Synergistic inhibition of the calcification of glutaraldehyde pretreated bovine pericardium in a rat subdermal model by $FeCl_3$ and ethanehydroxydiphosphonate: pre-incubation and polymeric controlled release studies

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Calcification is a frequent cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde-pretreated porcine aortic valves or glutaraldehyde-pretreated bovine pericardium (GPBP). We investigated the hypothesis that ferric chloride ($FeCl_3$) and sodium-ethanehydroxydiphosphonate (EHDP) may act synergistically to prevent bioprosthetic tissue calcification. Pre-incubations and controlled release systems were studied individually. $FeCl_3$–EHDP polymeric controlled release matrices were formulated using silicone rubber and evaluated for *in vitro* release kinetics at pH 7.4 and 37°C. The effects of Fe–EHDP synergism on GPBP calcification were investigated with 21 d subdermal implants in 3 wk-old male rats. Results demonstrated that levels of Fe^{3+} and EHDP uptake, measured in GPBP tissues pre-incubated first in an $FeCl_3$ solution (10^{-5} M) followed by an EHDP solution (0.1 M), were higher than in the reverse order of incubation. In the first series of rat implants, GPBP was pre-incubated in either $FeCl_3$ or Na_2EHDP solutions, or sequential pre-incubations of first $FeCl_3$ and then Na_2EHDP solutions, or the reverse. The inhibition of calcification was greatest when $FeCl_3$ (first pre-incubation, 10^{-5} M) was combined with Na_2EHDP (second pre-incubation, 0.1 M) ($1.78 \pm 0.2\ \mu g$ of Ca^{2+}/mg of dried tissue) compared with the other pre-incubation groups: EHDP (first pre-incubation) combined with $FeCl_3$ (second pre-incubation) (21.7 ± 6.4), $FeCl_3$ solution alone at 10^{-5} M (27.9 ± 10.7), Na_2EHDP solution alone at 0.1 M (52.3 ± 11.9) and the control group (72.3 ± 10.2). In a second series of implants, GPBP specimens were co-implanted with individual controlled release systems containing one of the following formulations (weight percentage in silicone rubber): 1% $FeCl_3$, 20% CaEHDP, 20% protamine sulphate, 1% $FeCl_3$–20% CaEHDP, and 1% $FeCl_3$–20% protamine sulphate. The 1% $FeCl_3$–20% CaEHDP silicone-rubber matrices were the most effective for inhibiting GPBP mineralization ($13.7 \pm 3.0\ \mu g$ Ca^{2+}/mg of dried tissue) compared with non-drug silicone co-implant controls ($74.7 \pm 5.58\ \mu g$ Ca^{2+}/mg of dried tissue) and other polymeric treatment groups (32.3 ± 2.3–80.0 ± 19.7). No adverse effects on bone or overall growth of any treatment protocols were noted. Thus, combinations of $FeCl_3$ and EHDP, using either pre-incubations or polymeric controlled release, were synergistic for inhibiting GPBP calcification.

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Calcific degeneration of bioprosthetic heart valves fabricated from glutaraldehyde-pretreated porcine aortic valves or bovine pericardium (GPBP) is a frequent cause of clinical failure[1]. Investigations of the initial calcific foci in bioprosthetic calcification have shown[2, 3] that the first mineral deposits are localized within glutaraldehyde-

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devitalized cells, and within various organelle membrane sites[3]. Collagen calcification occurs later in the development of experimental subdermal GPBP calcification[4]. There is no completely successful strategy for preventing the intrinsic deposition of calcium phosphates within these cardiovascular implants.

Various anticalcification agents, including metallic salts[2,5], detergents[6] and diphosphonates[7-9] have been used in a variety of therapeutic schemes including systemic administrations, pre-incubations or co-implanted polymeric controlled release drug delivery systems. Regional controlled release of anticalcification drugs from drug-polymer composites (known as controlled release matrices) has been demonstrated experimentally to inhibit bioprosthetic calcification. This approach allows local site-specific administration of the drug, thus minimizing dose requirements, while avoiding the possible development of systemic adverse effects.

In previous studies, GPBP pre-incubated in dilute aqueous solutions of either $AlCl_3$ or $FeCl_3$, did not develop pathological mineralization when implanted subdermally in rats[2,5]. The mechanism of Fe^{3+} or Al^{3+} inhibition of GPBP calcification is incompletely understood, but may be due to inhibition of calcium phosphate crystallization or a reduction in alkaline phosphatase (AP) activity, or both. AP is an enzyme of recognized importance in physiological calcification of bone[6], and is present in GPBP calcification. Its activity is also inhibited by GPBP pretreatment with $AlCl_3$ or $FeCl_3$ in rat subdermal implant studies[3]. Similarly, co-implanted controlled release polymeric matrices containing $AlCl_3$ and $FeCl_3$ also inhibit GPBP calcification in other rat subdermal studies[7]. However, the mechanism of action of these agents may also be related to their interactions with phosphorus loci within membranes of devitalized cells[2] and other phosphorus-rich organelles present in GPBP; these sites are thought to be the initial sites of calcium phosphate formation[4,10].

Another group of agents, the diphosphonates, has been investigated for use in the prevention of GPBP calcification[8]. The diphosphonates, pyrophosphate analogues, can bind to developing calcium phosphate crystals, and may effect calcification inhibition due to crystal poisoning effects[11], with resultant limiting of crystal growth. Pre-incubation studies of covalent binding of aminopropanehydroxydiphosphonate (ADPD) to residual aldehyde groups, resulting from glutaraldehyde fixation of GPBP, markedly inhibited calcification of GPBP in rat subdermal implants[9]; however the progressive leaching of ADPD from GPBP implants probably explains the lack of inhibition of calcification in long-term sheep circulatory implants[12]. In related research, sodium ethanehydroxydiphosphonate (Na_2EHDP) controlled release matrices were co-implanted subdermally with GPBP tissue, and also inhibited calcification[13]. However, this same drug used in pre-incubation studies with GPBP did not prevent the mineralization in rat subdermal implants[14]; this lack of efficacy was most likely due to a diffusion of EHDP out of GPBP tissue.

The goals of the present study were to assess the possible synergistic effect of diphosphonates and metallic salts on inhibiting bioprosthetic tissue mineralization. Therefore, the effects of $FeCl_3$ combined with EHDP (either by successive GPBP pre-incubations or co-incorporation in silicone-rubber matrices) were investigated on GPBP calcification in rat subdermal implant experiments.

MATERIALS AND METHODS

Materials

Ferric chloride (reagent grade) and potassium phosphate were purchased from Fisher (Fairlawn, NJ, USA). Protamine sulphate (PS), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES) and calcium chloride were obtained from Sigma (St Louis, MO, USA). Non-radiolabelled and radiolabelled sodium ethanehydroxydiphosphonate ($Na_2[^{14}C]$ EHDP, specific activity of 48.9 μCi/mmol) were donated by Procter and Gamble (Cincinnati, OH, USA). The scintillation fluid, Ecolume, was purchased from ICN Biomedicals (Irvine, CA, USA). A polydimethylsiloxane Silastic Q-74840 was obtained from Dow Corning (Midland, MI, USA). Ketamine hydrochloride (Aveco, Fort Dodge, IA, USA) and Rompun® (Haver, Shawnee, KS, USA) were used for anaesthesia.

Pericardium from mature bovines was obtained fresh at slaughter, and was cross-linked as previously described[4,10] in a 0.6% glutaraldehyde (diluted from an 8% aqueous solution, Polyscience, Warrington, PA, USA) solution buffered with 50 mM HEPES at pH 7.4, then transferred after 24 h to a 0.2% glutaraldehyde solution in the same buffer for storage at 4°C for 1 wk.

Methods

In vitro pre-incubations

GPBP specimens (1 cm^2) were rinsed free of glutaraldehyde with sterile saline and successively pre-incubated at 37°C in the $FeCl_3$ solution (10^{-5} M, pH 3 for 24 h), then in the $Na_2{}^{14}C$-EHDP (0.1 M, pH 7.4 for 24 h), or vice versa. Control specimens of GPBP were pre-incubated in 50 mM HEPES buffer, pH 7.4 for 24 h at 37°C. Afterward, the samples of pre-incubated GPBP were withdrawn, washed with distilled water and lyophilized. Half of the specimens were dissolved in tissue solubilizer (Solvable, DuPont, Boston, MA, USA) at 55-60°C and the $Na_2{}^{14}C$-EHDP was measured. The other specimens were minced, hydrolysed in 6 N HCl as previously described[4,10], and the Fe^{3+} levels were determined by atomic absorption spectroscopy[15].

Controlled release polymer formulation and in vitro *release studies*

All the agents (wt%/matrix) were incorporated as a matrix dispersion in Silastic Q-74840 prepolymer. The silicone-rubber formulations containing the cocipients were prepared by levigating the A and B components of Silastic Q-74840 thoroughly with the drug, followed by compression moulding and curing at 250°C for 1-2 h using a hydraulic press (70.2×10^5 Pa).

The *in vitro* release of agents from slab materials (1 cm^2) was conducted at 37°C, using either an excess of 50 mM HEPES buffer pH 7.4 or K_2HPO_4 buffer (pH 7.4). At each time point, 1 ml samples were withdrawn and the drug release was estimated. The Fe^{3+} content was measured chemically by atomic absorption spectroscopy[15]. The Na_2EHDP content was determined by measuring the

released radioactivity of $Na_2{}^{14}C$-EHDP with a liquid scintillation counter (Model LS 3801, Beckman, USA), and the PS release was analysed by using Lowry protein assay[16].

Implant and retrieval methods

Male weanling rats (50-60 g, CD, Sprague-Dawley, Charles River Laboratories, Burlington, MA, USA) were anaesthetized by an intramuscular injection of Ketamine and Rompun. Six subdermal pouches were dissected as previously described[4, 10] on each rat (four ventral, two dorsal) and GPBP specimens (1 cm^2), washed with distilled water to remove residual glutaraldehyde, were implanted in these pouches, either as isolated GPBP implants or as co-implants with polymeric matrices (1 cm^2, approximately 100 mg), with or without drugs, linked by surgical staples. The rats (two per group) were killed by CO_2 asphyxiation after 21 d. Explanted GPBP were washed with saline and distilled water, lyophilized and hydrolysed in 6 N HCl, as previously described, for calcium analysis by atomic absorption spectroscopy[4, 10].

Morphological analysis

Representative samples of each explanted group were immediately fixed in 2.5% glutaraldehyde, 2% paraformaldehyde and cacodylate buffer, pH 7.4 (Karnovsky's fixative)[17]. Representative specimens of femurs were fixed in 10% neutral buffered formalin. The specimens of valves and bones were dehydrated in graded ethanol solutions and embedded in JB-4 glycolmethylmethacrylate medium (Polysciences, Warrington, PA, USA). Sections 2-3 μm thick were stained with haematoxylin and eosin (for overall morphology) and Von Kossa's reagent (for calcium phosphate).

RESULTS

Maximum uptake of Fe^{3+} and EHDP, and Fe^{3+} dissociation from GPBP tissues

The results presented in *Table 1* demonstrate that the sequence of pre-incubation had a significant effect on the Fe^{3+} and Na_2EHDP content of the GPBP tissues. Initial pre-incubations of GPBP tissues in the $FeCl_3$ solution, followed by the Na_2EHDP solution, resulted in Na_2EHDP uptake an order of magnitude greater than the reverse sequence of pre-incubation.

The Fe^{3+} release from the successive pre-incubations by GPBP tissues are shown in *Figure 1*. Both conditions studied demonstrated their most rapid elution rates during the first 24 h. After this time, a slower nearly constant release rate of Fe^{3+} was noted to be roughly equal in both types of specimens. The Fe^{3+} release rate from the GPBP tissues pre-incubated initially in the $FeCl_3$ solution, then in the Na_2EHDP solution, was almost twice as rapid during the first 24 h as for the GPBP tissues pre-incubated in the reverse order.

In vitro releases of EHDP and Fe^{3+} from the polymeric matrices

The results of *in vitro* release studies of the polymeric matrices containing $FeCl_3$ with CaEHDP or PS are

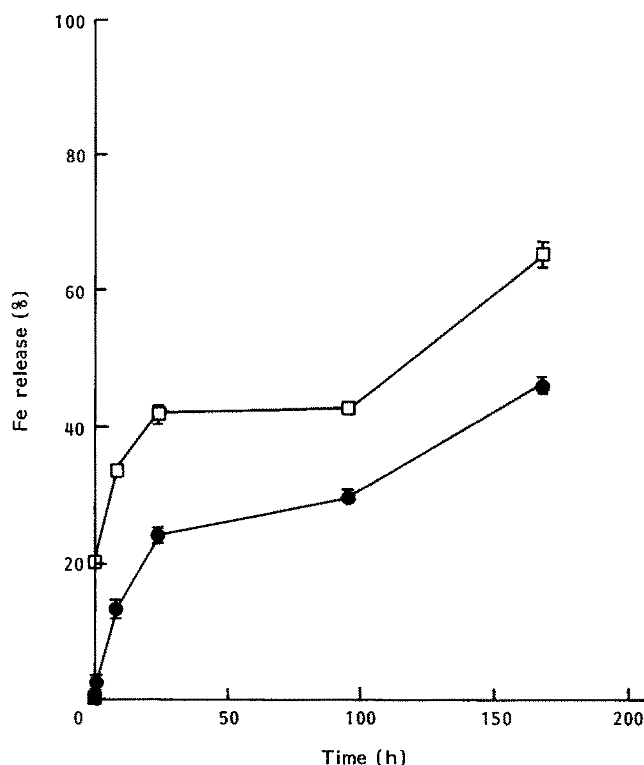

Figure 1 Fe^{3+} release profiles as cumulative percentage from pre-incubated GPBP tissues *in vitro*. Each symbol represents the mean of three specimens. □, $FeCl_3$ (10^{-5} M/first pre-incubation)/Na_2EHDP (0.1 M/second pre-incubation); ●, Na_2EHDP (0.1 M/first pre-incubation)/$FeCl_3$ (10^{-5} M/second pre-incubation).

Table 1 Fe and NaEHDP uptake data before implant and calcium levels after 21 d in GPBP pretreated material

Pre-incubation		Fe uptake*	NaEHDP uptake*	Calcium** following implantation
First	Second			
Control	-	-	-	72.3 ± 10.2 (10)
10^{-5} M $FeCl_3$	0.1 M EHDP	2.5 ± 1.0 (5)	184.3 ± 14.700 (5)	1.8 ± 0.2† (10)
0.1 M EHDP	10^{-5} M $FeCl_3$	2.1 ± 1.1 (5)	16.7 ± 1.100 (5)	21.7 ± 6.4† (10)
10^{-5} M $FeCl_3$	-	1.3 ± 0.2 (5)	-	27.9 ± 10.7 (10)
0.1 M EHDP	-	-	0.1 ± 0.006 (5)	52.3 ± 11.9 (10)

Mean ± SEM (*n*).
*nM/mg dried tissue.
**μg/mg dried tissue.
†$P < 0.0006$ compared with control.

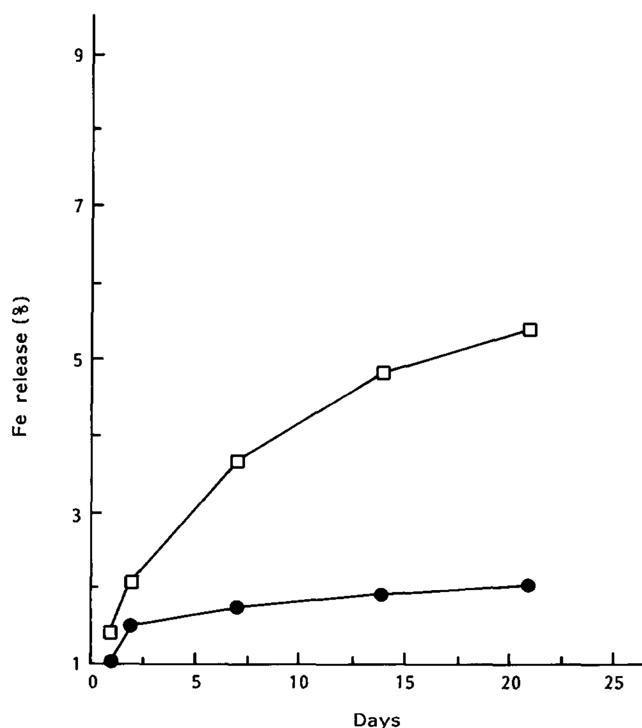

Figure 2 Fe^{3+} release profiles as cumulative percentage from □, 1% $FeCl_3$-20% CaEHDP and ●, 1% $FeCl_3$-20% protamine sulphate (PS) matrices (Silastic rubber polymer) during 21 d.

depicted in *Figure 2*. Relatively slow sustained release of Fe^{3+} was noted with both types of matrices. After 21 d, the Fe^{3+} release from the polymeric matrix containing 1% $FeCl_3$-20% CaEHDP (5%) was almost three times that of the 1% $FeCl_3$-20% PS (2%).

In vivo studies

The calcium levels of 21 d subdermal implantations of GPBP tissues pre-incubated in either $FeCl_3$ or Na_2EHDP solutions, or $FeCl_3$ combined with Na_2EHDP are presented in *Table 1*. Pre-incubation in Na_2EHDP (0.1 M) did not significantly inhibit the calcification (52.3 ± 11.9 μg Ca^{2+}/mg dried tissue) while $FeCl_3$ (10^{-5} M) pre-incubation partially inhibited the calcium accumulation (27.9 ± 10.7 μg Ca^{2+}/mg dried tissue) compared with the control group (72.3 ± 10.2 μg Ca^{2+}/mg dried tissue). The greatest inhibition was observed when the GPBP tissue was first pre-incubated in $FeCl_3$ (10^{-5} M for 24 h) and then in Na_2EHDP (0.1 M for another 24 h) (1.8 ± 0.2 μg Ca^{2+}/mg dried tissue). The reverse order of pre-incubation inhibited calcification less effectively than the previous group (21.7 ± 6.4 μg Ca^{2+}/mg dried tissue). The later calcium level was similar to the $FeCl_3$-only pre-incubation group (*Table 1*).

Co-implantation results of the $FeCl_3$-CaEHDP controlled release systems with GPBP tissues are presented in *Figure 3*. The extensive calcification of the GPBP tissue co-implanted with matrices containing only low-level loadings of $FeCl_3$ or CaEHDP or protamine was comparable with that of the control group, where a non-drug polymer co-implant was used (range of Ca^{2+} = 58.1 ± 10.2-74.7 ± 5.6 μg/mg). The matrices containing 1% $FeCl_3$, with either 20% CaEHDP or 20% PS in the polymer co-implant studies, demonstrated that

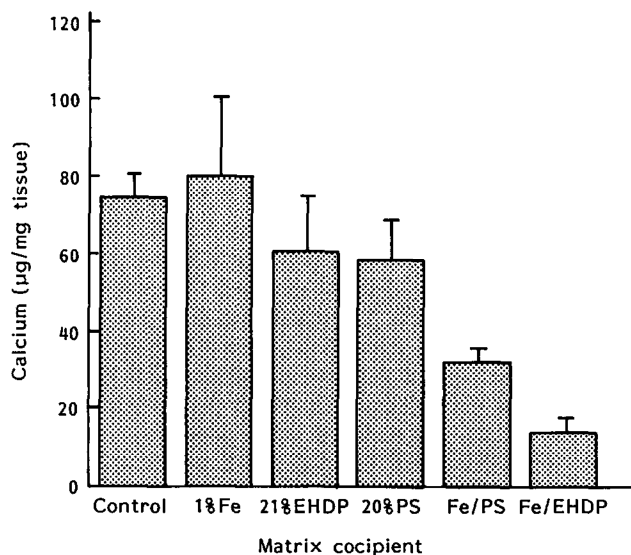

Figure 3 Calcium levels from GPBP tissue explants after co-implantation with various drug-polymer matrices (1% $FeCl_3$, 21% CaEHDP, 20% protamine sulphate (PS), 1% $FeCl_3$-20% protamine sulphate (Fe/PS), 1% $FeCl_3$-20% CaEHDP (Fe/EHDP)) from rat subdermal implants (21 d). Data are shown as means with standard error bars.

the co-drug CaEHDP plus $FeCl_3$ was significantly more efficient in calcification inhibition (13.7 ± 3 μg Ca^{2+}/mg) than the PS (32.3 ± 2.3 μg Ca^{2+}/mg) or the control polymer (non-drug) group (74.7 ± 5.6 μg Ca^{2+}/mg).

Additionally, rat growth was not significantly impaired by either the pretreatments or the silicone-rubber co-implants (weight gain range 95.9-99.1% of control). The morphology of the explanted tissues corroborated the chemical analysis. Tissue co-implanted with the polymer containing CaEHDP plus $FeCl_3$ had markedly less calcification than that of tissue in the control polymer group (*Figure 4*). Stains of this tissue for iron revealed moderate staining for iron in some specimens, localized to intrinsic pericardial cells (result not shown).

DISCUSSION

The present study demonstrated that $FeCl_3$ and EHDP acted synergistically to inhibit bioprosthetic tissue calcification in the rat subdermal model (21 d), whether administered by GPBP pre-incubation or controlled release co-implants. The prevention of GPBP calcification was significantly reduced in both types of inhibition studies: pre-incubations of GPBP tissues in successive $FeCl_3$ (10^{-5} M) and then Na_2EHDP (0.1 M) solutions; and the $FeCl_3$ (1%)-CaEHDP (20%) controlled release system.

The $FeCl_3$-Na_2EHDP pre-incubation synergism results point out the importance of the pre-incubation sequence. The optimal incubation order, $FeCl_3$ followed by Na_2EHDP, allowed a higher uptake of both drugs, $FeCl_3$ and Na_2EHDP, into the GPBP tissue. The reasons for the importance of the sequence of pre-incubation are not clear. Diphosphonates and water molecules may have formed complexes, as previously described[18], and therefore the Fe^{3+} ions are probably less easily incorporated into the tissue in the presence of these complexes. Alternatively, Fe^{3+} may form complex precipitates with

Figure 4 Markedly diminished calcification of tissue co-implanted with polymer containing CaEHDP plus $FeCl_3$ relative to control tissue. Tissues were implanted for 21 d subcutaneously in young rats. **a,** Tissue adjacent to polymeric implant; **b,** control tissue. Von Kossa stain (calcium phosphates black) (original magnification ×150).

GPBP phosphates, which then readily adsorb EHDP, due to ionic attraction. Prior work by our group has in fact used electron energy-loss spectroscopy to demonstrate colocalization of Al^{3+} (as an $AlCl_3$ pre-incubation) to GPBP phosphorus sites in cell membranes. Initial incubation with EHDP may block the metal–phosphorus interaction, leading to lower metal ion and EHDP uptake.

Controlled release of Na_2EHDP has been demonstrated to be effective for inhibiting calcification in the rat subdermal model[13, 19, 20], while the GPBP pre-incubation in Na_2EHDP did not inhibit calcification[14]. Our data (*Figure 3*) show that 21% CaEHDP alone loaded into silicone-rubber matrices did not significantly inhibit GPBP mineralization compared with the control. The calcium salt of EHDP is 1000 times less soluble than the sodium salt[14], so less drug will be released from CaEHDP matrices. Furthermore, other related work involving co-implantation of Fe^{3+} (10% loaded) or Al^{3+} (1 or 10% loaded) controlled release systems with GPBP tissue in the rat subdermal implants (21 d) demonstrated anticalcification efficacy[7]. In this study, Fe^{3+} was loaded at a lower percentage (1%) than the previous studies just cited, so the Fe^{3+} release profile (*Figure 1*) also showed significantly slower release rates compared with earlier results[7]. Nevertheless, efficacy was evidently due to EHDP synergism.

PS is an inhibitor of GPBP calcification, but only when covalently bound to the tissue[21]; its mechanism is hypothesized to be due to charge modification. A previous paper by our group also reported the failure of controlled release PS for calcification inhibition using 10% loading PS in Q-74840 matrices[7]. Therefore, the controlled release PS used in the present study may be viewed as a control cocipient, since our earlier results demonstrated no effective inhibition of GPBP calcification using PS controlled release matrix co-implants.

Pre-incubations of bioprosthetic tissue in trivalent metallic salts such as $FeCl_3$ or $AlCl_3$ prevented GPBP implant calcification[2, 5] in rat subdermal studies. At the same time, explant AP activity was reduced due to $FeCl_3$ or $AlCl_3$ pre-incubation during the first 72 h following implantation compared with the control[3]. In the rat subdermal model of GPBP calcification, AP activity normally has a peak activity at 72 h, at which time calcium phosphate accumulation is initiated. Morphological studies showed that Al^{3+} and Fe^{3+} are localized on the GPBP devitalized cells[2], which are the loci of the initial calcific deposits. Comparable results from bone research studies showed that matrix vesicle mineralization, also associated with AP, was inhibited by metal ions[22]. Matrix vesicles, membrane-enclosed structures, are known to be involved in the initiation of calcification in both endochondral bone and pathological mineralization[23-25], including dystrophic calcifications occurring in atherosclerosis and stenotic native aortic valves[26].

Previous work from our laboratory has demonstrated that the first mineral deposits in the bioprosthetic heart valve subdermal model were most often localized on devitalized cell and organellar membrane locations[4]. Electron energy-loss spectroscopy studies have demonstrated that those organelles are rich in phosphorus[2]. Therefore the presence of the metallic ions and phosphorus at the same site in the tissue suggests that metallic salts may act by inhibiting the initiation of calcification, since phosphorus-containing compounds, such as acidic phospholipid complexes, have been shown to be involved as mineralization initiators in bone and dystrophic calcification[27-29]. Others studies have shown that both Al^{3+} and Fe^{3+} ions inhibit hydroxyapatite formation and dissolu-

tion *in vitro*[30,31]. EHDP synergism must build on this already known phenomenon of Fe^{3+} inhibition, by formation of additional complexes resistant to calcification.

Dystrophic calcification is a multifactorial process, and therefore the best therapeutic strategy may be to seek drug action at different stages of the calcium phosphate deposition. Since the metallic salts are probably involved in suppressing the calcification initiation, and the diphosphonates probably act in the progression stage of mineralization processes, the combination of these two drugs resulted in a synergistic prevention of GPBP calcification. The incorporation of these two types of anticalcification agents in the GPBP tissue by pretreatment or controlled release polymeric matrices may offer a new viable solution to the clinical problem of dystrophic calcification. The controlled release system has the advantage of avoiding adverse systemic side-effects[7,12], and enhancing local drug concentrations. Further studies combining other drugs may help the understanding of the pathological calcification in bioprosthetic heart valves.

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European Society for Biomaterials

10th European Conference on Biomaterials

Davos, Switzerland 8-11 September 1993

The Conference will consist of keynote lectures delivered by invited speakers, thematically selected oral presentations, and lectures from ESB-Award winners. The oral component of the conference will be accompanied by poster presentations, including "last minute" contributions (deadline - end of July). Time has been allotted for the discussion of specific topics.

○ **Conference topics**

- Systemic effects due to implants
- Biodegradable materials
- Interface mechanics
- Interface biology
- Interactions between polymers and cells/tissues
- Polymer-protein interactions
- Bioactive polymers
- Composites & hybrid materials

The scientific program will be supplemented by workshop discussions with a limited number of participants.

○ **Workshop themes**

- **Clinical evaluation of biomaterials**. Coordinator: J. Cordey
- **New trends in internal fixation of fractures**. Coordinator: S.M.Perren
- **Biodegradable implant materials**. Coordinator: S. Gogolewski
- **High resolution techniques in biomaterial evaluation**. Coordinators: S. Downes & H.K. Koerten

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