Inhibition of calcification of glutaraldehyde pretreated porcine aortic valve cusps with sodium dodecyl sulfate: Preincubation and controlled release studies

Danielle Hirsch,[1] Joelle Drader,[1] Theresa J. Thomas,[1] Frederick J. Schoen,[2] Judith T. Levy,[3] and Robert J. Levy[1,*]

[1]Department of Pediatrics, Kresge II, R5014, University of Michigan, Ann Arbor, Michigan 48109; [2]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; [3]Chemistry Department, Eastern Michigan University, Ypsilanti, Michigan 48197

Calcification of bioprosthetic heart valves fabricated from glutaraldehyde pretreated bovine pericardium or porcine aortic valves (PAV) is a frequent cause of the failure of these devices. Of all strategies considered thus far, only detergent preincubations using compounds such as sodium dodecyl sulfate (SDS) inhibited PAV bioprosthetic mineralization in circulatory sheep bioprosthetic valve replacements. The present study sought to characterize the mechanism of action of SDS preincubation. Results of transport and material characterization studies showed that SDS had a relatively high affinity for PAV, with a maximum uptake of 167.1 ± 6.8 μg SDS/mg tissue over 24 h at 37°C with a partition coefficient of 19.3. The PAV diffusion of SDS was 1.95 ± 0.35 10^{-6} cm^2/sec. The principal effect of SDS on PAV was phospholipid extraction. The residual organic phosphate in the SDS pretreated tissue was 2.22 ± 0.72 nmol/mg tissue compared to the control untreated group with 18.52 ± 2.1 nmol/mg tissue. Incubations of PAV specimens in a 1% SDS solution for 24 h significantly inhibited calcification after 21 days in subdermal implants in 3-week-old male rats (PAV Ca^{2+} = 18.0 ± 11.8 μg/mg) compared to control (177.8 ± 6.0 μg/mg). In contrast, coimplants of 30% SDS silicone rubber polymers, for regional sustained SDS administration, did not impede PAV calcification in 21 day implants Ca^{2+} = 166.0 ± 14.0 μg/mg compared to the nondrug silicone matrix controls, Ca^{2+} = 173.0 ± 6.6 μg/mg). Thus, we conclude that the mechanisms of SDS inhibition of PAV calcification is due to material effects which occur during preincubation, and is not facilitated by sustained SDS administration.

INTRODUCTION

Dystrophic calcification of bioprosthetic replacement heart valves fabricated from glutaraldehyde pretreated porcine aortic valves (PAV) is the most frequent cause of their clinical failure.[1-3] The calcific deposits, composed of calcium phosphates, are intrinsically localized in the gluatraldehyde pretreated PAV connective tissue cells and collagen.[4,5] Animal model studies have also shown their initial calcific deposits to be cell-associated, with the later involvement of collagen bundles. The proliferation of the calcific depots alters the PAV implant architecture and leads to prosthesis failure because of stenosis or regurgitation, or both.

*To whom correspondence should be addressed.

Although a number of therapeutic strategies involving agents such as diphosphonates,[6-8] $FeCl_3$,[9,10] $AlCl_3$,[9,10] or detergents[11] have been effective in subdermal studies, thus far, only detergent pretreatment of PAV using various individual detergents, such as sodium dodecyl sulfate (SDS)[11,12] or aminooleic acid,[13] have been effective for preventing the mineralization of PAV bioprostheses implanted in the circulation in sheep studies. However, the mechanism of action of SDS and other detergents is not well understood.

Sodium dodecyl sulfate is an anionic detergent that can hypothetically modify the bioprosthetic tissue in a variety of ways including, perhaps, extraction of various component lipids or proteins, protein denaturation, or charge modification. *In vitro* studies of metastable calcium-phosphate solutions have demonstrated SDS prevention of the hydroxyapatite for-

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mation in the presence of acidic phospholipids and calcium-phosphate complexed lipids.[14]

The present study was carried out to investigate the mechanism of SDS-mediated inhibition of PAV calcification. The effects of SDS on PAV tissues were characterized in terms of uptake, diffusion, affinity, and phospholipid and protein extraction. Inhibition of PAV calcification in rat subdermal implants due to SDS pretreatment of PAV was also compared to the regional sustained release of SDS using SDS-silicone rubber coimplants.

MATERIALS AND METHODS

Materials

Sodium dodecyl sulfate (purity of approx. 99%), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and phosphate standard were purchased from Sigma (St. Louis, MO). Radiolabeled sodium dodecyl [^{35}S]-sulfate with a specific activity of 62 μCi/mg was obtained from Amersham (Arlington Heights, IL). [^{14}C]-Phosphatidylcholine (L-α-[choline-methyl-^{14}C]) was supplied by New England Nuclear (specific activity = 153 mCi/mmol). The Fisher Scientific Company (Fairlawn, NJ), supplied the following reagent grade chemicals: L-ascorbic acid, methanol, chloroform, toluene, *n*-pentanol. Concentrated sulfuric acid and 30% hydrogen peroxide were purchased from VWR Scientific. Sodium sulfite was obtained from JT Baker Chemical (Phillipsburg, NJ). The scintillation fluid, Ecolume, was purchased from ICN Biomedicals (Irvine, CA). Ammonium molybdate (VI) was obtained from Aldrich (Milwaukee, WI). Ketamine hydrochloride (Aveco, Fort Dodge, IA) and Rompun® (Haver, Shawnee, KA) were used for anesthesia. Porcine aortic valves, crosslinked with glutaraldehyde and stored in 0.2% glutaraldehyde at pH 7.4, were donated by Medtronic Inc. (Irvine, CA).

Methods: *in vitro* studies

Diffusion studies

The diffusivity, D, for [^{35}S]-SDS diffusion across PAV leaflets was measured using two chamber diffusion cells at 37°C with constant shaking. Each PAV sample was mounted between the two cells after measuring the thickness at three points with a Mitutoyo (Japan) micrometer. The donor solution was a physiologic buffer (50 mM HEPES adjusted to pH 7.4, 0.1M NaCl) containing 1% w/v SDS and preserved with 0.5% sodium azide. The receptor chamber contained the buffer without SDS. At each time point, the contents of the receptor cell were removed, [^{35}S]-Activity was quantitated with a liquid scintillation counter (Model LS 3801, Beckman, USA) and the SDS diffused calculated as a function of time. The receptor cell was also periodically replenished with fresh solution.

Maximum uptake of [^{35}S]-SDS

The PAV samples (approx. 1 × 1 cm) were incubated in 1%, 0.5%, or 0.1% w/v [^{35}S]-SDS solutions (see above) at 37°C, with constant shaking. Periodically, the samples were removed, rinsed twice with 1 mL of distilled water (with a background of 6.9 ± 1.06 μg SDS/mg dry weight) and lyophilized. Then, the samples were dissolved in tissue solubilizer (Solvable, Dupont, Boston, MA) at 55°–60°C overnight, and then counted for [^{35}S]-activity (see above).

SDS-PAV partition study

To study the affinity of SDS for native PAV leaflet tissue, the latter was placed in a 1.5 mL Eppendorf vial and immersed in 500 μL buffered solution (see above) of 1% w/v [^{35}S]-SDS at 37°C on a shaker (100 rpm). Control vials were prepared containing the radioactive [^{35}S]-SDS solution but without tissue; after 48 h, 100 μL samples were withdrawn from each vial and counted for radioactivity. The partition coefficient (K_p) was calculated as the ratio between the concentrations of the drug in the tissue and in the buffer, at equilibrium.[15] The concentration of SDS in the buffer was calculated from the control vials.

Phospholipid extraction and analysis

The lyophilized, minced samples (10 mg) were homogenized in 1 mL of 50 mM HEPES buffered saline (pH 7.4) with a 1 mL Econo-grind homogenizer (Radnoti Glass Technology, Inc., Aracadia, CA) for approximately 1 h on ice. Then, 20 μL of the [^{14}C]-labeled phosphatidylcholine solution was added to the sample homogenate in order to quantitate the percentage recovery of the homogenization and extraction procedures. The extraction followed the method of Bligh and Dyer[16] with the modifications of Merrit et al.[17] The phospholipid content of the tissues was determined as inorganic phosphate in digested samples. Aliquots of 500–900 μL of the redissolved phospholipid residue (in 1 mL chloroform) were dried and 300 μL of concentrated H_2SO_4 were added; then the acidic solutions were heated until a brown color developed and left overnight at 25°C. We added 50 μL of 30% H_2O_2 and the tubes were heated until no more gas evolved. The following solutions were added sequentially with mixing: 4 mL of 0.825% Na_2SO_3, 1 mL of 2% $(NH_4)_6(Mo)_7(O_2)_4 \cdot 4H_2O$ and 0.1 mL of a 10% L-ascorbic acid solution. After heating the samples of 90°C for 10 min and cooling, the solutions were extracted with 300 μL of *n*-pentanol

and absorbance of the blue pentanol layer was read at 795 nm.

Amino acid analysis

Aliquots of solutions of acid-hydrolyzed porcine tissues[18,19] were analyzed quantitatively for amino-acid content on a Beckman Spinco 121M Automated Amino Acid Analyzer (Palo Alto, CA).

Formulation of SDS-silicone rubber matrices and SDS release from polymeric matrices

Lyophilized radioactive SDS (30% w/v) was incorporated into prepolymerized MDX4-4210 Silastic (Dow Corning Corporation, Midland, MI) with mixing by uniform levigation. Then, 10% weight per matrix of the curing agent MDX4-4210 Silastic was added and the mixture was thoroughly admixed. The polymerization took place at 37°C using a 650 μm thickness mold. Polymerized silicone rubber [^{35}S]-SDS matrices (1 × 1 cm, 55–60 mg) were incubated in 5 mL of a physiological buffer (50 mM HEPES, pH 7.4, 0.1 M NaCl) at 37°C on a shaker stand at 100 rmp. At each time point, matrices were transferred into a fresh buffer solution and the previous releasing solutions were diluted with Ecolume and counted for [^{35}S]-SDS with a liquid scintillation counter (Model LS 3801, Beckman).

Methods: *In vivo* studies

Implant and retrieval methods

Three-week-old (50–60 g) male Sprague-Dawley rats (CD strain; Charles River Laboratories, Burlington, MA) were anesthetized by an intraperitoneal injection of xylazine (0.008 mg/g) and ketamine hydrochloride (0.057 mg/g). Two subdermal pouches were dissected on the dorsal side of each rat. Preincubated 1 × 1 cm pieces of PAV (in 1% or 0.005% SDS), or these same pieces attached with sutures to controlled release polymers of similar size, were rinsed with distilled H_2O and implanted into the subdermal pouches. These animals were maintained on a diet of Rodent Laboratory Chow (Purina Mills, Inc., St. Louis, MO) for 21 days. At this time the animals were sacrificed by CO_2 asphyxiation, and the tissue pieces explanted and rinsed with 0.9% saline. Tissues were individually rinsed with distilled H_2O, freeze-dried, and minced. An acid hydrolysis procedure was used to prepare each sample for calcium analysis by atomic absorption spectroscopy[18,19] and for a phosphorus assay.[20]

Microscopic analysis

Representative samples of expanded tissue were placed in Karnovsky's fixative (cacodylate buffered 2.5% glutaraldehyde, 2% paraformaldehyde at pH 7.4). Femurs of these same animals were excised at the time of sacrifice and placed in 10% neutral buffered formalin. Specimens of both tissue and bone were embedded in JB-4 glycomethacrylate medium (Polysciences, Warrington, PA) and sectioned to 2 μm. these sections were stained with hematoxylin and eosin for general morphology and von Kossa stain for calcium phosphates.

RESULTS

In vitro transport studies

Diffusion studies

The value of D was calculated from equation 1:

$$D = l^2/6 \cdot t_{lag} \quad (1)$$

where t_{lag} is the lag time (sec), l is the tissue thickness (cm).[21] The lag time for SDS diffusion through PAV leaflet was experimentally calculated by back extrapolation to the x-axis of the steady-state portion of the graph (Fig. 1). The lag times for SDS diffusion through porcine specimens were calculated from Figure 1 and then incorporated in Equation 1. The value of D for SDS was determinated to be $1.95 \pm 0.35 \cdot 10^{-6}$cm^2/sec for PAV, based on two replicate studies.

SDS PAV affinity and partition

The maximum uptake of SDS by PAV leaflet was 167.1 ± 6.8 μg/mg dried material under the assay

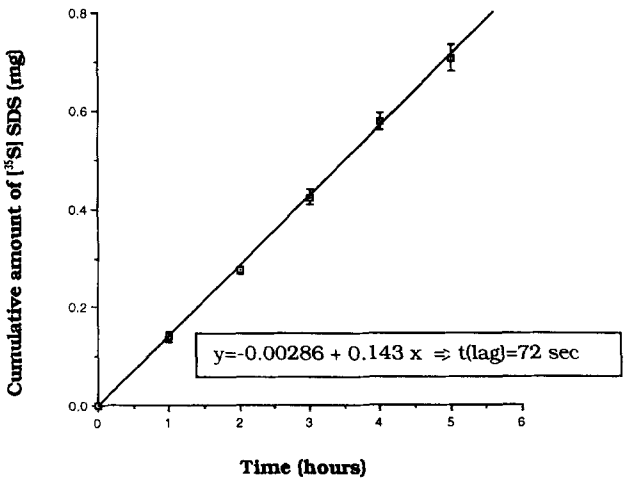

Figure 1. Diffusion of SDS through PAV leaflet membranes tissues.

system used in these studies with 1% SDS solution. The maximum uptake of SDS by PAV leaflet in a 0.5% SDS solution (166.3 ± 3.6) was similar to the SDS level measured in a 1% SDS solution. In a 0.1% SDS solution the maximum uptake of SDS measured dropped to 119.4 ± 1.2 μg/mg dry PAV. Furthermore, the partition constant $K_p = [C_{leaflet}]/[C_{solution}]$ of SDS between the PAV leaflet and buffer solution was 19.3

Protein and organic phosphate contents in SDS treated and nontreated PAV leaflets before implant

The results of protein and organic phosphate determinations in unimplanted SDS-treated and nontreated PAV leaflets are presented in Table I. Based on complete amino acid analyses, the average value for the protein level in the unimplanted SDS-treated valves (67.9 ± 4.4 mg protein/100 mg dried material) was not significantly different from the protein level in the unimplanted control valves (79.6 ± 11.8 mg protein/100 mg dried material). However, the organic phosphate level in SDS-treated valves (2.2 ± 0.7 nmol/100 mg dried material) was significantly lower as compared to the unimplanted nontreated control valves (18.5 ± 2.1).

Polymer formulation and characterization

The release profile of [^{35}S]-SDS (30% w/w) from silicone-rubber matrices is shown in Figure 2. Initial release rate results revealed 6.7% of the SDS eluting after 24 h, but only an additional 20.7% was released after 1 week. After the initial burst of SDS release, the release rate declined exponentially, reaching a relative plateau by 15 days. The cumulative amount of SDS released at 21 days was 45.15 ± 2.82 mg SDS/slab (or 2.15 mg/day/coimplanted PAV), corresponding to 45.1% of the incorporated SDS.

In vivo studies

Table II shows that preincubations in 1% SDS of PAV tissue significantly ($P < .05$) inhibited calcification of rat subdermal implants, as demonstrated by calcium and phosphorus analysis (Table II). PAV preincubation in 0.005% SDS was not effective for completely inhibiting the calcification, although the calcium level was significantly lower than the control. The porcine tissue coimplanted with 30% SDS-silicone rubber matrices for 21 days accumulated a similar level of calcium (166.0 ± 6.6 μg Ca^{2+}/mg dry weight) as compared to the respective control group (173.0 ± 6.6 μg Ca^{2+}/mg dry weight) (Table II).

Histological studies of explanted specimens confirmed the chemical results (Fig. 3). Calcification of the SDS-treated specimens was markedly reduced relative to control (nontreated) tissue, but had a similar pattern to that typically noted in calcified bioprosthetic tissue, suggesting cell and collagen involvement.[18,19] Interestingly, there was a suggestion of a zone of relatively diminished calcification in proximity to the SDS-containing polymer suggesting a very localized inhibiting effect, despite the overall severe calcification noted in the SDS-polymer sample. Femurs from animals with either treated tissue or drug release implants showed no alterations relative to femurs from animals not receiving drug.

DISCUSSION

This study showed that the SDS pretreatment of PAV significantly inhibited calcification of rat subdermal implants. Conversely, the SDS-silicone rubber polymer matrices coimplanted with the bioprosthetic tissue were not effective for preventing PAV mineralization. Thus, these results suggest that an extraction or material modification during SDS preincubation is responsible for preventing PAV calcification, rather than the continuous presence of SDS at what should have been at least comparable levels to the SDS content in preincubated PAV based on the partition constant results (see above).

The principal effect of SDS on PAV was phospholipid extraction (Table I) from membrane sites where they are prevalent. Acidic phospholipids are recognized to be involved in the initiation of both calcification in bone and dystrophic calcification,[18,19,22–24] possibly, according to Boskey,[24] as calcium-acidic phospholipid-phosphate complexes. These complexes have also been shown to be membrane constituents. [25,26] *In vitro* these same complexes induce

TABLE I
Comparison of Protein and Organic Phosphate Contents in Unimplanted SDS-treated (1%) and Nontreated PAV Leaflets

	Organic Phosphate (nmol/mg dry material)	Total Protein (mg protein/100 mg dry material)
Nontreated leaflet	18.5 ± 2.1 (5)	79.6 ± 11.8 (3)
SDS-treated leaflet	2.2 ± 0.7 (7)	67.9 ± 4.4 (3)

*Normalized by using C-phosphatidylcholine internal standard to 100% recovery for homogenization and extractions procedures; hydrolyzed by the procedure described in Methods.

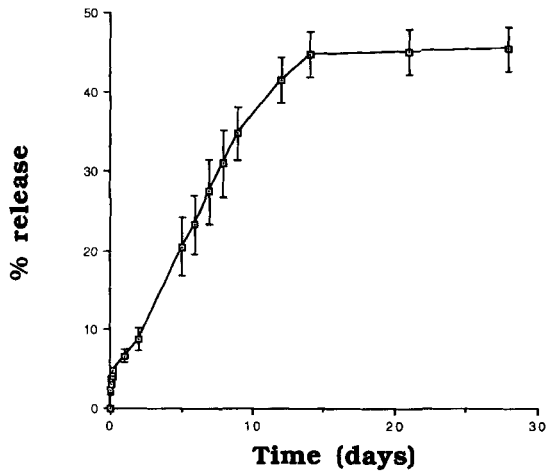

Figure 2. SDS release from silicone-rubber matrices at 37°C. Each point was the average of five measurements.

hydroxyapatite precipitation from metastable calcium phosphate solutions.[27] Another related *in vitro* study showed that SDS had a higher affinity for liposomes than proteins.[28] In addition, our data demonstrated that the protein level of the bioprosthetic tissue before and after SDS treatment was similar; again, this result indicated the affinity of SDS for lipid extraction rather than proteins of porcine tissues.

Therefore, we propose that SDS preincubation disrupts membranes, thereby releasing phospholipids that are involved in the calcification development. In this hypothesis, SDS solubilizes some membrane phospholipid components of connective tissue cells that have been shown to be the site for early mineralization in bioprosthetic tissue.[9,18,19,29] Interestingly, a recent study investigated the light and electron microscopic morphology of clinical porcine aortic valve bioprostheses which were subjected to an antimineralization pretreatment consisting of 1% SDS exposed for 24 h.[30] This study did not demonstrate any detectable alterations in morphology or ultrastructure due to SDS, comparing SDS pretreated bioprostheses to controls.[30]

The SDS-membrane related mechanisms for inhibiting bioprosthetic calcification may also be related to the role of alkaline phosphatase in bioprosthetic mineralization, since SDS has been demonstrated to extract alkaline phosphatase from matrix vesicles, thereby completely inhibiting matrix vesicle mineralization studies of *in vitro* bone mineralization.[31,32] Prior investigations by our group have demonstrated that alkaline phosphatase is present in both porcine aortic valve leaflet and bovine pericardial bioprosthetic tissue, even after gluataraldehyde fixation.[33] Furthermore, studies of subcutaneous implants of glutaraldehyde pretreated bovine pericardium have shown that alkaline phosphatase activity increases to maximal levels by 72 h postimplantation, coincident with the onset of bioprosthetic mineralization, and declines gradually by 21 h during continuing bulk mineral accumulation.[29] Alkaline phosphatase investigations were beyond the scope of the present studies concerning SDS pretreatment of porcine aortic valve calcification. However, preliminary results from our laboratory using rat subcutaneous explants of SDS pretreated and control bovine pericardial bioprosthetic tissue demonstrated no significant differences between SDS-treated and control tissues in terms of explant extractable alkaline phosphatase activity despite the SDS inhibition of calcification (Table III).

Previously published results from our group have also shown no relationship between bioprosthetic alkaline phosphatase activity and the efficacy of a number of different types of inhibitors, including biophosphates and metallo-inhibitors.[34] Furthermore, local controlled release implants containing the alkaline phosphatase inhibitor levamisole did not decrease the level of calcification observed in bovine pericardial rat subdermal implants.[35] Thus, the relationship between alkaline phosphatase activity and the mechanism of action of SDS for inhibiting bioprosthetic calcification is unclear, and should be the subject of further investigations.

Sodium dodecyl sulfate may also have some role inhibiting the later events in PAV calcification, in which the collagen mineralization takes place. SDS

TABLE II
Calcium and Phosphate Data from PAV (SDS Pretreated and Coimplanted With Polymeric Matrices) Explanted After 21 days

Treatment	n	Ca (μg/mg dry material)	P (μg/mg dry material)	Ca/P (molar ratio)
Preincubations				
Control	36	177.8 ± 6.0	115.3 ± 5.4	1.19
SDS 1%	21	18.03 ± 11.82*	13.02 ± 6.92*	1.07
SDS 0.005%	10	102.3 ± 12.52*	123.2 ± 3.2	0.64
Controlled release studies				
Control	40	173.0 ± 6.6	130.6 ± 5.26	1.02
30% SDS	22	166.0 ± 13.97	102.4 ± 5.51*	1.25

*$P \leq .05$ compared to respective control groups.

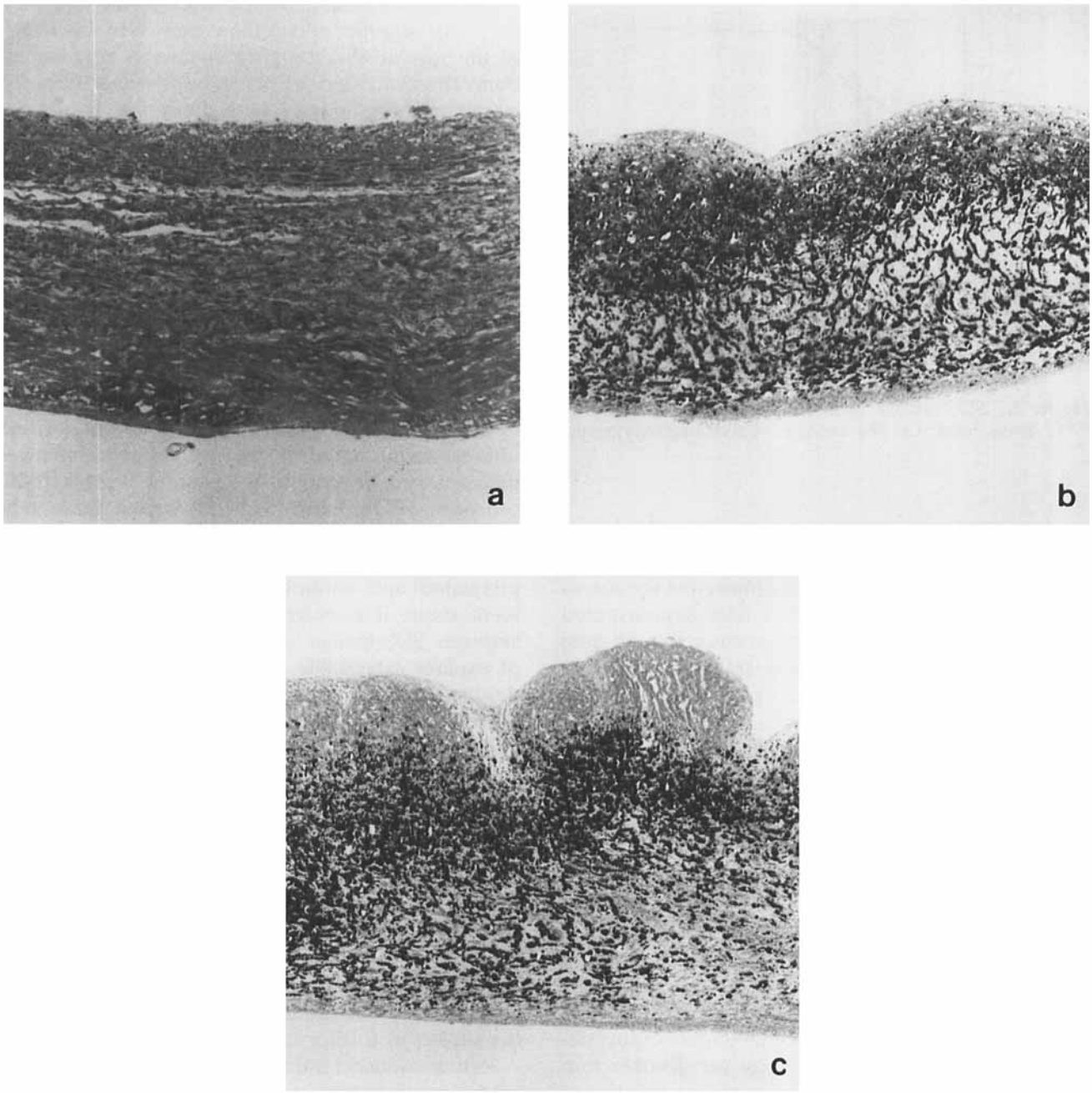

Figure 3. Morphology of explanted tissue, demonstrating inhibition of calcification by SDS pre-incubation but not by drug release in 21 day implants. a) SDS pre-incubated tissue, with minimal calcification. b) Tissue without incubation in SDS. c) Tissue adjacent to polymer containing SDS, with dense calcific deposits demonstrating somewhat reduced intrinsic calcification in proximity to the polymer. Von Kossa stain (calcium phosphates black); original magnification 150×.

has been demonstrated to alter collagen structure so that the resistance of collagen to proteolytic enzymes is annulled.[36] Perhaps SDS pretreatment similarly renders the bioprosthetic collagen secondary and tertiary structures less suitable as a template for calcium phosphate crystallization, and thus further inhibits bioprosthetic calcification.

CONCLUSION

Evidence in these studies indicates that SDS inhibition of PAV calcification is most likely due to modifications of PAV constituents essential to calcification rather than the binding of this compound to these constituents with a dose-response association.

TABLE III
Effects of 1% SDS Preincubation on Calcification and Alkaline Phosphatase Levels in Glutaraldehyde Pretreated Bovine Pericardial Rat Subdermal Explants (72 h and 32 days)

Type of Specimen	Calcium (μg/mg)	Alkaline Phosphatase*
SDS, 72 h	4.18 ± 1.27 (10)	774.46 ± 185.49 (15)
SDS, 21 day	29.16 ± 5.73 (13)	133.13 ± 38.87 (12)
Control, 72 h	4.76 ± 0.49 (11)	411.2 ± 75.81 (20)
Control, 21 day	102.9 ± 8.52 (10)	72.87 ± 12.53 (10)

*Expressed as nanomoles of paranitrophenolphosphate/minute/mg protein hydrolyzed under established alkaline conditions.[34] Numbers in parentheses are total samples analyzed.

Phospholipid extraction was most strongly associated with SDS preincubation, and thus further studies on optimal lipid extractions may not only shed light on the crucial events in PAV calcification and its inhibition, but also optimize preventive therapies.

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