Synergistic Inhibition of Calcification of Porcine Aortic Root with Preincubation in $FeCl_3$ and α-Amino Oleic Acid in a Rat Subdermal Model

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Abstract: Postimplant calcific degeneration is a frequent cause of clinical failure of glutaraldehyde crosslinked porcine aortic valve bioprostheses. We demonstrated previously in rat subdermal and circulatory implants that α-amino oleic acid used as a bioprosthesis pretreatment was highly effective in mitigating aortic valve cusp but not aortic wall calcification. In this study we investigated the feasibility of synergistically applying two proven anticalcification agents (α-amino oleic acid and $FeCl_3$) as pretreatments for mitigating both bioprosthetic cusp and aortic wall calcification. α-Amino oleic acid is hypothesized to prevent calcification by disrupting calcium phosphate formation kinetics, whereas suppression of alkaline phosphatase activity and ferric-phosphate complexation at cellular membrane initiation sites may be important factors in ferric ion's inhibition of calcification. *In vivo* implant studies (21-day rat subdermal model) indicated that individually $FeCl_3$ (0.01 or 0.1 *M* for 24 h) or α-amino oleic acid (saturated solution) treatments were equally effective in mitigating cuspal calcification (tissue calcium levels: 30.2 $\pm$ 10.2, 29.8 $\pm$ 2.7, and 31.6 $\pm$ 7.8 μg/mg tissue, respectively). However, sequential application of first α-amino oleic acid and then $FeCl_3$ synergistically reduced aortic wall calcification more effectively than either of the agents alone. The benefit of a synergistic application of two anticalcification treatments, α-amino oleic acid and $FeCl_3$, was demonstrated. However, the synergistic effect was observed on aortic wall only at a higher $FeCl_3$ concentration (i.e., 0.1 *M*). © 1997 John Wiley & Sons, Inc. J Biomed Mater Res (Appl Biomater) 38: 43–48, 1997

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INTRODUCTION

Calcific degeneration impairs the long-term clinical performance of bioprosthetic heart valves (BPHVs) fabricated from porcine aortic valves crosslinked with glutaraldehyde.[1] It was previously postulated that bioprosthetic calcification is a multifactorial process.[2] BPHV calcification occurs as an interaction of host, implant, and mechanical factors. Glutaraldehyde crosslinking is the most important implant factor—without this preparation step, subdermal implants of xenograft valve cusp materials do not calcify.[3] The most important host factor is young age, with immature subjects calcifying bioprosthetic implants more severely than mature subjects.[3] Therefore, immature animals are used in all experimental studies. Mechanical factors are of importance, because observations of circulatory implants indicate enhanced calcification at sites of greatest stress/strain phenomena.[3] Initial calcification occurs at the devitalized cell membranes, intrinsic to the glutaraldehyde crosslinked bioprosthetic tissue.[3,4] Following this, collagen calcification in aortic cusp and elastin calcification in aortic wall become progressively involved.[3,4] To date, anticalcification strategies have focused on prevention rather than therapeutic measures to arrest or cause regression of ongoing calcification. Although various strategies for counteracting this mineralization process have been investigated experimentally, no completely satisfactory method is currently available clinically for complete inhibition of BPHV calcification.

Recent studies using α-amino oleic acid pretreated bioprosthetic xenografts (either cusp or aortic wall) in rat

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subdermal implants,[5–7] sheep orthotopic mitral valve replacements,[8] and left ventricular apicoaortic shunts[9] demonstrated that α-amino oleic acid is effective in preventing calcification of cusp but not aortic wall. α-Amino oleic acid is hypothesized to bind to BPHV via its amino group reacting with residual glutaraldehyde (presumably forming a Schiff base or related compound[10–12]), thereby allowing α-amino oleic acid to remain in the tissue via chemical bonding. It was demonstrated previously that the presence of α-amino oleic acid on bioprosthetic tissues reduced the calcium ion diffusivity. Thus, calcium phosphate formation kinetics could be altered by this phenomenon.[7,9] Moreover, $FeCl_3$ preincubation was previously shown to be effective for inhibiting calcification of glutaraldehyde pretreated bovine pericardium in rat subdermal implants[13] and in a separate study to inhibit aortic wall calcification of glutaraldehyde pretreated rat aortic allograft circulatory implants.[14] Previous studies suggested that ferric–phosphate complexation at cellular membrane initiation sites could be a factor in the ferric ion mechanism of inhibition of calcification.[13] Alkaline phosphatase inhibition by $FeCl_3$ was also demonstrated to play a role in the anticalcification mechanism.[15]

The objective of the present study was to investigate the possibility of a synergistic use of α-amino oleic acid and $FeCl_3$ pretreatments for inhibiting calcification of both bioprosthetic cusp and aortic wall.

MATERIALS AND METHODS

Materials

Fresh and glutaraldehyde crosslinked porcine aortic valve tissues (both cusp and aortic wall prepared according to proprietary procedures approved for clinical use) were directly supplied by the Heart Valve Division of Medtronic Inc. (Irvine, CA). $FeCl_3$ was obtained from Aldrich Chemical Inc. (Milwaukee, WI). All other chemicals were reagent grade; distilled and deionized water was used.

α-Amino oleic acid was synthesized at Medtronic Inc. (Minneapolis, MN) and the α-amino oleic acid solution was provided to our laboratory as a saturated solution prepared under proprietary conditions also utilized to prepare clinically implantable heart valve bioprostheses.[16]

Treatment of Tissues (Cusp and Aortic Wall) with α-Amino Oleic Acid and $FeCl_3$

Glutaraldehyde fixed cusps were treated with a saturated α-amino oleic acid solution (approximately 1 mL α-amino oleic acid solution for 20 mg of wet tissue) for 72 h (at 37°C). Groups of α-amino oleic acid treated cusp tissues were subsequently incubated for 24 h with either 0.01 or 0.1 *M* $FeCl_3$ solutions at 37°C. Similarly, aortic wall samples were pretreated with the α-amino oleic acid solution and the $FeCl_3$ solutions in sequence. Two additional groups of tissues (not exposed to α-amino oleic acid) were similarly treated with 0.01 or 0.1 *M* $FeCl_3$ solutions.

TABLE I. Anticalcification Efficacy of α-Amino Oleic Acid and $FeCl_3$ on Cusp Tissue

Treatment	Calcium (μg/mg of Tissue) ± SEM
Control (no treatment)	163.4 ± 14.5
α-Amino oleic acid	31.6 ± 7.8*
α-Amino oleic acid/$FeCl_3$ (0.01 *M*)	59.8 ± 11.7*
$FeCl_3$ (0.01 *M*)	30.2 ± 10.2*
α-Amino oleic acid/$FeCl_3$ (0.1 *M*)	28.5 ± 2.4*
$FeCl_3$ (0.1 *M*)	29.8 ± 2.7

Data represent the average of 10 replicates ± SEM.
* $p < 0.01$ vs. control (see Results).

Efficacy of α-Amino Oleic Acid/Ferric Ion for Inhibiting Glutaraldehyde Pretreated Porcine Aortic Cusp and Aortic Wall Calcification in Rat Subdermal Model

Rat subdermal implants[17,18] were used to assess the anticalcification efficacy of α-amino oleic acid, α-amino oleic acid/$FeCl_3$, and $FeCl_3$ on both cusp and aortic wall. Tissues were implanted into male weaning rats (3-week-old, 50–60 g, Sprague-Dawley; Charles River Laboratories, Burlington, MA), anesthetized with a mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL), in subdermal pouches dissected in the ventral abdominal wall as described previously.[18] Each animal received two separate subdermal implants. After 21 days, the rats were euthanized by carbon dioxide inhalation and the tissue specimens were retrieved. All tissue specimens were rinsed with deionized water and lyophilized. Representative samples fixed with a combined glutaraldehyde–formaldehyde fixative[19] solution were processed for light microscopy using glycomethacrylate embedding medium and staining with hematoxylin and eosin (for overall morphology) and von Kossa's reagent (for calcium phosphate) according to previously described methods.[20] Acid hydrolysates of specimens were subjected to calcium analysis by atomic absorption spectroscopy using previously described procedures.[18,20]

Data and Statistical Analyses

Replicate data were calculated and expressed as mean ± standard error of the mean (SEM). Unpaired *t* tests were used to assess the significance of statistical differences between experimental groups and controls. Results were termed significant when the *p* value was less than 0.05.

RESULTS

The extent of inhibition of calcification on α-amino oleic acid/$FeCl_3$ treated cusp and aortic wall with respect to the concentration of $FeCl_3$ for tissue treatment, after 3 weeks of implantation, is illustrated in Tables I and II. Sequential application of the two agents did not enhance calcification

TABLE II. Anticalcification Efficacy of α-Amino Oleic Acid and $FeCl_3$ on Aortic Wall Tissue

Treatment	Aortic Wall Calcium (μg/mg of Tissue) ± SEM
Control (no treatment)	97.3 ± 10.5
α-Amino oleic acid	56.9 ± 2.7*
α-Amino oleic acid/$FeCl_3$ (0.01 *M*)	27.2 ± 9.0*
$FeCl_3$ (0.01 *M*)	129.2 ± 15.3
α-Amino oleic acid/$FeCl_3$ (0.1 *M*)	5.6 ± 1.5*
$FeCl_3$ (0.1 *M*)	19.1 ± 4.1*

Data represent the average of 10 replicates ± SEM.
* $p < 0.01$ vs. control (see Results).

inhibition of the cusp but it was efficacious in the aortic wall, especially when the concentration of $FeCl_3$ being used was at 0.1 *M*. Furthermore, the animals with various treated leaflets had body weights at termination that did not differ from the control, indicating a lack of adverse effects on overall growth.

Cuspal Effects

The results of the rat subdermal implant study indicated that $FeCl_3$ (at concentrations of 0.01 and 0.1 *M*) had an anticalcification effect on cusps (see Table I) and was at least as effective as that of α-amino oleic acid when applied alone ($p > 0.05$ as compared to control). At a concentration of 0.1 *M*, $FeCl_3$ alone produced the same anticalcification efficacy on cusps as applying α-amino oleic acid and $FeCl_3$ (0.1 *M*) in sequence ($p > 0.05$).

Aortic Wall Effects

Treatment with 0.01 *M* $FeCl_3$ appeared to slightly exacerbate aortic wall calcification as compared to control (Table II). However, the difference was statistically insignificant ($p > 0.2$). The sequential application of first α-amino oleic acid and then $FeCl_3$ (0.01 *M*) significantly reduced the calcification of aortic wall as compared to control ($p = 0.00006$). The 0.1 *M* $FeCl_3$ was also very effective in reducing aortic wall calcification, whereas α-amino oleic acid alone was significantly less effective ($p > 0.05$). Nevertheless, the sequential application of both α-amino oleic acid and $FeCl_3$ (0.1 *M*) significantly decreased aortic wall calcification to approximately one-third of the level noted with treatment with $FeCl_3$ (0.1 *M*) alone ($p = 0.0009$); synergism was clearly demonstrated.

Microscopy

The morphologic results were in agreement with the chemical measurements of calcification for the various groups (Fig. 1). Calcific deposits in cuspal tissue not treated with α-amino oleic acid or $FeCl_3$ were punctate to confluent. Calcification was markedly and equivalently reduced by pretreatment with α-amino oleic acid alone, by pretreatment with $FeCl_3$ alone (either 0.01 or 0.1 *M*), or with the combination α-amino oleic acid/$FeCl_3$ (0.1 *M*). Pretreatment with α-amino oleic acid/$FeCl_3$ (0.01 *M*) seemed to yield more calcification than α-amino oleic acid alone.

For aortic wall, the control (untreated) tissue calcified predominantly as bands of mineral near the intimal and the adventitial surfaces, characteristic of aortic wall implanted subcutaneously (Fig. 1). Although treatment with $FeCl_3$ (0.01 *M*) had little effect on the calcification morphology, treatment with $FeCl_3$ (0.1 *M*) or a combination α-amino oleic acid/$FeCl_3$ (0.01 or 0.1 *M*) markedly reduced aortic wall calcification. In aortic wall segments, as with the cusps, the least calcification was noted with the combination α-amino oleic acid/$FeCl_3$ (0.1 *M*). No inflammatory or other untoward effects on cusp or aortic wall were noted in the treated groups.

DISCUSSION

The long-term clinical use of BPHVs manufactured from glutaraldehyde crosslinked porcine aortic valve and bovine pericardial tissues is limited by calcific degeneration.[1] Some experimental strategies have been proven effective for mitigating calcification of cusps.[3,8,21] However, many of the strategies devised for preventing cuspal calcification have failed to effectively inhibit aortic wall calcification,[5] a potentially important problem with stentless valves.

The focus of this investigation was to establish the feasibility of a synergistic application of two different agents (i.e., α-amino oleic acid and $FeCl_3$), each with proven anticalcification efficacy on aortic cusp and aortic wall. The principal findings of this study were that additional use of $FeCl_3$ preincubations did not enhance the anticalcification effectiveness of α-amino oleic acid on the cusp, yet sequential application of α-amino oleic acid and $FeCl_3$ enhanced the anticalcification effectiveness of either agent applied alone to the aortic wall.

As described above, α-amino oleic acid is in part covalently bound to BPHV tissue, both cusp and aortic wall, presumably through an amino–aldehyde reaction. The mechanism of α-amino oleic acid inhibition of calcification is incompletely understood. Detergent pretreatments of BPHVs have been shown in a number of studies to inhibit calcification.[21] The mechanism of this effect is presumably due to surfactant extraction of phospholipids and other components mediating the calcification mechanism.[18,22] However, preliminary studies by our group indicate relatively little phospholipid extraction by α-amino oleic acid, thereby suggesting that the usual detergent mechanism for inhibiting calcification is not operative in the case of α-amino oleic acid. Other research by our group demonstrated pretreatment with α-amino oleic acid results in a significant diminution of the diffusivity of calcium through BPHV

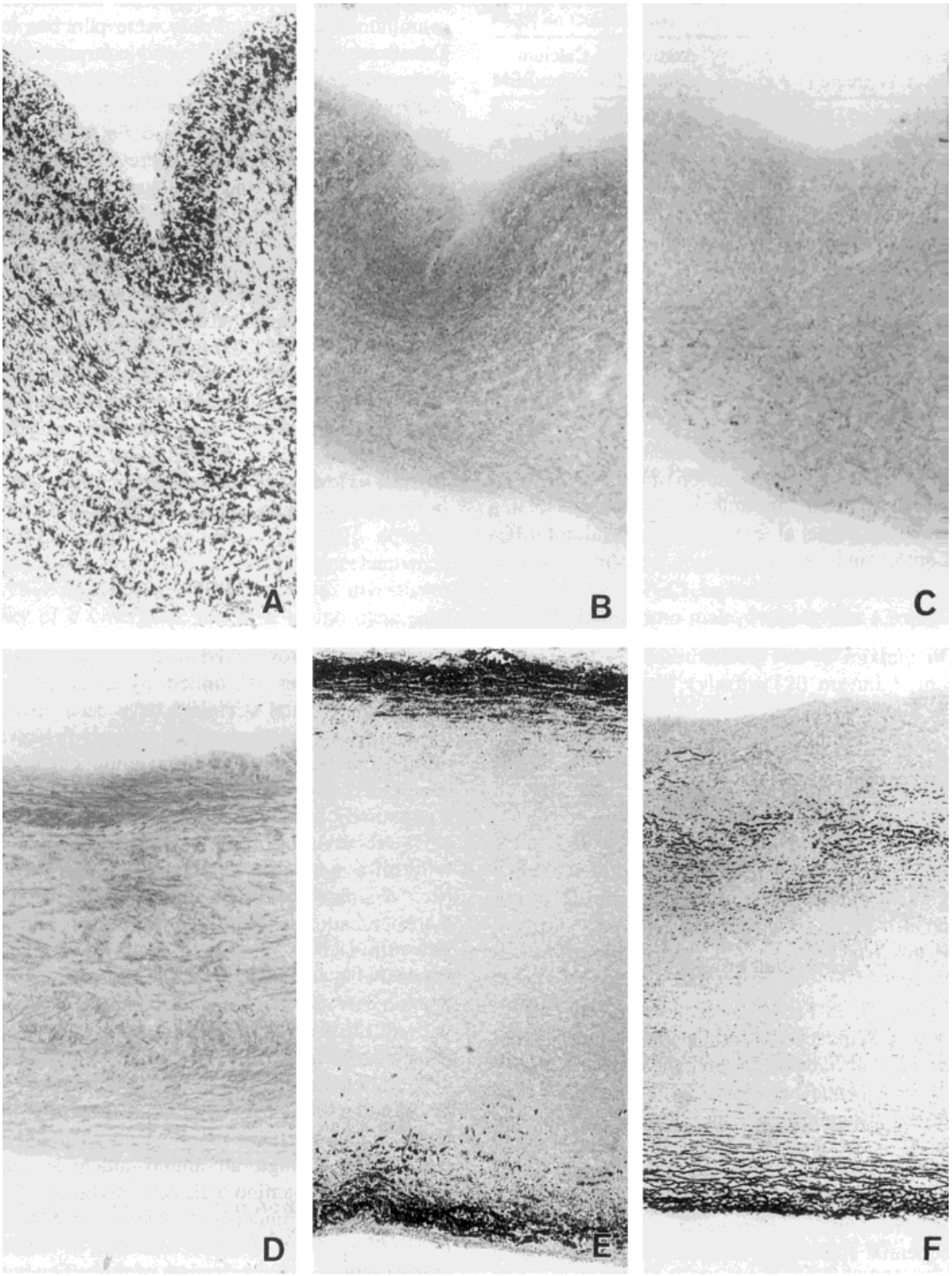

Figure 1. Morphology: (A)–(D) cusp, (E)–(H) aortic wall; (A) untreated, (B) α-amino oleic acid treated, (C) $FeCl_3$ (0.1 *M*) treated, and (D) combination α-amino oleic acid/$FeCl_3$ (0.1 *M*). (E) Untreated, (F) α-amino oleic acid treated, (G) $FeCl_3$ (0.1 *M*) treated, and (H) combination α-amino oleic acid/$FeCl_3$ (0.1 *M*). (H) This sample appears to have more calcification than (G). All stained with von Kossa's reagent (calcium phosphate black). Magnifications: (A)–(D) $\times$70 and (E)–(H) $\times$35.

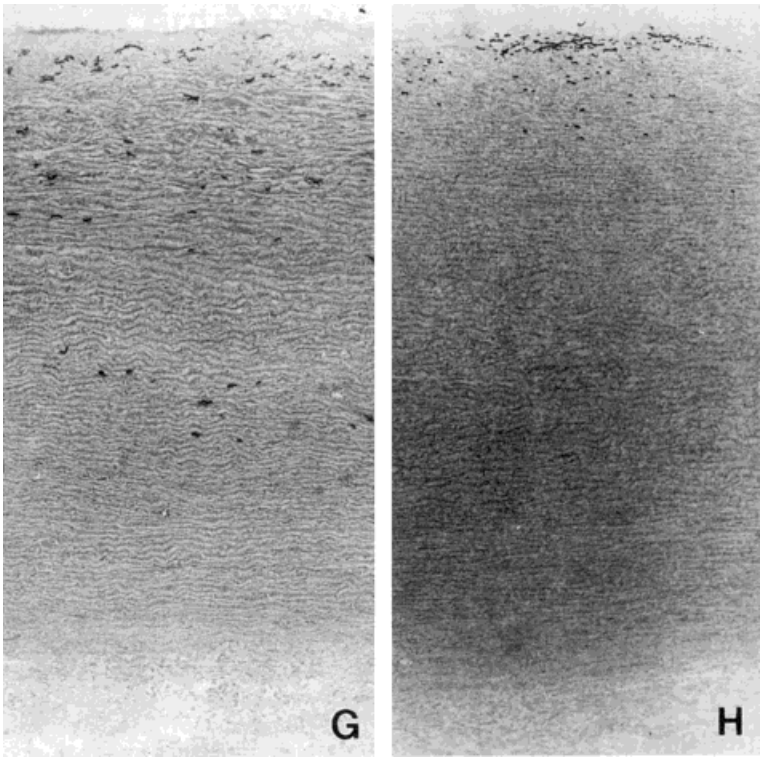

Figure 1. (continued)

leaflets. Thus, calcium phosphate formation kinetics may be significantly altered by this phenomenon, and this may explain the efficacy of α-amino oleic acid.

However, the mechanism of $FeCl_3$ inhibition of calcification would at least appear to be distinct from that of α-amino oleic acid. $FeCl_3$ pretreatment of glutaraldehyde crosslinked bovine pericardium, and in separate studies of aortic wall, was demonstrated to result in the inhibition of calcification of these tissues in subdermal implants (pericardium) and circulatory aortic allografts in rats.[14] $FeCl_3$ inhibition of bioprosthetic calcification is incompletely understood. Evidence from previous studies suggests that ferric–phosphate complexation at cellular membrane initiation sites may be an important factor in the ferric ion mechanism of inhibition of calcification. In addition, $FeCl_3$ pretreatment of pericardial bioprosthetic subdermal implants inhibits the alkaline phosphatase activity usually associated with the initiation and progression of calcification.[11] Studies of calcium phosphate crystallization indicate that ferric chloride is a potent inhibitor of calcium phosphate crystal growth.[23] Furthermore, ferric salts exposure was implicated in renal osteodystrophy, suggesting another avenue for interaction and interference with calcium phosphate formation.[24] It was proposed by Urry and Long that the calcium ion has high affinity toward elastin (a major component of aortic wall).[25,26] The coordination of calcium ion with elastin raises the hypothetical possibility that the attraction of phosphate ions to complexed calcium eventually results in elastin calcification. Ferric ion could inhibit the growth of calcium phosphate crystals on the elastin of aortic wall, thereby reducing calcification.[25,26]

α-Amino oleic acid and $FeCl_3$ synergism could occur due to the distinct separate actions of each of these agents, thereby combining to achieve a relatively greater inhibition of calcification. However, in our studies the synergy was only apparent for aortic wall calcification. Therefore, this potent combination of α-amino oleic acid and $FeCl_3$ may be most useful for the unique elastin oriented calcification characteristic of aortic wall mineral deposition. Although aortic wall calcification is not clinically significant in stented BPHV implants, it is potentially of importance in the mineralization of stentless valves because aortic wall calcific deposits could become large enough to mechanically impinge on the lumen of the aortic wall segment that is normally part of a stentless valve conduit implant. This phenomenon has not been observed as yet in the clinical use of stentless valves. However, aortic wall calcification has been reported to be a major cause of obstruction of aortic valve allografts in several clinical studies.[21] Thus, α-amino oleic acid–$FeCl_3$ synergy may be of relevance in avoiding this problem for the use of stentless valves. It should also be noted that previous work from our laboratory demonstrated that 21-day rat subdermal bioprosthetic leaflet implants result in calcium levels identical to those noted in failed clinical BPHVs. Although these same data are not as widely available for the aortic wall, a recent publication from our group indicates a similar result, that is, the level of aortic wall calcium in our 21-day subdermal

implants as comparable or greater to that noted in aortic wall in failed stent-mounted valves.[28] Thus, although longer term implants could be more revealing in terms of unmasking breakthrough calcification, the present study supports the view that inhibition of calcification comparable to that noted in clinical bioprosthesis failure is indeed possible with this approach.

CONCLUSION

The benefit of a synergistic application of two anticalcification agents, α-amino oleic acid and $FeCl_3$, was demonstrated in this study. However, the synergistic anticalcification effect was only observed on aortic wall (not cusp) and at a higher $FeCl_3$ concentration (i.e., 0.1 *M*). This approach could enhance α-amino oleic acid inhibition of aortic wall calcification, a prominent complication with stentless porcine BPHVs and aortic valve allografts.

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