestyle and Prima Plus porcine tissues, both of which are specifically treated against calcification.

Shrinkage temperature tests demonstrated acceptable crosslinking levels of the ADAPT-treated valve cusps, comparable to those of the commercially prepared valve cusps. However, ADAPT-treated aortic wall tissues showed a superior level of crosslinking compared to the commercially prepared Freestyle and Prima Plus wall tissues.

The resistance to enzymatic degradation of ADAPT-
treated, Freestyle and Prima Plus valve cusps further demonstrated excellent crosslinking levels in these tissues. The higher resistance to enzymatic degradation of ADAPT-treated aortic wall tissue was in accordance with the findings with regard to shrinkage temperature, and is best explained by enhanced crosslinking.

The absence of visible calcific deposits in ADAPT, Freestyle and Prima Plus valve cusps reflected the efficiency of the respective antimineralization protocols in mitigating calcification in this tissue. By contrast, the presence of moderate calcific deposits in Freestyle and Prima Plus aortic wall tissues demonstrated the inability of these antimineralization regimes to fully protect the aortic wall against progressive tissue calcification. The absence of visible calcific deposits only in ADAPT-treated aortic wall tissue reflected the additional protective effect of the ADAPT process towards aortic wall calcification.

Previous studies have shown that cusp and wall tissue calcification differ in nature because of morphological differences between the two respective tissues (35). Porcine valve cusp tissue contains a higher collagen and a lower elastin content than aortic wall tissue (36). These morphological differences not only affect the rate of calcification but also suggest that different mechanisms of calcification exist in the two types of tissue (36). The present results show that the short multi-step approach of ADAPT is effective in addressing both cusp and wall calcification, without losing the advantages of GA such as crosslinking and sterilization.

The effectiveness of ADAPT antimineralization on both cusp and wall tissue is best explained in terms of the enhanced crosslinking that occurs in both situations. This agrees with findings of a previous study (25) which indicated the important role of enhanced crosslinking in valve tissue calcification.

In conclusion, the ADAPT antimineralization process has shown distinctive reductions of calcification in GA-fixed porcine cusp and aortic wall tissues when implanted in a standard rat subcutaneous model (37). These findings inevitably give rise to a new generation of treated porcine tissues with superior antimineralization properties.

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References
6. Thubrikar MJ, Deck JD, Aoud J, Nolan SP. Role of mechanical stress in calcification of aortic biopros-


Meeting discussion

MR. RUSTY PHILLIPS (USA): You told us what you think ADAPT does. Can you tell us what it is?

DR. WILLIAM M. NEETHLING (Fremantle, Australia): Because of patent sensitivity, I can only give you a brief idea. It is a multi-step process which is partly a non-glutaraldehyde process, and partly a glutaraldehyde process. The sterilization is a glutaraldehyde process.

DR. PETER ZILLA (Cape Town, South Africa): From your shrinkage temperature and digestion results, you seem to have a higher crosslink density than in control tissues. I assume that you have also extracted the glutaraldehyde somehow. We have seen similar results with a higher crosslink density, but the price to pay is normally stiffness of the tissue. Is your tissue stiffer?

DR. NEETHLING: It is stiffer, and so we incorporated an additional process which forms part of ADAPT to restore the elasticity.

MS. CRYSTAL CUNANAN (USA): You say that in the ADAPT process you remove the residual glutaraldehyde, and all the bad parts of glutaraldehyde - yet you put the tissue back into glutaraldehyde. How can you be certain that there is no further incorporation of glutaraldehyde when you put the tissue back into it?

DR. NEETHLING: We have performed additional studies in which we stored the ADAPT tissue in non-glutaraldehyde substances, and the calcification was slightly lower. At this point we just wanted to compare the ADAPT process with commercially available tissue, and to show that glutaraldehyde can still be used as a storage medium. Later, we may decide to use non-glutaraldehyde storage to improve the quality and reduce calcification.

MS. CUNANAN: This implies that you are putting glutaraldehyde back into the tissue in some way, shape, or form, because your calcification was a little higher in that group. You may need also to look at the effects of storage.