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ABSTRACT

FACTORS EFFECTING CALCIFICATION OF BIOPROSTHETIC HEART VALVES

by
Sumei Yu

Calcification is the most frequent cause of clinical dysfunction of glutaraldehyde treated bioprosthetic heart valves. In this study, we compared calcification of bioprostheses of the No-React™ and the conventional glutaraldehyde treated pericardium by both in-vivo and in-vitro methods, and to further delineate the role of the host's inflammatory response in implant degeneration.

In the in-vitro study, the two types of pericardial samples were placed in individual polystyrene tubes containing physiological concentrations of calcium and phosphate and incubated for 21 days at 37°C. In the in-vivo study, the two types of pericardial samples were implanted subcutaneously in rats and subsequently retrieved at 15, 21, and 35 days postimplantation. Calcium analyses were performed on each specimen.

Experimental results showed that a significantly reduced in vitro calcification of No-React™ treated pericardium compared to conventional glutaraldehyde pretreated tissue (mean calcium content, 1.3 ± 0.2 μg/mg of No-React™ treated tissue versus 5.8 ± 0.7 μg/mg of glutaraldehyde pretreated tissue) ($p < 0.001$). In-vivo test showed progressive calcification of glutaraldehyde treated pericardium over 5-week period (mean tissue calcium content increasing from 49.6 ± 9.6 μg/mg after 2-week to 134.3 ± 9.1 μg/mg at 5 weeks postimplantation),

while No-React™ treated pericardial tissue calcified significantly less ($p < 0.05$) in 20~30 μg/mg level at each corresponding interval.

All these lead to the conclusion that the calcification of conventional glutaraldehyde treated pericardium is more severe than No-React™ treated pericardium both in vivo and in vitro tests.

FACTORS EFFECTING
CALCIFICATION OF BIOPROSTHETIC HEART VALVES

by
Sumei Yu

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
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APPROVAL PAGE

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This thesis is dedicated to my husband

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CHAPTER 1

INTRODUCTION

Heart valve bioprostheses were introduced about 35 years ago to cope with the major disadvantage of mechanical valves. After 35 years of valve replacement, it is fair to state that the operation can be considered as a cure for the short term, especially if large-size valves can be used, but only palliative for the long-term, and for a certain group of patients this operation can not be considered curative in any stage after the operation (children , women in child-bearing age, etc.). Valve replacement with a mechanical valve can never be considered curative because of the continuous need for anticoagulation as well as the continuous risk (although small) of sudden death, while a biological valve can give excellent quality of life. There is also ample evidence today that anti-platelets may be sufficient in cases of atrial fibrillation, or when patient had an episode of thromboembolism after valve replacement with bioprostheses. The quality of life with well-functioning biologic prosthesis is reported to be better than that with a mechanical valve.

Formaldehyde and particularly glutaraldehyde, are commonly used to control the physical and biological properties of a variety of collagen-based biomaterials such as heart valve and blood vessel prostheses, implantable collagen preparations and collagen dressings.

The first porcine xenograft valves were treated with formaldehyde, unfortunately they failed relatively early (1). Although formalin solution is an excellent sterilizing agent with profound tanning effects, the alternation of collagen by formalin is thought to be caused by polycondensation of protein chains (2), and the polymerization is reversed after implantation, thus the deterioration is accelerated by the original formalin effect. Formalin preservation of heterograft aortic valves has proved unsatisfactory in both experimental and clinical trials (1).

Malfunctioning valves examined after implantation are identical in both series, showing a diffuse process of deterioration. Changes of formalin concentration and storage conditions do not influence the findings significantly. This nonviable tissue is rarely repopulated by host tissue over the period of time studied, making the likelihood of satisfactory extended function improbable. So the use of formaldehyde has been abandoned fairly quickly (1).

The glutaraldehyde tanning method was proposed to solve the problem of availability with the allograft and tissue failure with the formalin treated xenograft. The use of weak glutaraldehyde solution did in fact provide more permanent fixation of porcine valve collagen, increasing tissue durability and enabling commercial marketing of this type of valves (3).

Bioprostheses fabricated from glutaraldehyde-preserved porcine aortic valve or bovine pericardium are widely used to replace diseased human cardiac valves. Glutaraldehyde is used to control physical and biological properties of collagen structure by means of intermolecular and/or intermolecular cross-linking of collagen molecules. Solubility, antigenicity, and biodegradation of naturally occuring or reconstituted collagenous matrices are effectively reduced by glutaraldehyde treatment. Glutaraldehyde treatment decreased inflammatory reaction by reducing antigenicity and destruction of the implant. Contrary to the formaldehyde, the cross-linking is more stable (3).

As the glutaraldehyde preserved porcine xenografts are widely used, durability remains the primary concern. Spontaneous, sterile deterioration of the glutaraldehyde preserved porcine xenograft appears to occur as a result of calcification, which may either stiffen and immobilize the valve leaflets or deform and stress them to the point of rupture. Calcification can be readily documented grossly by radiography and by histologic section, accurate measurement of the calcium content can be performed by atomic absorption spectrophotograpy. Calcification is proposed to occur by two basic mechanisms. One is the deposition

of crystalline material in denuded, disrupted exposed, or thrombosed regions of the leaflet, and the second is the presence of an innate, diffuse stippling of crystalline substances in the collagen bundles of the porcine leaflet itself (4, 5, 6, 7).

Several factors are proposed to be related to this process:

1. Calcium metabolism of the individual patient and host versus graft reaction,
2. Turbulence of blood flow through the valve,
3. The degree of valve tissue fixation,
4. Valve leaflet flexibility , and
5. The presence of thrombus on the valve leaflets.

Another possible mechanisms suggested that it might be an immunological response. In addition to these the calcification may also occur in valves implanted in patients with no apparent risk factors.

It was stated that the 5 year valve failure rate from spontaneous, sterile degeneration of valves manufactured from 1970 to 1975 is 7.1% (6/65) of those implanted (8). The number of patients is too small to predict a 10 year failure rate for valves implanted in the early 1970s, but there is some evidence to suggest that this failure rate is less than 20%. Nevertheless, commercial glutaraldehyde preserved porcine tissue valves have been shown to be functional in the twelfth year of implantation (3, 9)(reports of 20 years are available). At present , about 20 ~ 25% of adult patients and 50% of children patients have to be reoperated in less than 10 years. The undefined durability is the major drawback of bioprostheses. Clinical regurgitation, stenosis, or both, are frequently caused by calcification , with or without cuspal tearing, necessitating reoperation with valve removal or causing death of approximately 25% of patients with porcine bioprostheses within 10 years postoperatively.

While degeneration of bioprostheses is often a slowly progressing phenomenon that can allow for planned reoperation. The prosthetic valves made of

glutaraldehyde fixed porcine aortic valve and bovine pericardium have been used in more than 500,000 patients.

However, the main problems associated with the failure of bioprosthetic valves are calcification which can lead to stenosis or insufficiency. Calcification in children remains a serious problem. Bioprosthetic valves are practically contraindicated in children and mechanical valves are contraindicated in patients who can not receive anticoagulants. Socioeconomic factors constitute a relative contraindication to the use of mechanical valves, a high incidence of thromboembolic complications in patients with mechanical cardiac valvular prostheses during the late 1960s and early 1970s generated interest in valves made of biologic materials, e.g., fascia lata, duramater, porcine aortic valves, and bovine pericardium.

Glutaraldehyde treatment has been thought as main "villain" in calcification. Extensive clinical and pathological studies have demonstrated that these tissue valves treated with glutaraldehyde undergo degeneration and calcification, especially in children and adolescents (10, 11, 12).

Schoen et al. (13), reported that subcutaneously implanted porcine aortic valve showed calcification from 2 ~ 56 days, after which, up to 126 days, there is no further increase. And the subcutaneously implanted porcine aortic bioprosthesis develops collagen calcification after 21 days if pretreated with glutaraldehyde, with fresh valve cusps implants demonstrated only a minimal necrosis without calcification during same period of implantation (13).

Both porcine aortic valve and pericardium are rich in type I collagen, and it has been suggested that the inflammatory response is due to chemotactic potential of collagen peptides generated from the collagenase digestion.

Schoen et al. have concluded that the presence of glutaraldehyde is a prerequisite for calcium deposition (13).

In this study, we compared calcification of bioprostheses by in vitro and vivo methods to study the effect of conventional glutaraldehyde treated and the No-React™ anticalcification treatment on calcification, and to correlate inflammation and calcification.

The experiment consists of the following tests:

1. Static In-Vitro calcification tests.
2. Pronase digestion tests.
3. Rats subcutaneous implantation of pericardial strips.

This study is part of a larger investigation performed at the UMDNJ-NJMS Cardiothoracic Research Laboratory, to try to elucidate the different factors responsible for the degeneration and calcification of xenograft tissues.

CHAPTER 2

METHOD OF STATIC IN-VITRO CALCIFICATION TESTS

In-vitro systems are simpler, cheaper and more easily controlled than in-vivo systems. Static in-vitro test systems can be used to study small samples of material in large numbers relatively quickly, and has a useful role to play in the economic screening of new materials or modifications of existing materials prior to in-vivo testing . It may also aid the definition of the mechanism of calcification and hence the development of solutions to the problem.

In the test, solution composition was specifically chosen to be as simple and as close to physicologic concentrations as possible, and still achieve calcification, allowing the parameters involved in the process to be kept at a minimum.

In this test, we compared the calcification of conventional glutaraldehyde treated and No-React™ treated pericardium.

2.1 Preparation of Instruments and Solutions

1. Vials

clean , sterile, screw-top polystyrene sample vials.

2. Tissue samples

a. pericardium treated with conventional glutaraldehyde treatment,

b. No-React™ treated pericardium.

3. Solution

a. solution A:

15782 mg NaCl, 738.7mg $CaCl_2$ $2H_2O$, 326 mgKH_2PO_4, 20.9mg MOPS were weighed. Solution were made in 2 liters millipore water. Solution containing 135 mM NaCl, 2.88 mM $CaCl_2$ $2H_2O$, 1.2 mM KH_2PO_4, 0.05 mM MOPS. Certain amount of 0.25 M NaOH was added to adjust the pH to 7.40, while the solution

was stirred. Then, under aseptic condition, the solution was forced through a filter (0.2 μm) as a method sterilization.

b. solution B (saline solution):

0.9 % NaCl, pH = 7.40, autoclave to sterilize the solution

2.2 Procedure

1. Basin, scissors, clasps , towels etc were autoclaved.
2. Under laminar flow hood, the No-React™ treated pericardium was cut into 3 cm × 1 cm, washed with solution B in the sterile basin, stirred gently for 30 min. Same process for the glutaraldehyde treated pericardium samples in a different basin was performed.
3. Each sample was placed in individual vial with 20 ml solution A,
4. The experiments were divided by following groups:

Group 1----temperature of 37°C, 21 days, the solution was changed every week.

(5 vials of each type of tissue)

labelled as: G1-1 G1-2 G1-3 G1-4 G1-5

N1-1 N1-2 N1-3 N1-4 N1-5

where, G, represents the conventional glutaraldehyde treated samples,

N, represents the No-React™ treated samples,

Group 2----temperature of 37°C, 21days, the solution was not changed at any time.

(5 vials of each type of tissue).

labelled as: G2-1 G2-2 G2-3 G2-4 G2-5

(conventional glutaraldehyde treatment)

N2-1 N2-2 N2-3 N2-4 N2-5

(No-React™ treated tissues)

Group 3----temperature of 25°C, 56 days, the solution was changed every week.

(5 vials of each type of tissue)

labelled as: G3-1 G3-2 G3-3 G3-4 G3-5

(conventional glutaraldehyde treatment)

N3-1 N3-2 N3-3 N3-4 N3-5

(No-React™ treated tissues)

Group 4----temperature of 37°C, 56 days, the solution was changed every week.

(5 vials of each type of tissue)

labelled as: G4-1 G4-2 G4-3 G4-4 G4-5

(conventional glutaraldehyde treatment)

N4-1 N4-2 N4-3 N4-4 N4-5

(No-React™ treated tissues)

Group 5---- control group. Use solution B instead of solution A as a control solution for both glutaraldehyde and No-React™ treated pericardium samples. Temperature of 37°C, 21 days, the solution was not changed at any time.

(5 vials of each type of tissue)

labelled as : SG1 SG2 SG3 SG4 SG5

(conventional glutaraldehyde treatment)

SN1 SN2 SN3 SN4 SN5

(No-React™ treated tissues)

5. For group 1, the previous solution was replaced by a fresh solution every week, monitored every week. After 21 days the tissues were removed and washed with solution B, dried and digested with nitric acid and perchloric acid (with ratio of 3:1) , then analyzed for calcium content.

6. For group 2, after the period of 21 days, the concentration of calcium in the solution was monitored by atomic absorption spectroscopy. The tissue was removed, washed with solution B, and then dried, digested with nitric acid and perchloric acid (with ratio of 3:1), then analyzed for calcium content by atomic absorption spectroscopy.

7. For group 3 and 4, the old solution was replaced by a fresh solution, and monitored every week. After 56 days , the tissue was removed from the vials, washed with solution B, dried and digested with nitric acid and perchloric acid (with ratio of 3:1), then analyzed for calcium content by atomic absorption spectroscopy.

8. For the control group, the tissue was removed from the vials after 21 days, dried and digested, then analyzed for calcium content by atomic absorption spectroscopy.

CHAPTER 3

METHOD FOR PRONASE DIGESTION TESTS

Subcutaneously implanted porcine aortic bioprostheses develop collagen calcification after 21 days if pretreated with glutaraldehyde, while fresh valve cusp implants demonstrate only a minimal necrosis without calcification during same period of implantation. Both porcine aortic valve and pericardium are rich in type I collagen , and it has been suggested that the inflammatory response is due to chemotactic potential of collagen peptides generated from the collagen digestion.

Three groups of differently treated pericardiums were digested with pronase in this study to compare the weight lost by each group of pericardium during the pronase digestion process and correlate that information with the inflammation and calcification.

3.1 Materials

1. Chemicals

a. Pronase P5147 5.5 units/mg solid, Type XIV Bacterial
from Streptomyces griseus. Lot# 34H0331ordered from SIGMA Company

b. Glycine G6388 Lot# 84H05445 ordered from SIGMA Company

c. HEPES H9136 Lot# 64H5725 ordered from SIGMA Company

d. $CaCl_2 \cdot 2H_2O$

2. Pericardial samples

a. Fresh pericardium Lot# 941026

b. No-React™ treated pericardium Lot# 950102-II

c. Glutaraldehyde treated pericardium Lot# 950103

3. Sterile single-use polystyrene tubes

3.2 Procedure

1. Solution preparation

75 mg $CaCl_2.2H_2O$, 1.125 g glycine, 1g HEPES were weighed, mixed in 150 ml millipore water. Certain amount of 0.25 M NaOH was added to adjust the pH to 7.40, then 75 mg protease was added.

2. Pericardial samples preparation

The samples (n = 10 for each fresh pericardium, No-react™ pericardium and conventional glutaraldegyde-treated pericardium) were cut into 1cm × 3cm shape. These samples were labelled and blotted with clean, dry towel, then each piece was cut into two pieces, one was about 1cm × 1cm, another was about 1cm × 2 cm. Each piece was weighed simultaneously. The weights were recored as "initial wet weight of the large piece" and "initial wet weight of the small piece".

3. Dry sample

The small pieces were put into clean polystyrene plates (the plates were labelled from F1 to F10 for fresh pericardial samples, from N1 to N10 for No-React™ treated pericardial samples, from G1 to G10 for conventional glutaraldegyde treated pericardial samples), these plates (without cover) were put into 37°C oven and dried for at least 2 hours or until the weight was constant. Then the samples were weighed and recorded as "dry weight of the small piece".

4. Digestion

The large pieces were put into polystyrene tubes. 3ml solution made in procedure 1 was added into each tube. Tubes were covered, labelled and put in 50 °C shaking bath for 22 hours.

5. Weight recording

After 22 hours, the tubes were removed out of the shaking bath. The samples were taken out, blotted with clean towel to remove the free water, weighed and recorded as "wet weight of large piece after digestion". These samples were put in the clean polystyrene plates and dried in 37 °C oven for at least 2 hours or until constant weight. These weight were record as "dry weight of large piece".

3.3 Calculation

1. The "dry weight of small pieces" divided by "initial wet weight of small pieces" is the ratio of blotted/dry weight of the small samples, and this will be used to calculate the initial dry weight of the large samples based on their initial blotted weight. These steps are necessary because:

a. The samples can not be dried before the protease digestion,

b. During protease digestion, the hydrophilic ability of the tissue will change, thereby increasing considerably the blotted weight of the sample, leading to erroneous results.

2. "Initial wet weight of large piece" times "ratio of blotted/dry weight "gave the "initial dry weight of large piece", which was the weight of large piece before digestion.

3. The proportion of the final dry weight/initial dry large weight for the large pieces represented the weight left after digestion. And (1 - proportion) × 100% gave the weight loss during the incubation process due to the enzymatic degradation induced by the non-specific protease agent. The results were expressed for each experimental condition as mean ± SEM weight lost (% of initial weight).

CHAPTER 4

METHOD OF SUBCUTANEOUS IMPLANTS AND EXPLANTS IN RATS

This in-vivo study was performed to compare the calcification of conventional glutaraldehyde treated pericardium and No-React™ treated pericardium.

Samples of the two types of pericardiums were implanted in the rats under different side of the skin in abdominal wall. After few weeks, the samples were retrieved and analyzed. The pericardial samples were treated with the Biocor processes.

4.1 Preparation

1. Rats

20 male sprague-dawley rats of 4 weeks old, 60 ~ 80 grams

2. Pericardial samples

20 conventional glutaraldehyde treated samples, with size of 1cm × 2cm, Lot # 950202,

20 No-React™ treated samples , with size of 1cm × 2cm, Lot # 950102-II

3. Anesthesia reagents

The animals were injected intrabdominally with a cocktail of a total of 1~3 ml of Ketamine (20 mg/ml), Xylazine (2.5 mg/ml) and Pentobarbital.

4. Other

5-O coated vicryl suture,

1cc syringes ,

Scissors, clamp, basin, clean towel etc.

4.2 Procedure

4.2.1 Implantation Procedures

1. The surgical tools have been autoclaved.

2. Pericardial samples were washed in separate saline bath.

3. The rats were anesthesized with 1 ml ketamine cocktail, with the rats head down.

4. Once in deep sleep, the rats were prepared and wrapped.

5. Anterior abdominal wall was prepared sterilly with betadine solution and draped with sterile towels. Two subcutaneous pockets were created on anterior abdominal wall for accommodation of 1cm × 2cm pre-cut segments of conventional glutaraldehyde treated and No-React™ treated pericardium. The pericardium strips were implanted subcutaneously in the abdominal wall, the No-React™ treated tissue was implanted in the right side, while the conventional glutaraldehyde treated pericardium strip was implanted in the left side.

6. The skin was closed with labelled 5-O vicryl sutures.

7. The rats were fed Lab Rodent Diet (Purina Meals Inc.) and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society For Medical Research and the "Guide For the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by National Institutes of Wealth (NIH Publication No. 86-23, revised 1985).

4.2.2 Explantation Procedures

1. The rats were sacrificed by intraperitoneal overdose injection (300 mg /kg) of pentobarbital after 2, 3, 5 weeks, respectively.

2. The pericardial samples were retrieved from abdominal wall, labeled appropriately for both conventional glutaraldehyde treated and No-React™ treated pericardium as G(i) or N(i), where i stands for each rat.

3. Small strips were cut from each sample, placed in 10% buffer formalin for histological examination .

4. Each sample was washed with sterile 0.9 % NaCl before analysis.

CHAPTER 5

CALCIUM DETERMINATION BY ATOMIC ABSORPTION SPECTROPHOTOMETER

Both in-vitro and in-vivo test, the calcium content of samples were measured by atomic absorption spectrophotometry. Flame atomic absorption spectrophptometer was used to perform the analysis. The wavelength used to measure the calcium was 428 nm. National Institutes of standards and Technology bovine Liver (SRM 1577a, Gaithersburg, MD) was used as a quality control sample for all calcium content analyses.

5.1 Apparatus and Reagents

1. Apparatus

a. Flame Atomic Absorption Spectrophotometer

Perkin-Elmer model 603, Perkin-Elmer, Norwalk, CT.

b. Analytical balance

2. Reagents

a. Calcium standard 1000ug/ml, ordered from Fisher co.

b. Hydrochloric acid, ACS Reagent Grade, ordered from Fisher co.

c. Lanthanum oxide, ACS Reagent Grade, ordered from Fisher co.

d. Deionized-distilled water, made by Medical Preventive Lab, UMDNJ

e. Nitric acid (70% and 20%), ordered from GFS Chemicals, Columbus, OH

f. Perchloric acid (70%), ordered from GFS Chemicals, Columbus, OH

3. Glassware

a. Beaker (24) 40ml

b. Eppendorf/repipet (1) 12.5 ml reservoir

c. Graduated cylinder	(1)	25ml
d. Graduated cylinder	(1)	250ml
e. Volumetric flask	(24)	25ml
f. Volumetric flask	(7)	100ml
g. Volumetric flask	(2)	500ml
h. Volumetric flask	(1)	1000ml
i. Plastic tube	(48)	10ml

5.2 Solution Sample Acquisition and Preparation

This analysis was to monitor the calcium in the solution which were changed every week in the in-vitro test. The range of the atomic absorption analysis data from the spectrophotometer was 0 ~ 200 μg / dl. The reading data was proportional to the calcium concentration in the above range. Therefore, the solution sample need to be diluted to fall into the range if their concentration were higher than 200 μg / dl.

Since the calcium concentration was around 2.9 mM (i.e. 11600 μg/dl), the solution should be diluted at the ratio of 1:100.

5.3 Tissue Sample Acquistition and Preparation

Before being analyzed, tissue samples need to be digested with 3:1 nitric acid (70%) / perchloric acid (70%), diluted with 1% lanthanum. The preparation and analytical process takes 5 days. It was performed for 20 samples at the time. The processes were as following:

1st day:
24 beakers were soaked with 20 % nitric acid overnight;

2nd day:

a. The 20 % nitric acid was returned to the original acid bottle;

b. Each beaker was rinsed 5 times inside and outside thoroughly with distilled water;

c. Each beaker was labelled;

d. Each beaker was dried in 90 °C oven overnight;

e. 24 flasks of 25 ml volume were soaked with 20 % nitric acid;

f. 48 plastic tubes were soaked with 20 % nitric acid;

3rd day:

a. The beakers were removed from the oven quickly, placed in desiccator for 10 minutes;

b. Each beaker were weighed and recorded;

c. Each beaker was returned to oven for another 1 hours;

d. Steps a and b were repeated, till constant weight; the weight was recorded as W1;

e. The samples were placed into beakers;

f. 0.25g NBS was put in oven at 90 °C overnight;

g. All beakers were placed in oven at 90 °C overnight;

h. 20% nitric acid was returned from flasks and tubes to the original acid bottle. Flasks and tubes were rinsed 5 times with distilled water, then air dried.

4th day:

a. Beakers were removed from the oven and placed in desiccator to be cooled to room temperature (10 minutes);

b. Beakers were weighed and recorded;

c. Beakers were put in 90 °C oven for 1 hour;

d. Repeat steps a and b, till the weight was constant. Then the weight was recorded as W2, (the dry sample weight equal to W2 - W1);

e. Samples were digested at 150 ~175 °C with 10 ml mixture acid solution of 3 : 1 nitric acid (70%) / perchloric acid (70%), till 0.5 ml liquor left in the beaker;

f. The liquor was washed 5 times with millipore water and quantitatively transfer into 25 ml volumetric flask. Each time, the inside wall of beaker was washed as completely as possibl. Then the 25 ml volumetric flask was shaken to make the solution sample uniform;

g. The contents of volumetric flask was transferred to a plastic test tube.

5th day:

The sample solution was diluted with 1% lanthanum to prevent interference from silicon, aluminum, phosphate and sulfate, etc. Dilution ratio was chosen according to the calcium concentration in the sample.

5.4 Standard Solution Preparation

1. Preparation of 5% Lanthanum solution

a. 29.32 grams of Lanthanum Oxide was transferred to a 500 ml volumetric flask;

b. 25 ml of distilled water was added and swirled;

c. 125 ml of concentrated hydrochloric acid was added slowly to the 50 ml volumtric flask;

d. The solution was diluted with deionized-distilled water to 500 ml.

2. Preparation of calcium standards in 0.5 % Lanthanum solution

a. 100ml of 5% lanthanum solution was diluted with 900ml deionized-distilled water to form a 0.5% lanthanum solution;

b. 5.0 ml of calcium stock solution 1000 μg / ml was diluted to 100 ml in a volumetric flask with deionized-distilled water. The solution is equivalent to 50 μg calcium / ml ;

c. The 50 μg Ca/ml solution was diluted with 0.5 % Lanthanum solution according to the following ratios:

Ca Standard μg/100ml	Ca 50 μg/ml	0.5 % Lanthanum
0	0	100
50	1	99
100	2	98
150	3	97
200	4	96

5.5 Analytical Procedures

1. Samples and standard solutions were prepared;
2. The power was turned on, appropriate lamp and burner head were installed;
3. The lamp current, read on the Lamp Current Meter (in the lamp compartment), was set to the proper value with the lamp current control;
4. The slit and wavelength were set to proper values;
5. The SIGNAL control switch was set to CONC. (EM CHOP for flame emission measurements) and the MODE control switch to HOLD ;
6. The BKGD CORR switch was set to AA-BG;
7. The gas controls was set up and the gas was ignited;
8. An integration time was selected by entering the desired value through the numerical keyboard and the INT key was pressed (the initial integration interval was set at 0.5 second when the instrument was turned on) ;

9. A blank solution was aspirated and the A2 key was pressed to zero the digital display;

10. Calibration of the instrument.

a. The desired value for Standard 1 was entered through the numerical keyboard and the S1 key was pressed;

b. Step a was repeat for S2 and S3;

c. S1 was aspirated and the S1 key was pressed;

d. Step c was repeated for S2 and S3. The standards must be aspirated in order of increasing concentration, during the calibration of the instrument.

11. A sample solution was aspirated and the READ button was pressed. The indicator dot above the READ button would be lit until measurement is completed.

12. Analysis was performed at 428 nm using air-acetylene flame, and read on the Perkin-Elmer model 603 atomic absorption spectrophotometer.

5.6 Calculation

5.6.1 Calcium Content in Solution Samples

If the dilution ratio was 20 µl sample : 2000 µl 1% lanthanum and the reading on the atomic absorption spectrophotometer was 80 (µg / dl), then the calcium content in the solution sample would be :

$$\frac{80\ (\mu g/dl) \times (2000 + 20\ ) \times 10\ (dl/l)}{40\ (g/mol) \times 20 \times 1000\ (\mu g/mg)}$$

$$= 2.02\ \text{mM Ca}$$

5.6.2 Calcium Content in Tissue Samples

If the dilution ratio was X, the reading on the atomic absorption spectrophotometer was 80 (µg/dl), then the calcium content in the tissue sample would be:

$$\frac{X \times 80\ (\mu g/dl) \times 25\ ml}{100\ (ml/dl) \times (W2 - W1)}$$

with the unit of μg Ca / mg dry tissue.

CHAPTER 6

RESULTS

6.1 In Vitro Calcification

6.1.1 Solution Sample Analysis Result in Vitro Studies

Table 6.1 summarizes the results of the solution analysis for calcium concentration. Concentrations were expressed as mM. The average values were expressed as mean ± stantard error of the mean (SEM). Statistical significance was determined by two-tailed independent t-test.

The calcium concentration in the original fresh solution A was 2.88 mM. There were two groups for each kind of pericardium in this test. For group 1, the solutions were changed with fresh solution A and analyzed every week. The results show that the average calcium concentration in the glutaraldehyde group is significantly higher than that in the No-React™ group ($p < 0.01$). For group 2, the solution were not changed until the end of the test period (21 days). After 21 days, the solutions were analyzed, the calcium concentration in the glutaraldehyde group is also significantly higher than that in the No-React™ group ($p < 0.02$). All these illustrate that there is more calcium transfer to the glutaraldehyde treated pericardium from solution A than to the No-React™ treated pericardium.

6.1.2 Tissue Sample Analysis Result in Vitro Test

1. 21 days result

In this test, the calcium concentration of solution A was 2.88 mM. Table 6.2 summarises the result of the calcification of the two types of pericardium after 21 days incubation at 37°C. The average calcium content of the control (pre-incubation) conventional glutaraldehyde treated pericardial samples was 0.12 µg/mg dry tissue, the average calcium content of the control (pre-incubation) No-

Table 6.1 In-Vitro Calcification Data: Calcium Content in the Incubation Solution (mM)

Run #	1th week	2nd week	3rd week	Run #	3rd week
N1-1	2.70	2.80	2.80	N2-1	2.80
N1-2	2.88	2.88	2.75	N2-2	2.53
N1-3	2.88	2.78	2.85	N2-3	2.78
N1-4	2.80	2.88	2.88	N2-4	2.88
N1-5	2.88	2.83	2.88	N2-5	2.78
mean±SEM	**2.83±0.04**	**2.83±0.02**	**2.83±0.03**		**2.75±0.06**
G1-1	2.60	2.47	2.50	G2-1	2.63
G1-2	2.50	2.47	2.35	G2-2	2.50
G1-3	2.50	2.37	2.50	G2-3	2.42
G1-4	2.63	2.50	2.50	G2-4	2.53
G1-5	2.63	2.53	2.50	G2-5	2.40
mean±SEM	**2.57±0.03**	**2.47±0.03**	**2.47±0.03**		**2.49±0.04**

React™ treated pericardial samples was 0.11 μg/mg dry tissue. After 21 days incubation in solution A, the calcium content in group 1 of conventional glutaraldehyde treated sample was 5.84 ± 0.67 μg/mg, that of No-React™ samples was 1.28 ± 0.17 μg/mg. The difference is statistically significant ($p < 0.001$). The calcium deposition on conventional glutaraldehyde treated sample is higher than that on No-React™ samples. The data in group 2 shows that the calcium deposition in the group that the solutions were changed is much higher than that of the group which the solutions were not changed, demonstrating the calcium concentration of the solution as a factor which can affect the rate of calcium deposition. Statistc shows the difference is very significant ($p < 0.001$).

Figure 6.1 shows the test result.

2. 56 days result

In this test, calcium concentration of solution A is 1.8mM.

Table 6.3 summarises the result of calcification at different temperature.

Group 3 was incubated at 25 °C. The average calcium concentration in No-React™ group is 0.58 ± 0.04 μg/mg, while that in conventional glutaraldehyde group is 0.94 ± 0.07 μg/mg. The difference between these two types of percardium is very significant ($p < 0.01$).

Group 4 was incubated at 37 °C. The average calcium concentration in No-React™ group is 8.78 ± 1.02 μg/mg, while that in conventional glutaraldehyde group is 14.58 ± 1.40 μg/mg. The difference between these two types of percardium is significant ($p < 0.03$).

The results show that the higher temperature the higher calcification rate. The difference of calcification between two types of pericardium is more significant at 37 °C than that at 25 °C. The temperature at which the incubation is done plays a significant role in this calcification process in vitro test.

Figure 6.2 shows the result of the in-vitro test after 56 days incubation as well as other parameters.

6.1.3 Macroscopic Observation

In 21 days in-vitro test, from the 8th day of incubation, we began to watch the pericardium surfaces. There were some white calcium spots attached on both surfaces of the conventional glutaraldehyde treated pericardial samples. There was almost no spot could be observed on the No-React™ treated pericardial samples.

Table 6.4 is the record of the macroscopic observation. The spots on the conventional glutaraldehyde group 1 (solution were changed every week) were more than that on group 2 (solution were not changed during the test period).

6.2 Pronase Digestion Test

Table 6.5, 6.6 , 6.7 summarise the results of pronase digestion test of No-React™, glutaraldehyde , and fresh pericardial samples , respectively .

The results show that the weight loss of the above three types of pericardial samples are 10.3 ± 0.6%, 14.8 ± 1.1%, and 62.4 ± 1.3% respectively. The difference between any two of them is very significant ($p < 0.001$).

6.3 In-vivo Test---Subcutaneously Implants in Rats

Table 6.8, 6.9, 6.10 summarise the results of calcification in pericardial samples explanted at 15, 21 and 35 days, respectively. The calcium concentrations of the pericardial samples were expressed as microgram calcium per milligram dry tissue weight. Statistical significance was determined by two-tailed independent t-test. The mean calcium content ± SEM of conventional glutaraldehyde treated pericardium after 15, 21 and 35 days of subcutaneous implantation were 49.58 ± 9.60, 82.45 ± 10.40, and 134.32 ± 9.10 μg/mg, respectively. Comparatively, the

mean calcium content of No-React™ treated pericardium was signigicantly lower ($p < 0.05$) at each correstponding interval (19.58 ± 6.01, 32.27 ± 12.22, 21.43 ± 5.21μg/mg, respectively).

Figure 6.4, 6.5, 6.6 show the difference of calcificaiton in the two types of pericardial samples and show the significant variance among each individual rats. From these data, clearly the calcium content of the conventional glutaraldehyde treated pericardium is significantly higher than that of No-React™ treated pericardium. The calcium content increased significantly in conventional glutaraldehyde treated pericardium as the time of implantation is increased, but the No-React™ treated sample did not behave likewise.

Comparing these two types of pericardium, while for the conventional glutaraldehyde treated pericardium the calcium content have increased with the time of implantation, for the No-React™ treated sample at 3 weeks, the calcium content have increased slightly. For unclear reason, however, decreased at 5 weeks of implantation.

CHAPTER 7

DISCUSSION

In order to understand and interpret calcification both in-vitro and in-vivo, it is necessary to know some of the properties of the valve materials and the current hypotheses of normal and pathologic calcification processes.

Bioprosthetic valves --- bioprosthetic valves encompass all valves in which the leaflet function is carried out by materials derived from animal sources. These include homograft (allograft) valves derived from human cadavers, autografts derived from the recipient's own body (the utilization of the pulmonary valve in the aortic position) and heterografts derived from sources such as chemically-modified bovine pericardium or whole porcine valves. In general, these valves are mounted on some type of frame or stent for use. Therefore, they contain synthetic materials such as metals and / or polymers in addition to animal tissue that has usually been modified in some way.

The synthetic parts of these valves are more durable than the biologic parts. Stent failure is reported as less than 0.001% per annum, compared with tissue failure at a rate of 1% per annum. The latter is much higher than the failure rate reported for mechanical valves and raises serious questions about the long-term durability of these types of valves, with the exception of autograft valves which performed best overall, but are not widely used. Overall, the quality of life with a well-functioning biologic prosthesis is reported to be better than that with a mechanical valve and degeneration is often a slowly progressing phenomenon that can allow for planned reoperation.

Degenerated allograft valves have developed calcification, whereas autografts are reported to have no evidence of calcification processes. Some attempts have been made to construct valves out of human-source biomaterials

such as fascia lata or dura mater, but with limited success as both these materials tended to degenerate and calcify.

The first heterograft valves in use were chemically modified porcine aortic valves. These valves have good commissural supports but the valve orifice is increasingly restricted by a muscle shelf on the right coronary cusp, as valve size decreases. Improved valve orifice and pressure characteristics were achieved by using modified bovine pericardium to fabricate stent-mounted trileaflet valves. The dialdehyde, glutaraldehyde, is commonly used to introduce a stable cross-linking network into the tissue, reducing its antigenicity and sterilising the tissue.

All heterograft valves calcify at varying rates after implantation and this introduces a serious limitation on their useful life. This limitation is particularly stringent in the case of valves implanted into children when the rate of calcification of biologic prostheses is unacceptably high, resulting in early malfunction of the valve.

Normal and pathologic calcification --- Normal calcification essentially equates with bone formation. Bone is formed extracellularly in collagenous matrices with deposition of hydroxyapatite, a crystalline form of calcium phosphate containing 10 calcium atoms, six phosphate molecules and two hydroxyl groups. Under normal conditions, the body's extracellular fluid is in a metastable state i.e. spontaneous precipitation of calcium phosphate does not occur although sufficient quantities of calcium and phosphate are present to allow growth of crystal structures once precipitation is initiated. One hypothesis proposes that the normal mineralization process is initiated by matrix vesicles: small, membranous, extracellular particles which have been observed in physiologic calcification as well as in some pathologic processes (14).

Three classes of abnormal calcification in the body have been identified: heterotopic true.bone formation and two classes of soft tissue calcification, with no distinct bone matrix structure, i.e. metastatic calcification associated with

hypercalcaemia or hyperphosphataemia and dystrophic calcification associated with normocalcaemia and normophosphataemia (15).

Heterotopic bone formation is often associated with the deposition of cartilage and generation of new osteoblasts. The initial stimulus for this process is unknown but may involve specific proteins e.g. bone morphogenic protein. Alkaline phosphatase is often elevated. Heterotopic bone formation after hip replacement surgery is more common in male than female patients. Studies of cartilage calcification have observed deposition of the protein chondrocalcin, identified as the c-propeptide of type II collagen, which is thought to bind to proteoglycan aggregates and is known to bind calcium and hydroxyapatite (16). Proteoglycan aggregates are not detected in non-calcifying cartilage. An ion-exchange mechanism of cartilage calcification has also been suggested (17); calcium is present in high concentration in cartilage, but, normally, is largely bound to anionic groups of proteoglycans and hence inhibited from precipitating. This hypothesis suggests a local increase in phosphate concentration to release calcium from the proteoglycans, thus raising the calcium-phosphate product above the threshold for hydroxyapatite precipitation.

Metastatic calcification occurs more frequently at sites where the local pH is abnormally high, with a higher probability of the calcium-phosphate product rising above the precipitation threshold. This commonly occurs in patients with renal disease and can be prevented by reducing plasma phosphate with ingested aluminum hydroxide. Ingestion of large amounts of phosphate has been shown to result in metastatic calcification in animal experiments and may do the same in humans (15).

Hypotheses of bioprosthetic heart valve calcification --- Calcification of artificial heart valves is of the dystrophic type and may be intrinsic (directly associated with the biomaterial) or extrinsic (superficial to the biomaterial and associated with debris attaching to the surface of the material) to the material

involved (18). The mechanism is obscure and there are several current hypotheses. It has been reported that calcium is deposited earliest in connective tissue cells of the valve material and later in collagen fibrils-calcification also occurred in acellular collagen sponge implants. It is likely, therefore, that calcification is not specific in terms of the gross picture of initiation sites, but may be specific to molecular binding sites available on a variety of macromolecular structures.

Calcification may be mediated by extracellular vesicles. These could be matrix vesicles as observed in physiologic mineralization, membranous cell fragments produced as by-products of tissue damage or mitochondria of damaged cells (14). Such vesicles have been proposed as a means of achieving locally high concentrations of calcium and phosphate, with the initial site of crystal formation being inside the vesicle. The presence of calcium-phosphate-acid phospholipid complexes has been demonstrated in matrix vesicles and membrane fractions (19). These complexes, in vitro, were capable of nucleating hydroxyapatite but, in vivo, there is no evidence of crystalline calcium phosphate being stored in intact vesicles. It is thought that magnesium present in vivo stabilises amorphous calcium phosphate in the vesicles and that nucleation of hydroxyapatite does not occur until the membrane is ruptured, releasing the magnesium and exposing the complex to the extracellular fluid.

When tissue damage occurs, the normal homeostatic mechanisms for removing intracellular calcium may break down resulting in a massive build-up of calcium within the damaged cell or cell fragment by passive diffusion from the extracellular fluid. The calcium-phosphate product is then exceeded and spontaneous crystal precipitation occurs within the membrane. This hypothesis could account for both intrinsic and extrinsic calcification observed in artificial heart valves: extrinsic calcification would occur when dead or damaged cells or cell fragments lodged on the surface of valve leaflets and then calcified, whereas intrinsic calcification would occur to the damaged cells present in the leaflet tissue

itself due to the pretreatments with chemicals such as glutaraldehyde, or because of mechanical damage resulting from leaflet flexion. By this mechanism, it would be obvious that calcification of valves made from artificial polymers e.g. polyurethanes could only be of the extrinsic type.

In leaflet samples implanted subcutaneously in rats and enclosed in Millipore filters, calcification still occurred, implying that host cellular factors, at least, are not involved although damaged cells present in the leaflet material may still be a factor. This work also implies that calcification is not related to host immunologic processes, as does work with athymic mice in which implants calcified as readily as in normal mice. In vitro experiments have shown that collagen is capable of taking up calcium and phosphate.

The main sites of calcific deposition in porcine bioprostheses were shown to be within the cuspal connective tissue and in small surface thrombi (20). The initial form of deposition was amorphous calcium phosphate and the concept proposed was that phosphate formed covalent bonds connecting inorganic and organic components through the E-amino groups of lysine and hydroxylysine in collagen. Many hydroxylysine groups are normally glycosylated and it was suggested that proteoglycans and glycoproteins normally mask such binding sites and that these are lost during glutaraldehyde processing thus unmasking calcium phosphate binding sites in the tissue. Calcium phosphate uptake by collagen has also been associated with proteolipid bound to collagen. Collagen plus proteolipid calcified in a metastable calcium phosphate solution whereas collagen minus proteolipid failed to calcify and proteolipid alone did calcify. The proteolipids concerned were composed of hydrophobic protein with acidic phospholipid and had high affinity for collagen. Neither the hydrophobic protein nor the acidic phospholipid fraction was capable of initiating calcium phosphate crystallization alone and, even in complex, some of the lipid components seemed to possess an inhibitory function and may perform a regulatory function in vivo. Proteolipids, of

course, are present in all membrane structures and may be relevant to the proposed mechanism of nucleation of calcification by membranes and matrix vesicles.

Proteins, such as osteocalcin, which contain the amino acid gamma-carboxyglutamic acid, have a strong affinity for calcium. The synthesis of such proteins are dependent on vitamin K. They have been found in close association with regions of extending mineralization, and quantities of these proteins in implants have been found to increase proportionally with calcium (21). Warfarin, however, as an inhibitor of vitamin K-dependent processes did not block calcification, although osteocalcin found in implants was reduced (22). This protein may act as a metabolic control of calcification by binding calcium and hence making it unavailable for proliferation of hydroxyapatite. Another gamma-carboxyglutamic acid-containing protein, atherocalcin, has been found in atherosclerotic plaques and, unlike osteocalcin, had been observed to enhance hydroxyapatite formation.

Studies of calcified human aortas have shown significant increases in the cross-linked peptide, histidinoalanine, which occurs in an acidic protein probably associated with collagen or elastin. The histidinoalanine was concentrated in the mineralized regions of the aortic tissues. It is unknown whether this peptide is part of the calcified elastin in the aortic tissue or is part of a discrete peptide present in the tissue (23).

The concept of "neutral binding site/charge neutralization" has been proposed to explain calcification of aortic elastin. This hypothesises that the binding sites for calcium are the carbonyl oxygens of the peptide backbone. The positively charged bound calcium would then sequester phosphate to maintain charge neutrality, thus developing the potential for hydroxyapatite formation. Support for this concept was obtained by chemically blocking purified elastin to achieve neutrality and obtaining significant binding of calcium: exposure of blocked elastin to calcium and phosphate solutions resulted in calcification of the

elastin distributed throughout its bulk (24). It is also suggested that , normally in vivo, elastin is protected from calcification by the close association of proteoglycans and glycoproteins. Model peptides were shown to bind calcium via the carbonyl oxygens of their peptide backbones. Collagen has the potential to react similarly.

It has been suggested that calcification results from the removal of specific inhibitors. This mechanism implies that, under normal physiologic conditions, calcification is regulated by the presence of inhibitors and that mineralization only occurs locally where these inhibitions have been removed. Proteoglycans have previously been noted in this context. Nucleotide di- and tri-phosphates, low molecular weight metabolites with two ester phosphates, and pyrophosphate inhibit the transformation of amorphous calcium phosphate into hydroxyapatite (25). Alkaline phosphatase reverses this inhibition and has been suggested as a possible agent for promotion of mineralization. The action of alkaline phosphatase is of particular interest in view of the accelerated calcification of artificial heart valves implanted in children and the elevated amounts of the 'bone' isoenzyme of alkaline phosphatase found in this group compared with the adult population (26).

Early experimental work on synthetic elastomeric heart valves indicates that calcification may also be a problem for these materials. A polytetrafluoroethylene valve developed progressive calcification associated with an expanded form of the material and infiltration of the material by host cells (27). Polyurethane valves implanted in calves calcified primarily at the material surfaces. Similar valves calcified in sheep but no descriptive detail was reported (28). The problem has also been observed in polyurethane-coated blood pumps, such as ventricular assist devices and artificial hearts, in which the calcification has been associated with stresses and defects in the material. The association of calcification in such valves with the material itself (intrinsic calcification) or with host factors attaching to the material (extrinsic calcification) is not yet clear. If the synthetic elastomer is

directly involved, then the calcification mechanisms suggested so far seem inadequate. The features common to all these valve types include flexibility, permeability and the ability to adsorb / absorb certain blood components.

A physico-chemical hypothesis of calcification has been proposed by Bruck (29), and it relates to the penetration and absorption of blood components by flexing, deforming elastomers. Relevant factors in this process include thermodynamic solubility parameters, domain structures, defects and porosity. All these factors are functions of chemical composition, molecular weight and its distribution, branching and cross-linking, the presence of amorphous and crystalline domain structures, chain stacking and kinking. Absorption of blood substances into the elastomer is closely related to the thermodynamic solubility parameters of the biomaterial and the absorbed substance. By this principle, absorption of water, native proteins and phospholipids by either valve type is unlikely. Glutaraldehyde-treated tissue valves could absorb lipid-soluble vitamins as could polyurethanes. The polyurethane soft segments, however, would also absorb triglycerides, cholesterol and esters. Bruck concludes that these materials will always absorb some blood components and that this process, over time, will lead to biomaterial degradation. The direct relationship to calcification is not clear, but it has been suggested that lipid components absorbed in this way by polyether soft segments of polyurethanes initiate calcification in vivo by complexation.

The effect of polyurethane porosity has been studied. As porosity of the material increased, the degree of tissue ingrowth increased, but there was no relationship between porosity and calcification, at least in materials implanted intra-muscularly in rats up to 12 weeks. Specimens seeded with calcium, however, showed extensive calcification with limited tissue ingrowth (30).

Several groups postulate a close connection between the mechanical stress applied to the xenograft valve and the calcification process. It is unclear whether damage caused by the mechanical stress initiates calcification or calcification

caused increased stress resulting in membrane rupture. It has been shown that calcification occurs in regions of highest stress-strain (31).

This relationship has been more closely investigated by Deck et al. (32) and Thubrikar et al. (33), who postulated that collagen breakdown is initiated by excessive wear caused by mechanical stress. These authors implanted porcine or pericardial valves into calves with or without radio-opaque markers tagging the leaflets. Calcification occurred at regions of highest stress for both types of valve, but there appeared to be a difference in mechanism. The porcine valves developed visible calcification later than the pericardial valves and appeared to be subject to a mode of deformation resembling pure bending i. e. with the formation of internal voids within the leaflet tissue in which the calcium was initially deposited. Pericardial valves, in contrast, showed early visible calcification and a deformation mode of internal shearing, creating clefts between layers of collagen with calcification of the collagen along the planes of shear. Suggested mechanisms for the calcification include exposure of calcium-binding sites of collagen, or the appearance of collagen breakdown products capable of initiating calcification. Simple surface damage to the leaflets as caused by the radio-opaque tags did not appear to enhance calcification.

Work with valved ventricular assist devices implanted in calves found microscopic calcification within the pseudoneointimal layer which was closely associated with the flexing region of the pump. It was proposed that intrinsic calcification was of mechanical origin and that high cyclic strains disrupted the pseudoneointimal structure, thus initiating calcification in some way. They tested several tissue valves, finding that bovine pericardial valves gave the highest incidence of calcification compared with porcine or human dura mater valves.

The relationship between mechanical stress and calcification is supported by reports that implants in the right side of the heart are less subject to calcification than those in the left side (34). The stress levels on closed valves in

the mitral position are greater than in the aortic position, both of which are greater than those in the tricuspid position. One study of bioprosthetic valves explanted from juvenile sheep contradicts theses findings, with valves explanted from the tricuspid position being more heavily calcified than valves from the mitral position (35).

An accelerated rate of mechanical damage, caused by increased stresses due to relatively small valve orifices combined with higher resting heart rates in children compared with adults may contribute to the accelerated calcification of bioprosthetic valves seen in this group.

Finite element analysis has been used to show a similar strong correlation between stress-strain distribution and calcification in polyurethane diaphragms of blood pumps, with surface defects forming in the flexing regions. It has been further suggested that substantial heat is generated by flexing polyurethane membranes which would accelerate any chemical process, e.g. calcification, occurring in the vicinity. It is also possible that disruption of the surface structure of the polymer during flexion would expose calcium binding sites and thus enhance calcification. This is important particularly in view of the propensity of polyurethane materials to creep over time, resulting in thinning of the polymer structure and ,probably, greater exposure of segment components normally buried within the polymer which might have increased affinity for calcium phosphate. This process is likely to be enhanced in flexing structures.

There are many hypotheses relating to the process of biomaterial calcification. Many of these would seem to be more in the nature of promoting or inhibiting factors affecting the process, and the primary event has not yet been clearly defined. It is not clear whether calcification is intrinsic to the biomaterial itself or due to extrinsic factors, e.g. calcification of cell debris attached to biomaterial surfaces. In the latter case, procedures to improve biocompatibility, particularly with polyurethane materials, might prove beneficial.

It would seem logical to expect a similar mechanism of calcification to operate, whatever the biomaterial involved and if, of course, that material calcifies. Thus a likely candidate for a general calcification mechanism is cation chelation by groups present in the material, containing lone pairs of electrons susceptible to interactions with electron-deficient species. Such groups are available in both biologic and synthetic elastomeric materials: the carbonyl oxygens of the backbone of collagen and the polyether/polyester oxygens of the soft segments of polyurethanes. Carbonyl oxygens of the urethane and urea linkages of polyurethanes potentially could also be involved. Phosphate would be carried along in the process as the accompanying anion to maintain charge neutrality.

This mechanism could explain both intrinsic and extrinsic calcification, as cell debris causing extrinsic calcification would have similar binding sites to initiate calcification. By this mechanism, other factors would simply shift the equilibrium of the calcium-binding process in one direction or the other; if the shift were towards calcium binding , then the degree to which that occurred might influence mineralization by increasing the local calcium-phosphate product above the level needed for precipitation. Once hydroxyapatite was formed the reaction would be irreversible as the product is insoluble.

A further implication of this hypothesis is that calcification would not be dependent on biologic processes but should also occur in vitro under relatively simple reaction conditions. If this proved to be the case, it should be possible to obtain a clearer definition of the primary process involved in calcification. The development of controllable in vitro calcification systems should enable the advance of understanding of calcification and its control.

The results presented here indicate that much greater calcification is produced by in vivo than in vitro methods, although significant calcification is produced in vitro. Calcification in vivo occurs relatively rapidly and thus it is difficult to produce controlled low degrees of calcification for investigation of the

early stages of the process. Selection of specific degrees of calcification is much simpler in the in vitro systems in which the specimens may be observed continually.

Static tests in vitro did produce calcification, but the degree of calcification produced under these test conditions was extremely low, although significantly greater than uncalcified control material. The testing time could extended and the test solutions replenished at intervals, thus maintaining the supply of calcium and phosphate to the test material over long periods of time and increasing the degree of calcification achievable by this system. The prime disadvantage of such static systems is their inability to test the biomaterial in similar configuration and stress conditions under which it would be expected to function as a heart valve.

Calcification has been studied both in vivo and in vitro. The in vitro tests have demonstrated that specific blood factors are not required for calcification to take place, although theses may influence the rate of reaction. The hypothesis of Levy et al (35). regarding the role of localized tissue phosphate as a focus of initial calcification may be supported by the erratic early uptake of phosphate from the calcifying solution in this study, which does not begin to parallel calcium uptake until the calcification process is well established.

The association of calcification with surface trauma may be a result of exposure of free binding sites for calcium within the pericardial tissue, previously protected by the intact pericardial surface. It is not known whether this is a feature of damage to collagen fibres perse or related to a difference in the quality of chemical modification of the pericardium, by glutaraldehyde, between the surface and sub-surface regions of the tissue. It is worth, however, emphasizing the need for careful handling of bioprostheses prior to and during implantation, to minimize the likelihood of compromising the lifetime of the valve.

The No-React™ anticalcification treatment as well as the conventional glutaraldehyde process are two different modifications of the pericardial tissue. In

this test series, both in vitro and vivo test show that glutaraldehyde treated pericardium sample calcified to a much greater extent than No-React™ treated pericardium sample. This also illustrates the ability of the No-React™ treatment to significantly delay the degenerative mineralization of bovine pericardial tissue. Therefore, the way of modification plays an important role in the calcification process.

The pronase digestion test shows that the No-React™ samples have less weight loss after digestion, and glutaraldehyde samples are richer in type I collagen. It has been suggested that the inflammatory response is due to chemotactic potential of collagen peptides generated from the collagenase digestion. Furthermore, our experiments show that although initiation of tissue mineralization does not require host mediated response, the in-vivo milieu has potentially an important role in accelerating the calcification process. This phenomenon is further illustrated by the intense inflammatory reaction and the resulting tissue disruption noted in our morphological examinations of glutaraldehyde treated pericardium, and the remarkable absence of such destructive features in No-React™ treated tissue. Probably, this is one of the reasons that No-React™ treated samples are less calcified than that treated by glutaraldehyde. Further experiments needed to be performed to investigate the mechanism of the calcification and their difference between the two kind modifications.

Materials which calcify rapidly in these systems are unlikely to do better in humans. Material which show promise in these test systems may be selected for more intensive investigation in-vivo to decide whether or not their promise will extend to the ultimate, human implanted valve.

CHAPTER 8

SUMMARY

In this study, we compared calcification of bioprocess of No-React™ and conventional glutaraldehyde treated pericardium by both in-vivo and in-vitro methods. The results presented here indicate that much greater calcification is produced by in-vivo method than by in-vitro method. Static test in-vitro did produce calcification maintaining the supply of calcium and phosphate to the test material increase the calcification. Temperature is one of the factors affecting calcification. The calcification of glutaraldehyde treated pericardium is more severe than No-React™ treated pericardium both in-vivo and in-vitro test. The in-vivo test also shows that there is a substantial variance in the rate of calcification of tissues implanted in apparently the same group of animals (rats).

APPENDIX A

Appendix A contains the tables of the experimental results in Chapter 6.

Table 6.2 In-Vitro Calcification Data: 21 Days Incubation Results (Tissue Samples)

Run #	Solution change	tissue dry weight(mg)	initial Ca (ug / mg)	final Ca (ug/mg)	Ca increase percentage
G1-1	yes	80.9	0.12	7.48	6031%
G1-2	yes	83.4	0.12	6.66	5372%
G1-3	yes	87.2	0.12	4.29	3459%
G1-4	yes	76.1	0.12	4.55	3672%
G1-5	yes	101.5	0.12	6.20	5004%
mean±SEM				**5.84±0.67**	**4707%**
G2-1	no	72.5	0.12	4.82	3885%
G2-2	no	101.5	0.12	1.65	1333%
G2-3	no	102.8	0.12	2.94	2373%
G2-4	no	69.1	0.12	4.38	3530%
G2-5	no	87.2	0.12	3.94	3179%
mean±SEM				**4.02±0.34**	**2860%**
N1-1	yes	58.1	0.11	1.47	1287%
N1-2	yes	94.1	0.11	0.94	820%
N1-3	yes	77.5	0.11	1.85	1619%
N1-4	yes	77.1	0.11	1.07	939%
N1-5	yes	66.4	0.11	1.08	945%
mean±SEM				**1.28±0.17**	**1122%**
N2-1	no	61.6	0.11	1.03	901%
N2-2	no	69.2	0.11	1.19	1046%
N2-3	no	77.8	0.11	0.92	806%
N2-4	no	73.6	0.11	1.05	918%
N2-5	no	58.3	0.11	1.13	993%
mean±SEM				**1.06±0.05**	**933%**

Table 6.3 In-Vitro Calcification Data: 56 Days Incubation Results (Tissue Samples)

Run #	Temperature (C)	Ca (ug/mg) No-React group	Ca (ug/mg) glutaraldehyde group
3-1	25	0.44	0.69
3-2	25	0.67	0.92
3-3	25	0.54	0.98
3-4	25	0.66	1.14
3-5	25	0.58	0.96
mean±SEM		**0.58±0.04**	**0.94±0.07**
4-1	37	7.54	18.78
4-2	37	5.78	11.48
4-3	37	9.57	11.71
4-4	37	9.12	16.45
4-5	37	11.87	14.49
mean±SEM		**8.78±1.02**	**14.58±1.40**

Table 6.4 In-Vitro Calcification Data: Calcium Spots on the Pericardium Samples

day #	1	8	9	12	13	14	15	16	19	21
G1-1	0	5	6	8	9	10	11	11	11	12
G1-2	0	2	2	5	5	5	6	6	7	8
G1-3	0	5	7	7	8	8	9	9	9	9
G1-4	0	3	3	4	4	4	5	5	6	6
G1-5	0	2	2	3	4	4	4	4	4	4
G2-1	0	7	8	8	8	8	8	8	8	9
G2-2	0	0	0	0	2	2	2	2	3	3
G2-3	0	0	1	2	2	2	2	2	3	4
G2-4	0	1	2	3	3	3	3	3	3	3
G2-5	0	6	6	6	6	6	6	6	6	6
N1-1	0	0	0	0	0	0	0	0	0	0
N1-2	0	0	0	0	0	0	0	0	0	0
N1-3	0	0	0	0	0	0	0	0	0	0
N1-4	0	0	0	0	0	0	0	0	0	1
N1-5	0	0	0	0	0	0	0	0	0	0
N2-1	0	0	0	0	0	0	0	0	0	0
N2-2	0	0	0	0	0	0	0	0	0	0
N2-3	0	0	0	0	0	0	0	0	0	0
N2-4	0	0	0	0	0	0	0	0	0	0
N2-5	0	0	0	0	0	0	0	0	0	0

G1-1 to G2-5 -- Conventional glutaraldehyde treated pericardium

N1-1 to N2-5 -- No-React treated pericardium

Table 6.5 Pronase Digestion Data: No-React™ Treated Sample

Run No.	initial wet big	wet small	dry small	Ratio of small dry/wet	initial dry big	final dry big	after digest wet big	Ratio of fin/ini dry big	Weight loss
1	107.8	38.1	13.2	0.35	37.3	32.8	95.7	0.88	**12.2%**
2	99.4	34.5	12.5	0.36	36.0	31.5	93.6	0.87	**12.5%**
3	128.5	35.8	12.8	0.36	45.9	41.5	111.3	0.90	**9.7%**
4	154.6	77.4	25.6	0.33	51.1	47.4	147.8	0.93	**7.3%**
5	125.4	42.5	15.5	0.36	45.7	41.8	124.8	0.91	**8.6%**
6	126.9	39.2	13.7	0.35	44.4	40.1	115.3	0.90	**9.6%**
7	128.0	53.1	17.9	0.34	43.1	38.7	130.0	0.90	**10.3%**
8	128.7	43.6	16.6	0.38	49.0	43.3	129.1	0.88	**11.6%**
9	154.1	78.4	29.3	0.37	57.6	52.5	169.7	0.91	**8.8%**
10	119.8	50.3	16.7	0.33	39.8	34.9	114.2	0.88	**12.3%**
mean±SEM									**10.3±0.6%**

Table 6.6 Pronase Digestion Data: Glutaraldehyde Treated Samples

Run No.	initial wet big	wet small	dry small	Ratio of small dry/wet	initial dry big	final dry big	after digest wet big	Ratio of fin/ini dry big	Weight loss
1	157.8	34.8	9.2	0.26	41.7	37.3	129.0	0.89	**10.6%**
2	196.0	76.3	20.8	0.27	53.4	46	160.2	0.86	**13.9%**
3	104.7	46.3	13.1	0.28	29.6	27	94.6	0.91	**8.9%**
4	142.0	58.1	16.2	0.28	39.6	32.3	118.8	0.82	**18.4%**
5	134.6	46.6	13.5	0.29	39.0	31.1	120.1	0.80	**20.2%**
6	210.2	67.4	18.7	0.28	58.3	50.1	180.2	0.86	**14.1%**
7	98.2	40.7	12.6	0.31	30.4	25.8	94.6	0.85	**15.1%**
8	178.8	51.7	15.0	0.29	51.9	44.7	171.8	0.86	**13.8%**
9	143.5	49.6	14.3	0.29	41.4	34.8	112.5	0.84	**15.9%**
10	175.2	75.3	22.1	0.29	51.4	42.7	162.6	0.83	**17.0%**
mean±SEM									**14.8±1.1%**

Table 6.7 Pronase Digestion Data: Fresh Pericardial Samples

Run No	initial wet big	wet small	dry small	Ratio of small dry/wet	initial dry big	final dry big	after digest wet big	Ratio of fin/ini dry big	Weight loss
1	210.1	111.7	33.9	0.30	63.8	27.9	76.0	0.44	**56.2%**
2	179.2	67.3	21.9	0.33	58.3	20.1	40.7	0.34	**65.5%**
3	137.2	101.2	30.7	0.30	41.6	14.6	42.8	0.35	**64.9%**
4	117.2	49.5	17	0.34	40.3	15.1	40.8	0.38	**62.5%**
5	180.3	78.7	24.7	0.31	56.6	21.9	65.1	0.39	**61.3%**
6	309	101.6	32.2	0.32	97.9	42.7	136.0	0.44	**56.4%**
7	122.3	55.2	19.9	0.36	44.1	15.2	37.0	0.34	**65.5%**
8	315	144.7	44	0.30	95.8	38.9	191.3	0.41	**59.4%**
9	92.7	36.7	13.9	0.38	35.1	12.4	38.9	0.35	**64.7%**
10	79	41	13.6	0.33	26.2	8.5	20.9	0.32	**67.6%**
mean±SEM									**62.4±1.3%**

Table 6.8 In-Vivo Calcification Data: Comparison of Calcium Content between No-React and Glutaraldehyde Treated Tissue Samples (15 Days)

specimen #	initial Ca (ug/mg)	tissue dry weight(mg)	final Ca (ug/mg)
N1	0.11	39.7	18.63
N2	0.11	33.7	7.63
N3	0.11	42.6	34.62
N4	0.11	40.5	1.46
N5	0.11	49.7	35.56
mean±SEM			**19.58±6.01**
G1	0.12	43.6	87.13
G2	0.12	47.8	46.91
G3	0.12	49.6	40.31
G4	0.12	42.6	33.43
G5	0.12	29.7	40.13
mean±SEM			**49.58±9.60**

Table 6.9 In-Vivo Calcification Data: Comparison of Calcium Content between No-React and Glutaraldehyde Treated Tissue Samples (21 Days)

specimen #	initial Ca (ug/mg)	tissue dry weight(mg)	final Ca (ug/mg)
N1	0.11	49.6	60.07
N2	0.11	43.4	17.45
N3	0.11	42.4	44.66
N4	0.11	54.9	6.90
mean±SEM			**32.27±12.22**
G1	0.12	34.2	64.97
G2	0.12	43.1	64.01
G3	0.12	42.1	101.96
G4	0.12	47.1	98.85
mean±SEM			**82.45±10.40**

Table 6.10 Comparison of Calcification In-Vivo Test between No-React and Glutaraldehyde Treated Tissue Samples (35 Days)

specimen #	initial Ca (ug/mg)	tissue dry weight(mg)	final Ca (ug/mg)
N1	0.11	34.5	14.6
N2	0.11	39.7	29.1
N3	0.11	53.4	32.0
N4	0.11	32.2	14.0
N5	0.11	60.7	7.5
N6	0.11	50.8	8.9
N7	0.11	37.9	43.8
mean+SEM			**21.43+5.21**
G1	0.12	35.7	140.8
G2	0.12	68.3	125.1
G3	0.12	85.9	169.6
G4	0.12	47.4	91.2
G5	0.12	54.4	135.8
G6	0.12	69.0	128.9
G7	0.12	52.3	148.9
mean+SEM			**134.32+9.10**

APPENDIX B

Appendix B contains the figures of the experimental results in Chapter 6.

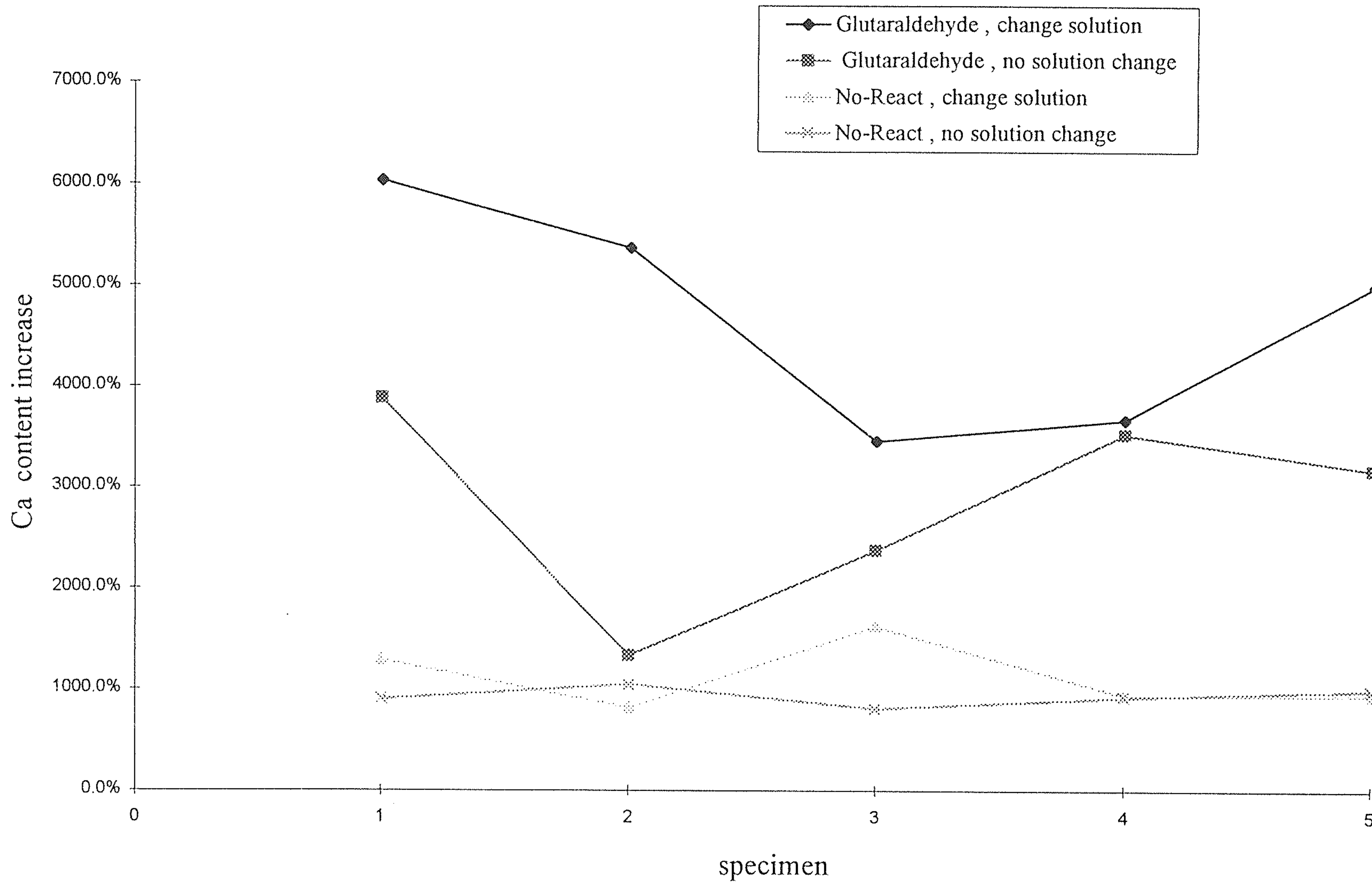

Figure 6.1 21 Days In-Vitro Tests (Pericardial Samples)

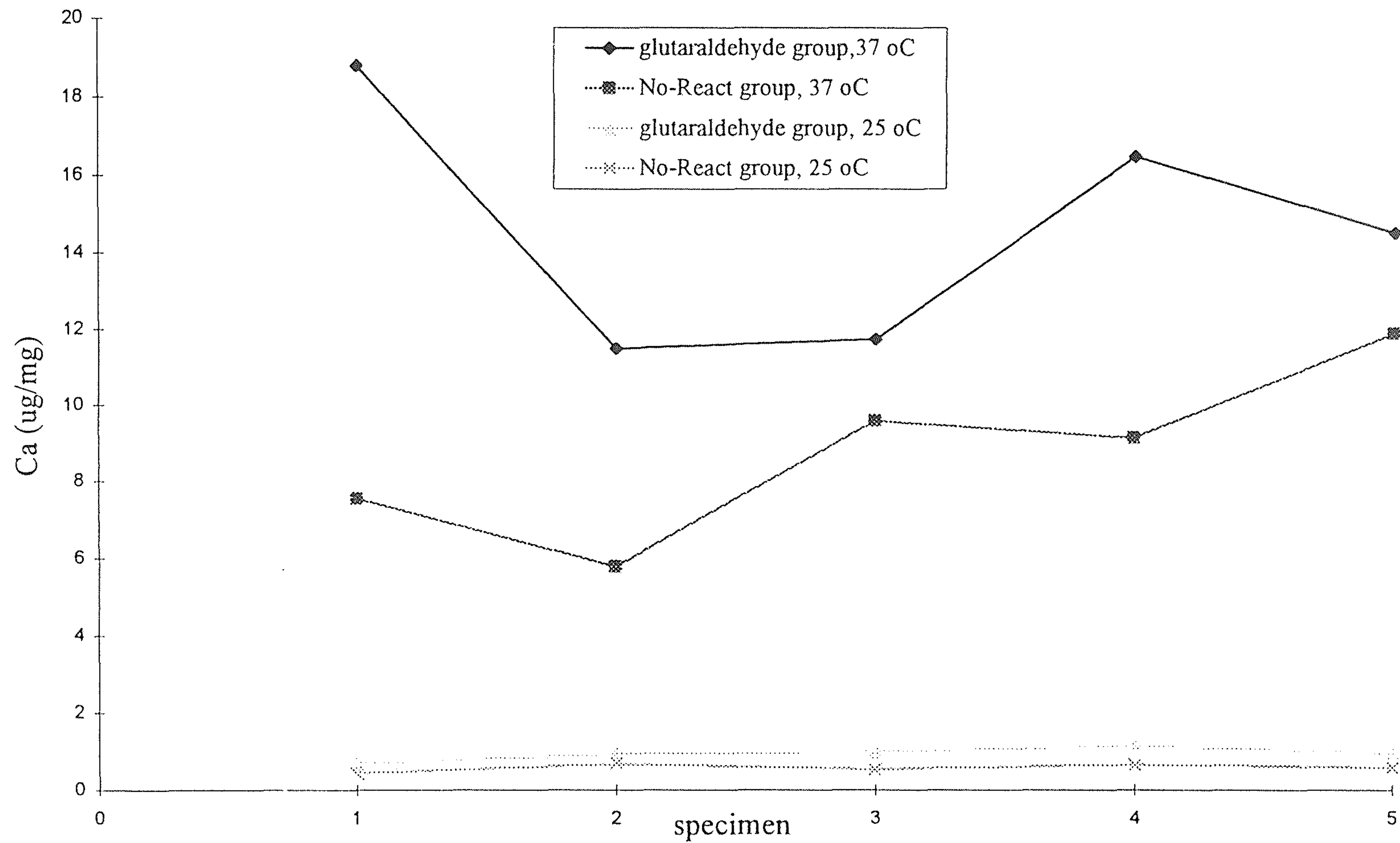

Figure 6.2 Comparison of Calcification Rate between No-React and Glutaraldehyde Treated Tissue Samples (56 days)

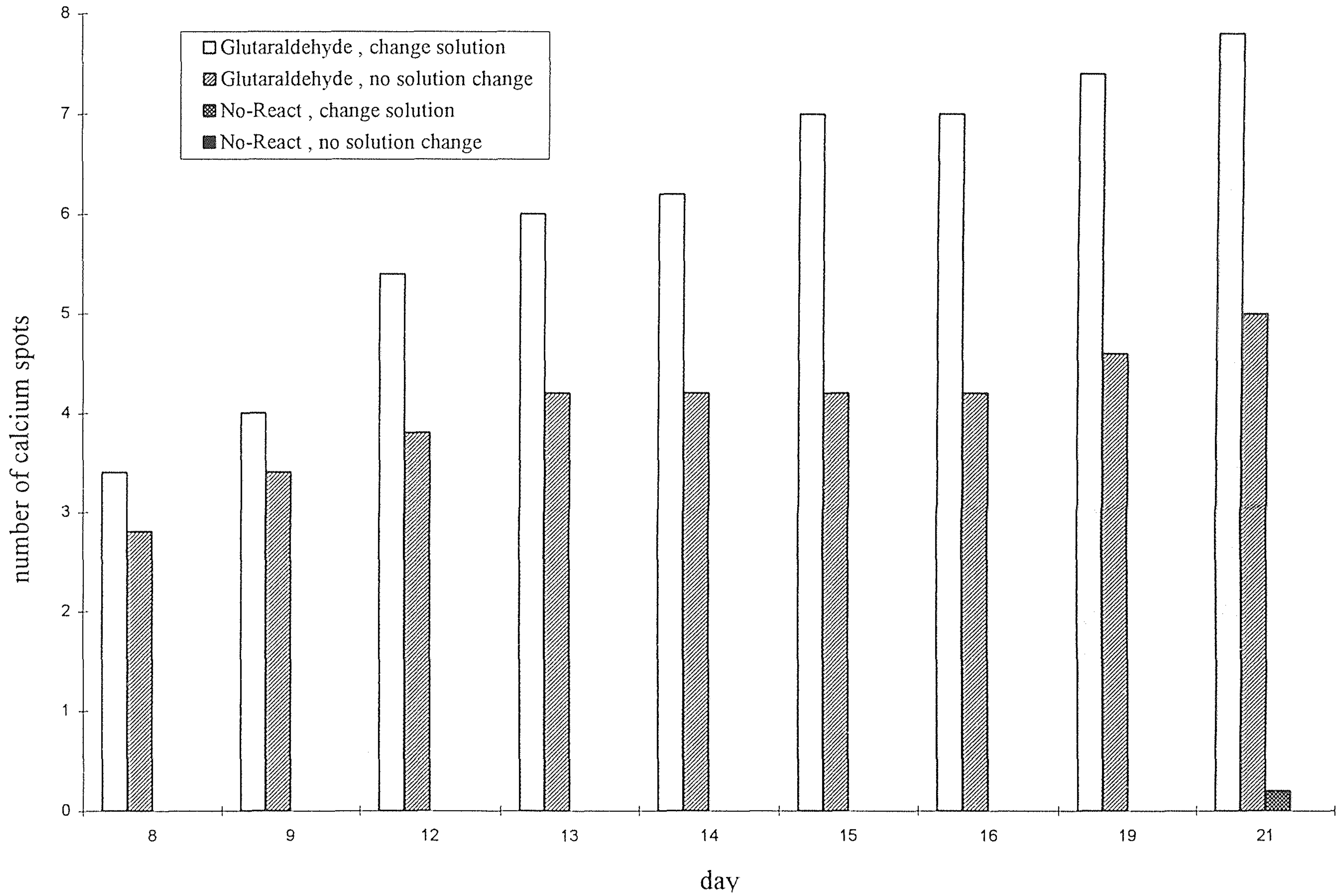

Figure 6.3 Calcium Spots on Pericardium Sample

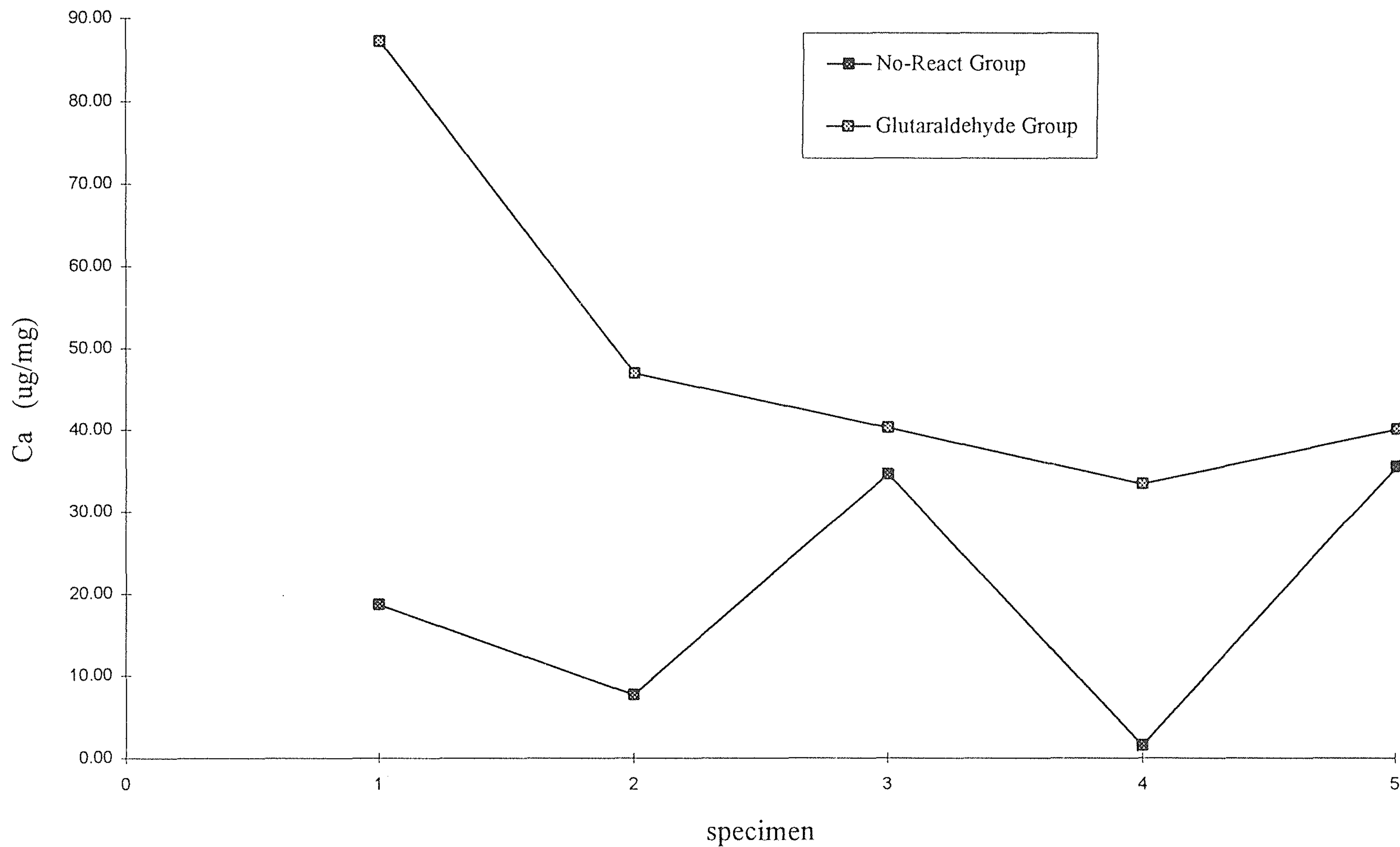

Figure 6.4 Comparison of Calcification Rate In-Vivo Tests (15 days)

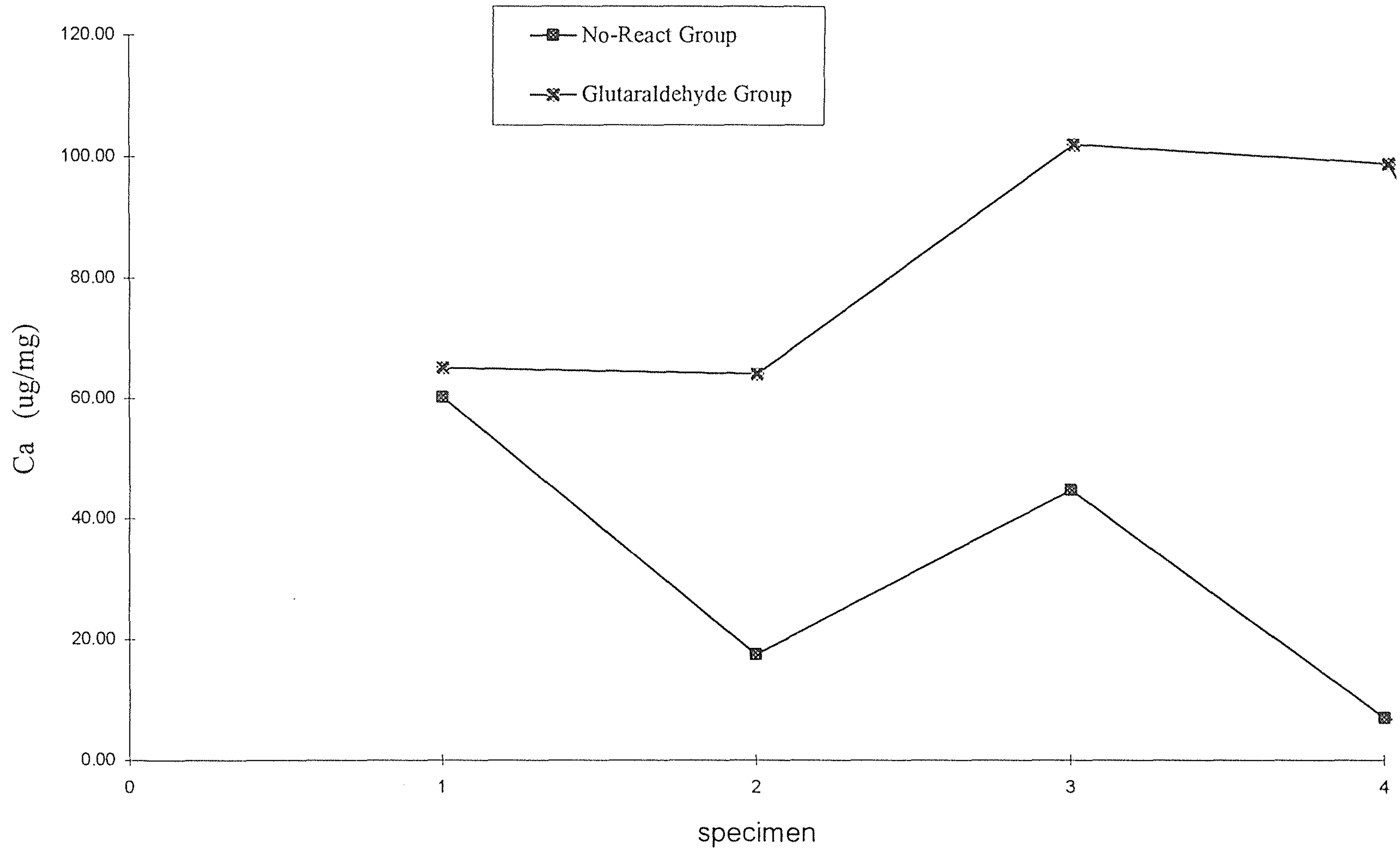

Figure 6.5 Comparison of Calcification Rate In-Vivo Tests (21 days)

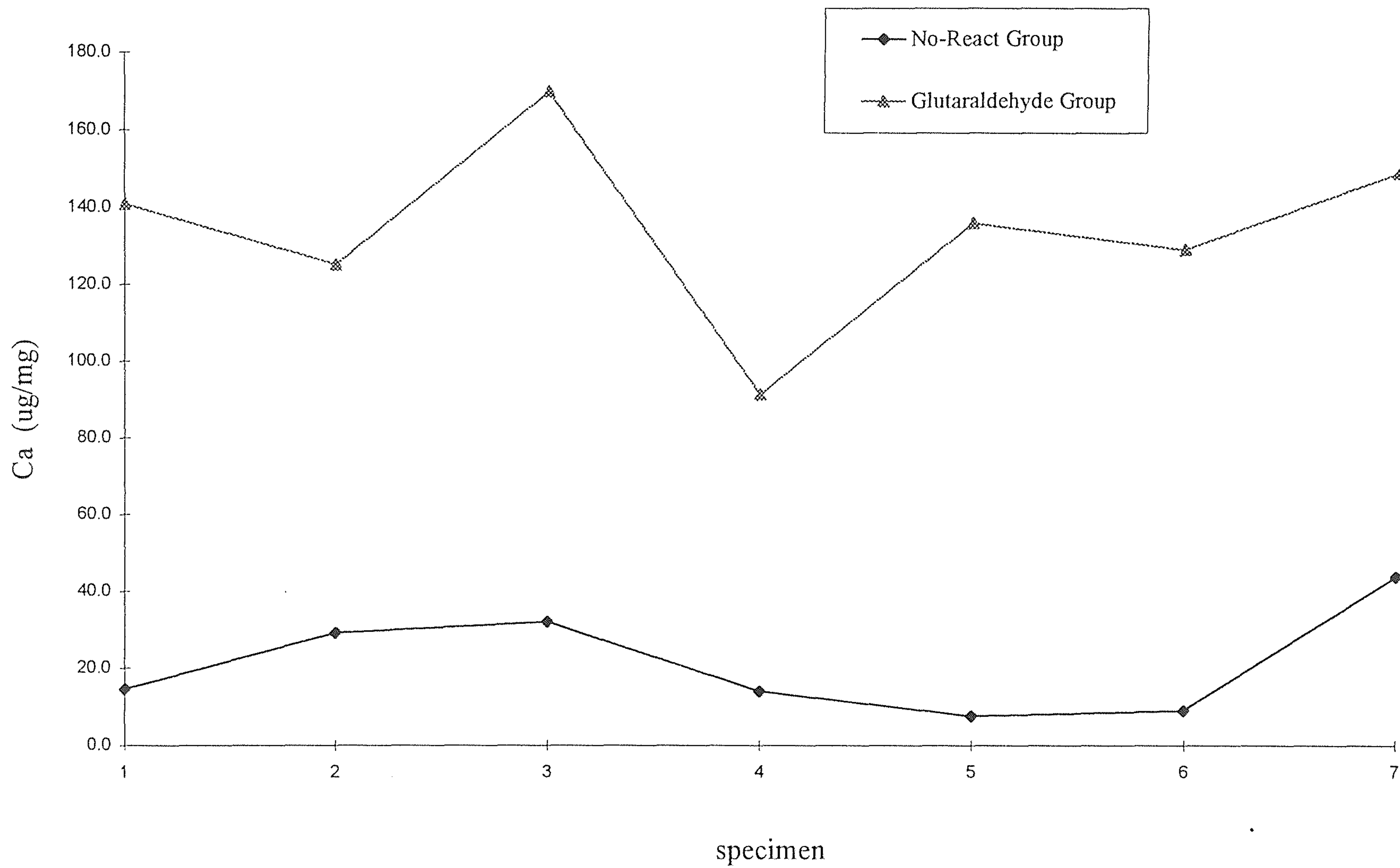

Figure 6.6 Comparison of Calcification Rate In-Vivo Tests (35 days)

REFERENCES

1. Buch, W.S., Kosek, J.C., Angell, W.W., "Deterioration of Formalin-treated Aortic Valve Heterografts", *J.Thorac Cardiovasc Surg*, 1970, 60:673-677

2. Inoescu, M.I., "Replacement of the Mitral Valve with Aortic Heterografts Mod", *Trends Cardiac Surg*, 1970, 2:112

3. Angell, W.W., Angell, J.D. Kosek, J.C., "Twelve Years Experience with Glutaraldehyde Preserved Porcine Xenografts", *J. Thorac Cardiovasc Surg*, 1982, 83:492-502

4. Ferrans, V.J., Spray, T.L., Billingham, M.E., Toberts, W.C., "Ultrastructure of Hancock Porcine Valvular Heterografts", *Circulation* , 1978, 58:Suppl 1:10-18

5. Spray, T.L., Roberts, W.C., "Structural Changes in Porcine Xenografts Used as Substitute Cardiac Valves", *Am J. Cardiol* ,1977, 40:319-330

6. Riddle, J.M., Magilligan, D.J., Stein, P.D., "Surface Morphology of Degenerated Porcine Bioprosthetic Valves Four to Seven Years Following Implantation", *J. Thorac Cardiovasc Surg* ,1981, 81:279-287

7. Schoen, et al., "Calcification of Bovine Pericardium Used in Cardiac Valve Bioprostheses, Implications for the Mechanisms of Bioprosthetic Tissue Mineralization", *Am J. Pathol* 1986, 123:134-145

8. Salomon, N.W., Stinson, E.B., Griepp, R.B., Shumway, N.E., "Mitral Valve Replacement: Long Term Evaluation of Prosthesis-related Mortality and Morbidity", *Circulation* ,1977, 56 (Suppl 2):94-100

9. Dewanjee, M.K., Fuster, V., Rao, S.A., Forshaw, P.L., Kaye, P.M., "Noninvasive Radioisotopic Technique for Detection of Platelet Deposition in Mitral Valve Prostheses and Quantification of Visceral Microembolism in Dogs", *Mayo Clin Proc*, 1983, 58:307-314

10. Yarbrough, J.W., Roberts, W.C., Reis, R.L., "Structural Alterations in Tissue Cardiac Valves Implanted in Patients and in Calves", *J.Thorac Cardiovasc Surg*, 1973, 65:364-375

11. Ionescu,M.I., Tandon, A.P., Mary, D.S., Abid,A., "Heart Valve Replacement with the Ionescu-hiley Pericardial Xenograft", *J.Thorac Cardiovasc Surg*, 1977, 73:31-41

12. Tandon, A.P., Whitaker, W., Inoescu, M.I., "Mutiple Valve Replacement with Pericardial Xenograft: Clinical and Hemodynamic Study", *Br. Heart J.*, 1980, 44:534-540

13. Levy, R.J., Schoen, F.J., Levy, J.T., et al., "Biologic Determinants of Dystrophic Calcification and Osteocalcin Deposition in Glutaraldehyde Preserved Porcine Aortic Valve Leaflets Implanted Subcutaneously in Rats", *Am J. Pathol*, 1983, 113:143-155

14. Anderson, H.C., "Normal and abnormal mineralization in mammals", *Trans Am Soc Artif Int Organs*, 1981, 27:702-707

15. Russell, R.G., Kanis, J.A., "Ectopic Calcification and Ossification. In:Nordin BEG, ed. Metabolic Bone Disease", *Churchill Livingstone*, 1984:344-365

16. Poole, A.R., Rosenberg, L.C., "Chondrocalcin and the Calcification of Cartilage" *Clin Orthop*, 1986, 208:114-118

17. Hunter, G.K., "An Ion-exchange Mechanism of Cartilage Calcification", *Conn Tiss Res*, 1987, 16:111-120

18. Schoen, F.J., Harasaki, H., Kim, K.M., Anderson, H.C., Levy, R.J., "Biomaterial-associated Calcification: Pathology, Mechanisms, and Strategies for Prevention", *J. Biomed Mater Res:Applied Biomaterials*, 1988, 22 A1:11-36

19. Wuthier, R.E., Gore, S.T., "Partition of Inorganic Ions and Phospholipids in Isolated Cell, Membrane and Matrix Vesicle Fractions: Evidence for Ca-Pi-acidic Phospholipid Complex", *Calcif Tiss Res*, 1977, 24:163-171

20. Ferrans, V.J., Boyce, S.W., Billingham, M., Jones, M., Ishihara, T., Roberts, W.C., "Calcific Deposits in Porcine Bioprostheses: Structure and Pathogenesis", *Am J Cardiol*, 1980, 46:721-734

21. Lian, J.B., Levy, R.J., Bernhard, W., Szycher, M., "LVAD Mineralization and Gamma-carboxyglutamic Acid Containing Proteins in Normal and Pathologically Mineralized Tissues", *Trans Am Soc Artif Intern Organs*, 1988, 34:839-944

22. Bernacca, G.M., Tom, G, et al, "In Vitro Calcification of Bioprosthetic Heart Valves: Report of a Novel Method and Review of the Biochemical Factors Involved", *J. Heart Valve Dis*, 1992, 1:115-130

23. Fujimoto, D., Yu, S.Y., "Elevation of Histidinoalanine in Calcified Human Aortas", *Biochemistry International*, 1984, 8:553-560

24. Long, M.M., Urry, D.W., "On the Molecular Mechanism of Elastic Fiber Calcification", *Trans Am Soc Artif Intern Organs*, 1981, 27:690-696

25. Termine, J.D., Conn, K.M., "Inhibition of Apatite Formation by Phosphorylated Metabolities and Macromolecules", *Calcif Tiss Res*, 1976, 22:149-157

26. Zilva, J.F., Pannall, P.R., "Clinical Chemistry in Diagnosis and Treatment", 3rd ed. London: *Lloyd-luke (Medical Books) Ltd.*, 1979, 342-343

27. Nistal, F., Garcla-Martlnez, V., Arbe, E. et al., "In Vivo Experimental Assessment of Polytetrafluoroethylene Trileaflet Heart Valve Prosthesis", *J Thorac Cardiovasc Surg*, 1990, 99:1074-1081

28. Kolff, W.J., Yu, L.S., "The Return of Elastomer Valve", *Ann Thorac Surg*, 1989, 48:S98-S99

29. Bruck, S.D., "Possible Causes for the Calcification of Glutaraldehyde-treated Tissue Heart Valves and Blood Contacting Elastomers during Prolonged Use in Medical Devices: a Physico-chemical View", *Biomaterials*, 1981, 2:14-18

30. Pollock, E., Andrews, E.J., Lentz, D., Sheikh, K., "Tissue Ingrowth and Porosity of Biomer", *Trans Am Soc Artif Intern Organs*, 1981, 27:405-409

31. Gabbay, S., Bortolotti, U., Josif, M., "Mechanical Factors Influencing the Durability of Heart Valve Pericardial Bioprostheses", *Trans Am Soc Artif Intern Organs*, 1986, 32:282-287

32. Deck, J.D., Thubrikar, M.J., Nolan, S.P., Aouad, J., "Role of Mechanical Stress in Calcification of Bioprostheses", In: Cohn LH, Galluci V, eds. *Cardiac Bioprostheses: Proceedings of the Second International Symposium. New York: York Medical Books*, 1982, 293-305

33. Thubrikar, M.J., Deck, J.D., Aouad, D.M., Nolan, S.P., "Role of Mechanical Stress in Calcification of Aortic Bioprosthetic Valves", *J Thorac Cardiovasc Surg*, 1983, 86:115-125

34. Villani, M., Bianchi, C.T., Vanini et al., "Bioprosthetic Valve Replacement in Children", In: Cohn LH, Gallucci V, eds. *Cardiac Bioprostheses: Proceedings of the Second International Symposium. New York: Yorke Medical Books*, 1982, 248-255

35. Levy, R.J., Schoen, F.J., Flowers, W.B., Tyler, S.S., "Initiation of Mineralization in Bioprosthetic Heart Valves: Studies of Alkaline Phosphatase Activity and its Inhibition by $AlCl_3$ or $FeCl_3$ Preincubations", *J. Biomed Mater Res*, 1991, 25:905-935