Mechanism of Efficacy of 2-Amino Oleic Acid for Inhibition of Calcification of Glutaraldehyde-Pretreated Porcine Bioprosthetic Heart Valves

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Background Calcification is a frequent cause of the clinical failures of glutaraldehyde-pretreated bioprosthetic heart valves (BPHV) fabricated from glutaraldehyde-cross-linked porcine aortic valves. 2-Amino oleic acid (AOA) has been shown in previous in vivo studies to be a promising anticalcification agent. Our objective was to investigate the mechanism of calcification inhibition mediated by AOA pretreatment of porcine aortic valve bioprostheses.

Methods and Results BPHV tissues were treated with an AOA solution for 72 hours before experimentation. The diffusion of AOA across both cusp and aortic wall was evaluated. The lag time for AOA to diffuse across the aortic wall was prolonged compared with that of the cusp. An extraction study was performed to determine the stability of AOA binding; the results indicated that the binding was relatively stable regardless of solvent extraction conditions. The interaction between ionic calcium and AOA on treated tissue also was investigated by evaluating the patterns of calcium diffusion across both treated and untreated tissues.

The results showed that AOA significantly reduced the diffusion of calcium. AOA inhibition of aortic valve calcification (calcium level, 5.5±3.0 mg/g of tissue compared with control; calcium level, 91.2±19.5 mg/g of tissue) but not aortic wall (calcium level, 158.7±10.3 mg/g of tissue compared with control; calcium level, 157.5±7.9 mg/g of tissue) was demonstrated on representative specimens from valves implanted in left ventricular aicoaortic shunts explanted after 150 days. Conclusions AOA covalently binds to glutaraldehyde-pretreated bioprosthetic heart valve tissue, presumably as the result of an aldehyde-amino reaction. Covalently bound AOA diminishes Ca\(^{2+}\) diffusion compared with non-AOA-pretreated bioprosthetic tissues. This may explain in part the anticalcification mechanism of AOA. Furthermore, AOA inhibits calcification of porcine BPHV cusps in the circulation. (Circulation. 1994;90:323-329.)

Key Words • calcification • glutaraldehyde • aortic cusp • aortic wall

Calcific degeneration causes most clinical failures of bioprosthetic heart valve (BPHV) replacements fabricated from porcine aortic valves cross-linked with glutaraldehyde. Procedures for inhibiting BPHV calcification have been investigated in animal model studies involving either subdermal implants in rats or circulatory implants in large animals (sheep or calves). Thus far, all of the inhibition strategies studied included pretreatments with diphosphonate or metallic salt solutions, polymer incorporation into leaflet structures, and others, only detergent pretreatments have inhibited calcification in both subdermal and circulatory animal model studies. The mechanism of action of detergent pretreatment for inhibiting bioprosthetic calcification is incompletely understood at present. The present investigations focused on a bio-

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from 150-day, left ventricle–to–descending aorta valve grafts in sheep.

Methods

Materials

Amino oleic acid was provided to our laboratory by the Medtronic Heart Valve Division (Irvine, Calif) as a saturated solution (0.171% AOA) prepared under proprietary conditions also used to prepare the clinically implantable heart bioprostheses used in the animal models described below. Control bioprosthetic incubations used the identical solvent used to prepare the saturated AOA solution. [14C]-labeled (specific activity, 1.6 mCi/mM, labeled at the beta-carbon) AOA was synthesized at American Radio Chemical Inc (St Louis, Mo) using procedures established by Girardot.12 Nonradioactive AOA was synthesized by the same procedures by Medtronic Inc. Fresh and glutaraldehyde–cross-linked porcine aortic valve tissues were supplied by the Heart Valve Division of Medtronic Inc. Glutaraldehyde–pretreated porcine aortic valves were prepared according to the clinical pretreatment protocol used by Medtronic Heart Valve Inc, following proprietary procedures. Non-AOA-exposed porcine bioprosthetic valve tissues were used in all control studies. Frozen bovine serum and Tween-80 (for use in extraction studies, see below) were purchased from Sigma Chemical Co and Fisher Chemical Inc, respectively. Porcine aortic valve bioprostheses (valved grafts, without stents) with and without AOA pretreatment,8,12 also exposed either to AOA plus proprietary solvent or proprietary solvent alone, were provided to Crawford Long Hospital (Emory University, Atlanta, Ga) for sheep implant study by the Heart Valve Division of Medtronic Inc. A 0.1% of sodium azide (Sigma Chemical Co) was added to the bovine serum as a preservative. All other chemicals were reagent grade; distilled and deionized water was used.

A saturated AOA solution (0.171%) was prepared under proprietary conditions by the Heart Valve Division of Medtronic Inc following the method established by Girardot.8,12 A 0.1% [14C]-AOA stock solution was prepared by the same procedures.3 The [14C]-AOA stock solution was spiked directly into AOA solution for all experiments. Porcine bioprosthetic methodology for AOA linking followed established methodology at 37°C for 72 hours under proprietary conditions (as above).8,12 Tissues pretreated with [14C]-AOA were washed extensively with an excess of normal saline until the radioactivity of the wash liquid was indistinguishable from background. Similar methodologies were applied to treat fresh tissues.

Determination of AOA Content in [14C]-AOA–Treated Tissues

Sample tissues were lyophilized separately, weighed, and incubated in Solvable (New England Nuclear Research Products Inc) for 24 hours at 60°C to 65°C in a water bath. Each digested sample then was combined with an adequate volume of Ecolume (ICN Biomedical Inc), and [14C] radioactivity was determined by liquid scintillation counting (Beckman LS3801 liquid scintillation counter). The AOA content of tissue was assessed by comparing its dpn value with that of the [14C]-AOA solution prepared for tissue treatment. The amount of AOA incorporated was expressed as nanomoles per milligram of dried tissue.

Diffusion of AOA Across Tissues

Representative thicknesses of all tissues were measured at four sites using a micrometer (Bel-Art Instruments) before experimentation, and data were reported as mean±SEM. For diffusion studies, each piece of cusp tissue was mounted on a microdiffusion cell. One milliliter of [14C]-spiked AOA solution was pipetted into the donor chamber, and 1 mL of non-AOA-containing buffer was immediately added to the recipient chamber. The diffusion cell was placed on a shaker rotating at 150 rpm; temperature was maintained at 37°C. After 15 minutes, the recipient chamber was evacuated and replenished with 1 mL of fresh AOA buffer. Additional samples were withdrawn at 30 and 45 minutes and 1, 2, 3, 4, 5, and 6, 7 hours. Each sample was combined with 19 mL of Ecolume, and [14C] radioactivity was assessed by liquid scintillation counting. The AOA level was determined by comparing the sample dpn value with that of the [14C]-spiked AOA solution prepared for the diffusion study. The AOA level detected was expressed as the cumulative percent AOA diffused into the recipient half-cell. The lag time for AOA diffusion was estimated by back-extrapolating the linear phase of the plot (cumulative % AOA diffused across versus time) to the time axis. Similar methodologies were applied to determine the diffusion parameters of glutaraldehyde–cross-linked aortic wall tissue. The sampling intervals were 1, 2, 3, 4, 6, and 7 hours and 1, 2, 3, 5, 6, and 7 days. Their diffusivities were calculated based on steady-state equilibrium as described by Johnston et al.13

\[
D = \frac{1}{2} \ln \left( \frac{t_{lag}}{t_{lag}} \right)
\]

where D is diffusivity, I is thickness of tissue, and t_{lag} is lag time.

Diffusion of Calcium Across Tissues

The thicknesses of all cusp samples were measured before experimentation as described above. A piece of AOA-treated cusp tissue was mounted on a microdiffusion cell. One milliliter of 0.2% (18 mol/L) calcium chloride solution was pipetted into the donor chamber, and 1 mL of water was immediately added to the recipient chamber. The diffusion cell was placed on a shaker rotating at 150 rpm; temperature was maintained at 37°C. After 5 minutes, the recipient chamber was evacuated and replenished with 1 mL of water. Additional samples were withdrawn at 10, 15, 20, 30, 45, and 60 minutes. The calcium contents of all samples collected (after proper dilutions) were determined by atomic absorption spectroscopy (Perkin-Elmer model 2380 spectrophotometer) at a wavelength of 422.8 nm. Similar methodologies were applied to evaluate the pattern of calcium diffusion across aortic wall. The sampling intervals were 4, 6, 12, 24, 28, and 32 hours. Diffusivity constants were calculated as described above.14,15

OA extraction Study

[14C]-AOA extraction studies were carried out with an array of solvents including pH 7.4 phosphate-buffered saline, 20% aqueous Tween-80 solution, and bovine serum. Samples of AOA-treated tissue were transferred to a vial of pH 7.4 phosphate buffer placed on a shaker (rotating at 150 rpm) at 37°C for 1 hour. Each BPHV specimen was then transferred to another vial containing fresh phosphate buffer. The same procedure was repeated at 2, 4, 6, 8, 12, and 24 hours, then 2, 4, 6, 8, 12, 16, 24, and 30 days. Samples were withdrawn from each vial and combined with Ecolume, and the [14C] level was assessed by liquid scintillation counting. AOA content was determined by comparing its dpn value with that of the [14C]-spiked AOA solution used for treating tissue.

At the conclusion of the extraction study, each tissue specimen was retrieved and digested with Solvable; residual [14C] radioactivity was determined by the procedures previously described involving saline washes to remove residual radioactivity followed by lyophilization. The residual dpn value was added to the total cumulative counts gathered during the study; this number was stipulated as the initial total counts on the tissue before the experiment. The extraction study results were thereby expressed as cumulative % AOA leached versus time. Similar methodologies were applied to conduct extraction studies using both bovine serum and Tween-80 as extraction media. Unpaired t tests were used to assess the significance of statistical differences from controls.
Calcium Analysis of Explanted Bioprosthetic Tissues

Fifteen sheep were used for valve implantation: seven of them received AOA-pretreated, stentless valves, and the rest received control valves (ie, non-AOA-pretreated). Representative samples (one sample from each leaflet and adjacent aortic wall tissues) from 150-day sheep valve grafts (implanted as left ventricular apicoaortic shunt) were assessed upon retrieval by one of us (F.J.S.) as part of an investigation of the hemodynamic performance and calcification inhibition of AOA-pretreated bioprosthetic heart valves.16 Retrieved aortic cusps and aortic wall samples were separately analyzed for calcium as detailed below. Cuspal and aortic wall tissues were lyophilized, weighed, and acid hydrolyzed according to established procedures.17,18 Tissue calcium contents were determined by atomic absorption spectroscopy at a wavelength of 422.8 nm. ANOVA was used to test statistical significance.

Results

AOA Diffusion

When glutaraldehyde-cross-linked porcine aortic valve tissue was used as a diffusion barrier (Fig 1A), a significant amount of AOA (>1% of the AOA from the donor chamber) penetrated the tissue after only 1 hour. The results of AOA diffusion across cusps tissue and aortic wall are illustrated in Fig 1A and 1B, respectively; the corresponding diffusion lag times and thicknesses are summarized in Table 1. The thickness and the lag time determined were 0.73±0.11 mm and 0.56 hour, respectively. When aortic wall was used as a diffusion barrier (Fig 1B), the time required to detect a significant amount of AOA (>1% of the AOA from the donor chamber) drastically increased to about 48 hours. The corresponding tissue thickness and extrapolated lag time were 2.59±0.14 mm and 88.14 hours, respectively. The diffusivities of cusp and aortic wall tissues are 0.157±0.001 and 0.013±0.001 mm²/h, respectively. The implication is that AOA does not penetrate aortic wall as readily as cuspal tissue.

Stability of AOA Binding

Extraction studies of AOA-treated tissues (cusp and aortic wall) were performed to determine the stability of AOA binding. Initially, pH 7.4 phosphate buffer was used as a medium for the study. To better simulate the in vivo environment that a BPHV valve would encounter after implantation, an AOA extraction study was also conducted using bovine serum as an alternative medium. Furthermore, the poor solubility of AOA in water and hence in phosphate buffer may thus reflect its dissolution characteristics rather than its binding stability. Therefore, Tween-80, a nonionic surfactant, was used as a harsh dissociative medium to forcibly enhance nonbound AOA dissociation.

The results of the 1-month extraction study using both cusp and aortic wall are illustrated in Fig 2A and 2B, respectively. In general, an initial burst followed by a greatly diminished rate of AOA extraction was observed in each study. The rate of extraction in phosphate buffer began to plateau at 24 hours compared with that of Tween-80, which leveled off within 8 hours. As illustrated in Fig 2A, using Tween-80 as the extraction medium, approximately 60% of the AOA was lost from the cusps after the first 12 hours. Although a similar pattern was observed with aortic wall (Fig 2B), a significant amount of AOA was found to remain on this tissue as well at the conclusion of the study.

Table 2 summarizes the initial and residual AOA levels on the tissues (both cusp and aortic wall) used for the extraction study. For comparison, fresh tissue (after AOA pretreatment) was used as a control. It should be noted that 42.2±2.3 nM of AOA was present on each milligram of fresh cusps tissue compared with 128.6±1.1 nM/mg of its glutaraldehyde-cross-linked counterpart (before extraction). Therefore, the residual glutaraldehyde appears to play an important role in linking AOA molecules to tissues.13 Each milligram of cusps tissue, after leaching in phosphate buffer, retained 95.5±5.2 nM of AOA; the high residual AOA level may simply reflect poor aqueous solubility of AOA. The presence of a surfactant (ie, Tween-80) greatly enhanced aqueous solubility of AOA; thus, 35.6±1.0 nM of AOA remained on each milligram of tissue. When bovine serum was used as an extraction medium, each milligram of tissue retained 52.1±2.2 nM of AOA. Although it is possible that serum protein adsorption could have influenced this result, this seems unlikely, given the
2. TABLE

Extensive washing protocol described above followed by freeze drying. Nevertheless, the results of the bovine serum studies were highly reproducible (as shown) and internally consistent, suggesting high levels of AOA residual tissue binding after serum exposure.

**Calcium Diffusion**

The results of calcium diffusion across cusp (AOA-pretreated and untreated) and aortic wall (AOA-pretreated and untreated) tissues are depicted in Fig 3. In Fig 3A, after 1 hour of diffusion, the cumulative amounts of calcium diffused across the AOA-pretreated and untreated cusp tissues were 33.1±1.7 and 78.2±3.8 μg, respectively. Their corresponding diffusivities are 2.69±0.11 and 1.25±0.05 mm²/h. When aortic wall was used as a diffusion barrier (Fig 3B), the cumulative amount of calcium diffused across the AOA-pretreated and untreated tissues was 9.0±1.7 and 14.0±1.6 μg, respectively, after 24 hours. Their corresponding diffusivities are 0.21±0.04 and 0.12±0.02 mm²/h. Overall, the presence of AOA on both tissues appears to significantly retard the transport of calcium ions, and this implies that AOA does indeed interact with calcium.

**Calcium Inhibition in the Circulation**

Explanted porcine bioprosthetic aortic valve cusps had significantly less calcium (5.5±3.0 mg/g of tissue) than did cusps from untreated valves (9.1±1.95 mg/g of tissue). For comparison, unimplanted aortic valve cusps contained 0.6±0.2 mg of calcium per gram of tissue. However, porcine bioprosthetic aortic wall calcification was not significantly retarded by AOA pretreatment (calcium content, 158.7±10.0 mg/g of tissue) compared with untreated tissues (calcium content, 157.5±7.9 mg/g of tissue). Unimplanted aortic wall contained 0.7±0.2 mg of calcium per gram of tissue.

**Discussion**

The long-term success of all types of BPHV (manufactured from glutaraldehyde-cross-linked porcine aor-
calcific valve or bovine pericardial tissues) is limited by calcific degeneration.\textsuperscript{1} Calcification of glutaraldehyde–cross-linked BPHV is hypothesized to be a multifactorial process.\textsuperscript{12} The influx of calcium ions into bioprosthetic tissue after BPHV implantation almost certainly plays an important role in the initial nucleation of calcium phosphate crystals intrinsically within devitalized cells in the valve cusps.\textsuperscript{12,17–19} Previous work has demonstrated that the initial calcium phosphate–forming event in bioprosthetic leaflet calcification, which can occur as early as 48 hours after a rat subdermal implant, involves calcium diffusion into the phosphorus-rich ultrastructure of glutaraldehyde-devitalized cells.\textsuperscript{19,20} Ultrastructural calcium phosphate formation has been demonstrated to occur as a result of the coincidence of diffused calcium with immobilized phosphorus.\textsuperscript{19,20} Thus, an agent such as AOA, which significantly alters the kinetics of the calcium influx (see “Results”), could be hypothesized to retard calcification by reducing the potential rate of calcium phosphate formation.

Could AOA interfere with the initial nucleation of calcium phosphate deposits or prevent the long-term proliferation of calcification or both? The data in the present study demonstrate both inhibition of AOA in a short-term rat model (21 days) and longer-term sheep implants (5 months). Nevertheless, both types of animal models cannot be compared with clinical implants, which must be expected to function free of calcification for decades. The results of the present study do not shed any light as to the prognosis for the AOA-pretreated heart valve on a long-term basis. However, other related research concerning detergent inhibition of calcification may be helpful in this regard. It has been shown by Bosky et al\textsuperscript{21} that detergents interfere with nucleation by disrupting the calcium-phospholipid-phosphate complex necessary for initial calcium phosphate crystallization. Furthermore, detergents also interfere with crystal growth of preexisting calcium phosphate mineral.\textsuperscript{22} Thus, previous research as well as the present results indicate that AOA would hypothetically be effective for interfering with initial nucleation and for preventing calcification on a long-term basis.

Our investigation was focused on the anticalcification efficacy of AOA on BPHV using an AOA preparation (or identical solvent in control studies) currently under consideration for preparing bioprostheses for clinical use. The principal findings of this study were (1) tissue-bound AOA retarded the penetration of calcium ions through tissue; (2) a finite and significant amount of AOA remained bound to the tissue regardless of aggressive solvent extraction; (3) aortic wall was more resistant to AOA penetration than cusp; and (4) there was an anticalcification effect of AOA on aortic cusp but not aortic wall, as demonstrated by the sheep explant calcium analyses.

**Calcium Diffusion**

In our calcium diffusion studies, the presence of AOA on bioprosthetic tissue greatly reduced the immediate calcium ion influx. Therefore, covalently linked AOA could hypothetically retard the kinetics of initiating crystalline calcium phosphate formation in part by a similar mechanism. The low abundance of initial nucleation sites hinders further crystallization of calcium phosphate and thus the formation of hydroxyapatite. Moreover, the phospholipids (and thus its phosphate groups) in cell membranes also could serve as sites for attracting calcium ion, which could in turn result in initiating the process of calcification. AOA also could function as a surfactant to extract and perhaps exchange the phospholipid. This may thereby lessen the sites available for calcification.

**Stability of AOA Binding to Tissues**

The results of the extraction study demonstrated that a significant level of AOA remained bound despite harsh solvent conditions. Thus, the hypothesized amino-residual aldehyde bonding mechanism is supported by these results. Alternatively, AOA affinity (nonextractability) could be due to hydrogen bonding or nonspecific hydrophobic adsorption. Definitive studies on the possible chemical bonds occurring between AOA and aldehyde have not yet been carried out.

How can the actual covalent bonding of AOA to residual aldehyde be proven? Definitive amino aldehyde reaction studies have thus far only been possible in model systems using aqueous solutions of the compounds of interest.\textsuperscript{23–25} Cheung and Nimni\textsuperscript{23} have established the reactivity of glutaraldehyde with amino-containing model compounds and proteins. However, establishing covalent bonding with a cross-linked aortic valve leaflet, which is resistant to enzymatic digestion, remains a challenge to overcome; thus far, no study by our group or others has addressed this issue. A number of surface chemistry techniques have become available in the last decade, such as electron spectroscopy for chemical analyses and attenuated total-reflectance Fourier-transformed infrared spectroscopy. However, these techniques cannot be applied to hydrated biomaterials because desiccation of a tissue such as a heart valve leaflet would distort structural and surface relations as well as denature the protein matrix of interest. However, some of the questions concerning the covalent reactions of glutaraldehyde with AOA can be approached in model studies with purified chemical systems, and these should be the subject of future research.

Although the precise mechanism of AOA binding has not been fully elucidated in this study, a Schiff base covalent linkage is one likely possibility. A similar mechanism was proposed in a previous study in which aminopropanehydroxydiphosphonate (APD) was linked to residual aldehyde groups on either porcine\textsuperscript{26} or pericardial\textsuperscript{27} bioprosthetic tissue. Interestingly, APD covalent binding, although stable in vitro, was less stable in vivo. Furthermore, APD pretreatment resulted a dose-dependent inhibition of bioprosthetic calcification in subdermal studies but not in sheep mitral valve replacements,\textsuperscript{28} unlike AOA.\textsuperscript{8} Part of the previous subdermal study involved treating a control group of bioprosthetic cusps with lysine.\textsuperscript{27} Although lysine could be demonstrated to bind at higher levels than APD to bioprosthetic heart valve tissue, pretreatment with only lysine was not effective in inhibiting calcification of bioprosthetic tissue in subdermal implant studies. Thus, these prior studies demonstrate that simply reacting residual aldehydes in bioprosthetic tissue with amino-containing compounds is not sufficient for prevent BPHV calcification. Some other unique structural-functional relations must facilitate the efficacy of AOA in
circulatory studies compared with the other amino compounds investigated.

**AOA Diffusion**

Hypothetically, the amount of AOA interacting with residual aldehyde groups is determined by the concentration of AOA, the availability of free AOA molecules, and the extent of available free aldehyde groups. This phenomenon is in turn governed by the rate of AOA diffusing into tissue. Compared with aortic walls, the aortic valve cusp is relatively thin and composed of less dense collagenous tissue\(^{29}\) and thus AOA can apparently penetrate it easily. Therefore, AOA molecules and glutaraldehyde residuals react readily, and a high level of AOA per unit weight of tissue can be achieved in a relatively short period of time. In contrast, the greater thickness of aortic wall in conjunction with its denser and apparently less permeable composition for AOA may affect the amount of AOA bound.

**Differential Inhibition of Calcification of Cusps and Aortic Wall**

The results from the in vivo study indicated that AOA was effective in preventing calcification of cusp tissue but not aortic wall. Further examination of AOA diffusion results (ie, the lag times for AOA penetration) in the two types of tissues suggests a diffusion-based explanation. The longer lag time (>88 hours) required for AOA to diffuse at equilibrium conditions across the aortic wall compared with cusp tissue indicated that the duration of tissue AOA treatment appeared to be insufficient for achieving the desirable initial AOA level to resist the immediate calcium influx into aortic wall tissue soon after the implantation of a BPHV. Alternatively, AOA may not deter the pathophysiology of aortic wall mineralization (partially involving elastin calcification), which differs markedly from aortic cusp calcification, in which collagen rather than elastin calcification is very prominent.\(^{30}\)

Furthermore, the level of cuspal calcification observed in the non-AOA-pretreated explants (control) from the present left ventricular-to-descending aorta conduit model was comparable to that noted in mitral orthotopic explants from sheep\(^{28}\), as well as in previous studies of bovine left ventricular-to-descending aorta conduit.\(^{4}\) Thus, effective inhibition of calcification by AOA in both models was comparable in terms of the level of bulk mineral that would have been otherwise deposited.

**AOA Comparisons With Other Detergents**

Previous investigations by others have demonstrated a number of detergents to be efficacious for inhibiting calcification in BPHV implants either as orthotopic valve replacements or in left ventricular-to-descending aorta conduits. Of these other detergents, only polysorbate 80 pretreatment\(^{28}\) inhibited calcification at comparable levels to those reported in the present study. However, polysorbate 80 is not chemically bonded to bioprosthetic heart valve tissue, unlike AOA, and thus their similar efficacy in short-term animal implants, such as those described in the present study, may not reflect or predict comparable efficacy in long-term clinical implants.

A number of detergents found to be effective pretreatments for preventing BPHV calcification in animal model circulatory studies have been reported to cause deleterious effects on valve cusp integrity, such as delamination.\(^{28}\) Initial studies with AOA, while demonstrating efficacy for preventing calcification, also showed that the AOA pretreatment procedures caused damages to cuspal surface integrity.\(^{8}\) However, this effect has been eliminated by a filtration step in the proprietary preparation procedures used by Medtronic Heart Valve for eliminating particulate material from the AOA solutions, thus avoiding material degeneration.\(^{8,16}\) Morphology results related to these considerations may be found in these previous studies\(^{8,16}\) and were beyond the scope of the present experiments.

**Future Considerations**

The effectiveness of AOA also should be viewed with the perspective of other experimental anticalcification strategies. Pretreating bioprostheses with sodium dodecyl sulfate (SDS) in sheep circulatory implants,\(^{31}\) aluminum chloride and ferric chloride\(^{30}\) in rat subdermal studies, and diphosphonate compounds (eg, amino diphosphonate) in rat subdermal studies\(^{22}\) has proven to be efficacious. Interestingly, aortic wall calcification has not been assessed in prevention studies except for subdermal implant experiments in which Al\(^{3+}\) and Fe\(^{3+}\) ion and aminodiphosphonate all inhibited aortic wall calcification.\(^{22}\) In comparison to other detergents, the level of inhibition by AOA of cuspal calcification was either comparable or superior to the most optimal results reported previously,\(^{29}\) which were obtained using either SDS- or polysorbate 80-pretreated bioprosthetic heart valves as orthotopic mitral valve replacement in sheep.\(^{28}\) Furthermore, other detergents effective for preventing calcification such as n-lauryl-sarcosine or Triton X-100 resulted in primary material failure caused by destabilization of bioprosthetic tissue.\(^{28}\)

Thus, it is possible to speculate that a sequential application of optimal anticalcification agents such as AOA (and others) to treat BPHV before implantation could be a rational approach for compensation of limitation(s) of each agent, and this multiagent approach of BPHV treatment may result in a synergistic anticalcification effect for both aortic wall and valve.

**Conclusions**

AOA was demonstrated to be tightly associated to glutaraldehyde-pretreated BPHV tissue, possibly the result of an aldehyde-amino reaction, and AOA association stability was demonstrated despite various strenuous solvent extractions. AOA pretreatment diminished Ca\(^{2+}\) diffusion into bioprosthetic tissues. This may explain in part the anticalcification mechanism of AOA. Furthermore, sheep explant analyses demonstrated that AOA preincubation was an effective means for inhibiting the calcification of glutaraldehyde-pretreated porcine aortic valve cusps but not aortic wall.

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