Prevention of calcification of glutaraldehyde pretreated bovine pericardium through controlled release polymeric implants: studies of Fe^{3+}, Al^{3+}, protamine sulphate and levamisole

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Calcification is the principal cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde pretreated porcine aortic valves or bovine pericardium. The present study investigated controlled-release implants for prevention of the calcification of glutaraldehyde pretreated bovine pericardium in a rat subdermal model. Either Al^{3+} and Fe^{3+} (inhibitors of the growth and dissolution rate of hydroxyapatite crystals), levamisole (alkaline phosphatase inhibitor) or protamine sulphate (charge modifier) were individually incorporated into various polymeric carriers (either silicone rubber, polyurethane or silicone rubber–polyurethane copolymer). Polymeric implants were evaluated for *in vitro* release kinetics, which revealed that sustained drug release was obtained from 21 d to more than 90 d from various drug matrices. *In vivo* efficacy was studied by co-implanting the polymeric delivery systems with glutaraldehyde pretreated bovine pericardium for 21 d using a subdermal rat model; glutaraldehyde pretreated bovine pericardium calcium levels were quantitated by atomic absorption spectroscopy in the explanted tissues. Fe^{3+} and Al^{3+} polymeric implants were the most effective for inhibiting deposition of calcium mineral. Al^{3+} demonstrated 82% inhibition of calcification compared to controls and Fe^{3+} resulted in 80% inhibition of calcification. Specific histologic staining methods showed that Fe^{3+} and Al^{3+} were localized within the devitalized cells of the explanted glutaraldehyde pretreated bovine pericardium. No adverse effects on somatic growth or recipient bone morphology were noted following controlled-release drug administration. Controlled release of protamine sulphate or levamisole did not significantly inhibit glutaraldehyde pretreated bovine pericardium calcification. It is concluded that regional controlled release of Fe^{3+} or Al^{3+} inhibits glutaraldehyde pretreated bovine pericardium calcification in the rat subdermal model without adverse effects.

Keywords: Drug delivery, calcification, prosthetic heart valves

Calcification is the most frequent cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde pretreated porcine aortic valves or bovine pericardium (GPBP)[1-3]. There is no satisfactory means for preventing or treating this disorder. Experimental bioprosthetic heart valve calcification has been thoroughly investigated with circulatory implants in sheep and calves and with subdermal implant models in a variety of species, including rats, rabbits and

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mice[4–6]. Retrieved subdermal bioprosthetic implants yield calcification pathology comparable to that seen in clinical and experimental circulatory explants[3].

Circulatory and subdermal investigations of strategies for preventing bioprosthetic calcification have revealed that a disadvantage for the systemic use of anticalcification agents such as diphosphonates is unavoidable side effects on bone and overall growth. However, controlled-release polymer matrices containing ethanehydroxydiphosphanate (EHDP), co-implanted with GPBP, significantly reduce the extent of calcification in both subdermal and circulatory animal models without adverse effects[7–9]. Site-specific therapy with controlled-release matrices offers the therapeutic advantages of optimally low regional drug levels with minimal systemic side effects.

Other promising anticalcification agents previously demonstrated to be effective as tissue pretreatments have not been studied for their efficacy via controlled release. For example, Al^{3+} pretreatment of GPBP prevents mineralization without generalized side effects[10] in rat subdermal implant studies for 21 and 60 d, and short-term inhibition was also observed with Fe^{3+} pretreatment of the GPBP[11]. The mechanism of action of these metallic ions may be due to their propensity to inhibit the growth of hydroxyapatite crystals[12] and partly due to their inhibitory effect on alkaline phosphatase[13]. Inhibitors of alkaline phosphatase, an important enzyme in physiologic mineralization, may also be of interest, since this enzyme has been shown to be present in porcine and GPBP bioprosthetic tissues both before and following the glutaraldehyde pretreatment[14]. Levamisole is a well known uncompetitive alkaline phosphatase inhibitor which has been shown to inhibit cartilage calcification *in vitro*[15]. Furthermore, covalent binding of protamine sulphate (PS) to GPBP inhibited calcification in the rat subdermal model and the mechanism of action is hypothesized to be due to charge modification[16]. Whether non-bound PS is similarly effective in inhibiting calcification is unknown.

The objectives of the present studies were to formulate and PS as anticalcification agents. Silicone rubber, polyurethane and silicone rubber–polyurethane copolymer were each studied as matrix substrates. *In vitro* drug release studies under perfect sink conditions at pH 7.4 and 37°C were followed by *in vivo* efficacy investigations using a rat subdermal model of GPBP mineralization.

MATERIALS AND METHODS

Reagent grade aluminium nitrate and levamisole were obtained from Aldrich (Milwaukee, WI, USA). Ferric chloride (reagent grade) was purchased from Fisher (Fairlawn, NJ, USA). PS was purchased from Sigma (St Louis, MO, USA). A polydimethylsiloxane Silastic Q-7 4840 and silicone–polyurethane copolymers Silastic 6605-41 and Silastic X-7 3029 were obtained from Dow Corning (Midland, MI, USA). Polyetherpolyurethane (Biomer®) was obtained from Ethicon (Somerville, NJ, USA).

Bovine parietal pericardium from mature bovines was obtained at slaughter and immediately placed in 0.6% glutaraldehyde in 0.05 M 4-2-hydroxyethyl-1-piperazine ethane-sulphonic acid HEPES buffer (pH 7.4) and transferred after 24 h to 0.2% glutaraldehyde in the same buffer for storage at 4°C.

Formulation of polymeric matrices

$Al(NO_3)_3$ and $FeCl_3$ (10 wt% per matrix) were incorporated into Silastic 6605-41 and Biomer by solvent casting. Levamisole was formulated as a matrix dispersion in Silastic 6605-41 (1 wt% levamisole) and Silastic X-7 3029 (10 wt% levamisole) and PS was incorporated as a matrix dispersion (10 wt% PS) in Silastic Q-7 4840 polymer. Silastic 6605-41 levamisole matrices were formulated by combining the appropriate ratio of drug to solid polymer using a 10% polymer solution in dimethyl acetamide. The polymer solution–drug dispersions were then cast as thin films and dried under vacuum at 50°C overnight. Silastic X7-3029 levamisole matrices were formulated by melt casting, first heating the polymer to 80°C, then levigating in the drug at the desired ratio.

For solvent casting, the anticalcification agent and the polymer were separately dissolved in dimethyl acetamide. The solutions were thoroughly mixed together and the films cast were dried under vacuum at 50°C overnight. The dried films were 0.54 ± 0.03 mm thick. The silicone-rubber formulation containing PS was prepared by levigating the prepolymer Silastic Q-7 4840 thoroughly with the drug, followed by compression moulding at 703 070 kg/m^2 at 250°C for 1–2 h using a hydraulic press to form films of 0.87 ± 0.04 mm thickness. Levamisole was incorporated in Silastic X-7 3029 by melt casting at 70°C. The molten mixture was poured into a stainless steel mould and allowed to come to ambient temperature, forming films which were 0.87 ± 0.04 mm thick.

In vitro release

In vitro release was carried out incubating slab materials (1 × 1 cm) at 37°C under perfect sink conditions in either 0.05 M K_2HPO_4 buffer pH 7.4 for levamisole and PS polymeric matrices of 0.05 M HEPES buffer pH 7.4 was used for Al^{3+} and Fe^{3+} matrices. Samples (1 ml) were periodically withdrawn from the vials for levamisole and PS release studies. Levamisole release was estimated by spectrophotometric detection at 213 nm using a Perkin Elmer Lambda 3B spectrophotometer (Norwalk, CT, USA); the PS content was determined by using the Lowry protein assay[17]. For either Al^{3+} or Fe^{3+}, at each time interval a small piece of each polymeric matrix was removed, dried under vacuum and weighed accurately. The Fe^{3+} or Al^{3+} content was then determined on each sample by neutron activation analysis[18].

In vivo studies

Male rats (CD strain, Charles River, Burlington, MA, USA) 21 d old were anaesthetized with an intraperitoneal injection of ketamine and Rompun®. Subcutaneous pouches were created over the anterior abdominal wall, 1 × 1 cm GPBP pieces were implanted in these pouches, either as isolated control of coimplants with polymeric matrices (1 × 1 cm), with or without agents attached with surgical staples. After 21 d rats were killed with CO_2 asphyxiation and the GPBPs explanted, lyophilized and hydrolysed in 6 N HCl, using established procedures[19]. Calcium levels were determined on aliquots of the hydrolysates by atomic absorption spectroscopy[19].

Morphologic assessment

Representative samples of each explanted group were fixed using 2.5% glutaraldehyde and 2.0% paraformaldehyde in cacodylate buffer (pH 7.0) Karnovsky's fixative[20]. Fixed specimens were dehydrated in graded ethanol and embedded in glycolmethylethacrylate. Sections 3 μ thick were stained

with haematoxylin and eosin for light microscopy. The von Kossa stain was used to demonstrate calcium phosphates and the Prussian blue stain was used to demonstrate iron deposition. Representative samples of the femurs obtained were fixed in neutral buffered formalin and examined for their morphology after both von Kossa and haematoxylin and eosin staining. The polymeric matrices pre- and post-*in vivo* release were studied for their morphology with scanning electron microscopy (SEM). Polymer surfaces were gold sputtered-coated and imaged using a Hitachi, Model S-570 (Santa Clara, CA, USA) SEM.

RESULTS

In vitro release

Release kinetic studies showed that all formulations exhibited an initial burst phase of release, followed by an exponentially decreasing release rate. The results of the *in vitro* release studies of the polymeric matrices are depicted in *Figures 1* and *2* for the Al^{3+} and Fe^{3+} polymers and the levamisole and PS polymers, respectively. Release rates were more rapid from Biomer matrices containing the Al^{3+} and Fe^{3+} than from the Silastic 6605-41. Furthermore, Fe^{3+} was completely released sooner than Al^{3+} from both types of polymers studied (Biomer and Silastic 6605-41). Fe^{3+} and Al^{3+} were released in a sustained manner for up to 80 d (*Figure 1*). Levamisole and PS matrices were observed to have relatively less rapid early release compared to the Al^{3+} and Fe^{3+} matrices. However, there was no significant difference in the release pattern of levamisole from the two different copolymers studied, i.e. Silastic 6605-41 and Silastic X-7 3029. The PS formulation demonstrated a minimal early burst effect and PS was released in a sustained fashion for more than 100 d. By comparison, levamisole was depleted in 70 d from the Silastic 6605-41 and Silastic X-7 3029 matrices.

Average release rates *in vitro* over 21 d and the estimated average dose in rats were calculated, based upon release data (*Table 1*). These data indicated that the average doses of Fe^{3+} and Al^{3+} were comparable, regardless of the polymer matrix. However, in view of the relatively rapid release of Al^{3+} and Fe^{3+} from Biomer (*Figure 1*), the predicted dosage per implant was expected to be completely delivered midway through the 21 d implant period. Furthermore, the two levamisole preparations provided estimated dosages which differed twenty fold.

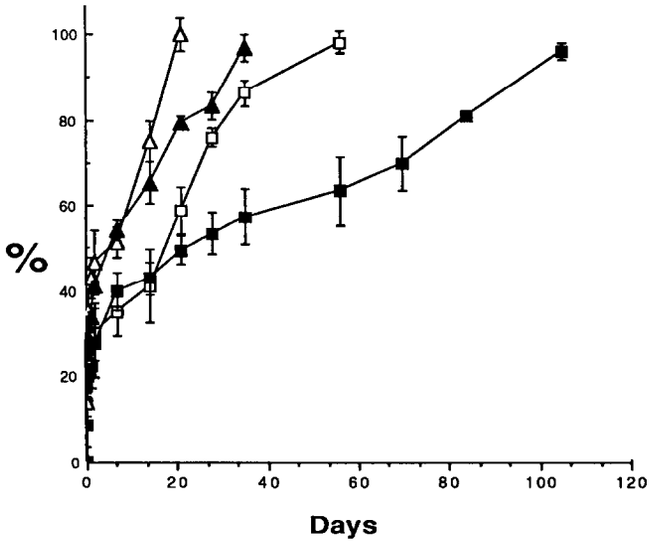

Figure 1 Fe^{3+} *and* Al^{3+} *cumulative release profiles from silicone rubber polyurethane copolymer and Biomer® matrices. (□)* $FeCl_3$ *(10%) in Silastic 6605-41, (△)* $FeCl_3$ *(10%) in Biomer®, (■)* $Al(NO_3)_3$ *(1%) in Silastic 6605-41, (▲)* $Al(NO_3)_3$ *(10%) in Biomer.*

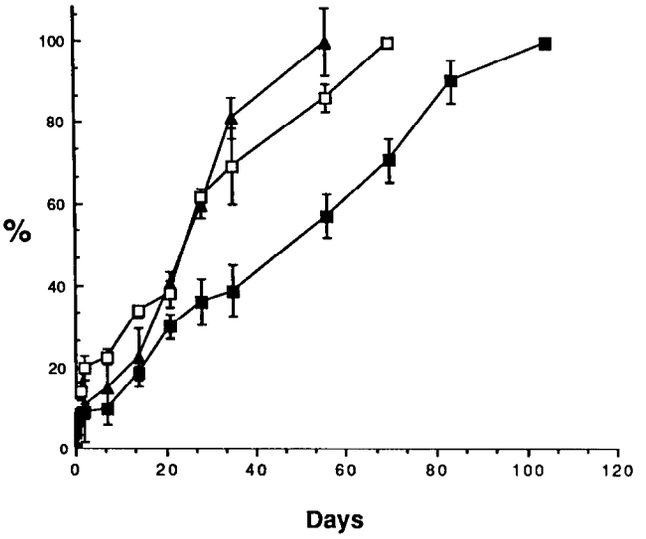

Figure 2 Levamisole and PS release profiles from silicone rubber-polyurethane copolymers and silastic rubber-polymer matrices. (▲) Levamisole (10%) in Silastic 6605-41, (□) levamisole (10%) in Silastic X-7 3029, (■) PS (10%) in Silastic Q-7 4840.

Table 1 Mean release rates and estimated dosages from slab matrices in vitro *(1 × 1 × 0.05 cm)*

Agent	Polymer	*N*	Average *in vitro* release rate (μg/24 h ± s.d.)	Estimated average dose in rats (μg/kg/24 h)
Fe^{3+}	Biomer	5	214.49 ± 41.69	857.96
	Silastic 6605-41	5	156.59 ± 53.48	626.36
Al^{3+}	Biomer	5	178.14 ± 29.72	712.56
	Silastic 6605-41	5	141.69 ± 9.97	566.76
Levamisole	Silastic 6605-41	5	10.43 ± 1.86	41.72
	Silastic X-7 3029	5	232.24 ± 19.67	928.96
Protamine sulphate	Silastic Q7-4840	5	154.38 ± 23.39	617.52

In vivo studies

Results of *in vivo* evaluation of different polymeric implants are presented in *Table 2*. There was significant inhibition of calcification due to either Fe^{3+} or Al^{3+} ($P < 0.001$), when incorporated in the Silastic 6605-41 matrices. When compared with the control values, > 80% calcification inhibition occurred, due to either 6605-41 matrix system of Fe^{3+} or Al^{3+}. In addition, it was also observed that when Al^{3+} but not Fe^{3+} was incorporated into Biomer matrices, there was also significant calcification inhibition, although less than observed with the 6605-41 matrices. The Ca levels were 60 and 38% of control for explant for the animals receiving Fe^{3+} and Al^{3+}, respectively, in Biomer matrices. Ca^{2+} levels in the GPBP, implanted in close proximity to either the levamisole or PS matrices, were not statistically different from the control explanted GPBP. Further, none of the animals experienced any adverse effects on somatic growth of bone development due to the administration of the controlled-release drug administration.

Table 2 Controlled release calcification inhibition: results of glutaraldehyde pretreated bovine pericardium (GPBP) subdermal implant studies in rats

Cocipient	Polymer	N	Calcium levels in explanted GPBP μg/mg of dried tissue	% calcification in the explanted GPBP compared to control
$FeCl_3$	6605-41	10	14.25 ± 2.91	19.26*
$FeCl_3$	Biomer	10	44.67 ± 8.04	60.38
$Al(NO_3)_3$	6605-41	10	14.28 ± 5.78	17.87
$Al(NO_3)_3$	Biomer	10	31.04 ± 5.48	38.85
Levamisole	6605-41	10	39.96 ± 4.81	77.15
Levamisole	X-7 3029	10	31.69 ± 7.12	61.18
Protamine sulphate	Q-7 4840	10	71.04 ± 5.29	83.45
No drug	6605-41	30	52.27 ± 9.35	74.70
No drug	Biomer	20	64.20 ± 10.24	91.16
No drug	Q-7 4840	10	68.08 ± 16.66	96.67
No drug	X-7 3029	10	48.04 ± 8.37	68.21
Control GPBP	No polymer	40	69.97 ± 10.76	100.00

*$P < 0.001$.

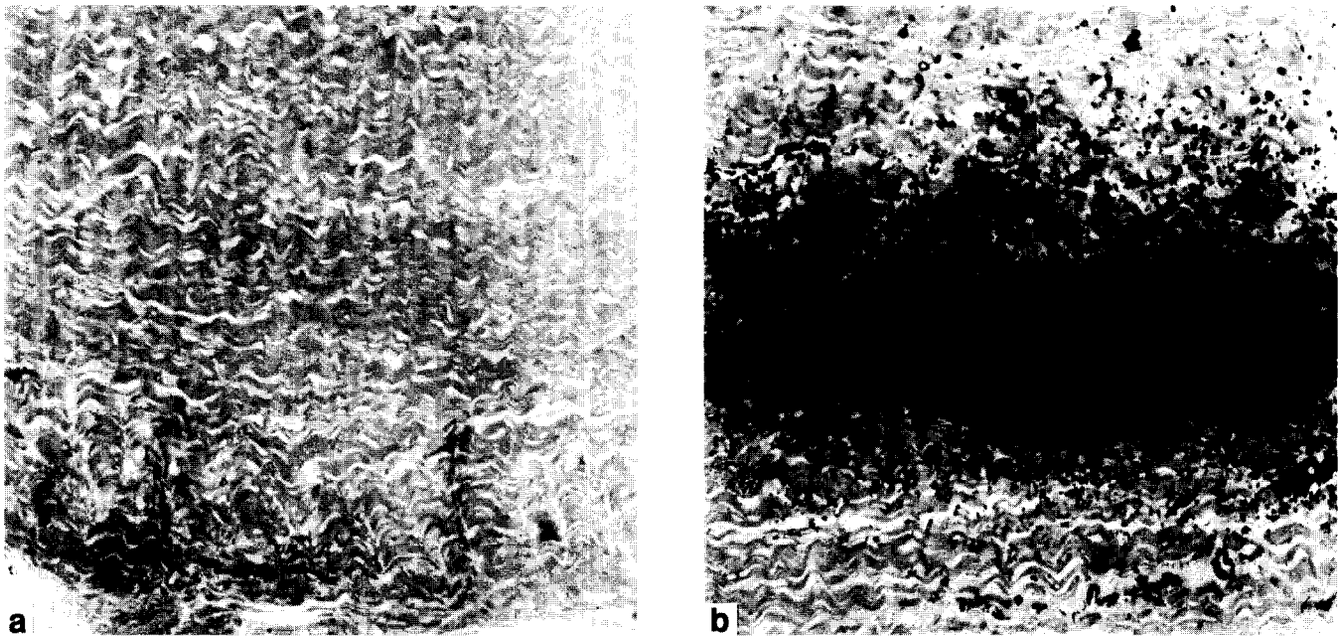

Figure 3 Inhibition of calcification by incubation of GPBP in solutions of iron-containing compounds, demonstrated by removed specimens of bovine pericardium, implanted subdermally in rats for 21 d. (a) $FeCl_3$ (0.1 M) plus citrate (0.1 M) treated implant[11] with no visible mineralization, (b) control implant with severe intrinsic mineralization. Both stained with von Kossa's reagent (calcium phosphates in black) (original magnification × 375).

Light microscopic examination of the explanted GPBP revealed a typical intrinsic distribution of the calcium phosphate deposits in the control material and, to a lesser extent, in the explants exposed to calcification inhibitors (*Figure 3*). Fe^{3+} specific staining demonstrated Fe^{3+} localization within the devitalized cells of the GPBP (*Figure 4*). Further, no abnormalities were noted in the femoral specimens from any of the treatment groups compared to controls.

The SEM study of the polymeric matrices (*Figure 5*) showed that the anticalcification agents were uniformly distributed in the matrix materials before release (*Figure 5a* for Fe^{3+} matrix). The post-*in vivo* Fe^{3+} matrix SEM showed partial depletion of the agent from the matrix material, with features demonstrating cocipient particle swelling, dissolution and cavitation.

DISCUSSION

Superior anticalcification efficacy was noted for both Al^{3+} and Fe^{3+} matrices. Previous work has shown that GPBP tissue pre-incubated in Al^{3+} or Fe^{3+} calcified significantly less than the control. The mechanism of action of Al^{3+} may be due to localization of Al^{3+} within the devitalized cells of the GPBP as demonstrated previously[10] in GPBP pre-incubated in Al^{3+}. Previous work also has demonstrated that the intrinsic cells of GPBP, which are fixed in place by glutaraldehyde, are the initial sites of calcification[21]. Thus, Al^{3+} perhaps bound at phosphorous loci within the devitalized cells may serve to block calcium phosphate formation or arrest crystal growth, as demonstrated by others *in vitro*[22]. Fe^{3+} localization within the devitalized cells in GPBP was demonstrated in the present study and suggests that Fe^{3+} may act to inhibit GPBP calcification by a mechanism similar to Al^{3+}.

The *in vitro* release kinetics observed for various matrix systems were useful in understanding some of the differences noted in the *in vivo* results. Comparing the *in vitro* and *in vivo* data suggests that 80% of the Fe^{3+} and Al^{3+} was released from the Biomer implants within 21 d, of which > 50% of the drug release was provided in the first few hours, due to the burst effect. This could lead to inadequate levels of Fe^{3+}

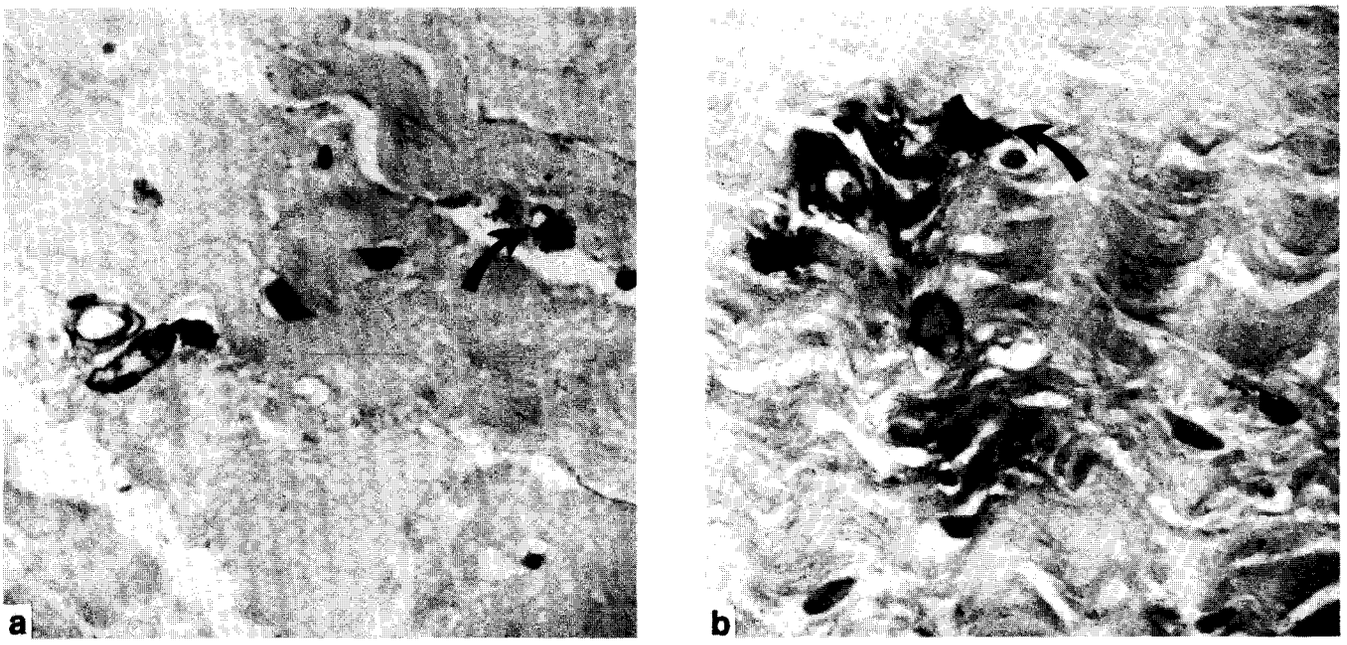

Figure 4 Light histologic demonstration of iron localization in GPBP incubated in iron solutions and implanted 21 d subdermally in the rat. (a) Pretreatment (24 h) with $FeCl_3$ (0.1 M) and (b) pretreatment with $FeCl_3$ plus citrate (0.1 M) to enhance uptake[11], iron is visualized in the connective tissue cells, as fine, punctate, cell-oriented deposits (arrows). Both stained using the Prussian blue technique (iron black) (original magnification × 600).

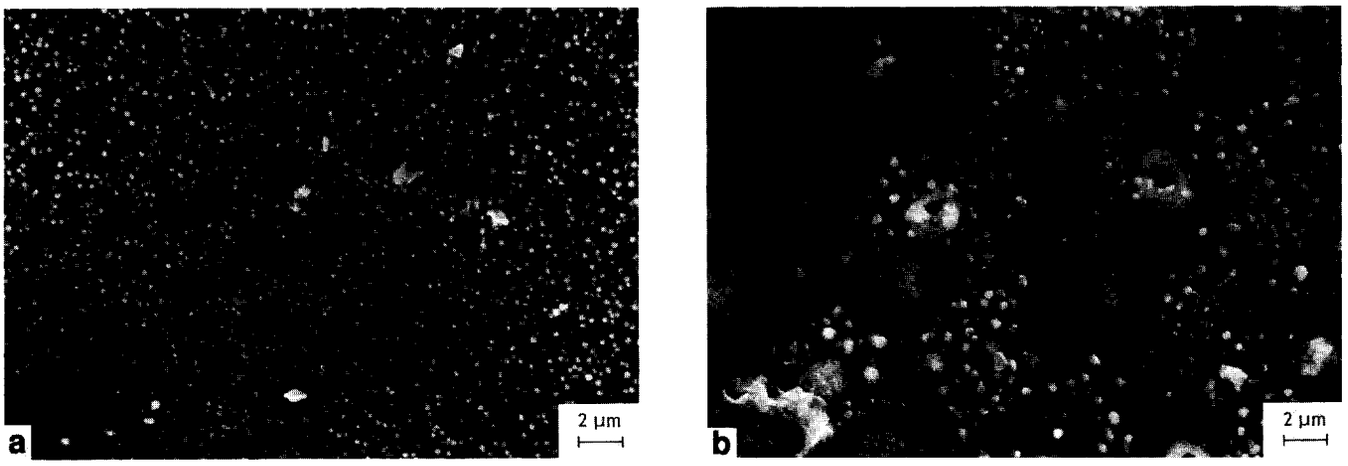

Figure 5 SEMs of Fe^{3+} silicone rubber-polyurethane copolymer matrix: (a) 10% $FeCl_3$ in the matrix material before release and (b) polymer explanted after 21 d in the rate subdermal study. The post-release matrix demonstrates cocipient particle swelling, dissolution and cavitation.

and Al^{3+} for sustained anticalcification activity and may explain the higher Ca^{2+} levels in the explanted pericardial tissues from the rats in which Al^{3+} and Fe^{3+} Biomer matrices were implanted next to GPBP. However, the Fe^{3+} and Al^{3+} Silastic 6605-41 matrices demonstrated that only 50% of the total drug was released in 21 d and hence there would be predicted to be a continuous supply of the Fe^{3+} and Al^{3+} leading to significantly lower Ca^{2+} levels in the GPBP explants exposed to these dosage forms. Thus, release kinetics varied, depending on the type of matrix polymer and also reflected the rectangular slab matrix geometry in terms of the plateau phenomenon noted during depletion. SEMs confirmed the release mechanism to be due to particle dissolution-related events previously noted for monolithic matrices[8, 9].

Levamisole, a reversible non-active site inhibitor of alkaline phosphatase, did not significantly inhibit calcification when administered by controlled release. However, the drug release form the levamisole matrices was nearly constant and continuous after 21 d *in vitro*. Thus, perhaps either a more potent alkaline phosphatase inhibitor than levamisole or higher dosages of levamisole would be more effective.

Previous work demonstrated that PS too effectively inhibits GPBP calcification when covalently bonded to the cross-linked GPBP. The mechanism of action of PS has been hypothesized[16] to be due to charge modification, i.e. imparting a net positive charge to GPBP, tending to repel Ca^{2+}. The work failed to find an inhibitory effect of controlled-release PS. Thus, direct covalent modification of GPBP by compounds such as PS may be required in order for this therapeutic approach to be effective.

Further research is needed to establish the efficacy of Fe^{3+} delivery system for longer durations as well as in the circulatory system. Fe^{3+} controlled release would be preferred over Al^{3+} since, despite the very low doses of Al^{3+} used, Al^{3+} is of concern as a central nervous system toxin which may have a causal role in Alzheimer's Disease and other neurological disorders[23]. The previous results have showed that there is synergism between Al^{3+} and EHDP, preventing bioprosthetic valve calcification. If this synergism is possible

between Fe^{3+} and EHDP, then this drug combination might be more effective as an anticalcification formulation than either agent alone. Furthermore, since Fe^{3+} pretreatment has been shown to be effective in short-term subdermal implants[11], an optimal approach might be to preload the GPBP leaflets with Fe^{3+} as a primary dose and couple this with a controlled-release matrix for continuously available Fe^{3+}.

In conclusion, controlled-release matrices containing Fe^{3+}, Al^{3+}, levamisole and PS were successfully formulated. However, only the Fe^{3+} and Al^{3+} with the most sustained release properties optimally inhibited GPBP calcification. Furthermore, since Fe^{3+} or Al^{3+} was localized to the devitalized cells of the explanted GPBP, their mechanism of action may be to inhibit the initial calcific deposits in these sites.

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