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Inhibition of bovine pericardium calcification: A comparative study of Al^{3+} and lipid removing treatments

E. JORGE HERRERO, M. P. GUTIÉRREZ, C. ESCUDERO, J. L. CASTILLO-OLIVARES
Service of Experimental Surgery, Clínica Puerta de Hierro, Madrid, Spain

The biological heart prostheses present midterm and long term problems owing to the progressive deterioration and calcification of the tissue. In the attempt to study the latter problem, we have compared the effectiveness of anticalcification treatments in calf pericardium samples implanted into female Wistar rats after undergoing the following procedures: Group I, control, treatment with glutaraldehyde; group II, treated with 0.1 M Al^{3+} for 24 h; and group III, subjected to lipid removal by chemical treatment with chloroform/methanol for 1 h. Positive results were obtained with both treatments, but the results after 60 days of implantation were more favourable with lipid removal than with Al^{3+} treatment.

1. Introduction

The major problem with biological cardiac bioprostheses continues to be the calcification of the tissue of which they are made. In their initial development, in the early 70's, it was observed that in spite of their being an improvement over mechanical prostheses in haemodynamic and thrombogenic aspects, their lesser durability due to degeneration and calcification of the biological tissue was a great disadvantage and the cause of an estimated 60% of failures in these prostheses [1].

The causes of dystrophic or metastatic calcification in these prostheses are not very clear, and it is thought that the origin is multifactorial. Chemical treatments [2], the immune response [3], mechanical stress [4] and the deposition of proteins and circulating cells [5, 6] have been implicated, among other factors.

To date, attempts to develop a chemical treatment that prevents the calcification of the tissue by interacting with the blood have been unsuccessful. Numerous studies dealing with chemical anticalcific treatments have been carried out in this respect, beginning with SDS-type detergents for lipid removal [7, 8] and diphosphonate hydroxyapatite poisons – administered systemically, and tissue-linked [9–11]. Recently, interest in treatments with aluminium salts has increased given the positive results obtained. Through a yet unknown mechanism, these salts retard the mineralization of the tissue in subcutaneous implants in young rats [12, 13]. We decided to compare this treatment with the results of the selective extraction of lipids from the bovine pericardial tissue using chloroform/methanol. With respect to the function of the lipids, it is known that they play a role in physiological mineralization [14]. To date, some authors have referred to lipids as promoters of tissue mineral deposition and, using alternative chemical treatments such as detergents, have focused their efforts on these tissue components [7, 8]. The effects of the treatments are controversial since their mechanisms of action are unknown; moreover, the results obtained in circulatory systems [15] generate numerous doubts concerning their effectiveness, mainly questioning whether they are able to resist the mechanical stress to which the valves are subjected. In this sense, it is important to keep in mind, that certain chemical treatments, while reducing calcification, can damage the integrity of the tissue, reducing its mechanical resistance, and thus, its durability. The onset of calcification could originate from an electrostatic attraction between the acid phospho-lipids of the connective tissue and calcium [16]. Some authors [17] consider this process to be limited to the initial phases of calcification. Here, we have attempted to ascertain whether the interaction of the calcium cation with contra-ions present in the connective tissue is a prerequisite for the process of tissue calcification.

2. Material and methods

The pericardium of 6 to 12 month old calves was obtained directly from the slaughter house and transported in ice and saline solution with HEPES buffer (4–(2–hydroxyethylpiperazine-1) ethanesulphonic acid); pH 7.4. It was cleaned manually to eliminate remains of fat, and placed in a shaker bath of Hanks' solution for 4 h to eliminate the soluble proteins.

For the study of tissue calcification, four week old female Wistar rats, weighing 80–100 g, received separate subcutaneous implants of four pieces (approximately 0.5 cm × 0.5 cm; 400–700 μm thick; mean thickness: 550 μm) of chemically-treated bovine pericar-

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dium in their abdominal wall. The animals used were divided into three groups on the basis of the different treatments applied to the tissue. For each treatment, two subgroups of three rats each were set up to determine calcification at 21 and 60 days.

2.1. Control group

Twenty four hour treatment with 0.6% glutaraldehyde prepared from a commerical solution of 25% glutaraldehyde (Merck) in 0.1 M sodium phosphate buffer (pH 7.4), at room temperature, as previously described [18].

2.2. Group I

The initial treatment was the same as that of the controls, followed by additional treatment in a shaker bath with 0.1 M $AlCl_3$ for 24 h, at room temperature, in a proportion of 1/100 (w/v) of $AlCl_3$ [12, 13].

2.3. Group II

Removal of lipids was performed with chloroform/methanol in a proportion of 1/5 (v/v) and in a proportion of 1/30 (w/v), under N_2 atmosphere for 1 h, after which chemical treatment with 0.6% glutaraldehyde (pH 7.4) was carried out for 24 h. The prior extraction of lipids does not modify the characteristics of the tissue [19], and thus, we consider it preferable to treat the tissue with chloroform/methanol prior to glutaraldehyde fixation. The lipids removed were filtered and concentrated in a vacuum (Rotavapour), and introduced into a previously weighed tube to be N_2-dried until dryness. The extract was then quantified by gravimetry and analysed by one and two dimensional thin layer chromatography.

Prior to their implantation into rat, all tissue samples were washed in abundant saline.

After implantation times of 21 or 60 days, the rats were sacrificed with ether and the tissue samples were removed, cleaned of debris, dried and weighed. Determination of calcium in each of the samples removed was performed by atomic absorption spectroscopy and the results were expressed in mg Ca g^{-1} dry tissue.

For the statistical analysis of calcium accumulation, the different groups were compared by the Mann–Whitney U contrast for unpaired data [20]. This test is an alternative version of the Wilcoxon rank sum test, for the comparison of two groups in independent samples. Both are non-parametric methods (that is, distribution-free methods).

3. Results

The lipids quantified after the 1 h extraction time were 3.47 ± 0.15 mg g^{-1} wet tissue.

The phospho-lipids separated by one and two dimensional thin layer chromatography, phosphatidylethanolamine (PE), phospatidylserine (PS) and phosphatidylcholine, were identified by their migrations on the chromatogram with respect to phospho-lipid patterns applied to the plate (data not shown). The use of ninhydrin revealed the presence of spots corresponding to PE and PS, while spots corresponding to the lyso-derivated forms were not observed, a result which is similar to those reported by other groups [21].

The studies of calcium accumulation were performed after 21 and 60 days postimplantation. In the samples studied (Table I), after 21 days, the highest levels of calcium were observed in the control group (48.31 ± 15.00 mg g^{-1} dry weight). After 21 days, the groups with aluminium treatment and lipid-free tissue presented lower levels of calcium (0.12 ± 0.02 and 0.34 ± 0.23, respectively) which in both cases were significantly different with respect to the control ($p < 0.05$). When compared with each other, however, the degrees of calcification of the two treated groups ($p < 0.05$). When compared with each other, however,

For the 60 day implantation period, in the control group, the levels of calcium were considerably increased (79.46 ± 6.65 mg g^{-1} dry weight), while in group I they had increased slightly and group II undergoes almost no change (3.11 ± 1.67 and 0.43 ± 0.22, respectively). The difference between the two groups and that resulting from their comparison with the controls after 60 days were also statistically significant ($p < 0.05$).

Nevertheless, the results obtained in groups I and II after 21 days as well as after 60 days show a significant inhibition of the calcification, especially in the lipid free tissue.

4. Discussion

In recent years, an attempt has been made to develop a chemical treatment that would prevent bioprosthesis calcification. One such treatment employs aluminium salts which, according to different groups of workers, result in a lesser accumulation of calcium in the tissue after 21 days of implanatation [12, 13]. With this in mind, we have attempted to determine the effectiveness of this treatment, comparing it to the effect of the selective extraction of lipids (Table I). The latter treatment might considerably reduce the electrostatic factor which would determine the interaction with ionic calcium and the precipitation of calcium salts. On the other hand, the aluminium might act as a positive contra-ion of the tissue, blocking the affinity sites for calcium.

In the lipid free tissue, the results obtained showed much lower levels of calcium than in the control group, pointing to phospho-lipids as possible promoters of calcification. Our results in models of subcutaneous implantation are comparable to those

TABLE I Accumulation of calcium with the two anticalcification treatments

Treatment	Ca^{2+} (mg g^{-1} dry weight tissue)	
	21 days	60 days
Control	48.31 ± 15.00^a	79.46 ± 6.65^a
Aluminium	0.12 ± 0.02^a	3.11 ± 1.67^a
Chloroform/methanol	0.34 ± 0.23^a	0.43 ± 0.22^a

[a] mean $\pm$ standard deviation of 12 samples.

obtained by other authors employing SDS-like surfactants [7, 8]. The method of action of treatments using surfactants of chloroform/methanol appears to be based on the extraction of the acid phospho-lipids from the tissular matrix. Some authors implicate phosphatidylserine in certain types of *in vivo* calcification [22, 23]. This phospho-lipid is present in bovine pericardium [21].

On the other hand, the mechanism of action of aluminium is as yet unknown, and successive studies will be necessary to determine whether its effect is related to the inhibition of the formation and propagation of hydroxyapatite crystals [24] or it competes with the calcium cations to bind to the negative contra-ions present in the tissue [12, 13]. From the results, it would appear logical to question whether these binding sites might be the phospho-lipids of the tissue themselves. Gross and Strunz [25] compared a broad range of bioactive materials, finding that the introduction of some metallic ions impedes bone development. Aluminium would exert its effect on the phospholipids of the tissue, blocking their interaction with the calcium, perhaps by the formation of strong ionic bonds with the negative charges of the tissue. In either case, the kinetics of aluminium treatments still remain to be determined in major aspects such as their incorporation into the tissue, stability and resistance to mechanical stress in long term studies performed in prostheses. The question that arises is whether in a variable period of time, calcium salts (solubility product: 1×10^{25} mol l^{-1}) could displace aluminium from its binding sites in the tissue, originating the formation of calcium deposits which would easily be precipitated in their most stable form into hydroxyapatite salts. For this reason, in selecting a chemical treatment, it is necessary to consider its effectiveness over an extended period of time, to decide whether it would be preferable to eliminate those components that might be major causes of the deposit of calcium salts, as is the case of the lipids.

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