Calcification of Bovine Pericardium Used in Cardiac Valve Bioprostheses

Implications for the Mechanisms of Bioprosthetic Tissue Mineralization

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Calcification of bioprosthetic heart valves fabricated from glutaraldehyde-pretreated bovine pericardium has not been investigated. The objectives of this study were to characterize pericardium before and after glutaraldehyde pretreatment and to study the pathophysiology of mineralization of glutaraldehyde-preserved pericardium. Pericardial protein was approximately 90% collagen, predominantly Type I. Glutaraldehyde incorporation was complete following 24 hours' incubation (151×10^{-9} mol/mg). Bovine pericardium pretreated in buffered 0.6% glutaraldehyde, implanted subcutaneously in young rats for 24 hours to 112 days, was analyzed chemically (calcium and phosphorus) and morphologically. Mineralization, detected at 48 hours' implantation, was initially associated with pericardial connective tissue cells and later also collagen. Mean calcium content was 114 μg/mg at 21 days and 199 μg/mg at 112 days. The morphologic features and the kinetics and degree of mineral accumulation in glutaraldehyde-pretreated bovine pericardium were strikingly similar to those previously determined for porcine aortic valve. These results predict that calcification will critically limit the late durability of clinical pericardial bioprostheses and suggest generalized mechanisms of bioprosthetic tissue mineralization which are probably dependent on modification of implant microstructure by glutaraldehyde pretreatment. (Am J Pathol 1986, 123:134–145)

BIOPROSTHETIC heart valves fabricated from glutaraldehyde-pretreated porcine aortic valves or bovine pericardium are commonly used to replace diseased human cardiac valves.[1,2] Primary tissue degeneration due to intrinsic cuspal calcification is the most frequent cause of clinical failures of porcine aortic valve bioprostheses, necessitating reoperation or causing death in 20–25% of adult recipients within 7–10 years postoperatively[2–5] and substantially more frequently in children.[6] Bioprosthetic heart valves fabricated from bovine pericardium, introduced into general clinical practice in 1976, are now widely used.[7] Although failures related to calcification are now beginning to be reported,[8–10] it is yet unclear to what extent degenerative mechanisms, especially calcification, will ultimately limit the long-term durability of these bioprostheses. Furthermore, and importantly, it is unknown whether the mechanisms of mineralization of glutaraldehyde-pretreated bovine pericardium and porcine aortic valve are similar.

Specific implant and host factors regulating the calcification of glutaraldehyde-preserved bioprosthetic tissue have been clarified,[11–13] but underlying mechanisms of such mineralization are essentially unknown. The present study initially characterized pericardial tissue with respect to kinetics of glutaraldehyde incorporation, collagen content and predominant collagen type, and changes in amino acid composition resulting from glutaraldehyde pretreatment and implantation. Subsequent studies were designed to identify early events of pathologic calcification and follow the temporal progression of mineralization in glutaraldehyde-pre-

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treated pericardium implanted subcutaneously in rats. The hypothesis was tested that the kinetics and pathology of calcification of this material would be equivalent to those previously determined for porcine aortic valve in this model.[11,12] This study found that despite differences in infrastructure of porcine aortic valve and bovine pericardium, the biochemical, kinetic, and morphologic features of mineralization of these materials were virtually identical. The findings suggest not only that clinical bovine pericardial bioprostheses, like porcine aortic valves, are likely to fail frequently through calcification, but also that there are shared underlying mechanisms of mineralization of bioprosthetic tissues which depend on specific modification of constituent cells and collagen by glutaraldehyde.

Materials and Methods

Pericardium

Parietal pericardium from 2–3-week-old calves was immediately placed in 0.6% HEPES-buffered glutaraldehyde (pH 7.4) and transferred after 24 hours to 0.2% glutaraldehyde in the same buffer for storage at 2 C. Superficial fat was dissected from the external surface. Phosphate-buffered 0.6% glutaraldehyde-treated adult bovine pericardium was received from Shiley, Inc. (Santa Ana, Cal). These pretreatments are similar to those used in the fabrication of clinical valves. A previous study demonstrated equivalence of calcification in HEPES and phosphate-buffered glutaraldehyde-treated porcine aortic valve.[13] The remainder of the experimental protocol, including extensive preimplantation washing of tissue, was identical to that used in previous studies.[11,12] For biochemical studies, pericardium from the same source was treated as described below.

Previously described nomenclature for the structure of bovine pericardium will be followed in this paper.[14–16] Bovine parietal pericardium has three layers: 1) the serosa, or mesothelial cell layer closest to the heart; 2) the fibrosa, formed by diversely oriented, wavy bundles of collagen and by elastic fibers, and 3) the epipericardial connective tissue layer, which is partly continuous with the pericardiosternal ligaments.

Collagen Characterization Procedures

Collagen was extracted from freeze-dried pericardium suspended in 0.1 M acetic acid (Baker, Phillipsburg, NJ) with 1 mg/ml pepsin (Sigma, St. Louis, Mo) at a concentration of 10 mg/ml for 48 hours at 2 C.[17] The suspension was centrifuged at 10,000*g* for 60 minutes, and the supernatant and pellet were freeze-dried for amino acid analyses and electrophoresis procedures. Type I collagen, purified from tail tendon from 6-week-old rats, was used as a standard.[18]

Complete amino acid analyses were done on 6 M HCl hydrolysates of specimens of freeze-dried pericardium and collagenous extracts,[19] with the use of a specially programmed Beckman-Spinco 121 M automated amino acid analyzer (Beckman Instruments, Berkeley, Calif). Sensitivity was 0.5 nM, with reproducibility to within <5.0% on triplicate samples.

Electrophoretic procedures utilized a modification of the method of Laemmli[20] with a 3% acrylamide stacking gel and a 7% acrylamide running gel. Electrophoreses with sodium dodecyl sulfate were carried out on nonreduced and dithiothreitol (0.1 M) reduced samples.[17] Ten percent trichloroacetic-acid–fixed gels were rinsed with 10% acetic acid, stained with 0.25% Coomassie blue in 10% methanol–10% acetic acid, and destained in 10% methanol–10% acetic acid.

Glutaraldehyde Incorporation Studies

Fresh pericardium was rinsed free of blood with normal saline and then incubated in tritium-labeled (specific activity 183 cpm/μmol) 0.2% glutaraldehyde (New England Nuclear, Billerica, Mass) at pH 7.4 in 0.05 M HEPES, 0.9% NaCl, at 2 C. After incubations for 1, 2.5, 6, 12, 17, 24, 48, 72, and 192 hours, cusps were rinsed free of glutaraldehyde with HEPES buffer (as above), and the glutaraldehyde reaction products were stabilized by incubation for 16 hours in 0.01 M $NaBH_4$ in the same buffer.[21] Specimens were subsequently exhaustively washed free of radioactivity until background levels were obtained on the washings. They were then freeze-dried, and the lyophilized material (dry weight approximately 20 mg) was incubated in 1.0 ml of Protosol (New England Nuclear) for 24 hours at 50 C. The clear digests were then combined with 10.0 ml of Econofluor (New England Nuclear) and counted for tritium radioactivity in an Intertechnique SL32 Liquid Scintillation counter (IN/US Service Corp., Fairfield, NJ), and glutaraldehyde content was calculated from a series of identically quenched specimens. Glutaraldehyde incorporation is expressed in micromoles (ie, 10^{-9} moles) per milligram dry weight.

Subcutaneous Implantation in Rats

Sprague–Dawley rats (CD strain, Charles River Laboratories, Burlington, Mass) were used at age 3 weeks (60–80 g). The rats were fed Lab Chow (Ralston-Purina, St. Louis, Mo). Ether-anesthetized animals each received two 1.5-cm squares of bovine pericardium in separate subcutaneous pouches separated by at least 2 cm dissected in the ventral abdominal wall. For each

Table 1—Amino Acid Analyses of Pericardium*

Amino acid	Fresh	Glutaraldehyde-pretreated	Implanted and calcified†
Hydroxyproline	96	91	68
Proline	114	115	102
Glycine	257	334	295
Alanine	117	114	114
Lysine	31	8	15
Hydroxylysine	10	4	4

* Residues/1000 amino acids.
† Twenty-one-day implant.

time interval, 24, 48, and 72 hours, and 7, 14, 21, 28, 56, and 84 days, 10 specimens of calf pericardium were removed; at 112 days, 6 specimens were taken. Because most clinically used valves are fabricated from adult pericardium, adult bovine pericardium implanted for 21 days was also studied (12 specimens), so that we could ensure that pathologic features of mineralization were not dependent on the age of the source. Unimplanted glutaraldehyde-pretreated material and portions of all explants were chemically assayed for mineral content and examined by light microscopy. Representative specimens implanted for 48 and 72 hours (calf pericardium) and 21 days (both calf and cow pericardium) were also examined by transmission electron microscopy.

Mineral Analyses

Retrieved pericardial tissue was rinsed in sterile saline, washed free of salts with distilled water, lyophilized, pulverized to a fine powder, and dried to constant weight in a desiccator oven. Mineral analyses of 6 N HCl hydrolysates consisted of tissue calcium (Ca) determined by atomic absorption spectroscopy[22,23] and tissue phosphorus (P) assayed according to the methodology of Chen.[24] Elemental concentrations are expressed throughout as micrograms per milligram dry tissue weight (mean ± standard error of the mean [SEM]).

Morphologic Analyses

Samples removed for morphologic evaluation were fixed immediately in 0.1 M cacodylate-buffered 2.5% glutaraldehyde, 2% paraformaldehyde, pH 7.2,[25] dehydrated following 24 hours of fixation, and stored in 100% ethanol. Specimens for light microscopy were embedded in JB-4 glycolmethecrylate medium (Polysciences, Inc., Warrington, Pa), sectioned to 2–3 μ, and stained with hematoxylin and eosin (H&E) (for overall morphology) and with von Kossa stain (for calcium phosphates).

Representative specimens fixed, dehydrated, and stored as above were rehydrated, postfixed in OsO_4, dehydrated, and embedded in Poly/Bed 812 medium (Polysciences). Ultrathin sections unstained and stained with uranyl acetate and lead citrate were examined with a JEOL JSM-100 transmission electron microscope (JEOL, Nutley, NJ).

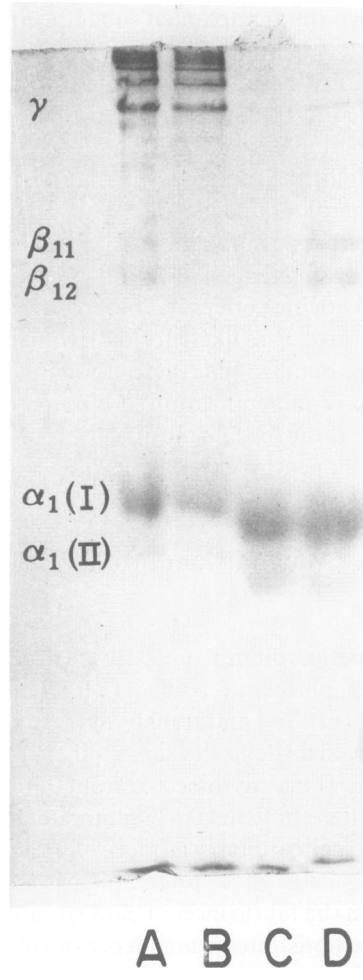

Figure 1—Polyacrylamide (5%) sodium dodecylsulfate electrophoretic gels of pericardial collagen (acetic acid–pepsin solublized; see Materials and Methods). **A**—Pericardial pepsin digest (2 mg/ml), nonreduced. **B**—Pericardial pepsin digest, reduced with dithiothreitol. **C**—Type I collagen (rat tail tendon), nonreduced. **D**—Type I collagen as in **C**, reduced with dithiothreitol. Stained with Coomassie blue.

Results

Biochemical Characterization of and Glutaraldehyde Incorporation by Pericardium

Complete amino analyses revealed unimplanted pericardium to contain approximately 90% collagen on the basis of the hydroxyproline content, hydroxylysine content, and the high ratio of hydroxyproline to hydroxylysine[17] (Table 1). Calcified pericardium had markedly lower hydroxyproline content, compared with

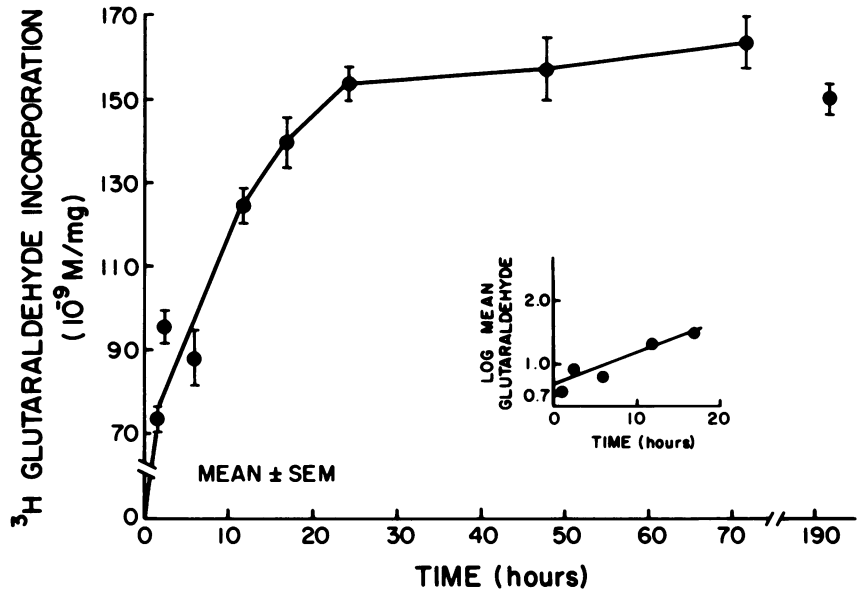

Figure 2—Kinetics of incorporation of tritrium-labeled glutaraldehyde into bovine pericardium. Nearly half is reacted within 1 hour, and the maximum level (approximately 150 × 10^{-9} mol/mg) is reached by 24 hours. Initial incorporation follows first order kinetics.

untreated and glutaraldehyde-preserved pericardium, which indicated reduced collagen of calcified and implanted pericardium, presumably due to absorbed protein and collagen breakdown. The relative diminution of lysine and hydroxylysine noted after glutaraldehyde pretreatment (Table 1) reflects the presence of acid-stable compounds resulting from lysyl amino-glutaraldehyde reactions.

Polyacrylamide gel electrophoresis demonstrated Type I collagen predominance in the digest of the non-glutaraldehyde-pretreated pericardium. This is evident from the characteristic band pattern, compared with a Type I rat tail tendon collagen standard, and the lack of alpha chain intensification upon reduction,[17] indicating the presence of little if any Type III collagen (Figure 1).

During 1 hour of incubation, 73.6 nmol/mg of glutaraldehyde were incorporated, representing half of the maximal incorporation (151 nmol/mg), obtained after 24 hours of incubation. Comparable levels of glutaraldehyde were present in specimens examined thereafter (Figure 2). Furthermore, a linear relationship between the logarithm of initial glutaraldehyde concentration and incubation time indicates first-order reaction kinetics (inset, Figure 2). This suggests that one aldehyde group reacts with one amino function.

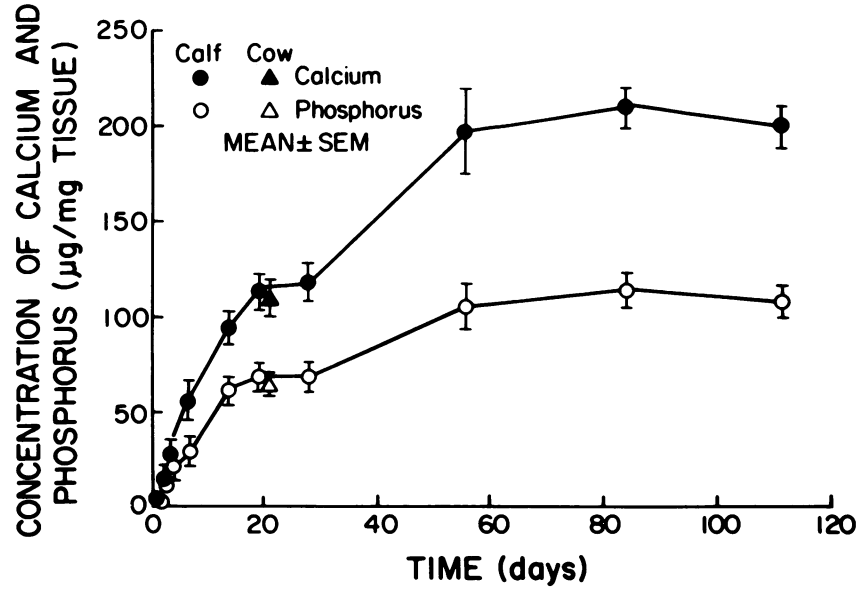

Figure 3—Kinetics of mineral deposition in glutaraldehyde-pretreated bovine pericardium subcutaneously implanted in rats. Calcium and phosphorus accumulation is initially noted after 48 hours' implantation and reaches maximum by 56 days. The maximum level of calcium is approximately 200–220 µg/mg tissue.

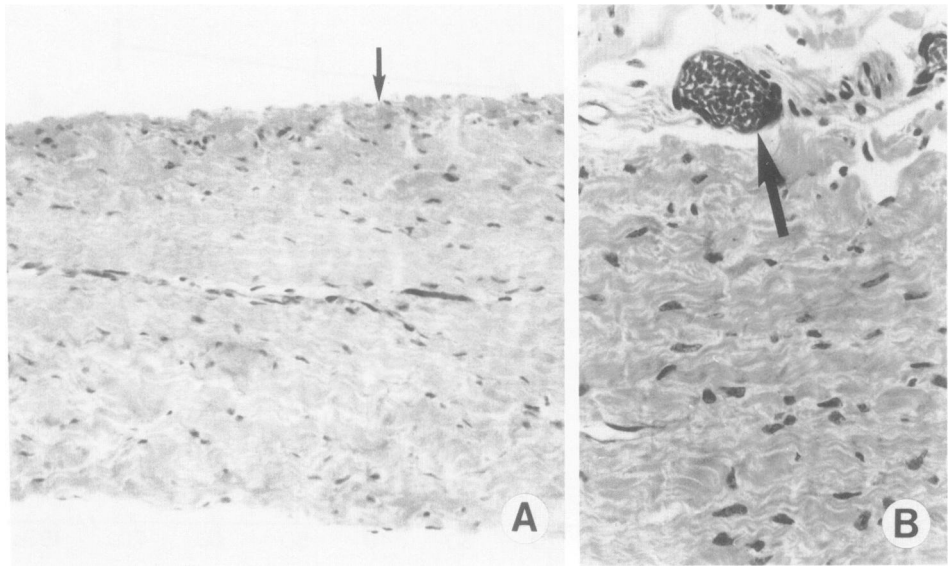

Figure 4—Histologic features of unimplanted but glutaraldehyde-pretreated bovine pericardium. **A**—Complete cross-section. The serosal surface is denoted by the *arrow*. **B**—Representative area of pericardium demonstrating a prominent blood vessel (*arrow*) near the serosal surface. (H&E; **A**, ×180; **B**, ×300)

Mineral Analyses

The kinetics of mineralization of subcutaneously implanted glutaraldehyde-pretreated bovine pericardium are illustrated in Figure 3. Mineral was undetectable, either chemically (Ca, 1.2 ± 0.1 μg/mg; P 1.4 ± 0.1 μg/mg) or morphologically, in glutaraldehyde-treated but unimplanted tissue. Although mineral content was not increased after 24 hours' implantation, significant accumulation of calcium and phosphorus was noted after 48 hours' implantation (Ca, 12 ± 1; P, 9 ± 1); mineral content increased progressively over the following 8 weeks. At 21 days, calcium and phosphorus content (114 ± 8 and 67 ± 4, respectively) were approximately half the maximum levels, attained by 84 days (221 ± 8 and 128 ± 6, respectively). Neither calcium nor phosphorus content changed between 84 and 112 days, which suggested saturation at approximately 200–220 μg calcium/mg tissue. For purpose of comparison, cusps of clinically failed porcine aortic bioprostheses have 202–234 μg/mg calcium.[2,6] Mineral content of adult pericardium after 21 days implantation (Ca, 110 ± 7; P, 64 ± 0) was equivalent to that of similarly treated calf pericardial implants.

Morphology

The morphology of unimplanted pericardium is illustrated in Figure 4. Deposits of calcium phosphate were initially noted by light and electron microscopy in specimens harvested 48 hours after implantation; mineral deposits were not observed in specimens implanted for 24 hours. Light-microscopic evidence of mineralization increased progressively over the duration of the experiment (Figure 5). Calf and adult pericardial implants had similar morphologic findings following 21 days' implantation (compare Figures 5E and F).

Light microscopy revealed that early calcific deposits were small and punctate (less than the size of connective tissue cells) and usually diffusely and randomly distributed within the tissue, although there was in many specimens a relatively preferred plane of deposition of mineral parallel to the implant surface in the pericardial fibrosa near the serosal layer parallel to the implant surface. Cells lining small pericardial blood vessels were frequently among early mineralization sites (Figure 5B and C). The number of calcified sites and the size of individual deposits increased with time; furthermore, there was progressive confluence of individual calcific deposits to nodules (at least approximately 50 μ in major dimension), ultimately destructive to the tissue (Figure 5G). The frequency of nodular calcific lesions increased with time; they were noted in 0/10 implants removed after 48 hours, 1/10 at 72 hours, 7/10 at 7 days, 10/10 at 14 days, and uniformly thereafter. Mineral nodules often ablated tissue architecture, ulcerating through the implant surface in some specimens as early as 14 days. Small mineral deposits and gross nodules stained intensely with the von Kossa stain.

The earliest mineral deposits were accommodated in the pericardial tissue without distortion, but later deposits caused expansion and thickening. Cuspal thickening with increasing duration of implantation is noted qualitatively in Figure 5 and quantitatively in Figure 6. From implantation to 112 days, the thickness of the tissue approximately doubled, from approximately 200 to 400 μ (Figure 6A). Additional causes for this

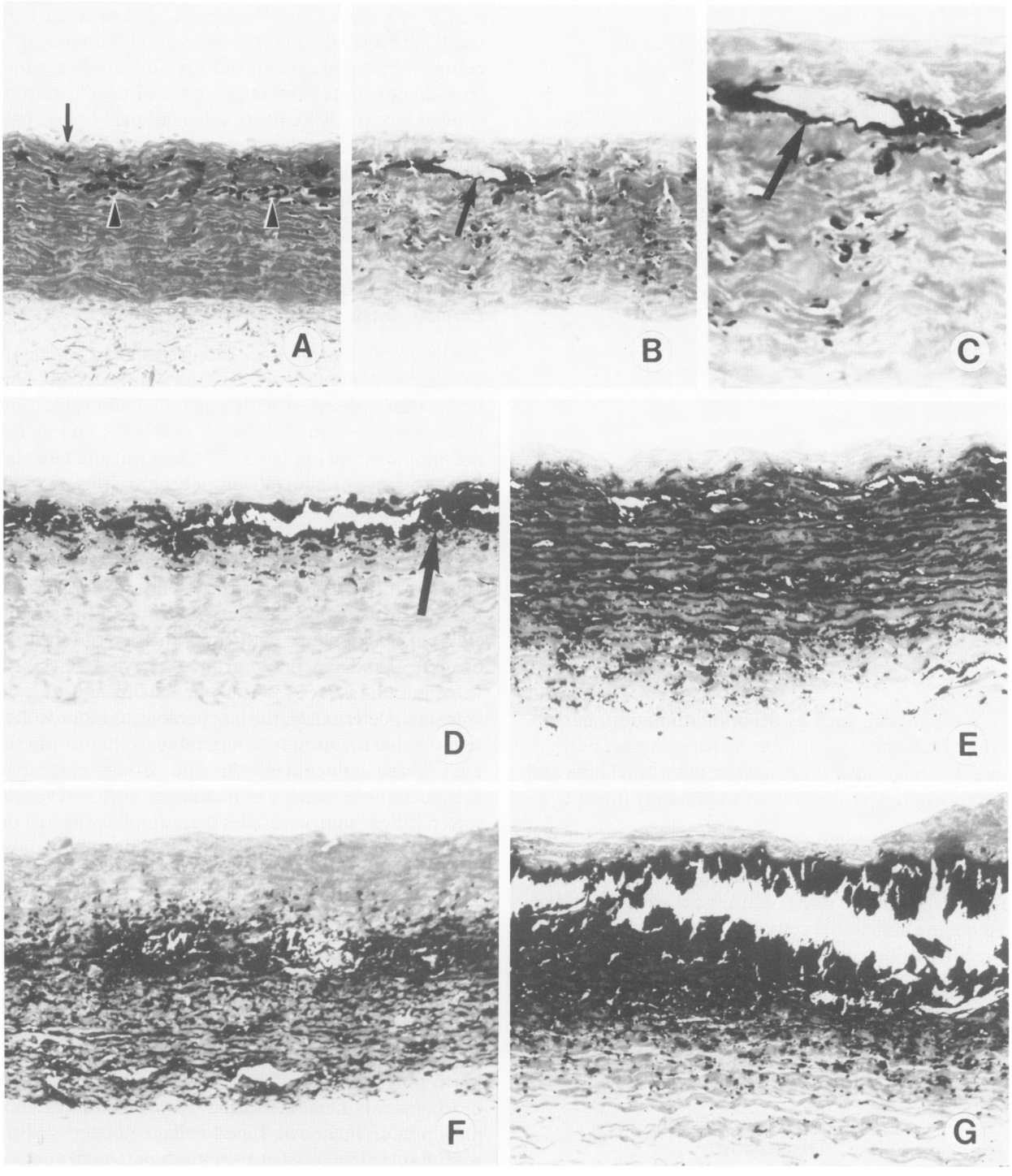

Figure 5—Onset and histologic evolution of calcium accumulation in glutaraldehyde-pretreated bovine pericardium. **A**—Implanted 48 hours. Multifocal calcific deposits (*black*) are noted, in an apparently planar configuration (*arrowheads*) near the serosal surface (*arrow*). **B** and **C**—Implanted 72 hours. Calcified blood vessel wall is denoted by the *arrow*. **D**—Implanted 7 days. Early formation of nodules by confluence of microdeposits is apparent (*arrow*). **E** and **F**—Calf pericardium and adult bovine pericardium, respectively, implanted for 21 days. Their patterns of calcification are similar. **G**—Implanted 56 days. This specimen demonstrates calcific nodules highly destructive to the tissue. Note the progressive thickening of the tissue with increasing time of implantation and accumulation of calcific deposits. In all cases the serosal surface is up. All sections above stained using von Kossa's reagent (calcium phosphate is *black*). (**A**, **B**, and **D**–**G**, ×185; **C**, ×375)

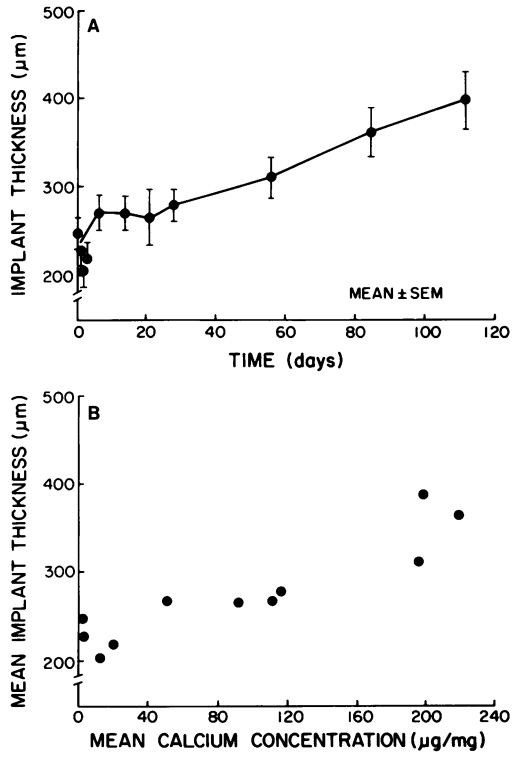

Figure 6—Progressive thickening of bovine pericardium with time (**A**) and calcification (**B**). Over the 112 days of the experiment the cross-sectional thickness approximately doubled. There was a linear relationship of tissue thickness to calcium content.

tissue expansion, such as deep inflammation, edema, or noncalcific fiber separation, were not noted. Furthermore, the relationship between implant thickness and calcium concentration was approximately linear (Figure 6B).

Ultrastructural examination revealed cell-associated and collagen-associated mineralization (Figure 7). Early calcific deposits (2–3 days) occurred primarily in devitalized bovine pericardial cells and their fragments, often dissecting among, but not involving, collagen bundles. Deposits in 21-day implants were not only increased in cells but were also associated with collagen. Calcific deposits were noted equally in unstained ultrathin sections as well as those stained with lead citrate and uranyl acetate.

Discussion

The present study characterized several biochemical features of bovine pericardium, measured the kinetics of glutaraldehyde incorporation, and documented the initiation and progression of calcification in this material, implanted subcutaneously in rats. The material studied is comparable to that used in clinical bovine pericardial bioprosthetic heart valves. This study shows considerable similarity between mineralization in glutaraldehyde-pretreated bovine pericardium and porcine aortic valves, and suggests identical underlying mechanisms for their mineralization. This investigation complements and extends our previous studies, which have demonstrated the importance of calcification in clinical bioprosthetic heart valve failures[2,4,6] and have defined host and implant determinants of bioprosthetic valve and purified Type I collagen calcification in well-characterized subcutaneous and circulatory experimental systems.[11,12,26–29]

Similarity of Calcification of Bovine Pericardium and Porcine Aortic Valve

The overall morphologic progression and kinetics of calcification of bovine pericardium were strikingly similar to those observed with porcine aortic valves, implanted in the same model[11,12] (Table 2), and as calf orthotopic mitral implants.[12,13] Chemical and morphologic evidence of mineralization began within 48 hours after subcutaneous implantation. Calcification was initially associated with transplanted connective tissue cells, devitalized by the glutaraldehyde pretreatment, and later included collagen fibrils. Observations localizing calcific deposits in bovine pericardium to cells and collagen are consistent with ultrastructural observations on clinical and experimental circulatory porcine aortic valve implants.[11,12,30–32] To some extent, and to a degree yet undetermined, the morphology of bioprosthetic tissue mineralization is comparable to that of physiologic bone mineralization, and other pathologic calcifications in which unit membrane enclosed vesicles (often called "matrix vesicles") accumulate the first detectable mineral deposits and collagen-associated mineralization may occur later.[33–37]

Collagen calcification noted in the pericardial implants in the present study resembles that previously noted in porcine aortic valve and bovine pericardium,[11,12,30–32] despite the differences in collagen composition between aortic valve and pericardium. The amino acid analyses indicated that collagen is the most abundant protein present in the pericardial tissue, similar to porcine bioprosthetic valve tissue.[38] Collagen is biochemically heterogeneous.[17,39,40] The almost complete predominance of Type I collagen in pericardium is a notable difference between this biomaterial and porcine aortic valve cusps, which contain Type I and Type III.[41] Whether, and if so how, collagen molecular structure or net composition influences calcification is unknown. Glutaraldehyde-preserved purified Type I collagen mineralizes after subcutaneous implantation in the rat,[29] but Type III collagen mineralization has not been investigated.

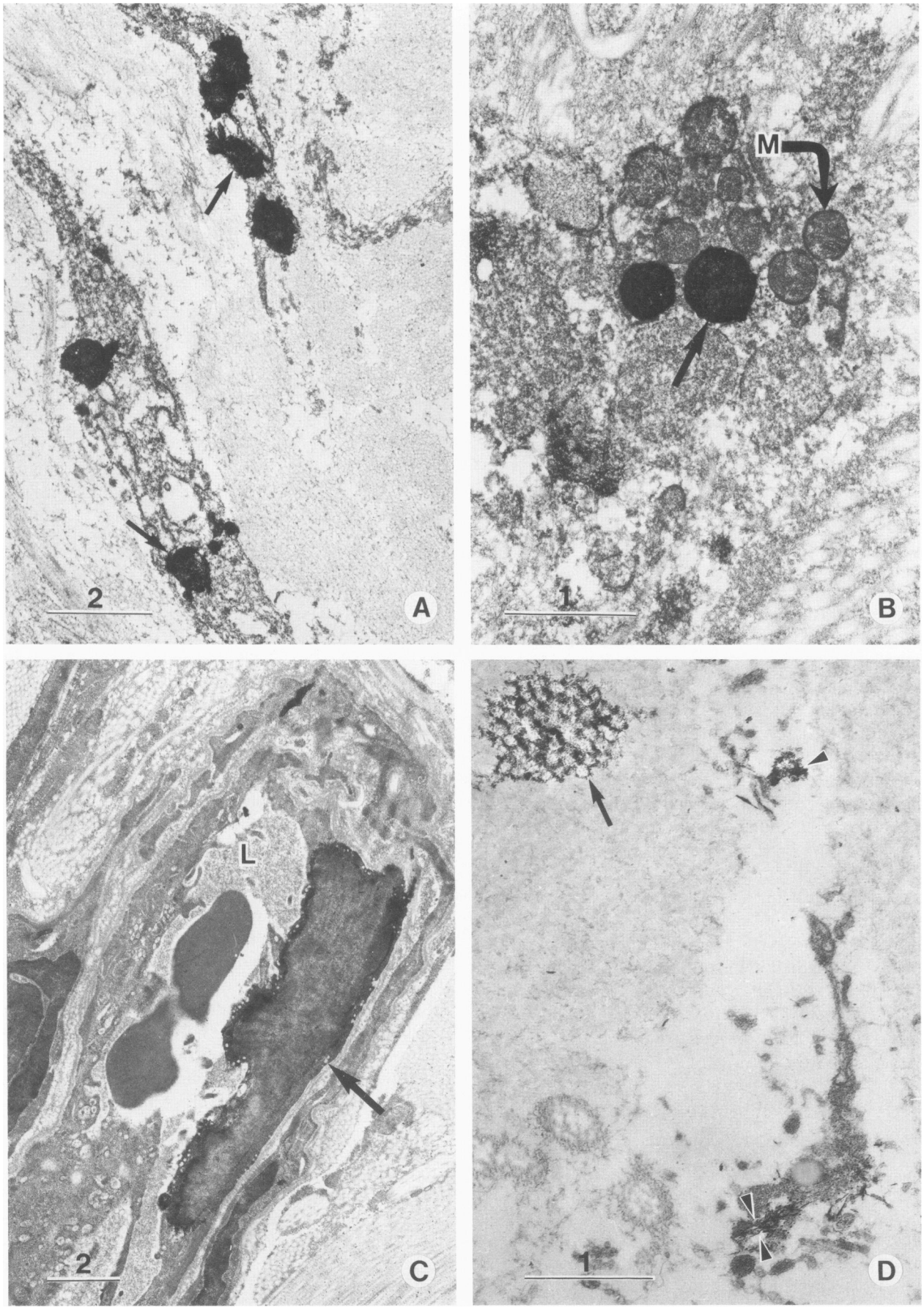

Figure 7—Ultrastructural features of calcification in glutaraldehyde-pretreated bovine pericardium. **A**—Early calcific deposits associated with residual connective tissue cells, and their fragments, of the fixed pericardial structure (*arrows*). Bar, lower left = 2 μ. **B**—Mineralization of mitochondria of residual cell fragments (*arrow*); adjacent mitochondria indicated by *M*. Bar, lower left = 1 μ. **C**—Calcification of endothelial cells of a small pericardial blood vessel (*arrow*). The lumen is indicated by *L*. Bar, lower left = 2 μ. **D**—Calcification associated with cell fragments (*arrowheads*) and collagen (*arrow*) in a 21-day pericardial implant. Fine mineral spicules characteristic of hydroxyapatite are noted at this magnification. Bar, lower left = 1 μ.

Table 2—Comparison of Pathologic Features of Calcification in Subcutaneously Implanted Bovine Pericardium and Porcine Aortic Valve

	Bovine pericardium	Porcine aortic valve*
Earliest mineral deposits	48 hours	48 hours
Calcium (μg/mg dry tissue, mean ± SEM)		
At 21 days	114 ± 8	114 ± 18
At 84 days	221 ± 8	226 ± 18
Overall calcification location	Intrinsic	Intrinsic
Initial mineral site	Cells	Cells
Ultimate mineral sites	Cells and collagen	Cells and collagen
Late calcific nodules	Yes	Yes

* Data from Schoen et al.[12]

A previous study in our laboratories demonstrated that early calcification in porcine aortic valves occurred equally in the valvar fibrosa and spongiosa, but eventual deposition predominated in the spongiosa,[12] a loose layer not present in bovine pericardium.[14-16] It was hypothesized that the more compact architecture of the collagen in the valvar fibrosa served as an internal mechanical constraint to the growth of mineral deposits, as has been postulated in the regulation of physiologic bone mineralization.[33] Bovine pericardium, which has a collagenous architecture different from and less compact than that of porcine valve fibrosa,[15] but certainly more dense than spongiosa, developed calcification equivalent overall to that of porcine valve.

As with porcine aortic valves, initial calcific deposits in bovine pericardium were small and punctate and were well accommodated in the normal architecture of the implant. However, calcific deposits progressively thickened the pericardium, causing an approximately 100% increase in tissue thickness after 112 days' implantation. Furthermore, there was late enhancement of small calcific deposits to confluent nodules which often ulcerated through the cuspal surface. These nodules, through cuspal stiffening or tearing or both, probably initiate clinical failures in porcine aortic valve bioprostheses.[2,12] They are therefore probably similarly detrimental to the integrity of bovine pericardial valves.

Mechanisms of Bioprosthetic Tissue Mineralization

Cross-linking appears to be a prerequisite for mineralization of implanted bioprosthetic tissue. Previous studies in this laboratory have shown that while specimens of glutaraldehyde-fixed porcine aortic valve, or glutaraldehyde- or formaldehyde-pretreated Type I collagen mineralize when implanted subcutaneously in rats, fresh implants undergo inflammatory organization without mineralization.[11,29] Because early clinical and experimental studies demonstrated that formaldehyde-cross-linked bioprostheses had grossly inadequate durability,[42,43] glutaraldehyde is now utilized virtually exclusively. The biochemical reactions of glutaraldehyde with tissues are complex,[44-46] and the molecular mechanisms by which these reactions permit calcification are not understood. However, acellular Type I collagen preparations undergo mineralization, and calcific deposits nucleated in association with cells in both porcine aortic valve and bovine pericardium clearly precede those localized to collagen fibrils. This suggests that calcific crystal nucleation in bioprosthetic tissue cells and collagen occurs by independent mechanisms.

The rate of glutaraldehyde incorporation by bovine pericardium has not previously been reported. In the present study, incorporation was rapid, with nearly half maximal incorporation after only 1 hour, and complete reaction occurring in less than 24 hours. The semilog plot of the kinetics of glutaraldehyde accumulation suggests first-order kinetics. The importance of the nature of the collagen-bound glutaraldehyde to the mechanism of calcific crystal nucleation is unknown.

It is likely that the initial and predominant mineralization of connective tissue cells of the bioprosthetic tissue matrix may be attributed to the unique calcium binding properties of cells and their compartments. Intact living animal cells have intracellular free calcium concentrations approximately 10^{-7} M, while extracellular free calcium is approximately 10^{-3} M, leading to a 10,000-fold gradient across the plasma membrane.[37,47,48] Thus, calcium entry into cells is passive, but cellular calcium is usually held low by energy-requiring metabolic processes such as a plasma membrane-bound Ca^{2+}-ATPase, which requires the energy of ATP hydrolysis to pump Ca^{2+} out of the cell, and intracellular binding by soluble cytosolic or membrane-bound proteins.[47,48] In contrast, calcium and phosphorus levels may be relatively high in membrane-bound organelles such as mitochondria,[47] and phosphorus is prevalent within the organellar and plasma membranes themselves, in the form of phospholipids, proteolipids, and enzymatic systems metabolizing high-energy phosphates.[34] These are the observed sites of primary nucleation of bioprosthetic tissue mineralization. In cells modified by aldehyde cross-linking, passive calcium entry probably occurs unimpeded, but the mechanisms for its removal are no longer active. It is hypothesized that this calcium influx leads to hydroxyapatite formation by reacting with the compartmentalized and bound phosphorus, and that these early crystal nuclei progressively accumulate additional mineral, eventuating in macroscopic calcific deposits. This imbalance between influx and efflux induced by loss of cell vitality is analogous to the mitochondrial calcification which occurs when cardiac myocytes irreversibly damaged by

severe ischemia are exposed to plasma during reperfusion.[49] The plasma membranes of these necrotic cells are incapable of excluding massive quantities of calcium ions, in the presence of active transport systems that are no longer functional. It is likely that these processes are also somewhat analogous to the late mineralization of unfixed tissues used to replace cardiac valves,[50,51] and to the senile degeneration of native aortic valves and aorta,[52] in which tissue damage results from longstanding cyclical stress and its resultant cell damage, probably in conjunction with local tissue hypoxia.

Clinical and Experimental Implications

Calcification of experimental bovine pericardial valve prostheses and other pericardial implants has been described,[32,53-55] and clinical failures due to calcification of bovine pericardial bioprostheses have been reported.[8-10] Although the calcium content of clinical bovine pericardial bioprosthetic valves has not been reported, the maximum concentration of calcium achieved in the present study, approximately 200 μg/mg, was similar to that previously encountered in failed clinical porcine bioprosthetic heart valves (202–234 μg/mg).[6] The level of mineralization achieved at 21 days (Ca, 113 μg/mg) was equivalent to that noted in orthotopic mitral porcine aortic valves in calves, removed electively after 69–142 days (Ca, 47–128 μg/mg).[2,12] Since calcium accumulation is associated with progressive cuspal thickening, sequential echocardiographic measurements demonstrating cuspal widening may identify a group of patients at high risk for calcification-associated valvar failure.[56] The present study, indicating equivalence of experimental mineralization in glutaraldehyde-treated bovine pericardium and porcine aortic valve, suggests that calcification will be as prominent a feature in the late pathologic findings in pericardial bioprostheses, as it has been in porcine aortic valves. Nevertheless, despite the previously demonstrated similarity of the pathologic findings obtained in the present model to those in both clinical and experimental circulatory implants, the differences in host (immature rats) and site (subcutaneous) relative to clinical valve replacements necessitate that the results be extrapolated with caution to the management of patients who have received bovine pericardial valves.

Both host and implant factors are contributory to degenerative bioprosthetic heart valve mineralization.[2,11-13] Studies in our laboratories and those of other investigators are seeking to determine pharmacologic therapies of the host and cuspal pretreatments which would inhibit this process.[53,54,57,58] The similarity of the pathology and pathophysiology of mineralization of bovine pericardium and porcine aortic valve suggests that either of these materials, or valves constructed from them, may be used interchangeably for such studies. Furthermore, the calcification prevention efficacy of host pharmacologic therapies or preimplantation chemical cuspal modification previously demonstrated with porcine aortic valve[57,58] should be equally applicable to bovine pericardial bioprostheses. However, a recent study of the efficacy of sodium dodecyl sulfate as an "anticalcification" agent in porcine aortic valves and bovine pericardial valves implanted in juvenile sheep found that mineralization of porcine valve but not pericardium was reduced by this pretreatment.[54] The reasons for the discrepancy, as yet unclear, could relate to the different efficiencies of the surfactant in altering tissue charge characteristics or in extracting constituents such as phospholipids from the materials or to different kinetics of reaccumulation of mineralization-potentiating molecular species from the blood.

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