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The efficacy of a dual anticalcification agent treatment in the prevention of calcification of glutaraldehyde–fixed porcine pericardium

E. KHOR, A. WEE*, B. L. TAN‡, T. Y. CHEW
*Departments of Chemistry and *Pathology, National University of Singapore, Kent Ridge, Singapore 0511*

The efficacy of a series of combined anticalcification treatments utilizing a chloroform–methanol mixture or a surfactant, sodium dodecyl sulfate, followed by a trivalent metal ion Fe(III) were evaluated with glutaraldehyde-treated porcine pericardium. The results indicate that the combined treatments are comparable in the reduction of calcification levels to that of the metal ion alone. Furthermore, pretreatment with the organic reagents improves the residence time of Fe(III) in the tissue. The results suggest that the combination approach offers a basis for improved mitigation of calcification in glutaraldehyde–treated tissue over that so far found in single systems.

1. Introduction

Bioprosthetic heart valves (BHV) have been shown to have desirable properties over mechanical heart valves (MHV) in some clinical situations. These include excellent hemodynamics; minimal occurrence of hemolysis and thromboembolism; and in most instances patients do not require postoperative anticoagulation therapy [1–3]. However, limited long-term durability is the major disadvantage of BHV. The primary cause of failure of BHVs is attributed to dystrophic calcification of the bioprosthetic tissue. This may lead to valve incompetence and stenosis, eventually requiring removal of the BHV [4].

Dystrophic calcification in bioprosthetic tissue is attributed to a multitude of factors believed to be a consequence of the glutaraldehyde fixation process used to stabilize the tissue [4]. The tissue undergoes structural changes such as the loss of proteoglycan material in the midsection of the leaflet, giving rise to empty spaces that can trap blood elements, predisposing the area to calcification [4–8]. This situation is further aggravated by mechanical stress, which leads to cuspal tears and perforations. Grabenwoger [5] noted that degenerative changes in BHVs were enhanced by mechanical stress and phagocytosis of collagen material in the vicinity of tears. However, glutaraldehyde fixation remains the most successful method for stabilizing biological tissue used in bioprostheses. Therefore, controlling calcification in glutaraldehyde-based bioprostheses remains the preferred option for the commercial viability of bioprosthetic heart valves.

To date, many potential methods have been identified to control the calcification of biological tissue intended for bioprostheses use in various *in vitro* and *in vivo* models. Predominant among the strategies are the use of chemical-based agents that are postulated to interrupt various points of the mineralization events [9]. Chemicals reported to show encouraging results in the prevention of calcification in small animal models include metal ions such as Fe^{3+} and Al^{3+} [10–13], surfactants such as triton X, sodium dodecyl sulfate (SDS) and Tween 80 [13–22] and the diphosphonates [23]. The mechanism of anticalcification action of Fe^{3+} is believed to be in competition with calcium in the binding of phosphates thereby reducing the formation of calcium phosphate crystals. In the case of anionic surfactants, removal of phospholipids, which attract calcium ions, possibly explains its mode of action. Alternative hypotheses for the anticalcification action of anionic surfactants include modification of surface charges, and prevention of influx of phosphates into the tissue. Jorge-Herrero *et al.* [16, 17] found that a mixture of chloroform and methanol, which is known to extract phospholipids from biological tissue, also reduces calcification.

Regardless of the mode of action, it is obvious that in order to control calcification effectively, a combined treatment with two or more chemical agents that would interfere with more than one mineralization process, would be the best approach to calcification prevention in glutaraldehyde-treated tissue. This idea has some support in recent work reported by Hirsch *et al.* [10] where treatment with iron was followed by on-site drug delivery of diphosphonates. Therefore, in order to gain an insight into the usefulness of a combination treatment, we report our study in using a surfactant or chloroform–methanol mixture followed in

‡ *Permanent address*: St. Vincents Meditech Pte. Ltd., 51A The Faraday, Science Park, Singapore 0511

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tandem with a solution of Fe^{3+} in the mitigation of calcification in glutaraldehyde-fixed porcine pericardium. Porcine pericardium was chosen because of its ready availability in this region.

2. Materials and methods

Freshly harvested porcine pericardium was trimmed of adherent fat, cut into 1 × 1 cm square pieces, rinsed with sterile HEPES-buffered saline and treated in one of the following ways:

0.45% glutaraldehyde [GA]	Treatment O
0.45% glutaraldehyde (24 h), 0.1 M $FeCl_3$ (1 h)	Treatment A
1% HEPES-buffered sodium dodecyl sulfate [SDS] (3 h), 0.45% GA (24 h)	Treatment B
Chloroform–methanol mixture [CM] 1:5(v/v) (3 h) in a nitrogen atmosphere, 0.45% GA (24 h)	Treatment C
1% SDS (3 h), 0.45% GA (24 h), 0.1 M $FeCl_3$ (1 h)	Treatment D
CM 1:5(v/v) (3 h), 0.45% GA (24 h), 0.1 M $FeCl_3$ (1 h)	Treatment E

All treatments were performed at room temperature except GA, which was carried out at 4 °C.

The variously treated pericardium pieces (15 per treatment, randomly distributed) were implanted subcutaneously in the abdominal region of 3-week-old male sprague dawley rats. Each animal received four implants. At 15 or 30 days, the specimens were retrieved and rinsed thoroughly with distilled water. Samples were subsequently dried at 110 °C for 24 h, weighed and hydrolysed in 2 ml of 6N HCl for 24 h at 85 °C. The hydrolysates were analysed for calcium content by atomic absorption spectroscopy, phosphate content by uv-vis spectrophotometry assay according to the method of Chen [25] and iron content by inductively coupled plasma atomic emission spectrometry. Statistical analyses were performed on the results of elemental analyses using SigmaStat® software. Kruskal–Wallis one-way analysis on ranks was used to determine statistical significance while the Mann–Whitney rank sum test was used for pairwise comparisons: $p < 0.05$ was considered statistically significant.

Histology was performed on retrieved samples washed in buffered saline and distilled water, fixed in 10% buffered formalin and routinely processed. Paraffin-embedded histological sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E). Selected samples were stained with Perls' prussian blue method for iron.

3. Results

3.1. Calcium analyses

The results of the calcium content analyses of the variously treated samples are summarized in Fig. 1.

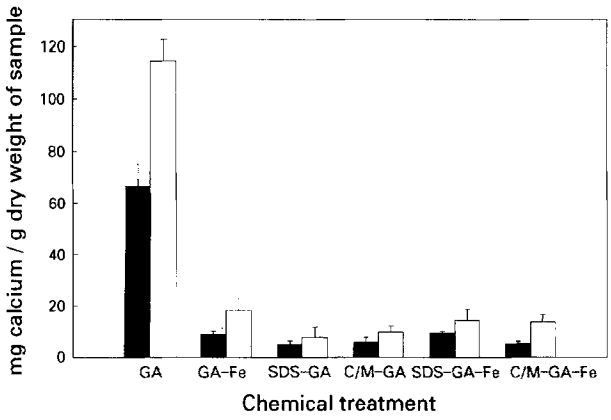

Figure 1 Calcium levels in retrieved porcine pericardium: 15 days ■; 30 days □ ($n \geqslant 10$; background calcium 0.96 mg/g).

All treatments (combined and individual) show a marked reduction in calcium content compared to the glutaraldehyde control (> 80%). Our results are in general agreement with those reported for Fe^{3+}, SDS and CM in other studies [13, 16–20, 22]. A comparison of the two time periods indicate that these treatments delay the onset, as well as reduce the extent of calcification, but do not prevent its absolute occurrence.

The Kruskal–Wallis one-way analysis on ranks evaluation was performed on the calcium analyses. The H statistic for 15-days time period was determined to be $37.145 > \chi^2$ distribution values with five degrees of freedom at 1% significant level = 20.517 and $p < 0.001$. The H statistic for the 30-days period was $34.942 > \chi^2$ distribution value with five degrees of freedom at 1% significant level = 20.517 and $p < 0.001$. The evaluation confirms that there is a statistically significant difference in the median values among the treatment groups, greater than would be expected by chance for the data of the two time periods.

The results of the Mann–Whitney rank sum evaluation to contrast the pairs of treatment (Table I) show that the combined treatment D (SDS–GA–Fe^{3+}) does no better than that of the individual treatment A (GA–Fe^{3+}) and exhibits lower anticalcification behaviour than the individual treatment B (SDS–GA) for both time periods. However, for the 15-days' results, the combined treatment E (CM–GA–Fe^{3+}) shows significant improvement over that of individual treatment A, but is comparable to that of individual treatment C (CM–GA). In the 30-days' results, this combined treatment E is comparable to both individual treatments A and C.

TABLE I Mann–Whitney rank sum tests of calcium content between pairs of treatments

Comparison	Day 15	Day 30
A (GA–Fe^{3+}) versus D (SDS–GA–Fe^{3+})	NS[a]	NS
B (SDS–GA) versus D (SDS–GA–Fe^{3+})	S[b]	S
A (GA–Fe^{3+}) versus E (CM–GA–Fe^{3+})	S	NS
C (CM–GA) versus D (CM–GA–Fe^{3+})	NS	NS

[a] NS = no significant difference
[b] S = significant difference

3.2. Phosphate analyses

The results of the phosphate content analyses of the variously treated samples are summarized in Fig. 2. A similar trend to that of calcium is observed. The presence of phosphate in tissue does not necessarily reflect the extent of calcification as phosphate can exist as inorganic or organic phosphates.

3.3. Iron analyses

The results of the iron content analyses of the Fe^{3+}-treated samples are summarized in Fig. 3. The data shows that a significant amount of Fe^{3+} is taken up by treatment D tissue. The Fe^{3+} uptake of treatment A and E samples were found to be comparable. The Fe^{3+} retention for treatment A shows a steady decrease with time. However, specimens subjected to the combination treatments D and E show an initial drastic drop of Fe^{3+} between the 0 and 15-days period but retain Fe^{3+} much better over the next time period, with a drop of 13.9 % for treatment D and essentially no change for treatment E.

The Mann–Whitney rank sum test results (Table II) confirms these observations, i.e. significant uptake of Fe^{3+} ions for treatment D and large uptake for treatments A and E. Another conclusion which can be drawn from the test results is that for the 30-days' period, the loss in iron content in treatment A is significantly more than that for the combination treatments D or E.

3.4. Histology

Macroscopic examination of the dried GA-treated porcine pericardium samples showed a white layer attributed to calcium phosphates on the surface. This was not found for all the other samples (A–E).

For samples removed after 15 days implantation, all specimens except the control were generally intact with no evidence of dystrophic calcification. Extensive shrinkage of the collagenous tissue in the fibrous layers of specimens treated with SDS singly (treatment B) or in combination with Fe^{3+} (treatment D) was found. However, no adverse tissue effects were found for specimens from treatment C or E. A representative photomicrograph of the control (Fig. 4a) shows a peripheral zone of calcification indicated by the darker region. In contrast, a representative photomicrograph for the other samples (A–E) does not (Fig. 4b). Of the samples treated with Fe^{3+}, specimens from treatments A and E showed a linear brownish discoloration on the surface of the fibrous layer that stained positive for iron with Perls' prussian blue method. In the specimen from treatment D, the collagenous tissue stained diffusely blue with Perls' method for iron. For the 30-days' implantation, extensive shrinkage of the collagenous tissue was again found in specimens subjected to treatments B and D. Extensive band-like calcification was noted in specimens from the control (treatment O) and treatment C, whereas focal calcification was noted in the tissue subjected to treatment A (Fe^{3+}). The staining

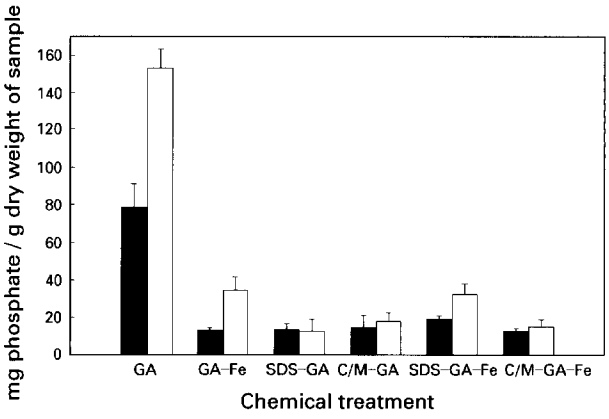

Figure 2 Phosphate levels in retrieved porcine pericardium: 15 days ■; 30 days □ ($n \geqslant 10$; background phosphate 0.78 mg/g).

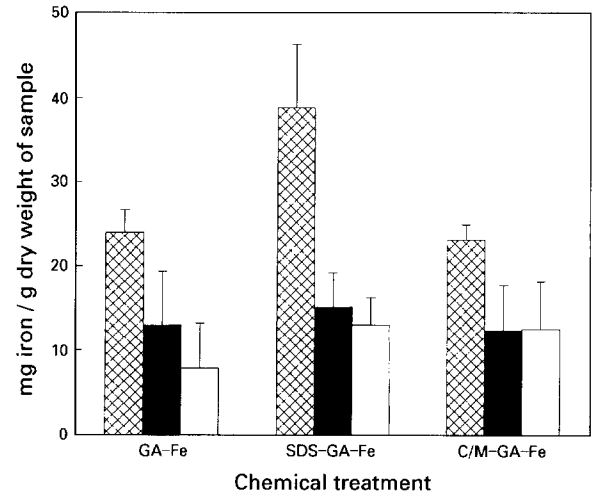

Figure 3 Iron (III) levels in retrieved porcine pericardium: 0 days ⊠; 15 days ■; 30 days □ ($n \geqslant 10$; background iron 0.0015 mg/g).

patterns for iron were similar to the respective specimens removed at 15 days.

A summary of the results is given in Table III.

4. Discussion

Considering first the comparison with SDS, the Mann–Whitney statistic shows no notable difference in the calcium levels between tissue from treatment D, i.e. SDS followed by Fe^{3+}, over the single treatment A with Fe^{3+} only. When compared to treatment B where only SDS is used, treatment D's anticalcification effect fares worse. This outcome is perplexing because the Fe^{3+} levels of the treatment D compared to treatment A are relatively high for both the 15 and 30 day periods. The results indicate first, that there is apparently no synergistic mechanism operative using these two agents in combination. Second, the presence of Fe^{3+} has a neutralizing effect, or masks the anticalcification action of SDS alone. Therefore, the degree that Fe^{3+} exhibits calcification prevention determines the onset of calcification in treatment D samples. That SDS is better at calcification prevention may indicate that removal of phospholipid is more efficient than repulsion of Ca^{2+} ions.

An interesting aspect of treatment D is the high concentration of Fe^{3+}. A possible explanation may lie

TABLE II Mann–Whitney rank sum tests of iron(III) content between pairs of treatments

Comparison	Day 0	Day 15	Day 30	Day 15 versus day 30
A (GA–Fe^{3+}) versus D (SDS–GA–Fe^{3+})	S[a]	NS[b]	S	—
B (SDS–GA) versus D (SDS–GA–Fe^{3+})	—	S	S	—
A (GA–Fe^{3+}) versus E (CM–GA–Fe^{3+})	NS	NS	S	—
C (CM–GA) versus E (CM–GA–Fe^{3+})	—	S	S	—
A (GA–Fe^{3+})	—	—	—	S
D (SDS–GA–Fe^{3+})	—	—	—	NS
E (CM–GA–Fe^{3+})	—	—	—	NS

[a] NS = no significant difference
[b] S = significant difference

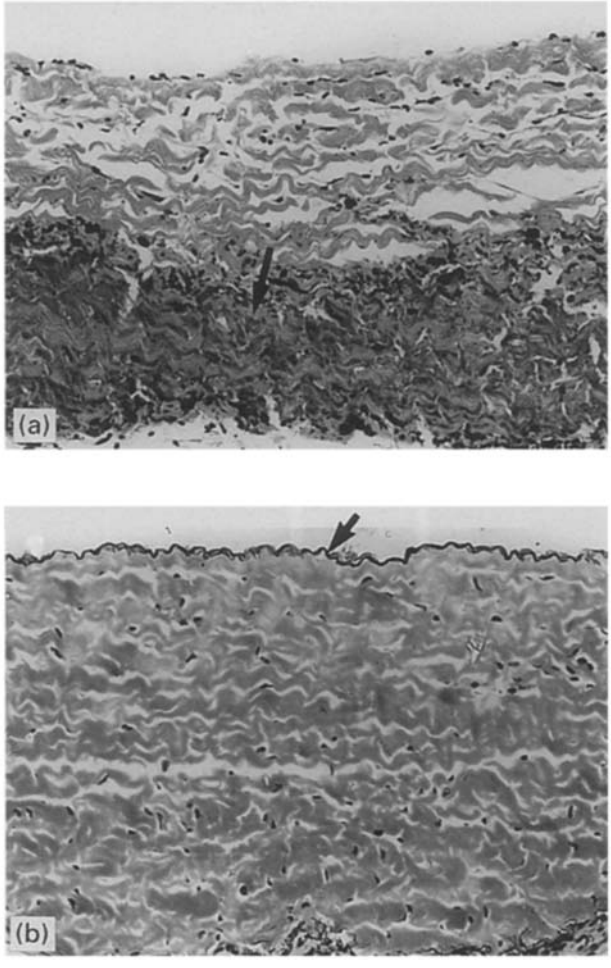

Figure 4 Photomicrograph of (a) glutaraldehyde-treated implant after 30 days of implantation showing band-like dystrophic calcification in the fibrous layer (H&E, ×130); (b) glutaraldehyde and Fe (III)-treated implant after 15 days of implantation showing intact collagenous tissue with no evidence of calcification. The linear brownish discoloration due to iron adsorption on the surface of the fibrous layer is indicated (arrow) (H&E, ×130).

in the effect of phospholipid removal by SDS. As noted in the histology of this sample type, the distribution of Fe^{3+} is more diffuse and no brown band was found. It has been reported that the use of SDS results in a less compact arrangement of fibres in the tissue [16] which can be channels for Fe^{3+}-rich solution to enter and in the process be deposited. The drastic drop in Fe^{3+} levels at day 15 can be accounted for if we consider that Fe^{3+} can exist in two forms in the samples. The first, is adsorbed by unbound Fe^{3+} ions that most probably diffuse into the extracellular fluid quickly when implanted in the animal model thereby reducing the Fe^{3+} level immediately. The second type of Fe^{3+} may be those that are chemically bonded by electrostatic interactions with the negatively charged side groups of amino acids on collagen such as the carboxyl and hydroxyl. This chemical interaction is strong enough to retain the Fe^{3+} ions at an almost constant level as supported by the Mann–Whitney analyses (Table II).

In contrast, the combination treatment E of chloroform–methanol followed by Fe^{3+} shows a statistical improvement over the treatment A of only Fe^{3+}. However, no difference was found between treatment E when contrasted to treatment C, i.e. chloroform–methanol singly. Furthermore, this slight combinatorial anticalcification effect is short-lived as the results of the 30-days Mann–Whitney test show no statistical significance between the members of this group. Therefore, in comparison to SDS, the two treatments behave similarly.

From the Fe^{3+} analyses, there is no difference between the 0 days Fe^{3+} content but a noticeable difference between the 30-days results. However, the histology of both samples show the brown discoloration typical of Fe^{3+} [12]. The difference is possibly accounted for by considering that treatment E does remove phospholipids similar to SDS and therefore the same explanation about Fe^{3+} retention can be invoked. That the initial adsorption of Fe^{3+} may not be as high may be attributed to the efficiency of phospholipid removal by the chloroform–methanol mixture.

The undesirable effect of SDS on the collagen fibres observed in the histological studies in this experiment can be attributed to the sequence of applying SDS. Typically, SDS treatment is applied only after GA treatment. We have chosen to treat tissue before GA so that comparison with chloroform–methanol can be performed. Therefore the observed degeneration found with SDS in this work need not reflect badly on this reagent. It is interesting to note that the adverse tissue effect was not found with the CM treatment either singly or in combination. Since removal of phospholipid may be more efficient prior to GA fixation than post fixation, CM could be a useful alternative to SDS that does not affect the structural integrity of the tissue.

TABLE III Summary of anticalcification treatment effects on explants

Code	Treatment solution	15 days		30 days	
		Calcium level	Tissue features	Calcium level	Tissue features
O	GA	medium	band-type calcification	high	band-type calcification
A	Fe^{3+}	low	normal tissue features	med–low	focal calcification
B	SDS-GA	low	tissue shrinks	low	tissue shrinks
C	C/M-GA	low	normal tissue features	low	band-type calcification
D	SDS-GA-Fe^{3+}	low	tissue shrinks	low	tissue shrinks
E	C/M-GA-Fe^{3+}	low	normal tissue features	med–low	normal tissue features

low $\leqslant 10$ mg/g
med–low $\geqslant 10$ mg/g; $\leqslant 20$ mg/g
medium $\geqslant 20$ mg/g; $\leqslant 100$ mg/g
high $\geqslant 100$ mg/g

5. Conclusions

The results of the elemental analyses for calcium indicate comparable levels of anticalcification behaviour of the combined treatments to the single treatments. Furthermore, pretreatment of tissue with SDS or chloroform–methanol favours the better retention of Fe^{3+} in the tissue compared to just treatment with Fe^{3+} singly. The high retention of Fe^{3+} has been attributed to the chemical agents' ability to wash out phospholipids thereby facilitating some chemical interaction of Fe^{3+} with functional groups on collagen which impedes its diffusion in the extratcelluar fluid. The results offer the opportunity to investigate the combination approach further as the variations in the order and time of treatment may lead to a system that would mitigate calcification in glutaraldehyde-treated tissue than so far found in single systems.

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