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Synergistic Effect of Released Aspirin/Heparin for Preventing Bovine Pericardial Calcification

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Abstract: Calcification is a frequent cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde pretreated bovine pericardium (GATBP). Aspirin, a potent antiplatelet drug, and heparin, an anticoagulant, are commonly used for postimplant complications such as thrombosis and thromboembolism. Aspirin and heparin were embedded in chitosan/polyethylene vinylacetate co-matrix to develop a prolonged release form. The effect of these drugs towards the bioprosthetic calcification was investigated by in vitro and in vivo models. In vitro and in vivo evaluation suggest that the released aspirin/heparin from the co-matrix had a synergistic effect in inhibiting GATBP calcification. In vivo subcutaneous coimplantation was performed with PEG-20,000 grafted bovine pericardium (PEG-GABP), aspirin, and heparin. Biochemical, histological, and scanning electron microscopic evaluation of retrieved samples demonstrated a significant reduction in calcium deposition and alkaline phosphatase activity on PEG-GABP compared to GATBP. It seems that the aspirin/heparin combination synergistically inhibits the pericardial calcification in addition to their antithrombotic function. **Key Words:** Bovine pericardium—Alkaline phosphatase—Calcification—Aspirin/heparin release.

Calcific degeneration of bioprosthetic heart valves fabricated from glutaradehyde pretreated porcine aortic valves or bovine pericardium (GATBP) is a frequent cause of clinical failure (1). Ultrastructural studies demonstrate that early mineral deposition occurs both in clinical and experimental pericardial tissues, and it is localized to transplanted connective tissue cells but not on extracellular collagen fibers. The collagen involvement of calcification occurs at a later stage (2–5). It is also proposed that the nucleus of calcification appears as a result of adhesion and death of cells that contain calcium, phosphate, phospholipids, lipoproteins, and enzymes (6,7). Hence, as a result of cell death, membrane fragments forming a vesicular matrix appear in the media, and calcium phosphate crystals are formed in this matrix (8).

The earliest events in mineralization of bioprosthetic connective tissue cells are hypothesized to result from glutaraldehyde induced cellular *devitalization* and resulting disruption of cellular calcium regulation (9). Moreover, in membrane-bound organelles such as mitochondria, calcium and phosphorus levels are relatively high, and the organellar and plasma membranes themselves contain considerable phosphorus, largely as phospholipids. Further, alkaline phosphatase (AP), an enzyme also associated with matrix vesicles involved in bone mineral nucleation, is present in bovine pericardium (BP) tissue mineralization sites (10). AP may hydrolyze cellular phosphoesters to increase the regional phosphate concentrations (10). These sources of phosphorus are the observed sites of early bioprosthetic mineralization. Unfortunately, the mechanism of induction and propagation of this disorder is not well defined.

Because dystrophic calcification is a multifactorial process, therapeutic strategy may require drug action at different stages of calcium phosphate deposition. Basically, two approaches have been employed to reduce bioprosthesis associated calcification: (a) implant modifications to prevent mineral-

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ization via the pretreatment of bioprosthetic cusps with detergents, metal ions, or diphosphonates, etc.,and (b) the implantation of a controlled release drug delivery system, which facilitates local site-specific anticalcification while avoiding systemic side effects. Local polymeric delivery systems have been investigated with several drugs, such as $FeCl_3$, $AlCl_3$, diphosphonates, by different groups (10–12). Recently, we have reported the in vitro and in vivo efficacy of prolonged release chitosan matrices containing protamine sulfate and ferric chloride to inhibit polyurethane and tissue associated calcification (13,14).

Reports suggest that aspirin, a well-known platelet antagonist, and heparin, a bioactive agent for curtailing thrombosis, appear to be effective as antithrombotic agents when immobilized on the polymer surfaces (15,16). Controlled release of aspirin/heparin from the chitosan/polyethylene vinylacetate co-matrix system has been indicated from our laboratory (17).

The released drug combinations have shown a substantial reduction in platelet surface attachment and anticoagulation. Further aspirin/heparin combinations can prevent platelet functions and thrombosis. Polyethylene glycol modified bovine pericardium, a hybrid tissue material, has shown resistance to calcification and cellular attachment (18). Hence, the basis of this study is to assess the possibility of aspirin/heparin in inhibiting bioprosthesis associated calcification by in vitro and in vivo models. Histological and biochemical evaluations have proposed that controlled delivery of aspirin/heparin on PEG-20,000 grafted bovine pericardium can significantly inhibit the alkaline phosphatase activity and tissue calcification.

MATERIALS AND METHODS

Materials

Chitosan [$\alpha(1\rightarrow4)$2-amino-2-deoxy-β-D glucan), one of the abundantly available polysaccharides in nature, was obtained as a gift from the Central Institute of Fisheries Technology (Cochin, India). Poly(ethylene-vinylacetate) 60% ethylene, polystyrene-butadiene (Polysciences, Inc., Warrington, PA, U.S.A.), heparin sodium injection, i.p. 25,000 IU in 5 ml (Biological E. Limited, Hyderabad, India), and aspirin, 300 mg, (Boots India Pvt. Ltd, Mumbai, India) were also used as received. Calcium phosphorus and alkaline phosphatase test combination kits were obtained from Miles India Ltd., Baroda. Glutaraldehyde and Triton X-100 were from Spectrochem (Bombay, India), and sodium dodecyl sulphate (SDS) was from Sigma (St. Louis, MO, U.S.A.) and all other chemicals were of analytical grade.

Tissue preparation

Bovine pericardial sacs were selected after removing excess tissue and fats. They were decellularized with 2 different detergents, Triton X-100 and SDS, as reported earlier (19,20). In short, the pericardium was initially treated by 0.1% solution of Triton X-100 containing 1 ml Phenyl Methyl Sulphonyl Flouride (PMSF) in saline for 24 h at room temperature. Next, a second detergent extraction was performed with 0.5% SDS and 1 m*M* PMSF in saline for 72 h at room temperature in a mechanical shaker at 100 rpm. Multiple detergent extraction was employed with the aim of removing cell components that were sites of initial calcium nucleation (19,20).

Tissue treatments

Glutaraldehyde treated BP (GATBP)

Tissue fixation was carried out with 0.6% glutaraldehyde in 0.1 *M* phosphate buffered saline (pH 7.4) and transferred after 24 h (2,3). Further, they were exposed to 0.2% glutaraldehyde for 2 weeks at 4°C then washed with distilled water to remove residual (GA) and used for these studies.

PEG grafting on GABP

The decellularization process was immediately followed by collagen crosslinking with (GA). Tissue fixation was carried out with 0.6% GA in 0.1 *M* phosphate buffered saline (pH 7.4) and transferred after 24 h. Further, they were exposed to 5% solution of PEG-20,000 (pH 5.5) for 5 h and then washed with distilled water (18).

Preparation of aspirin/heparin co-matrix system

A co-matrix system was developed to release aspirin/heparin as reported elsewhere (17). Briefly, chitosan dissolved in 2% acetic acid was blown into a NaOH–methanol solution by compressed air through nozzles, and the regenerated porous beads were washed by hot and cold water successively. Loading of aspirin to chitosan beads was carried out by incubating beads of uniform size in aspirin solution, pH 7.4, and stirred at room temperature. The preparation was evaporated to dryness.

A known amount of heparin sodium was dispersed in 15% solution of Poly (ethylene vinyl acetate) (PEVAc) in dichloromethane at room temperature. The solution was spread over a glass plate separated by shims, and the solvent evaporated slowly. The

aspirin-loaded chitosan beads were spread over the partially evaporated solutions. Further, more heparin–PEVAc was added, and the solvent was evaporated completely. By this process, a co-matrix system of 1 cm^2 was developed by incorporating 14.45 mg/cm^2 aspirin-loaded chitosan beads in 11.4 mg/cm^2 heparin-loaded polyethylene vinylacetate matrix. These co-matrices were further coated with polystyrene–butadiene (85:15) for slow release of the drugs (17).

Assay of the released drugs

Metachromic toluidine blue assay (21) was used to quantitate the amount of heparin released from the co-matrix system, developed from aspirin-loaded chitosan beads and heparin-loaded PE(VAc) (17). Aspirin concentration was measured at 260 nm by UV absorbance using spectrophotometer (Shimadzu, Tokyo, Japan) against a standard (17).

In vitro calcification

The calcification experiments were performed as reported earlier (13,22) with slight modifications. Briefly, in this system the calcium concentration (10.28 mg/100 ml) was similar to mean total serum levels (10 mg/10 ml), and the ratio of Ca/PO_4 was 1.67 as in hydroxyapatite (HAP). The concentration product of calcium ($CaCl_2 \cdot 2H_2O$) and phosphate (K_2HPO_4) in the incubation solution was 3.95 mM^2, 2.57 mM calcium, and 1.54 mM phosphate. Each salt solution was prepared in 0.1 M Tris buffer, pH 7.4 containing 0.03% sodium azide as preservative. Equal volumes of 2.57 mM calcium chloride solution and 1.54 mM K_2HPO_4 solution were mixed in a screw-capped bottle containing the pericardial tissues (13,22). These solutions were also incorporated with a protein mixture containing 25 mg% albumin, 15 mg% γ-globulin, and 7.5 mg% fibrinogen. One set of experiments was performed along with aspirin/heparin drug delivery by incubating a known amount of aspirin/heparin-loaded matrix along with a metastable solution of calcium phosphate.

At specific time points, the tissues were removed and rinsed with water to remove excess solution and loosely attached deposits. They were oven dried (2 h, 100°C), accurately weighed, and hydrolyzed in 2 ml of 2N HCl for 24 h at 50°C as reported elsewhere.

The calcium concentration was determined from HCl hydrolysate using a colorimetric method of O-cresolphthalein complexone obtained as standard kits.

Implant and explant methods

Three-week-old (50–70 g) male Wistar rats (animal facility maintained in SCTIMST) used for the implantation experiments were housed and managed according to the standards prescribed for care and management of laboratory animals. The rats were anesthetized by i.m. injection of ketamine and xylazine, and 4 subdermal pouches at least 1.00 to 1.5 cm lateral to the midline were dissected in the dorsal position on each rat. The glutaraldehyde treated BP (GATBP) specimens (1 cm^2) were washed with distilled water to remove residual glutaraldehyde, then implanted in these pouches and covered aseptically (14). Additional experiments were carried out in which the aspirin/heparin co-matrix was implanted close to the tissue implants in each subcutaneous pouch.

The rats were sacrificed; after particular time periods, explanted tissues were analyzed biochemically for the presence of calcium and alkaline phosphatase. Morphological changes were also assessed by light microscopy. The amount of calcium in the retrieved tissues was determined from HCl hydrolysate, as reported elsewhere (14), using the O-cresolphthalein complexone method.

Extraction and analysis of alkaline phosphatase

Explanted pericardial samples (25–30 mg) were collected in ice and homogenized with a mortar and pestle using 2.0 ml s butanol and 1.0 ml distilled water. The liquid was removed, and the homogenization and extraction in the butanol–water system was repeated twice. Extracts were pooled and centrifuged for 15 min at 1,500 g (10) at 4°C. The aqueous layer was removed and the protein concentration and AP were assayed.

The assay for AP was based on the enzymatic hydrolysis of the substrate, p-nitrophenyl phosphate to nitrophenol, which was measured quantitatively using visible light spectroscopy at 405 nm (10). A standard kit obtained from Miles lab was used for this assay. The protein concentration in the aqueous layer was determined by the phenol reagent method using human serum albumin as a standard (23). The AP activity was represented as the nano mole of p-nitrophenol liberated per minute per milligram protein (nm pnp/min/mg protein).

Scanning electron microscopy

The surface and/or internal morphology of bovine pericardial tissues and the drug-loaded co-matrix were examined using a scanning electron microscope (SEM, Model S-2400, Hitachi, Japan). Samples were mounted onto metal stubs using double-sided adhesive tape, vacuum coated with gold film, and then observed.

RESULTS

Inhibition of GATBP calcification via aspirin/heparin delivery: in vitro study

In vitro controlled-release drug delivery of aspirin/heparin from chitosan/PE(VAc) co-matrix and the amount of Ca^{2+} deposited on GATBP as a function of time are represented in Fig. 1. SEMs of 30 days in vitro calcified glutaraldehyde treated bovine pericardium, and in presence of aspirin/heparin delivery, are also depicted in Fig. 1. The deposition of calcium on GATBP was substantially reduced in the presence of aspirin/heparin. Further, the figure indicates that the infusion of aspirin/heparin molecules had inhibited the growth of calcium deposits on GATBP, in a concentration-dependent manner. The calcium phosphate crystals seen on the GATBP surface were roughly spherical in overall shape, as is evident from their SEM. However, the calcium deposition was substantially reduced on GATBP samples due to aspirin/heparin delivery in the media. The calcium crystals were rare, and only microgranular crystals were evident along with some released aspirin/heparin crystals on the surface of GATBP, as indicated in the SEM. These initial in vitro observations showed the anticalcification effects of antiplatelet aspirin/anticoagulant—heparin in their combinations. Further, in vivo implantation studies have been carried out in a rat subdermal model to evaluate the anticalcification effects of released aspirin/heparin on PEG grafted BP.

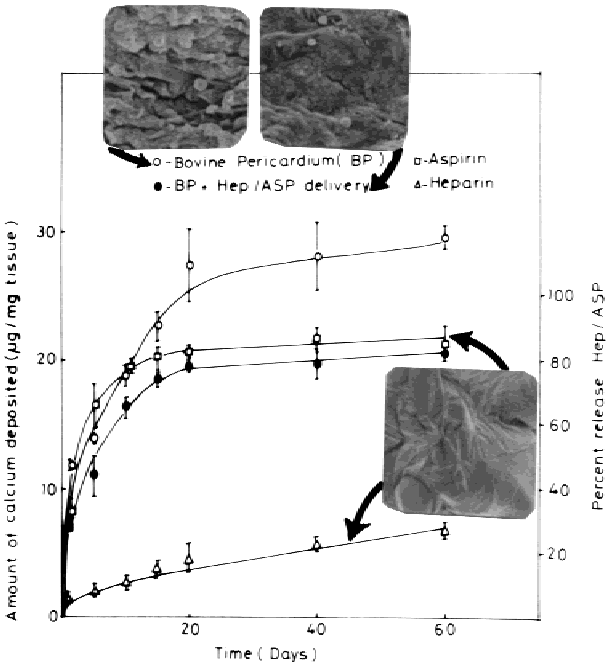

FIG. 1. Shown is the amount of calcium deposited to bovine pericardium against the released Hep/ASP as a function of time. The bar indicates 95% confidence limits (○,●), calcium deposited percent Aspirin (□), and heparin (△) released.

SEMs of calcified pericardial tissues after 21 day rat subcutaneous implantation are depicted in Fig. 2. Surface morphology of glutaraldehyde treated BP (Fig. 2) showed adhered platelets, calcified areas, and markable breakdown of collagen bundles whereas reduced platelet adhesion, calcification, and enzymatic digestion were observed on PEG grafted BP coimplanted with the aspirin/heparin loaded co-matrix. However, our previous in vitro and in vivo observations (24) have proven that PEG grafting on the pericardium reduces chances for calcification and enzymatic attack. Aspirin/heparin delivery on PEG modified BP further improved the stability of the pericardial tissue and produces a blood-compatible and anticalcifying interface.

Histological evaluation of subdermal implants

All implants of bovine pericardial tissues had normal architecture. PEG modified BP along with aspirin/heparin delivery showed significant reduction in calcification compared to GATBP. The calcified areas, which stain darkly with von Kossa, were present in most GATBP samples in the form of granules as well as confluent areas (Fig. 3A). Breakdown of collagen fibrils due to calcification were also evident (Fig. 3A) in GATBP after 21 days of implantation. Marked reduction in calcification was observed in PEG-20,000 grafted BP when stained with von Kossa (Fig. 3B).

Implants were surrounded by fibroblasts and fibrocytes with an infiltration of macrophages into the peripheral layers of the implant. Evidence of fibroblasts and fibrocytes were observed along 1 edge, and implants infiltered with numerous macrophages, fibroblasts, lymphocytes, and foreign body type of giant cells along with occasional foci of neutrophils were also observed in 21-day GATBP explant. In an earlier observation (24), PEG grafted BP showed highly biocompatible interface with less cell infiltration to the implant. The cellular infiltration to the implant had been reduced in the case of PEG modified BP along with aspirin/heparin delivery compared to GATBP and PEG-GABP samples. Table 1 provides the amount of calcium deposited to GATBP, PEG modified BP (24) aspirin/heparin delivery on PEG-GABP after 21 days subcutaneous implantation in rats. Glutaraldehyde treated bovine pericardium showed higher levels of calcium deposits. However, aspirin/heparin release from the co-matrix system had substantially inhibited the levels of calcium on PEG-GABP irrespective of the extent of calcification.

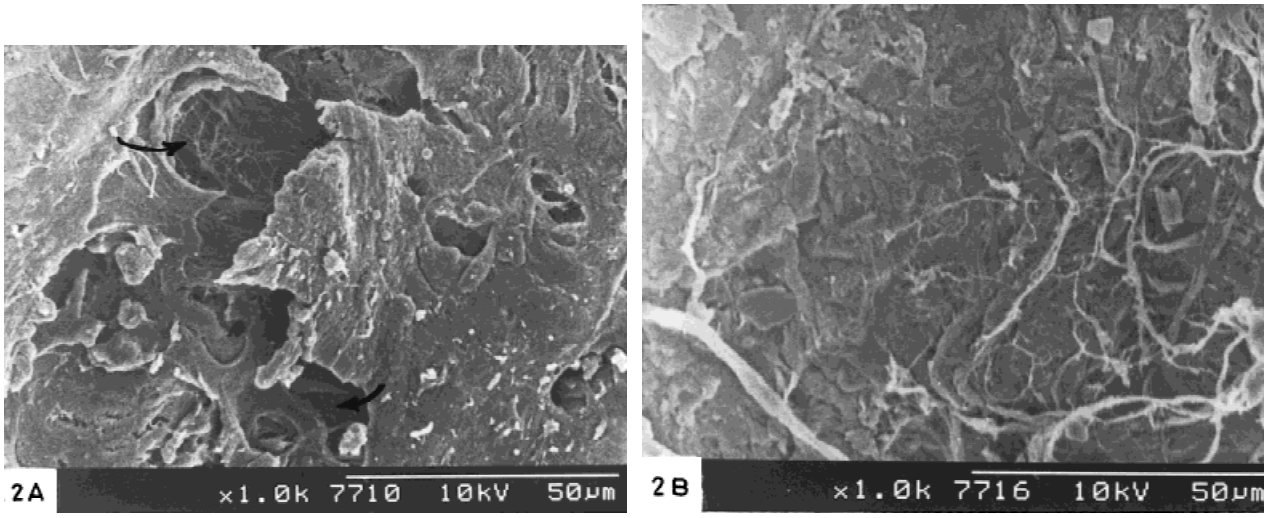

FIG. 2. Shown are scanning electron micrographs of BP, explanted after 21 days subcutaneous implantation in a rat: glutaraldehyde treated BP (GATBP) **(A);** PEG-GABP along with aspirin/heparin delivery from the co-matrix **(B).**

Inhibition of tissue calcification alkaline phosphatase involvement

Alkaline phosphatase activity reproducibly extractable from the explants (after 72 h and 21 days) GATBP, PEG-GABP, and aspirin/heparin on PEG-GABP are shown in Table 2. Explant analysis of PEG grafted GABP with aspirin/heparin delivery revealed that extractable AP activity showed marked reduction compared to GATBP during the period of study.

DISCUSSION

Calcification of biological tissues used to construct substitute cardiac valves has emerged as an important problem affecting their function and long-term durability (1–4). Golomb et al. (22) developed an in vitro model to study the effects of biomaterial associated calcification, and this in vitro model was quantitatively compared with in vivo subcutaneous models. The in vitro model had been sensitive enough to diagnose the biomaterial's propensity to calcify and could serve as a prescreening method to examine calcification mechanisms and methods of prevention. There are many limitations to this in vitro calcification model compared to that of in vivo conditions, which have been elaborated elsewhere (17,18). However, we have incubated major plasma proteins such as albumin, γ-globulin, and fibrinogen in the calcium metastable solution to make it closer to plasma.

The studies from this laboratory have shown that certain trace mediators (e.g., antibiotics, anesthetics, etc.) arriving at the blood material interface can modify the calcification profile and the interfacial phenomena (25,26). The present results suggest that aspirin and heparin can inhibit the deposition of cal-

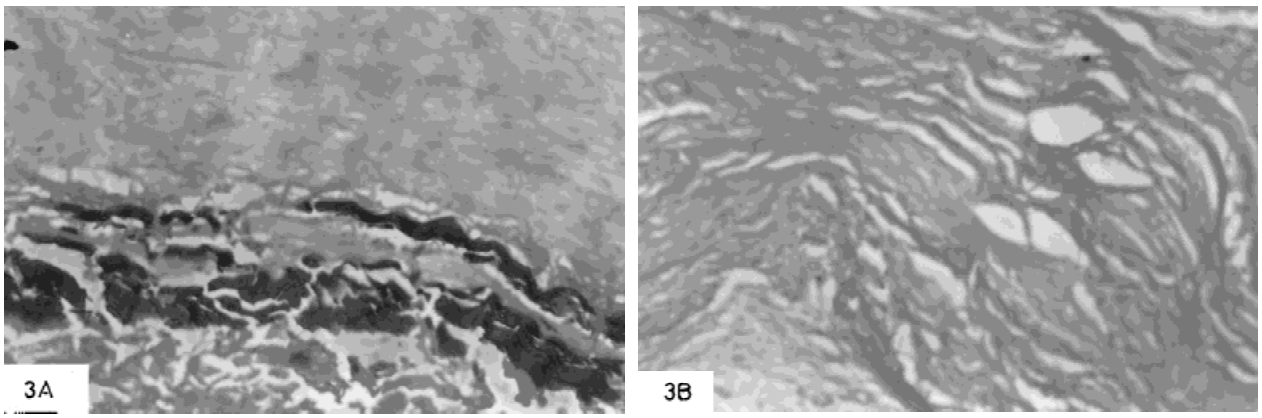

FIG. 3. Histological features of subcutaneous implants of bovine pericardium GATBP **(A)** and PEG-GABP **(B)** along with aspirin/heparin delivery for 21 day post implantation in rats stained with von Kossa are shown (calcium phosphate-Black, original magnification ×50).

TABLE 1. *Amount of calcium deposited on bovine pericardium after 21 days and 6 months of subcutaneous implantation in rats*

Surfaces	Amount of calcium deposited in μg/mg dry tissue	
	21 days	6 months
GATBP	52.4 ± 1.5	240.8 ± 1.6
PEG-GABP (24)	24.1 ± 0.8[a]	51.0 ± 0.2[a]
PEG-GABP + Asp/Hep	3.4 ± 0.6[a]	10.4 ± 2.1[a]

Values are expressed as mean + SD from at least 6 samples.
[a]$p < 0.005$ where values of PEG modified surfaces were compared to GATBP.

cium to GATBP and PEG-GABP, although the exact mechanisms are still unclear.

It is known that aspirin inhibits the platelet release reaction and subsequent adhesion induced by collagenous matrix in vitro. Additionally, aspirin prolongs the bleeding time. Heparin is a natural anticoagulant responsible for maintaining fluidity of blood (16). Therefore, it may be expected that aspirin and heparin can exert antithrombotic effects in those clinical conditions where platelets and proteins play a significant role in the pathogenesis of thrombosis (27). Calcium ions are required for most forms of platelet activation and thrombosis. Further, Vroman (28) indicated that platelets adhere where they find adsorbed fibrinogen. The present studies of aspirin–heparin with protein mixture (albumin, γ-globulin, and fibrinogen) in calcium phosphate solution demonstrated substantial reduction in GATBP calcification (Fig. 1). Further, SEMs of PEG grafted BP coimplanted with aspirin/heparin loaded co-matrix have shown substantial reduction in platelet adhesion as well as calcification compared to GATBP after 21 days of rat subcutaneous implantation (Fig 2A and B). Thus, it is assumed that aspirin/heparin can modulate the surface fibrinogen, and subsequent cellular attachment. This may be another factor for the inhibition of material-associated calcification through cellular involvements. Moreover, heparin carries high -ve charge due to sulfonate groups, which may in turn interact with Ca^{2+} ions blocking them to form HA crystals to some extent (29).

Studies have suggested that polyethylene glycol at the blood–material interface can increase surface hydrophilicity and can reduce protein adsorption as well as platelet adhesion and can develop a passive nonthrombogenic interface (30,31). Recent reports from our lab (18) indicated that PEG grafting to pericardium can modify or mask the platelet receptor sites for collagen, which results in the reduction of platelet collagen attachment. The PEG grafted pericardial matrix has also demonstrated enzymatic stability and reduction in pathologic calcification (24).

The localization of ultrastructural mineral deposits in calcified bioprosthesis to vesicular cell fragments and collagen is comparable to bone (32). Moreover, nearly all forms of cell oriented calcification occur by crystal formation on cell membranes. Extracellular matrix vesicles have both a high concentration of calcium binding acidic phospholipids within their membranes as well as high activity of phosphatases, especially alkaline phosphatase (AP).

The activity of AP is markedly increased in the first 72 h of the GATBP rat subcutaneous implants, during which calcification is initiated (Table 2). This may involve both intrinsic AP activity associated with the site of the initial calcific deposits and absorbed extrinsic AP, which could accrue from inflammatory cells or serum, or both. Adsorbed AP could rapidly hydrolyze the phosphate esters to provide a rapid rise in regional phosphate concentrations, leading to mineralization. This enhanced AP activity appeared to normalize within 21 days of implantation.

The mechanistic relationships between aspirin/heparin mediated mitigation of calcification and inhibition of AP are incompletely understood. However, this mechanism could, in part, be responsible for the reduction of calcification noted in this study. Aspirin/heparin release directly quenches AP activity. Biochemical studies revealed that 72 h explants have intense AP activity adsorbed into and near the surface of GATBP and on tissues with PEG modification. This may be due to the cellular injury associated AP activity due to surgery.

Deposition of extrinsic AP might be important to other types of biomaterial implant calcification. AP is present in blood extracellular fluid and urine; therefore, AP could be readily adsorbed on and absorbed into many implantable biomaterials. Calcification of bioprosthesis may occur in association with

TABLE 2. *Alkaline phosphatase activity of retrieved samples after 72 h and 21 days implantation in rats, represented as the nano mole of para nitrophenol liberated per minute per mg protein (unit)*

Surfaces	AP activity in nm pnp/min/mg protein	
	72 h	21 days
GATBP	280.5 ± 1.2	52.1 ± 1.8
PEG-GABP	225.7 ± 3.9	22.2 ± 1.4[a]
PEG-GABP + Asp/Hep	165.2 ± 16.6[a]	10.2 ± 0.8[a]

Values are expressed as mean ± SD from at least 6 samples.
[a]$p < 0.005$ where values of PEG modified surfaces were compared to GATBP.

the deposition of devitalized cells and cellular debris (6,8) and perhaps adherent blood platelets, which also contain high levels of AP activity. In this case, PEG grafting on BP in combination with aspirin/heparin delivery can substantially inhibit the cellular attachment and subsequently their extrinsic calcification. This may be one of the reasons for reduced AP activity (that localized to devitalized cells), which subsequently reduces calcification. Hence, aspirin/heparin delivery can inhibit the bioprosthesis induced thrombosis and calcification. However, more detailed investigations are needed to understand the possible interrelations of plasma proteins and platelets in surface mediated calcification.

CONCLUSIONS

This study proposes that PEG-GABP with aspirin/heparin delivery can inhibit substantially the tissue associated calcification. The released aspirin/heparin may interfere with the cellular activation or injury to reduce the extrinsic AP activity. This may reduce substantial reduction in AP activity and contribution to mineralization by multiple independent mechanisms. Thus, it is conceivable that a combination therapy via surface modification in parallel with synergistic drug delivery may inhibit tissue associated mineralization. However, this study lacks the in vivo hemodynamic effects in inhibiting calcification noted with aspirin/heparin. More detailed studies are needed to understand the involvement of plasma proteins and cellular components in tissue associated calcification to develop applications.

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