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BIOPROSTHETIC HEART VALVES: ULTRASTRCUTURE AND CALCIFICATION

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DECLARATION

I, Yinxing Zhang, claim that this is my original work and that it has not been submitted in this or in similar form for a degree at any university.

Signed by candidate

Date 21.6.18

DEDICATION

I dedicate this work to my Mother for the profound interest she always took in me, to my wife, Yapin for her generous concern and to our son, Tian Ling whose academic achievements at school are a continual source of encouragement to our family.

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ABSTRACT

Background:

Due to the geographic distance between abattoirs and commercial valve plants delays between harvest and fixation usually range from 48 to 72 hours. In order to assess the pre-fixation tissue damage arising from the hypoxic period and the resulting calcific degeneration after implantation, we used an ultrastuctural damage score and transmission electron microscopy.

Materials and Methods:

In a step by step manner, three major issues were clarified:

1)The degree of pre-fixation tissue damage was determined in the four most widely used commercially produced tissue heart valves. Since stentless bioprostheses represent the latest promising trend in the development of biological heart valves, stentless models of the following makes were compared: Baxter, Medtronic, St.Jude and Biocor. Due to the fact that the aortic wall component of these valves proved most resistant to all anticalcification treatments, aortic wall tissue stood in the centre of our analyses.

2) Subsequently, three main determinants of the fixation process namely: delay, temperature and fixative-concentration were varied with the goal of significantly improving the ultrastructural preservation of the bioprosthetic tissue.

3)Eventually, the influence of improved ultrastructural preservation on calcific degeneration was evaluated under in vivo conditions in the non-human primate and the rat model.

Results:

The comparison of the four most commonly used stentless bioprosthetic heart valves revealed a disturbing degree of

tissue damage in all valves. Using a damage score from 1 to 21 (21 being the worst), aortic wall tissue of commercial valves ranged from 10 to 18 and that of leaflet tissue from 12 to 20.

When fixation conditions were permutated, tissue damage could almost be abolished by immediate fixation (within 30 minutes of slaughter), low-temperature fixation(4°C) and high glutaraldehyde concentrations (>1%).

Our in vivo experiments confirmed that commercially used fixation (delayed fixation, room-temperature and low concentrations of glutaraldedhyde) with its concomitant high degree of tissue damage results in high levels of calcification. Apart from a distinctly improved calcification potential in ultrastructurally well preserved tissue, there was also an inverse correlation between tissue calcification and the concentration of glutaraldehyde used for fixation.

Conclusion:

We could demonstrate that commercially produced bioprosthetic heart valves uniformly show badly damaged tissue and that tissue damage contributes to the calcific degeneration of these valves. We were also able to determine ideal fixation conditions which in turn significantly reduced tissue calcification.

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BIOPROSTHETIC HEART VALVES: ULTRASTRCUTURE AND CALCIFICATION

A. *INTRODUCTION*

1. ROLE OF BIOPROSTHETIC HEART VALVES

Heart disease will still be one of the main causes of death in the next two decades (fig.1) according to the World Health Organisation's (WHO) human death statistics. More than 700,000 open-heart operations are performed each year and heart valve replacement is the most common and most frequently performed heart operation in developing countries.

Although mechanical heart valves have been successfully used to replace human heart valves during the last 20 years, their thrombogenicity necessitates chronic anticoagulant therapy. Even with anticoagulant therapy, however, the linearized embolic complication rate for mechanical valves may be as high as 5% per patient year and the bleeding complication rate from taking anticoagulants is similar[1]. Because of these problems an alternative to mechanical valves is often required particularly under circumstances which do not allow a regular follow up of anticoagulation. The most widely used alternative to mechanical valves are bioprosthetic heart valves which are manufactured

from tanned porcine or bovine tissue. Due to the much lower thrombogenicty of bioprosthetic heart valves, they do not require anticoagulation and can thus be used by a third world population with a lock of follow-up facilities, women who have not completed their families and by those in occupations requiring extreme physical activity. In summary, the main advantages of bioprosthetic valves are that they are often cheaper and can be used in a third world population. In order to render these valves non-immunogenic and prevent their rapid degradation, bioprosthetic heart valves require a tanning procedure which is achieved by the crosslinking of proteins. The most frequently used cross-linking agent is glutaraldehyde (GA).

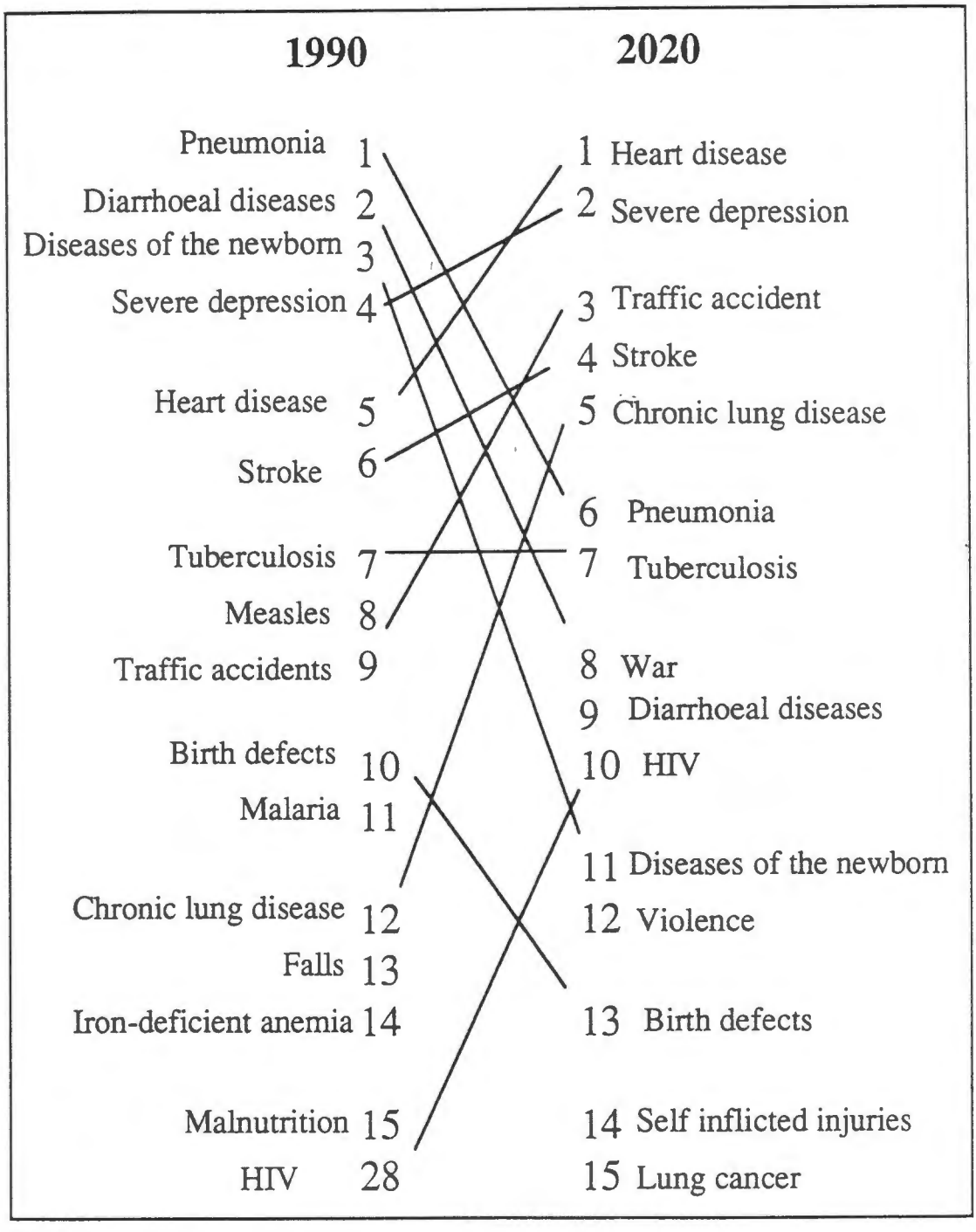


Fig. 1 WHO Predictions: A look at the changing occurrence of diseases that will affect people in the new millennium. It is apparent that heart disease will turn into the number one cause of death within the next 20 years.

2. MECHANISM OF GA-CROSSLINKAGE

Fixatives which are used for the treatment of bioprothetic heart valves have the purpose to: 1) reduce immunogenicity of materials, 2) increase resistance to degradation by host and bacterial enzymes and 3) sterilise the materials[2]. Due to its water solubility, small molecular character, and low cost, glutaraldehyde has been the most commonly used fixative[3] . Glutaraldehyde pre-treatment of xenogenic tissue was first introduced in 1968 [4], and the first glutaraldehyde cross-linked porcine aortic valve was implanted into humans in 1970 [1]. Apart from porcine valves, glutaraldehyde was also used for the fixation of bovine pericardium.

Chemical and physical character of GA

Glutaraldehyede has a molecular weight of 100.2, is characterized by a relatively low viscosity and a vapor pressure of 17 mmHg at 20°C. It is chemically known as 1,5-pentane dialdehyde, a five-carbon dialdehyde of relatively simple structure. It has a high aqueous solubility at physiological pH's.

Aqueous solutions of glutaraldehyde are highly reactive toward amino compounds. The glutaraldehyde molecule itself and its stoichiometric incorporation determines the level of material cross-linkages and stability[5]. At low concentrations, GA produces intermolecular crosslinks in collagen while at higher concentrations GA also forms intermolecular crosslinks

consisting of polymeric glutaraldehyde chains. Glutaraldehyde solution of 0.2% can crosslink up to 80% of the total sites available in leaflet tissue within fifteen minutes of fixation.

Advantage of GA treatment of bioprosthetic tissues

GA crosslinked collagen has a greater resistance to proteases, and a lower degree of swelling than uncrossliked collagen. Thus, crosslinking is an effective means of controlling the biodegradation rate of collagen-based biomaterials [2] .

Furthermore,GA not only neutralises antigenicity by cross-linking atigenic domains[6] but also inhibits enzyme action of the immobilized proteases by crosslinking and restricting them from the substrate interaction. Therefore, GA may also inhibit penetration of the endogenous proteases into the material and thus, further prevent degradation of the tissues. The resulting crosslink stability has been assessed by enzymatic digestion test, proving that [3] glutaraldehyde suppresses host reactivity and produces a material with good biochemical stability and minimal antigenicity [7] .

Disadvantage of GA treatment of bioprosthetic tissues

The chemical nature of GA-amino reaction products is complex. The simplest known reaction was confirmed to be a Schiff base resulting from the reaction of the ε-amino group of lysine with one of the aldehyde residues on the glutaraldehyde molecule [3,5]. However, Schiff bases are known in general to be reversible and

unstable, with advanced hydrolysis under acid exposure[3]. Although the exact nature of the GA crosslinking chemistry is still unclear, it is known that some of the polymeric GA species, notably oligomeric and polymeric hemi-acetals, are also unstable, and that crosslinks resulting from these species are therfore equally reversible.[2] Fifty percent of the apparently stable GA crosslinks in pre-treated pericardium eventually dissociated after three weeks of implantation in rats [8]. These degradation products of polymeric glutaraldehyde have been shown to be cytotoxic [3]. Concentrations as low as 3 parts per million are sufficiently toxic to kill endothelial cells in cell culture [9,10]. The relevance of this observation can be seen from the fact that free aldehyde groups released from bioprosthetic heart valve material are capable of preventing endothelial cell growth in vitro [10].
Furthermore, GA treatment does not inhibit enzymatic activities completely [2].

Pre-treatment of porcine aortic valve leaflets or bovine pericardium with glutaraldehyde also results in a noticeable colour change of these materials from white to yellow, which is thought to reflect the formation of cyclic-chromophores due to aldehyde-amino reactions [3]. Glutaraldehyde also changes the mechanical properties of the unimplanted material. In particular, unrestricted pericardial tissue for instance, shrinks and increases in extensibility after chemical modification with glutaraldehyde[7]. In the development of alternative tissue preservation procedures, particular attention is therefore paid to various pre and postfixation tissue treatments to offset the negative effects of glutaraldehyde [7].

In summary, glutaraldehyde treated tissue offers excellent hemodynamics and low thrombogenicty, but frequently fails because of tears or stiffening resulting from calcification and degradation [5].

3. FAILURE MODES OF BIOPROSTHETIC HEART VALVES

3.1 GENERAL OVERVIEW

Homograft-, xenograft-, and pericardical valves used for human aortic valve replacements may fail after several months of implantation, while others last for 15 years [11]. The causes of this failure are primarily implant factors, material factors, host biological factors and stress factors [12]. These include aspects such as calcification, thromboembolic events, bleeding, prosthetic endocarditis, immunoreaction, technical errors and design problems. Of these, calcification appears to be a main culprit in the failure of most bioprosthetic valves [13,14,15].

3.2 SPECIFIC FACTORS INFLUENCING VALVE FAILURE

3.2.1 CALCIFICATION

Bioprosthetic valve calcification is accelerated in children where it occurs in 50% within four years of implantation. Re-operation is also required in 10-20% of adult recipients within 7-10 years post-operatively[16]. Clinical studies have shown that calcification of implanted valves occurs sooner and more frequently under

conditions of altered or accelerated calcium metabolism, such as in patients with chronic renal failure, in growing children and in pregnant women[16,19].

Bioprosthetic tissue calcification is a process which affects both cellular remnants and extracellular components [12]. These initially diffuse calcific deposits increase in number and size to form calcific nodules which cause stiffening of the cusps or ulceration and tearing [12,17]. Calcified bioprosthetic valve tissues can be visuatised on the microscopy level by the von Kossa stain. Deposits in the cellular remnants and ground substance can be analyzed by electron microscopy.

3.2.1.1 MECHANISMS OF CALCIFICATION IN HEART VALVES

Physical and chemical characteristics of Ca^{++} deposits:

Vascular calcification is similar to that one physiologically occurring in bones [18] . The nucleus of calcification appears to be cellular remnants containing phospholipids, lipoprotein, and enzymes [2] . Usually, accumulating calcium phosphate is present in the form of apatite. Hydroxyapatite is also the most prevalent form of bone mineral, being composed of a crystalline lattice containing 10 calcium atoms, 6 phosphate groups, and 2 hydroxyl groups. Hydroxyapatite, $[Ca_{10}(PO_4)_6(OH)_2]$ is a rather insoluble compound in water at physiological pH [19] , but will dissolve in acid. The hydroxyapatite crystal lattice can be recognised by its characteristic diffraction pattern, or "fingerprint", when subjected to x-ray or electron diffraction [19]. HR-TEM observation

of vascular calcium phosphate particles prepared by different methods revealed very fine electron-dense dots of about 0.3-1.0 nm. Amorphous calcium phosphates prepared with differing Ca:P ratios showed that the basic structural unit is a sphere-like consisting of ion pairs of Ca^{++}-Ca^{++} and P^{+++}-P^{+++} in differing ratios or single Ca^{++}-P^{+++} ion [20].

Correlation of glutaraldehyde and calcification

Calcification of glutaraldehyde cross-linked bioprosthetic heart valves appears to be a multifactorial process. [21,17] Increased calcification for instance seems to correlate directly with the length of GA fixation time [22] . Although non-GA-crosslinked rat aortic homografts calcified within a very short time, GA crossliked aortic homograft tissue calcified even more severely[23].

Mechanical stress

Bioprosthetic valve tissues are subjected to a high dynamic mechanical stress that may lead to fatigue and abrasion. Various findings indicate that mechanical strain also accelerates mineralization and subsequently the life span of the valve [24 17,25] .

Thrombotic Surface Appositions

Thrombi at various sites are known to calcify, most notably in the left atrium of adults with mitral stenosis. Calcification in thrombi and vegetations is caused by platelets and leukocytes

that become trapped in the fibrin mesh. The intracellular components of those entrapped white cells and platelets that undergo calcification are the mitochondria. Crystal growth from these sites leads to the formation of large confluent and calcified masses [26] . Reducing the thrombogenicity may allow the endothelial cells to cover the surface of the bioprosthetic valve and prevent calcification [9] .

Cell membranes and organelles

Membrane-associated complexed acidic phospholipids play an important role in the process of both physiological and pathological calcification. Cytoplasmic organelles and plasma membranes of interstitial cells seem to serve as initial sites of calcification of bioprosthetic tissue [18] . The term membranous calcific deposits refers to the deposition of apatite along the membrane surfaces. Such deposits are especially present in fat necrosis and experimental calcification of liposomes. Membranous deposits imply that membranes may serve as a substrate for heterogeneous nucleation of apatite. Due to interaction with acidic phospholipids, Ca^{2+} condensation on the surface of the membranes may contribute to the membranous deposition of apatite. It has been reported that altered cellular membranous organelles in vascular tissues and neoplasms frequently develop a so-called "thick wall" with calcification. The "thick-wall" formation is apparently a slow process. Certain complex formations by the membrane with osmiophilic substances appear to form this "thick-wall". Alteration of the membranes by

phospholipases and non-membranous components of the cells are likely to be involved in the "thick-wall" formation[27].

Calcification of membranous structures occurs via the direct deposition of the microcrystalline apatites about a nanometer in size with their consequent side-by-side and end-to-end interactions. They form mature hydroxyapatite crystals which are distributed in the interstitial and succeeding molecular layer, and appear as needle-shaped or plate-like crystals [20] .

Proteolipids

Proteolipids which are usually integral membrane components have been shown to be the origin of the calcium phospholipid-phosphate complexes[17,19] . There is evidence that these complexes are present in the membranes of extracellular matrix vesicles and the membranes of cells in calcifying tissue [20] . Ca-phospholipid complexes serve as nucleating substrates for apatite formation in the cellular and organellar membranes. Calcium-binding proteins contain γ-carboxyglutamic acid residues. Other proteins, like alkaline phosphates and calcium-binding phosphoproteins were shown to be associated with crystal formation in mineralizing tissues. Osteopontin, for instance, a non-collagenous bone matrix phosphoprotein, is associated with several dystrophic cardiovascular calcifications[28]. Phospholipid and phosphoserine-containing proteins have been found in pathologically calcified tissues, while phospholipid membranes have been associated with hydroxyapatite crystal formation during pathological calcification [22] . Calcification of implanted valves has been

associated with comparatively high serum phosphate and osteocalcin levels and enhanced parathyroid hormone and vitamin D metabolism seen in the young [22, 23] .

Phosphate ions

Dissolved calcium and phosphate ions normally exist in the extracelluar fluid in a metastable equilibrium [29] . Calcium is mainly an extracellular ion whereas P_i is mainly intracellular [28] . Intact living animal cells have intracellular free calcium concentrations of approximately 10^{-7} M, while extracellular free Ca^{2+} is approximately 10^{-3} M, leading to a 10, 000-fold gradient across the plasma membrane. Thus, the Ca^{2+} entry into cells is passive [30,31] . Intracellular Ca^{2+} is bound by soluble cytosolic or membrane-bound proteins [31] . The calcium and phosphate ions are derived from the circulating blood and can come into contact with the calcifying matrix either directly, by simple diffusion through the matrix or after being absorbed and concentrated within connective tissue cells [26] . Concomitant increases in Ca^{2+} and P (inorganic-phosphate) in cell injury and in apoptosis provide an ideal milieu for apatite nucleation [27] . As ion concentrations increase in a solution, the incidence of collisions between solubilized ions, Ca^{2+} and P_i, increases, and the ions form clusters of critical size or nuclei. Addition or deletion of one more molecule to or from the nucleus will lead to crystallisation or dissolution of the nucleus. Once the nucleus is formed, the subsequent growth of crystals occurs very rapidly [27] .

Mitochondria and Endoplasmic reticulum

Isolated mitochondria have a large capacity to accumulate Ca^{2+}. Mitochondria are the major intracellular Ca^{2+} storage site. Coincidentally, the calcium deposits appear first in the mitochondria of traumatised cells [19] . In vivo mitochondrial calcification is usually limited to massive tissue necrosis. Mitochondrial calcification constitutes a basic mechanism for the initiation of intracellular calcification, and from mitochondria the deposits can spread and fill the entire cell [26] . Similarly, the endoplasmic reticulum serves as the main Ca^{2+} storage organelle, containing Ca^{2+}-binding proteins [27] .

Cell remnants and fragments

GA leads to the devitalization of the intrinsic connective tissue cells of bioprostheses, thus resulting in a breakdown of transmembrane calcium regulation and hence contributing to cell associated calcific deposits [28,23] . These deposits are associated with cell remnants and fragments in the nuclei, residual organelle-debris in the cytoplasm, or the plasma membrane [32,18] . From these primary sites, the process progresses into the intercellular space, adjacent to collagen fibrils and elastic fibrils.

Matrix vesicles are membrane-bound extracellular bodies secreted by connective tissue cells [18,19] . They contain calcium-acidic phospholipid-phosphate complexes in their membranes and possess significant alkaline phosphates activity [18] . Matrix vesicles which are rich in phospholipids may play a role in the

initial phase of mineralization [22] . If calcified, these vesicles contain small needle-shaped crystals of calcium phosphate enclosed by a membrane. The crystals grow in size as the membranes of the matrix vesicles rupture, and extension and coalescence of crystal growth lead to calcification of collagen [1] . Phospholipid phosphate complexes have also been found in the membranes of extracellular matrix vesicles [20] . Matrix vesicle-like cell fragments have been noted in several pathological calcification processes, including calcified degeneration of aged aortic valves [31] . In a study of the calcification of human aortic valves by Kim, products of cellular degradation as well as matrix vesicles appeared to be the early sites of calcium deposition [23] .

Collagen

Calcification of collagen can occur from crystal growth arising from the matrix vesicles or can be formed directly on the collagen fibres as previously reported [26] . Localized calcific deposit found in the interior of collagen fibrils support the second mechanism, indicating that collagen calcification can be an independent process, initiated through phosphate groups on the collagen proteins. Calcification occurring on the surface of collagen fibrils was almost identical to interstitial calcification. Calcification was observed only around the 'thick' collagen fibrils[29] , longitudinaly aligned along the collagen fibrils [33] . It appears that collagen calcification is primarily involved in determining the size, shape and orientation of calcific deposits in bioprosthetic heart valves [20,31] .

3.2.1.2 MORPHOLOGICAL OBSERVATIONS

Pathological-anatomy of bioprosthetic heart valve calcification

Calcific deposits in bioprosthetic valves after long-term implantation (>3 months) were first noted in the cuspal commissures and basal attachment sites. Calcific deposits also accumulate within and at points of attachment and coaption of the valve cusps [22] . The cuspal deposits have a pale yellow appearance, are discrete and usually raised and projected above both (in-and out-flow) surfaces of the leaflets forming irregularly shaped conical or bulbous crystals [26] .

Histology of bioprothetic heart valve calcification

The leaflets of the bioprosthetic arctic valves are arranged in three layers 1) fibrosa, 2) spongiosa, and 3) ventricularis. The base of the leaflet is thicker than the free edge. The inflow surface displays several prominent transversely oriented folds. The outflow surface is composed of dense bundles of collagen and overlying endothelial cells. The endothelium and adjacent fibrosa layer is rich in elastic fibers and collagen.

The aortic wall media contains elastic fibers and lamellae, smooth muscle cells, collagen fiber, and ground substance.

deposits were noted in the fibrosa, but later deposits expanded into the spongiosa [26] . The large calcific deposits in the spongiosa frequently displace the other layers of the bioprosthesis. These deposits are often multinodular, with adjacent nodules being separated from one another by fibrin deposits or by clear spaces [26] .Others have shown that the sites of calcification, as well as the size of the individual deposits , increased with time, principally in the spongiosa [36] . Tissue damage is not always associated with calcification, but calcification is always associate with tissue damage at the boundary between the organised core and damaged collagen. Calcification never originates on the leaflet surface; any calcium salts seen at this site originated in deeper layers [7] . Giant cell and macrophage reaction are rarely seen [34] . The earliest deposits at the edges of expanded nodules involve cell remnants. Mineral deposits are also diffusely found throughout the aortic media, with initial deposits apparently associated with devitalised smooth muscle cells of the aortic wall media between the elastic lamellae [26,37] . Later calcific deposits appeare to also affect the elastic lamellae themselves [32] .

Ultrastructure of bioprosthetic heart valves and calcification

Bioprothetic heart valve ultrastructure

- Aortic valve leaflet:

1) The endothelial cells form a discontinuous layer on both inflow and outflow surface of the valve. The cells of this monolayer are joined to one another by junctional complexes. The shape of the aortic valve endothelial cells is variable. Some are thin, elongated and attenuated with flatened nuclei.

2) The spongiosa: This layer contains loosely arranged connective tissue with fibroblasts, collagen fibrils and small elastic fibers.

3) Fibrosa: In this layer the collagen fibrils are much more abundant and more densely packed and show a tendency to form parallel bundles. Fibroblasts are present in both the fibrosa and the spongiosa.

- Aortic wall :

1) The endothelium is similar to that on the aortic valve leaflets. Endothelial cells extend through the fenestrations of the elastica interna and contact the underlying smooth muscle cells. Through the same openings, the ground substance of the intima is in continuity with that of the media.

2) The media is mostly muscular. Smooth muscle cells are surrounded by basal laminae and collagen fibers and are interspersed with isolated elastic fibers or lamellae wich are fenestrated and helically oriented. Smooth muscle cells are the only cellular component of the media.

3) The adventitia is thick with an inner dense and an outer loose part; it contains bundles of collagen and elastic fibers with longitudinal or helical orientation, sparse fibroblasts, adipose cells and longitudinal smooth muscle fibers.

Spherical calcium deposits

Spherical calcium deposits

The spherical calcific deposits consist of a central amorphous mass of flocculent material surrounded by a denser flocculent mass frequently covered by thin crystals of hydroxyaptite. Small dense deposits of a crystalline nature are often seen on elastic fibers but rarely on other structures. Amorphous granules are frequently observed abutting directly at the edge of elastic laminae. Spherical material, bearing no relationship to any formed elements, is frequently seen in the ground substance. The central area of the mass is made up of flocculent and spherical components. A lighter core surrounded by a dense area with crystals of hydroxyapatite at its edges and in contact with a patch of elastic tissue can also be observed[23] .

Calcification of cellular debris

The ultrastructure of bioprothetic tissue calcification shows early association with the connective tissue cells or association with dead smooth muscle cells of the aortic wall media. In the cytoplasm of pre-implantation samples residual organelles, cell debris, cell remnants and membrane fragments are regularly seen[2,4,22, 23,24,32,38] . The subsequent calcification is associated with necrotic aortic wall medial smooth muscle cells, particularly in the nuclei, the cytoplasm and the cell membranes[38] .

Calcification of extracellular components (Collagen, Elastin):

Collagen: A radial flower-like arrangement of small crystals is seen around both single and groups of collagen fibrils. The calcified fibrils are localized within groups of collagen fibrils showing washed-out borders with obliteration of interfasiculate spaces. These calcified masses of sharp spicules form irregular but sharply demarcated areas surrounded by an osmiophilic border[29,39].

The most often found calcific deposit patterns are: 1) The calcific deposits involve directly the collagen fibrils but do not extend into the interfibrillary spaces. 2) The calcific deposits are localised on the periphery of the collagen fibrils and in the interfibrillary spaces; however, the interior of the collagen fibrils are not involved and the fibrils therefore appear as electronlucent cylinders surrounded by and extremely dense calcified matrix. 3) Calcification deposits occupy both the interfibrillar spaces and the interior of the fibrils, thus giving the deposits a relatively homogeneous appearance. High resolution images of collagen-associated calcification masses reveal alterations of the characteristic of dark and light bands with their periodicity of 1.5 nm[20].

Elastin: This extracellular matrix compponent is a major site of extracellular calcific deposits in the aortic wall[38]. The first calcific deposits are usually small, growing between the elastic lamellae. The elastic fibers show a strange speckled appearance due to the deposition of starry crystals around their microfibrils which are filled with needle-shaped crystallites[29]. There is also a large number of membranous vesicles in apposition to the

elastic fibers. Calcific deposits are subsequently seen in both the vesicles and elastic fibers.[27] Ultrastructurally, elastic lamellae appear as dense, light-gray formations with occasional microfibrilar elements.

B. MATERIALS AND METHODS

1. COMMERCIAL VALVES

Four different companies donated three of their current aortic root prosthesis: Edwards Prima (Baxter Healthcare Corp, Irvine, CA), Medtronic-Freestyle (Medtronic Inc. Irvine, CA), Biocor No React (Biocor Industria Pesquisas Ltda, Nova Lima, Brasil), and St. Jude-Toronto SPV (St. Jude Medical, St.Paul, MN). Samples were taken from the central area of leaflets and aortic walls in the same manner as those for the other experiments.

2. LIGHT MICROSCOPY / IMAGE ANALYSIS

Fixed samples were embedded in paraffin wax and sectioned at 5 µm using e rotary microtome. These sections were then incubated overnight, at 37°C and thereafter a von Kossa stain was prepared, with van Gieson counter stain. The percentage of tissue calcification was then analysed using a Leice Q 500 MC system (Leica, Cam bridge Ltd, Cam bridge, UK).

3. SPECIMEN PROCESSING FOR TRANSMISSION ELECTRON MICROSCOPY

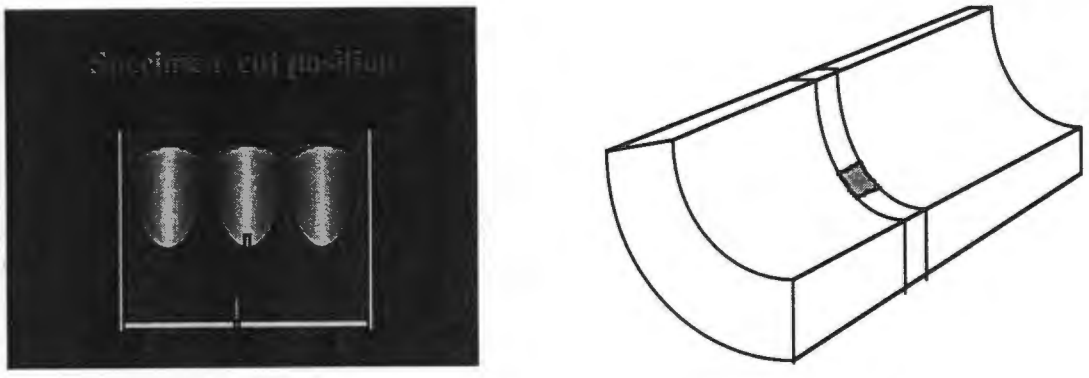
GA-fixed samples were incubated in 0.16M cacodylate buffer for 12 hours and then rinsed three times (2 minutes each) in distilled water. After osmification (2%; Electron Microscopy Science FT.

Washington, PA19034) for two hours and three rinses in distilled water (2 min. each), contrasting was performed in an Uranyl Acetate (Merck, Germany) solution (0.5% Uranyl acetate in 80% acetone) for 15 minutes. After graded dehydration in acetone (95% for 15 min and 100% for 30 min) samples were placed into a 1:1 mixture of 100% acetone and Spur resin (Electron Microscopy Science FT. Washington, PA19034) for two hours. This mixture was replaced by fresh pure Spur resin and incubated at 70°C for one hour. Subsequently samples were again transfered to fresh resin for embedding. The embedded samples were then trimmed and cut at ultrathin sections of 70 - 80 nm on a Leica Ultracut S microtome (Reichert, Vienna, Austria). Staining of the samples was done with Uranyl acetate (2%) and lead citrate (FT, Washington).

As indicated in the drawing (fig. 2) a small sample was taken from the base of the leaflet and the aortic wall.

All specimens from commercial heart valves were stanied by Toluidine blue [uniLAB.®SAARCHEM (PTY) LTD] for ultrastructural pre-examination of semi-thin sections.

Aortic wall ultrathin sections were taken from two different regions of the specimen block.
All of the ultra-sections of leaflet and aortic wall samples were investigated under the standard magnification of 3,000X, 10,000X, 50,000X .
Specimens were viewed in a JEOL-100 S transmission electron microscope (Jeol, Tokyo, Japan).



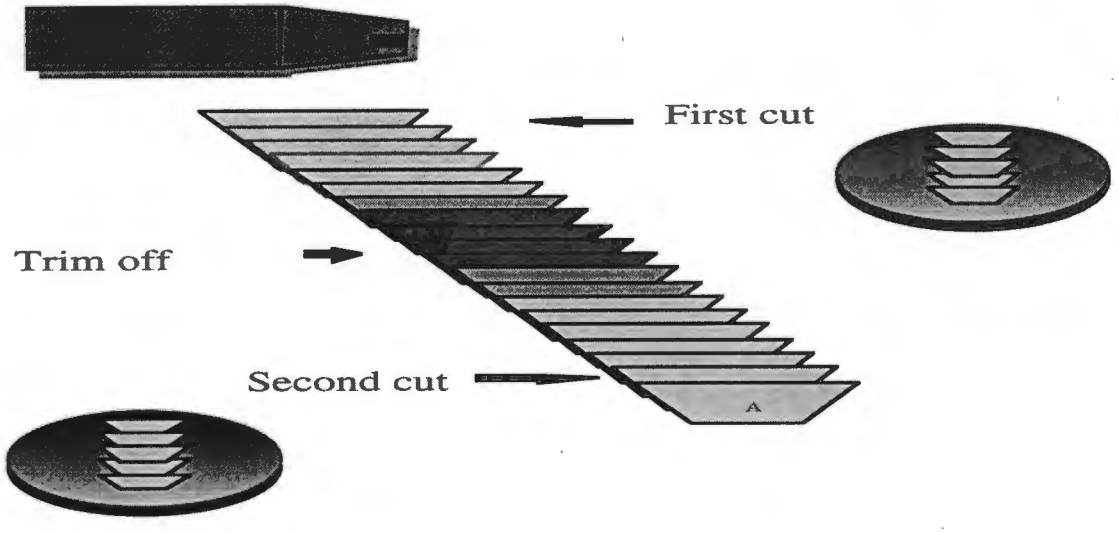


Fig.2 Ultra section collection

4. DAMAGE SCORE

Glutaraldehyde fixation procedures were evaluated on porcine aortas. The integrity of the ultrastructure of the tissue was assessed by transmission electronmicroscopy (TEM). In order to grade the extent of tissue damage, a 21-point scoring system was developed (figs. 3-9). Seven different cellular and extracellular components were evaluated, namely: the fragmentation/splinting of collagen, intercellular water spaces, membrane integrity,

cytosolic flocculation, intracellular vacuoles as well as swelling and disintegration of mitochondria and endoplasmic reticulum. Depending on the extent of damage, up to three points were awarded to each component. While zero points meant perfect ultrastructural integrity of the respective structure, a value of one indicated minimal damage, of two moderate damage and of three severe damage.

Damage scores of each of the three groups (n=6) were compared using student's paired t-test.

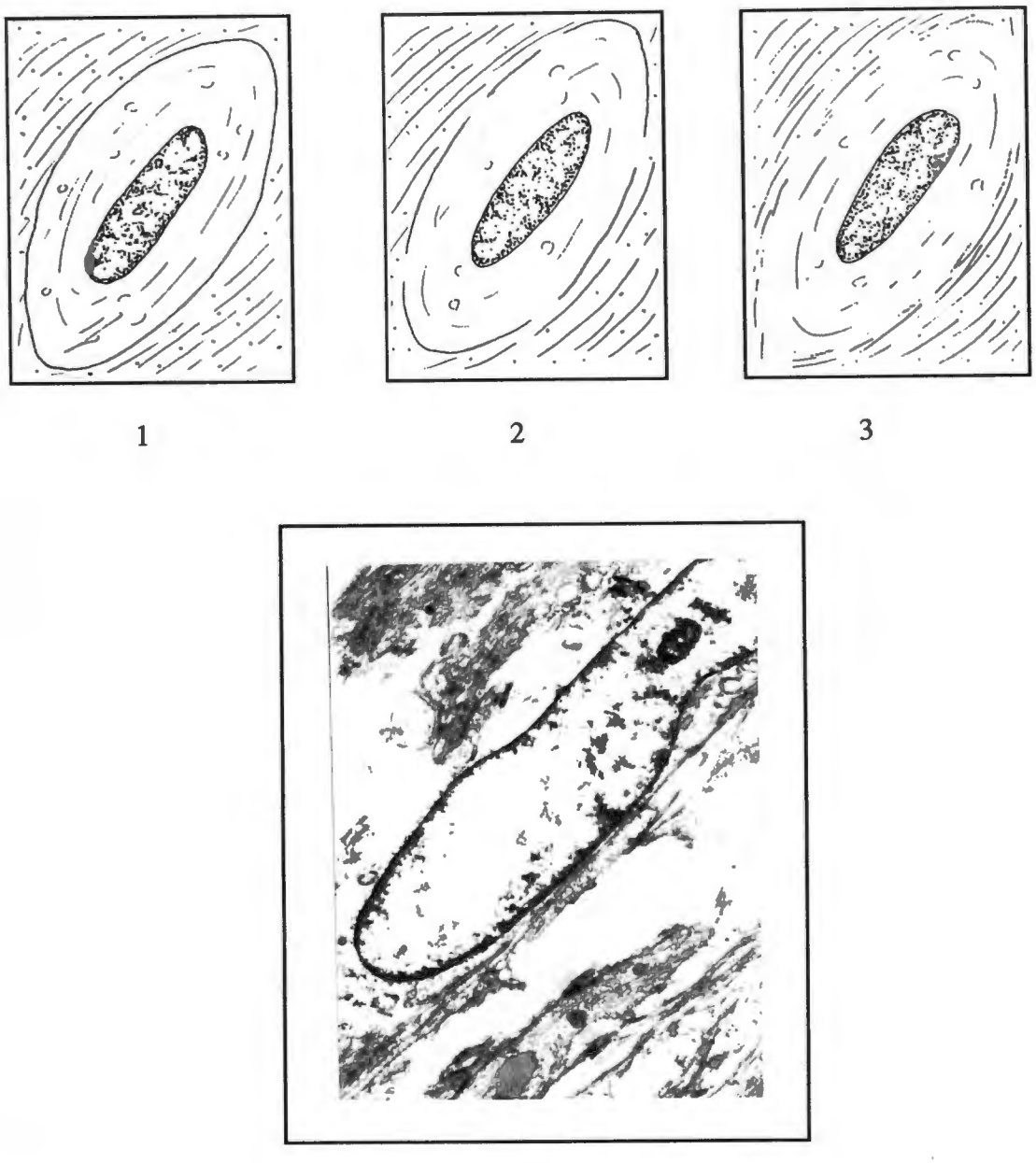


Fig. 3 Damage score of membranes 3,000X to 10,000X

1 2 3

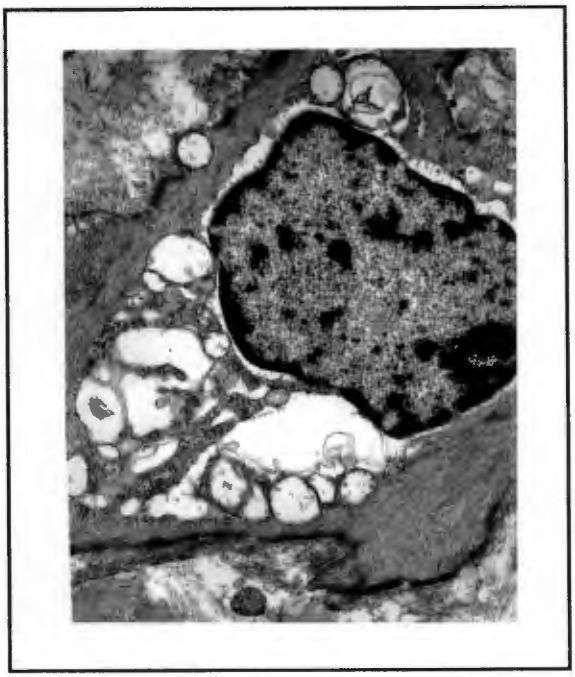
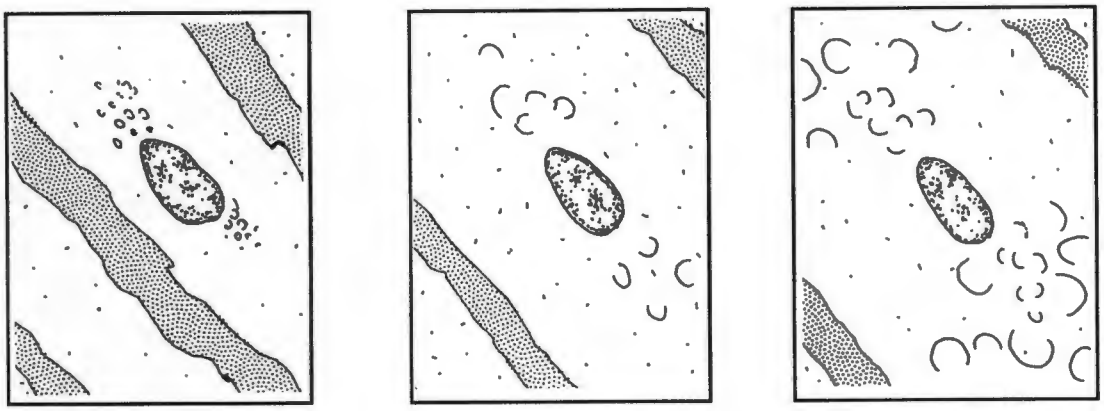


Fig. 4 Mitochondrial damage score 3,000X to 10,000X



1 2 3

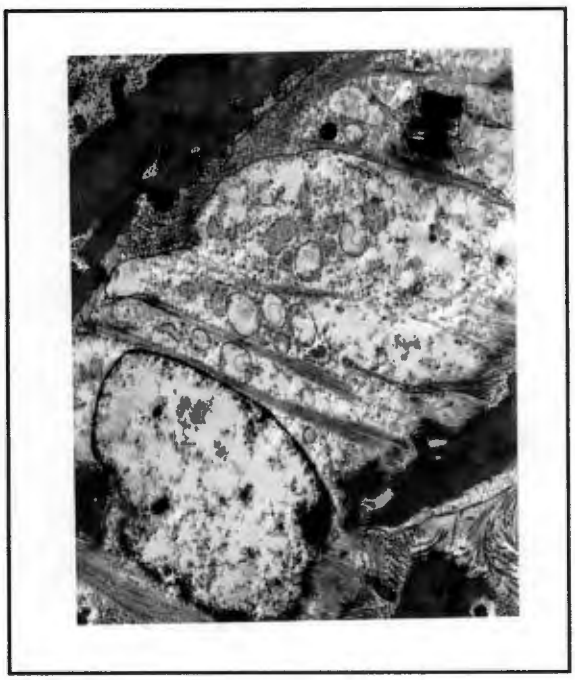


Fig. 5 Damage score of extracellular matrix 3,000X to 10,000X

1 2 3

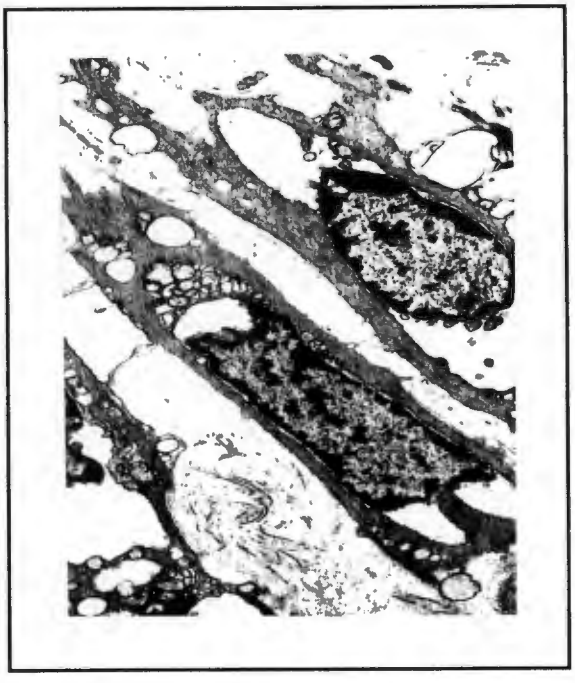
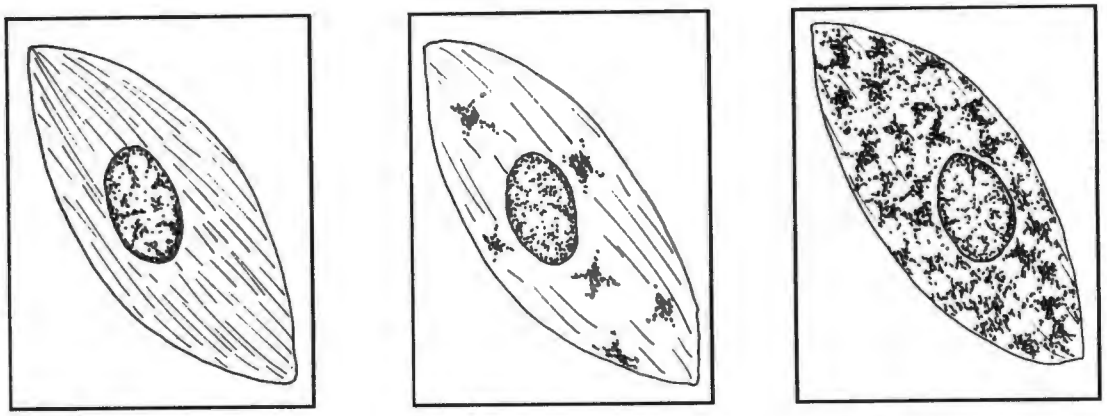


Fig. 6 Peri-nuclear vacuoles 3,000X to 10,000X



1 2 3

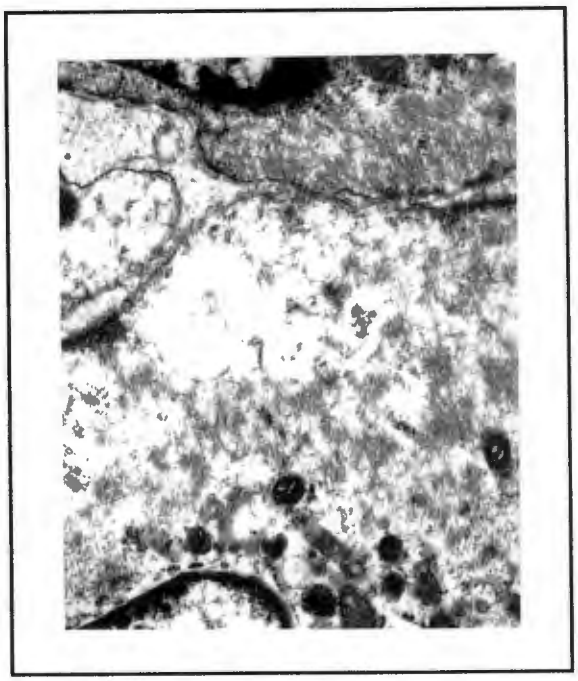
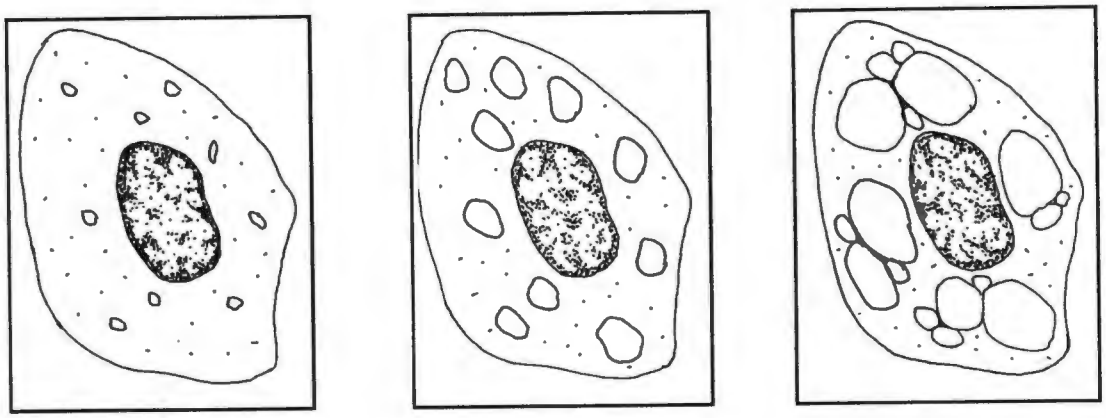


Fig. 7 Damage score of cytosol flocculation 3,000X to 10,000X



1 2 3

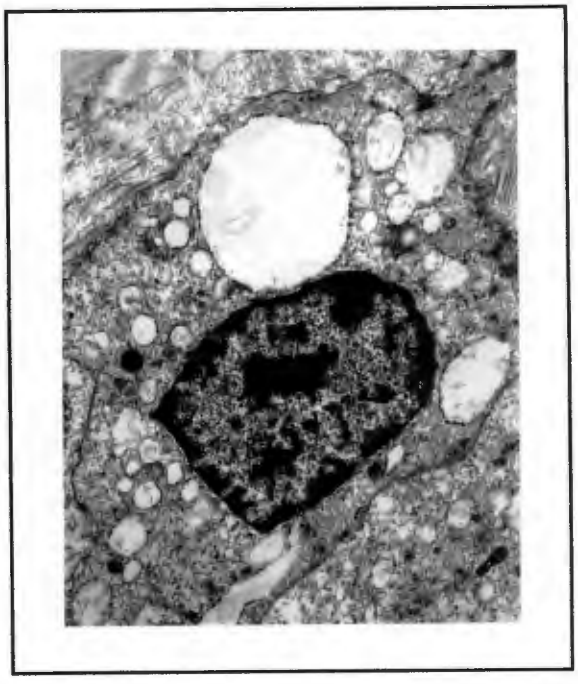
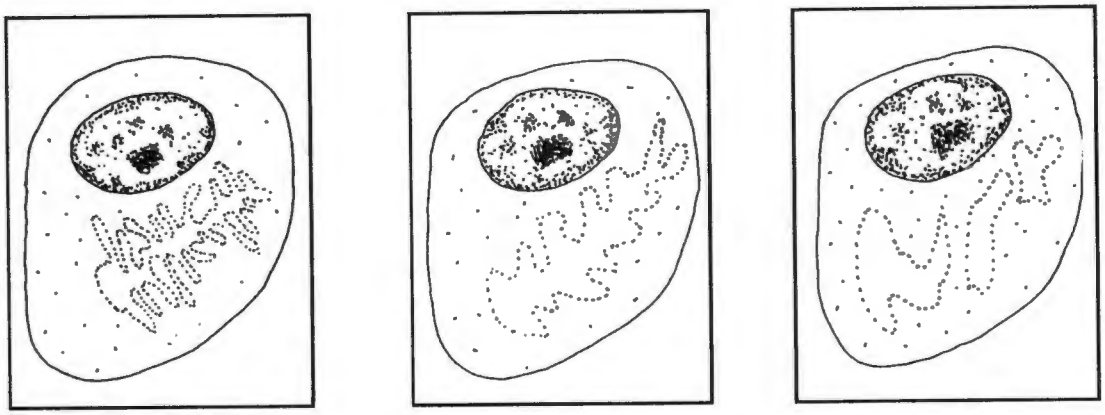


Fig. 8 Cytoplasmic vacuoles 3,000X to 10,000X



1 2 3

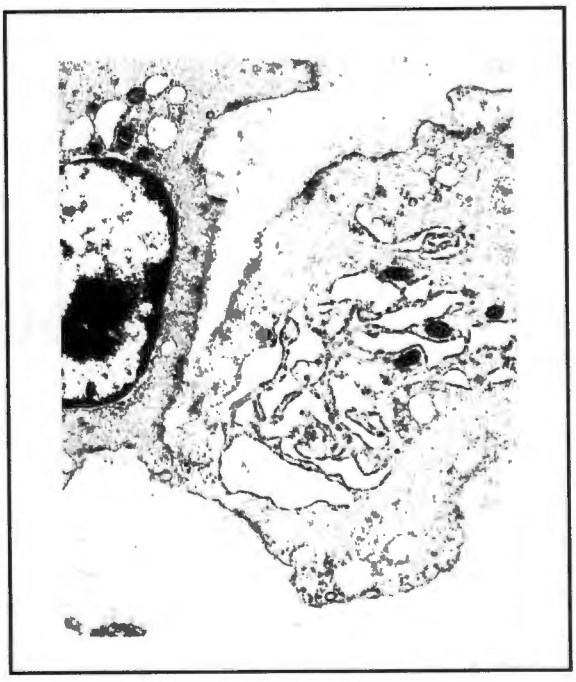


Fig. 9 Damage score of endoplasmic reticulum 3,000X to 10,000X

5. FIXATION STUDIES AND TISSUE COLLECTION

Porcine aortas were obtained from a local abattoir from freshly slaughtered pigs. Harvesting was performed as aseptic as possible. The aortas were dissected from the mediastinal block by removing all adjacent tissue, fat and para-aortic lymph-nodes. Aortas with a diameter of 2 to 2.5 cm and a wall thickness of 3 mm (measured with a vernier) were selected and cut into 2 cm segments. These segments were then either placed into phosphate buffered saline (PBS) or glutaraldehyde.

5.1 INFLUENCE OF FIXATIVE CONCENTRATION

Seven different concentrations of glutaraldehyde (Saarchem, Pty Ltd, South Africa) in PBS were prepared: 0.2%(V/V), 0.5%, 0.65%, 1%, 2%, 3%, and 4%. The temperature of the solutions was kept at 4°C and the pH buffered to 7.4. The osmolarity of each solution was measured before and after fixation (Semic-micro osmometer, Knauer, Germany). Fixation of tissue from six different aortas (n=6) per concentration was performed on site by placing the aortic segments into the different glutaraldehyde solutions immediately after dissection (30ml/g tissue). The entire procedure between slaughter and fixation did not take longer than 5 minutes. On day one, the fixative was replaced by fresh fixative. Samples were kept in the fixative at 4°C for all together 7 days. According to Hayat [40] the osmolarity of the buffer used for the fixative is more important than that one of the final glutaraldehyde solution. Therefore, PBS with an osmolarity of 290 mOsm was used for all fixatives.

5.2 INFLUENCE OF FIXATION TEMPERATURE

In each temperature group samples from 6 different aortas (n=6) were fixed at the abattoir, immediately after dissection. All samples were fixed in 3% glutaraldehyde (PBS; 30 ml/g tissue) at temperatures of 4°C (n=6), 22°C (room temperature) (n=6) and 37°C (n=6). The initial fixative was always replaced with fresh one on day two. Fixation was performed for 7 days.

5.3 INFLUENCE OF DELAYED FIXATION

Six samples from different aortas were fixed in each time-group using 3% glutaraldehyde (PBS; 30ml/g tissue) at 4°C. Specimens were either immediately fixed or kept in PBS at 4°C prior to fixation for 30 min , 1h , 4h , 8h , 16h , 64h and 128h. Fixation was again continued for 7 days at 4°C with renewal of fixative on day one.

After seven days of fixation, aortic segments were opened by a longitudinal incision and discs with a diameter of 0.6 cm were punched from the aortic wall. The outer and inner 1mm of the discs were trimmed off by a specially designed trimming device and two $1mm^3$ blocks of tissue were cut out of the remaining middle 1mm of tissue. Specimens collection was followed by routing TEM sample processing. Specimens were viewed in a JEOL-100 S transmission electron microscope (Jeol, Tokyo, Japan) and electron micrographs were taken systematically of the entire section at magnifications of 3000X, 6000X and 10,000X.

6. VERIFICATION OF ASSUMED IMPROVEMENTS IN VIVO

6.1 BIOPROSTHTIC TISSUE SAMPLING

Descending porcine thoracic aortas (ca. 8 cm in length) were roughly dissected immediately following slaughter and subsequently transferred without delay at the abattoir into low temperature (4°C) solutions of either phosphate buffered saline (PBS, pH 7.4 0.1M) or PBS containing glutaraldehyde (GA, Saarchem electron microscope grade) (150 ml per sample). Following transport on ice to the laboratory the tissue was fixed for seven days and then either cut into discs (1.2 cm diameter, trimmed to a standard thickness of 1.5 mm with the luminal surface left untouched, weight ca. 150-200 mg) or sewn into tube-grafts of six centimeter length. Groups were defined as follows:

6.1.1 SINGLE STAGE GA FIXATION : Groups I to IV

In contrast to the tissue collected in glutaraldehyde (0.2% GA, Group II; 1.0% GA, Group III; or 3.0% GA, Group IV), fixation of aortic tissue collected in PBS was delayed for 48 hours at 4°C before the tissue was eventually transferred to the fixative (0.2% GA in PBS, Group I). This latter treatment of group I reflects the standard fixation procedure used by certain commercial manufactures of bioprosthetic heart valves. All tissue was kept in fresh fixative (30 ml / g tissue) for seven days. Isopropyl

alcohol (1%; Merck. Cat No. 9634), in accordance with standard commercial fixation procedures [41], was added during the last 3.5 hours of fixation in Group I only. All tissue samples were then rinsed in PBS for 24 hours at 37°C and stored at low volume in PBS at 4°C until implantation.

6.1.2 TWO STAGE GA FIXATION Groups V to VIII

Following immediate fixation in glutaraldehyde for 48 hours in PBS at 4°C, aortic tissue (tubes or discs) were transferred to 0.1M L-Lysine solution (Sigma, catalogue No. L- 5501, pH 7.6 at 37°C) [42] for an additional 48 hours (30 ml/g tissue) before being returned to the corresponding glutaraldehyde fixatives (0.2% GA with initial 48 hour delay, Group V; 0.2% GA, Group VI; 1.0% GA, Group VII; or 3.0% GA, Group VIII) in PBS for five days at 37°C. Finally the tissue was rinsed in PBS for 24 hours at 37°C and stored at low volume in PBS at 4°C until implantation.

6.2 ANIMAL IMPLANTS

All experimental protocols were approved by the Animal Research Review Committee of the University of Cape Town, and all procedures complied with the "Principles of Laboratory Care" and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23).

In all specimens the delay between completion of the fixation and the subsequent implantation was limited to a maximum of ten days.

6.2.1 RAT SUBCUTANEOUS IMPLANTS

Aortic wall discs were implanted subcutaneously into the abdominal wall of weaned outbred Sprague Dawley rats (200-250g). Four randomly assigned discs were implanted per animal. For each group six different samples were implanted. After 12 weeks the tissue was retrieved for subsequent histological, electron microscopical and quantitative tissue calcium analysis.

6.2.2 BABOON ILIAC IMPLANT MODEL

Aortic tubes were bilaterally interpositioned in an end-to-end fashion into the iliac arteries of baboons (*Papio ursinus*). Six grafts per group were implanted into male animals (22.6 $\pm$ 3.9 kg). After six weeks the animals were sacrificed and the tissue fixed through perfusion fixation of the lower extremities. For this purpose the abdominal aorta was cannulated, and proximally cross-clamped. The inferior vena cava was then opened and 2 litres of warm (37°) Ringer's Lactate followed by 1 litre of 10% formalin in PBS were infused at a pressure of 80 mm Hg. The tissue was then removed and processed for histology, electron microscopy and quantitative tissue calcium analysis.

Explanted samples were processed for Transmission Electron Microscopy (see section 1.1.2) and viewed as described in section 1.2.3.

7. TISSUE CALCIUM MEASUREMENT

Following explantation tissue was excised and stored at -20°C until quantitative analysis of tissue calcium by atomic absorption spectroscopy could be performed. The tissue was then dried at 104°C for 24 hours, weighed and ashed at 560°C for 12 hours in a Muffle furnace. Ashed tissue was diluted in 20% hydrochloric acid (BDH) at a ratio of 10mg dried tissue:1ml HCl. A final dilution of this solution was made in 0.5% lanthanum chloride (BDH, Spectrosol) and the absorption measured at 422.7 nm on an atomic absorption spectrophotometer (Varian Techtron, model AA1275). A standard curve was obtained using a standard calcium solution (Titrisol, Merck, catalogue No. 9943) in 0.5% lanthanum chloride. The level of calcium in the solution was expressed as μg calcium per mg dry weight of tissue.

All tissue calcium results were expressed as mean ± standard error of the mean. Analysis of tissue calcium in the baboon model was done by one factor analysis of variance with replicates. Analysis of tissue calcium in the rat model was performed by two factor analysis of variance with replicates. The significance level was set as $p<0.05$. Post-hoc multiple comparisons in both groups were done according to the method of Scheffè.

Cross sections of the mid-segment of each graft were subjected to image analysis using a Leica Q 500 MC system (Leica Cambridge Ltd, Cambridge, UK). The percentage of cross-section area which was positively stained for calcium was determined.

8. TEM ANALYSIS

Explanted samples were processed for Transmission Electron Microscopy (see section 3) and viewed as described in section 5.3.

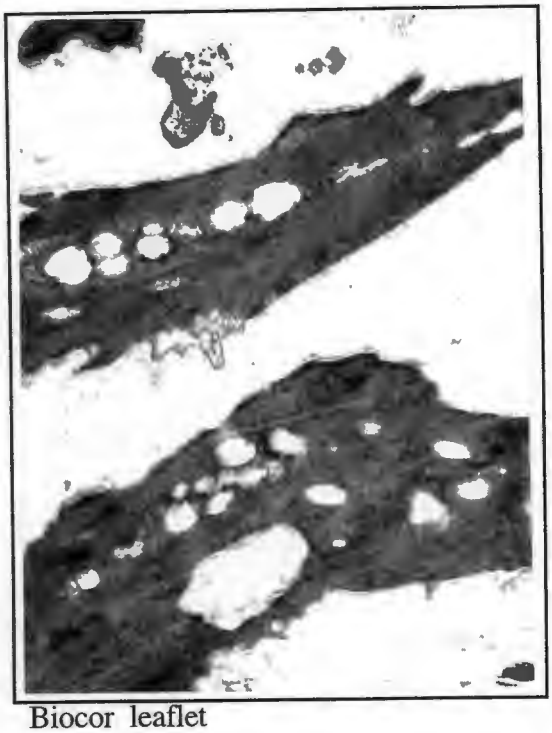
C. RESULTS

1. COMMERCIAL HEART VALVES

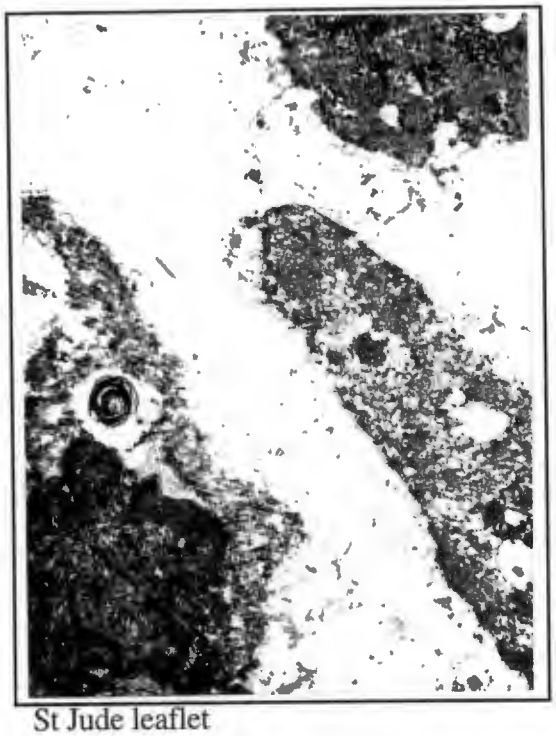
Ultrastructural Assessment of Commercial Aortic Root Prostheses

All nine commercial bioprosthetic aortic walls showed signs of severe ultrastructural disintegration which were least pronounced in the Biocor No React valve (damage score 10.75). Cytosol flocculation, intracellular vacuoles, swollen mitochondria and intercellular waterspaces were uniformly found in all valves, whereas membrane disintegration and mild signs of collagen fracture and splinting were only detected in Baxter's Edwards Prima (damage score 17.33), Medtronic's Freestyle (15.83) and St.Jude's SPV valve (15.5) (Fig.10, Fig.12).

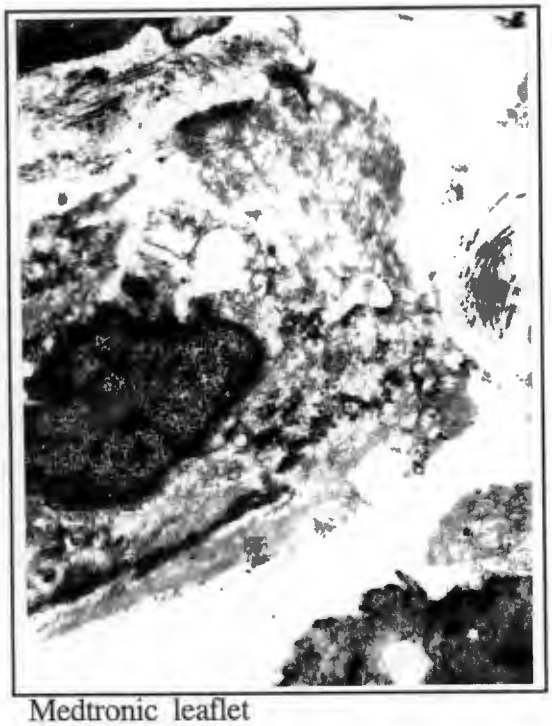
The leaflets of commercial valve tissue were more seriously damaged than the aortic wall. All the cells were distorted and electron-dense, and cytosol flocculation was seen. Edema was evident in the extracellular matrix. Collagen consisted of loosely woven bundles of filaments that appeared to spiral around one another. The average damage scores of leaflets were: Biocor No React: 19.5; Baxter: 18.3; Medtronic : 19.3; and St. Julde: 12.0 (Fig. 11, Fig.12)



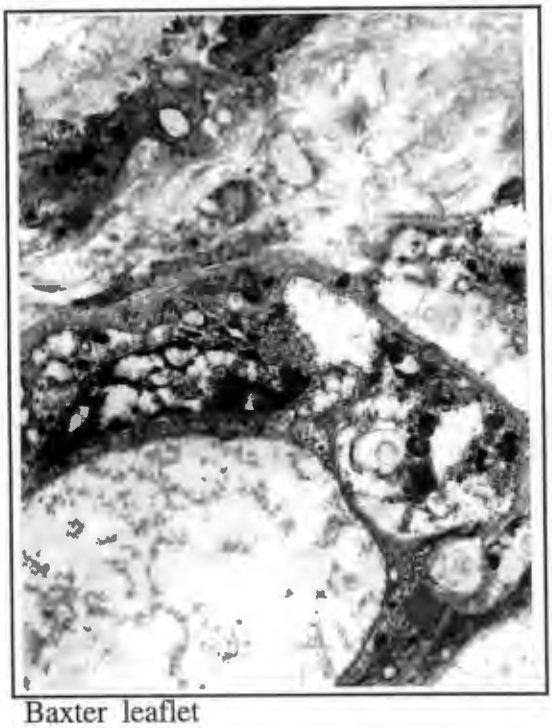
Biocor leaflet



St Jude leaflet



Medtronic leaflet



Baxter leaflet

Fig. 10 Ultrastructure of aortic wall of commercial heart valves: All four types of prostheses primarily shoued cytosol flocculation, massive intracellular vacuolization and huge intercellular oedema . Except for Biocor, all valves showed extended disintegration of the cell membrane.

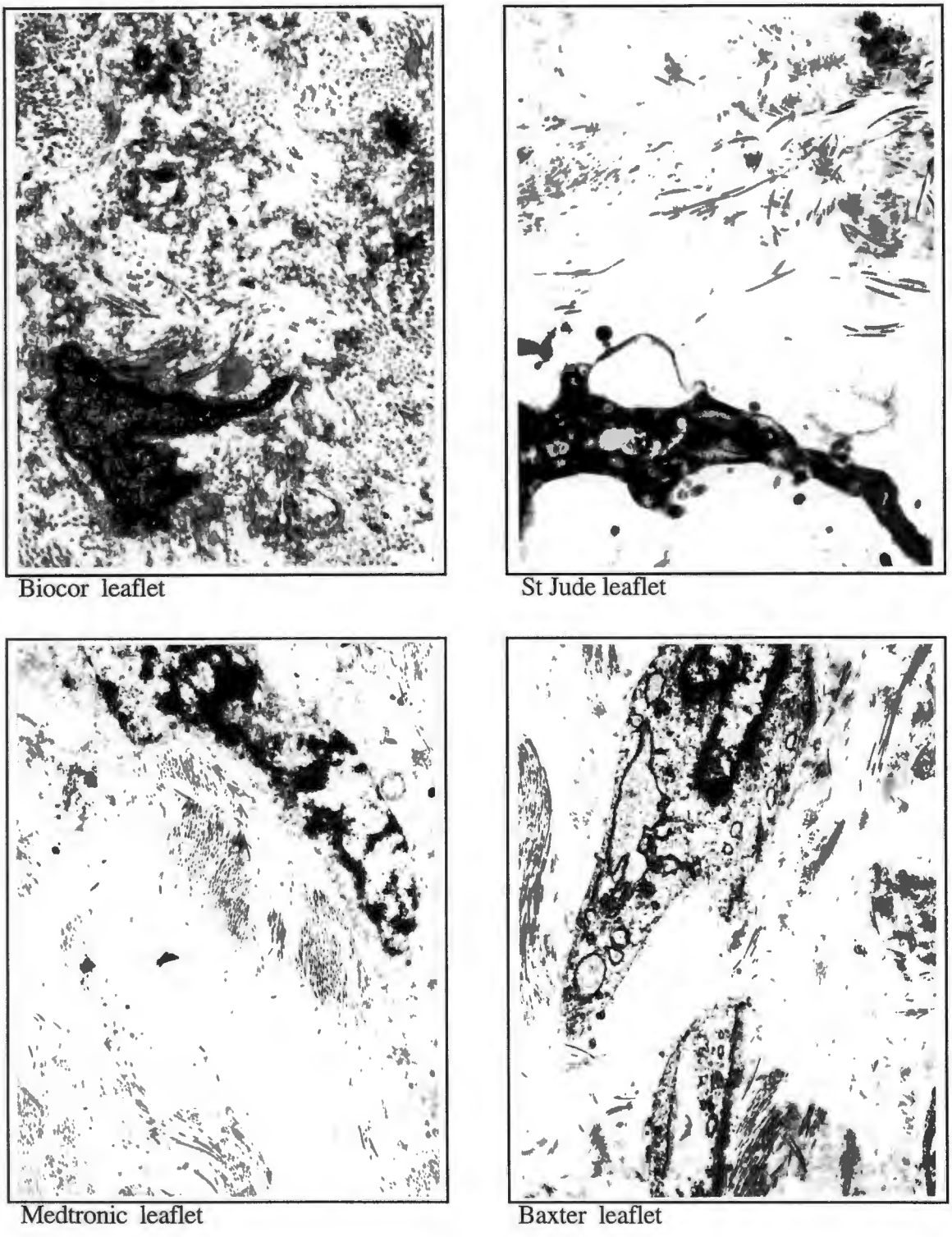


Fig. 11 Leaflets of commercial heart valves ultrastructure. All four types of bioprosthtic valves show oedema in the extracellular matrix and cytosol flocculation. Nuclei are surround by organelle debris and discontinual membranes.

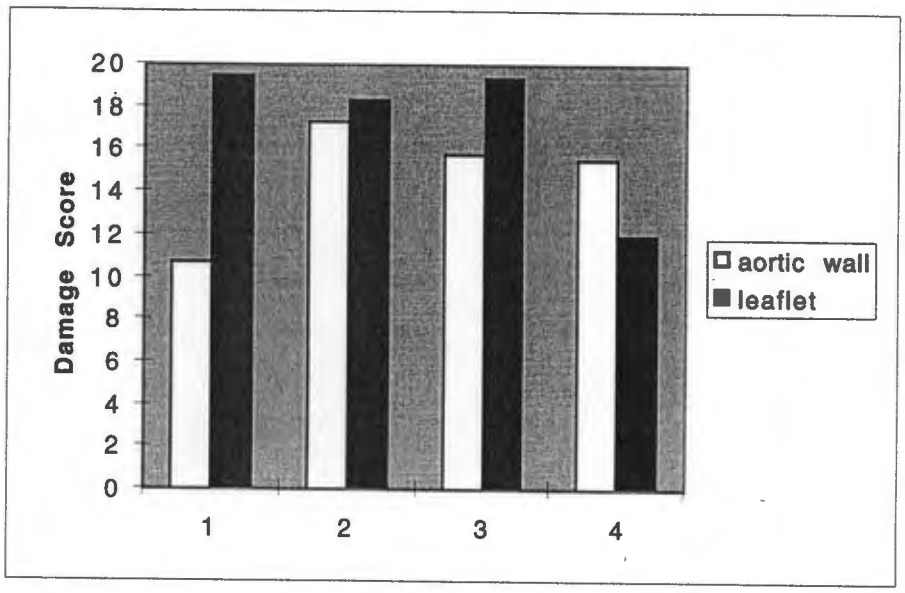
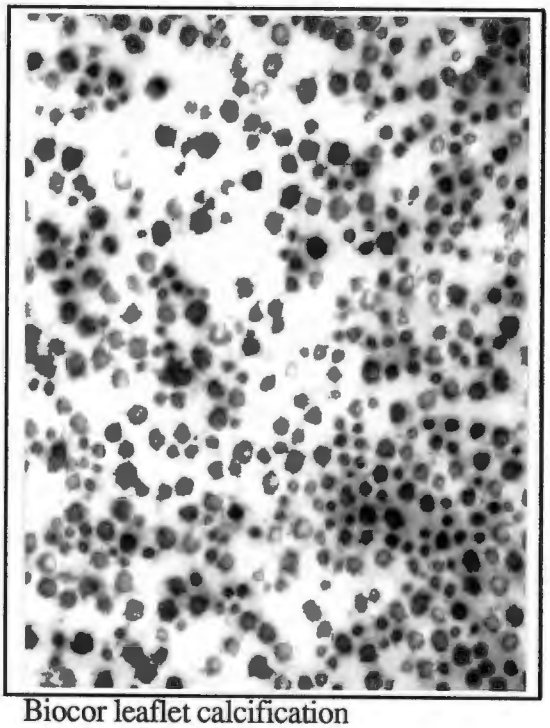


Fig.12 Ultrastructural damage score of commercial heart valves 1) Biocor. 2) Baxter. 3) Medtronic. 4) St.Julde.

Pre-Implantation calcification of a Biocor Prosthesis

Of the four types of commercial valves investigated, calcium deposits were only found in one Biocor prosthesis. Both the leaflets and aortic walls of these valve was calcified. Membranes were discontinuous around the shadows of the cells. Granules of abnormally high electron-density appeared on hazy edges or empty cores in the cytoplasm. The organelles of cells were swollen or broken and the ER had thick walls. The extracellular matrix contained whole regions which were dominated by of water spaces and tears. Calcified amorphous granules were found in the ground substance of the aortic wall. Smooth muscle cells were less damaged than fibroblasts (Fig 13 Biocor aortic wall).

Biocor aortic wall calcification



Biocor leaflet calcification

Fig. 13 Calcification of Biocor-1 heart valve in aortic valves: calcified spherical material between elastin and collagen in the extracellular matrix with no relationship to any elements.

Leaflet: a cross section of collagen fibres shows that calcium crystallization is present on all fibres. A number of fibres show a pale core surrounded by dense ring-like structure.

The collagen of this Biocor leaflet contained irregularly calcified arrangements of small crystals (Fig 13). The calcified collagen fibrils appeared either as dense cores or as light cores surrounded by dense deposits surrounding light cores.

2 OPTIMIZATION OF FIXATION PROCEDURES

Influence of Fixative Concentration (Fig. 14 , Fig. 15).

When tissue was immediately fixed at the abattoir at low temperature (4°C) samples fixed in glutaraldehyde concentrations which are currently used for commercial valve production (0.2%-0.65%) showed a similar degree of tissue damage to commercial valves. The most prominent features of tissues fixed at these concentrations were disintegrated cell membranes, large intracellular vacuoles and swollen mitochondria which lost their cristae-structure. Furthermore, significant flocculation of the cytosol and intercellular oedema were found. A significant improvement of ultrastructural preservation occurred at a concentration of 1% (from 5.83 ± 2.56 to 3.33 ± 1.03; $p=0.01$). Although the trend towards improved tissue preservation continued between 1% and 4% glutaraldehyde, it was modest (3.33 ± 1.03 to 2.50 ± 0.55; $p=0.046$). None of the samples in the 1% to 4% group showed signs of membrane disintegration or cytosol flocculation. The most sensitive parameters were intracellular vacuoles and swollen mitochondria, which were also found at higher concentrations. None of these specimens (1.0% to 4.0%) showed signs of swelling or disintegration of the endoplasmic reticulum.

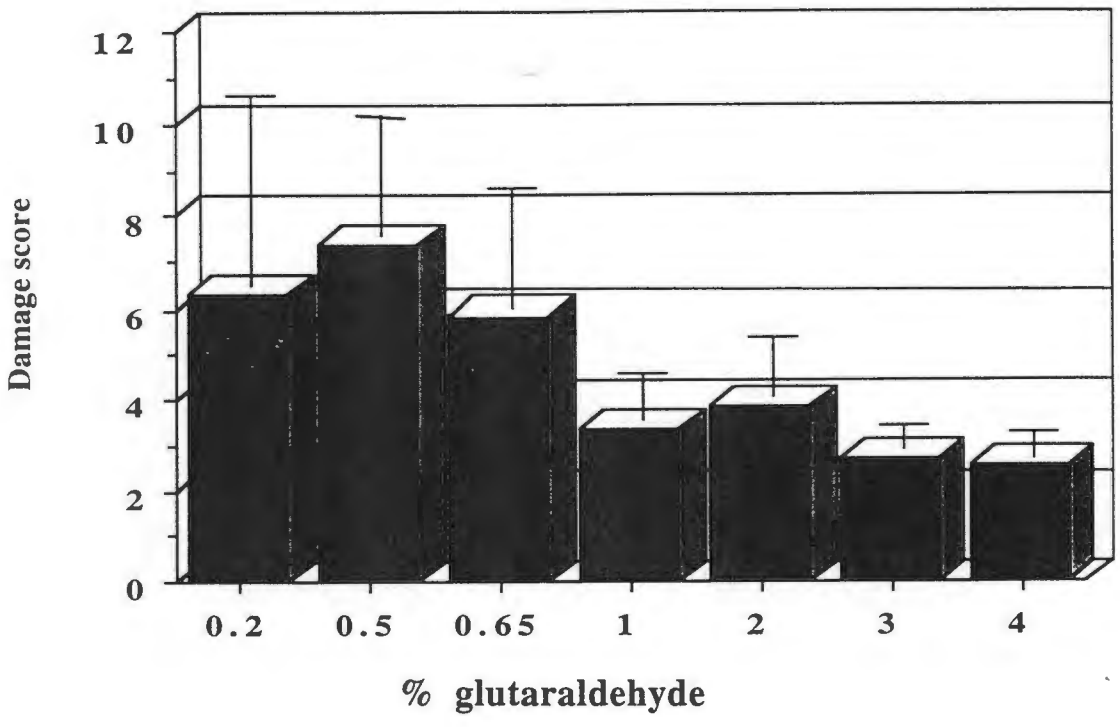


Fig.14 Immediate low temperature fixation of aortic wall tissue. Significantly improved ultrastructure is demonstrated from 1% upwards.

Table. 1 Standard deviation and p value of damage score of tissues fixation using seven different concentration of glutaraldhyde

	Mean	SD	P value						
			0.20%	0.50%	0.65%	1.0%	2.0%	3.0%	4.0%
0.20%	6.33	4.13		0.342	0.417	0.092	0.097	0.043	0.035
0.50%	7.33	2.58	0.342		0.068	0.003	0.024	0.002	0.003
0.65%	5.83	2.56	0.417	0.068		0.013	0.124	0.01	0.008
1.0%	3.33	1.03	0.092	0.003	0.013		0.271	0.051	0.046
2.0%	3.83	1.33	0.097	0.024	0.124	0.271		0.055	0.061
3.0%	2.67	0.52	0.043	0.002	0.01	0.051	0.055		0.305
4.0%	2.50	0.55	0.035	0.003	0.008	0.046	0.061	0.305	

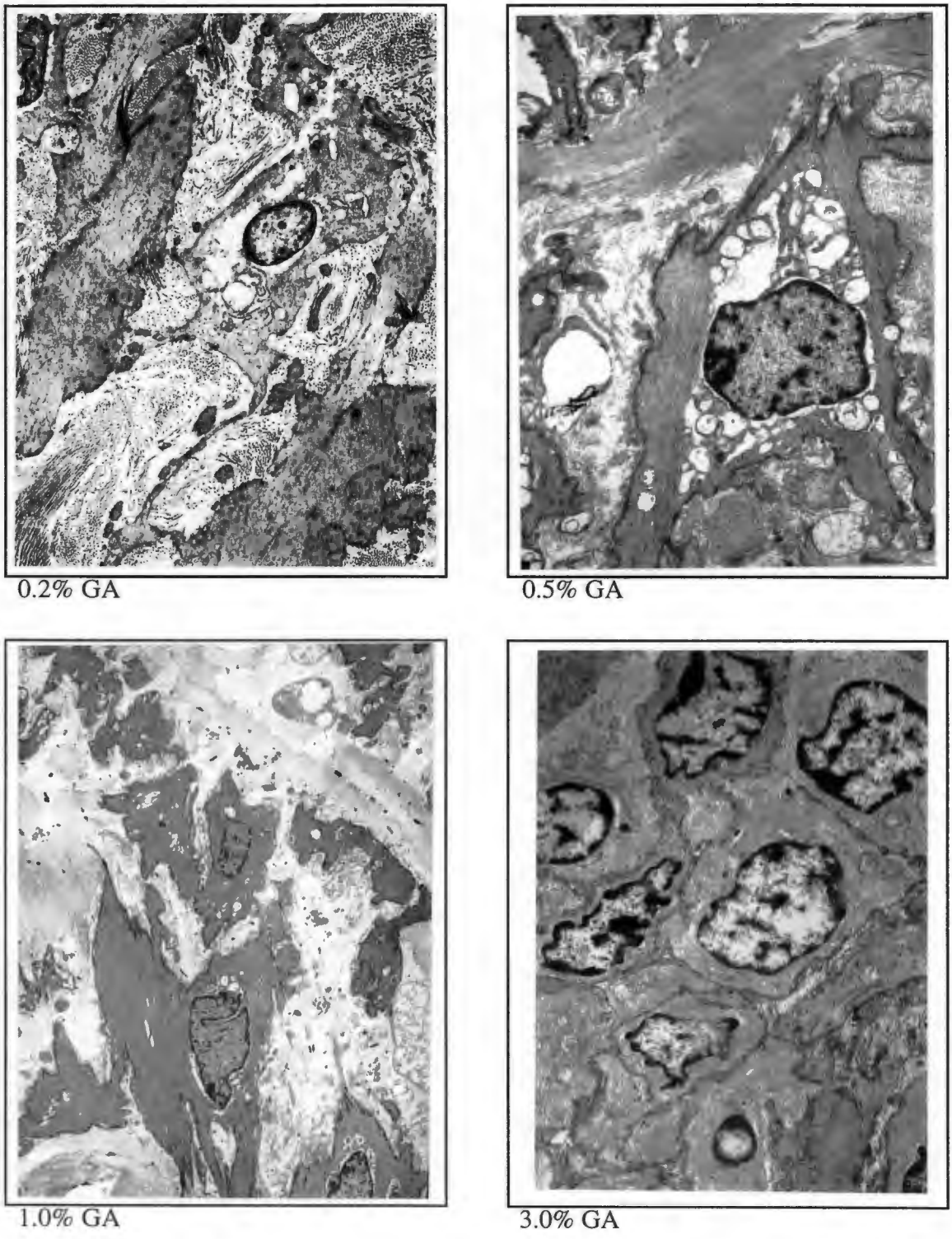
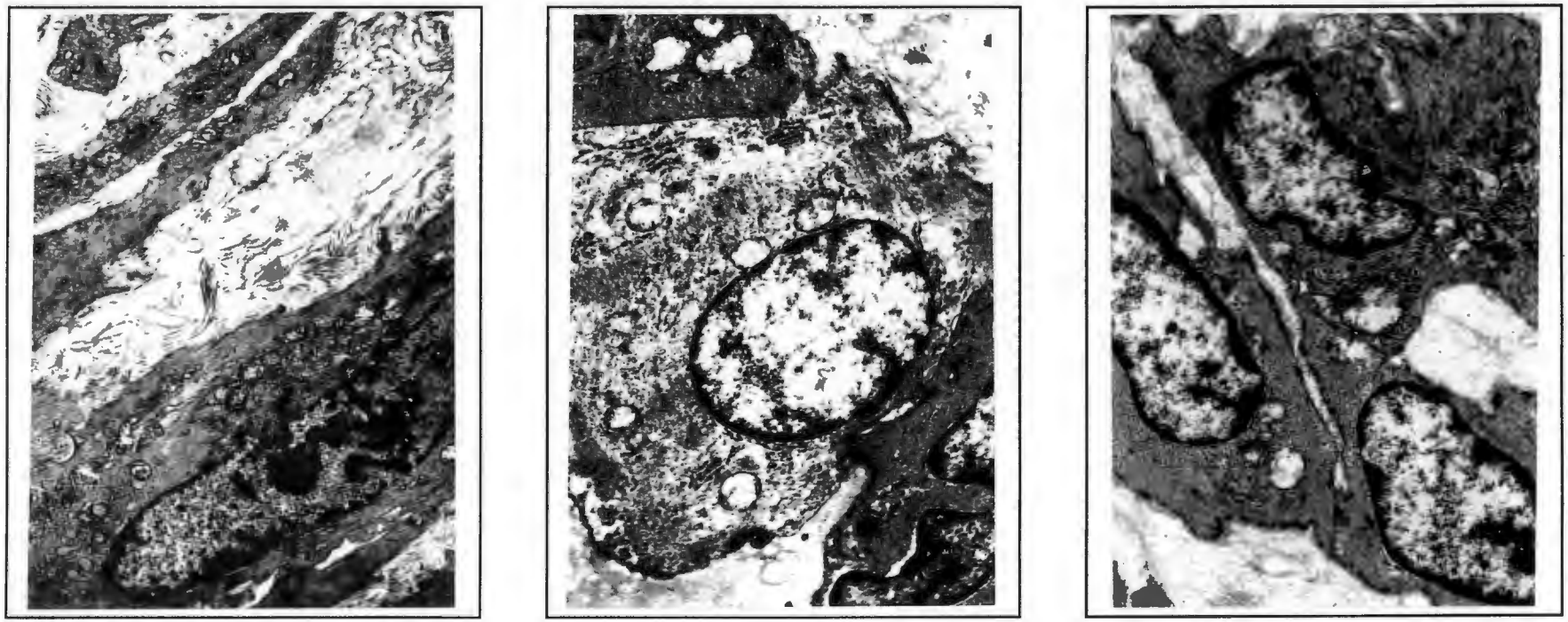


Fig. 15 At standard commercial concentrations (0.2% GA. 0.5% GA), moderately damaged cell membranes, intracellular vacuoles and swollen mitochondria dominate the picture. Significantly improved ultrastructure from 1.0% GA upwards, where only intracellular vacuoles and swollen mitochondria are found. At higher concentrations of 3% the cellular and extracellular ultrastructure is almost ideally preserved.

Influence of Fixation Temperature (Fig. 16 , Fig 17)

Using immediate fixation in 3% glutaraldehyde, the best ultrastructural tissue preservation was found at low temperatures of 4°C. With a damage score of 9.20 $\pm$ 4.49, room temperature fixation showed significantly the worst results (versus 3.67 $\pm$ 2.58 at 4°C; p=0.03 and versus 7.33 $\pm$ 3.93 at 37°; P=0.05). Particulatly membrane disintegration was found in tissue fixed at 22° whereas collagen fracture/splinting and disintegration of the endoplasmic reticulum was prevalent after fixation at 37°C.



4°C 3.0% GA

22°C 3.0% GA

37°C 3.0% GA

Fig. 16 Influence of fixation temperature on ultrastructural integrity. The worst damage happens at room temperature (22°C), with disintegrated cell membranes, flocculated cytoplasm, intercellular vacuoles and huge intercellular waterspaces.

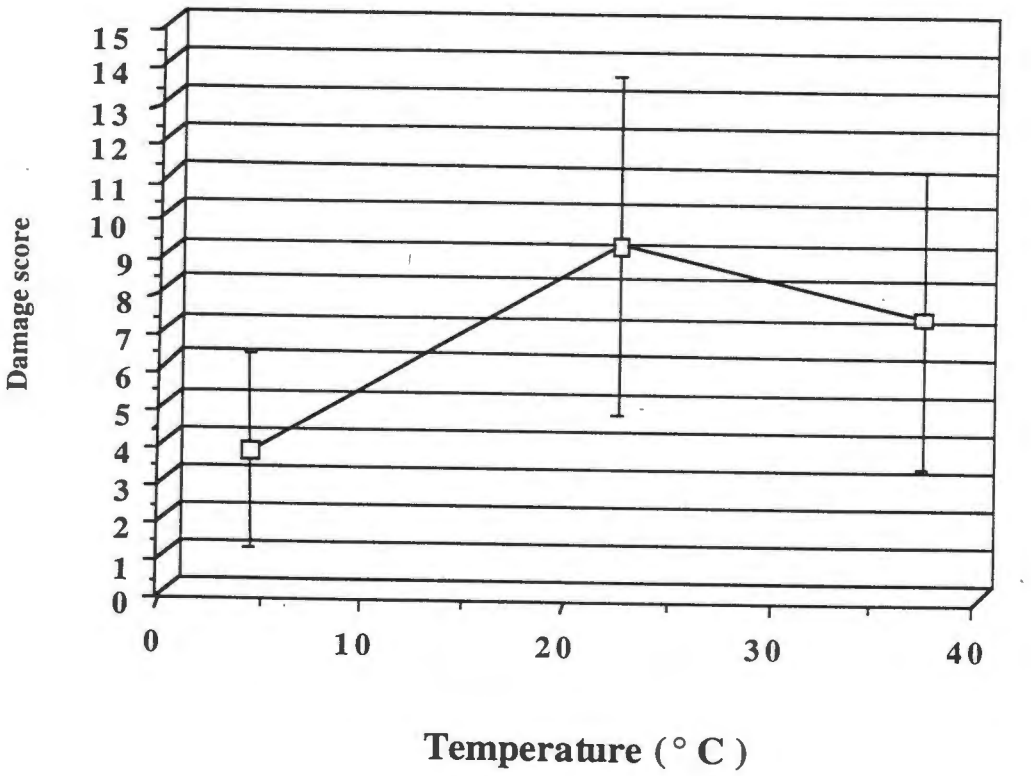


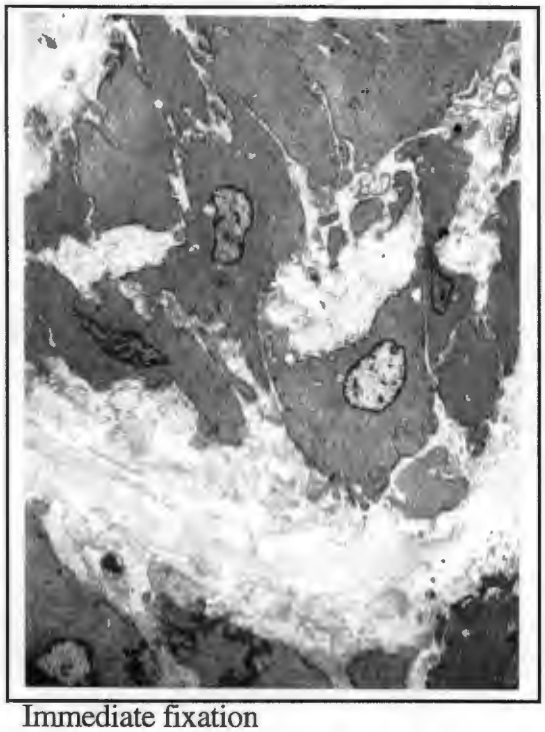
Fig. 17 Influence of fixation temperature. Ultrastructural integrity of aortic wall tissue after immediate fixation with 3.0% glutaraldeghde at 4°C, 22°C, and 37°C. The worst tissue preservation occurs at 22°C.

Table. 2 Standard deviation and p value of damage score of tissues fixed at different temperatures.

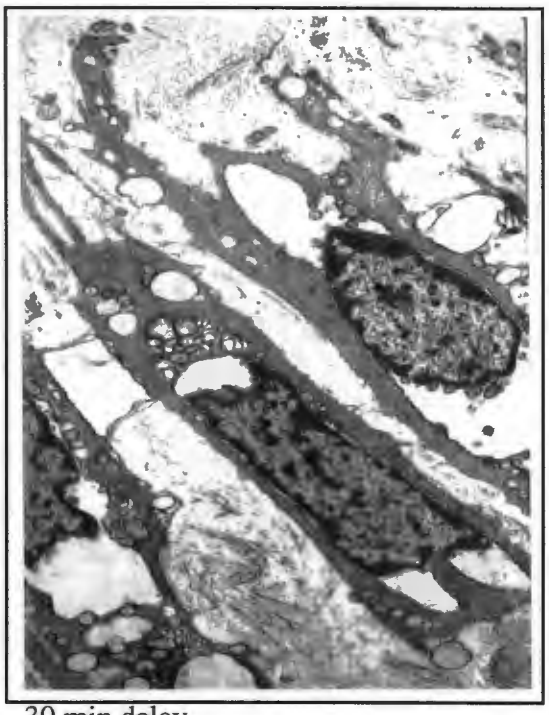
	Mean	SD	P value		
			4°C	22°C	37°C
4°C	3.67	2.58		0.03	0.021
22°C	9.20	4.49	0.03		0.05
37°C	7.33	3.93	0.02	0.05	

Influence of Delayed Fixation (Fig. 18, Fig. 19)

Even after immediate fixation (which was commenced within 5 minutes after slaughter), the samples showed signs of cell damage resulting in a damage score of 2.33 $\pm$ 2.07. This base-line tissue-damage was primarily confined to the presence of intracellular vacuoles and swollen mitochondria, although there were also areas which showed a loss of cellular integrity and intercellular architecture. The most significant increase in tissue damage occurred within the initial 30 minutes (7.40 $\pm$ 5.54; p=0.026) plateauing over the subsequent 8 hours. During this time interval moderate membrane disintegration, perinuclear vacuoles and cytosol flocculation were found together with the appearance of large extracellular water spaces. A second significant deterioration was found after 16 hours (from 5.67 $\pm$ 2.33 at 4 hours to 9.67 $\pm$ 3.08 at 16 hours; p=0.017). Between 16 hours and 128 hours of storage on ice hardly any additional tissue damage was seen. All samples investigated during this period of time showed severe cell membrane disintegration, cytosol disruptions, huge intracellular vacuoles and extracellular water spaces. From 32 hours of delayed fixation onwards, collagen damage and disintegration of the endoplasmic reticulum was found.

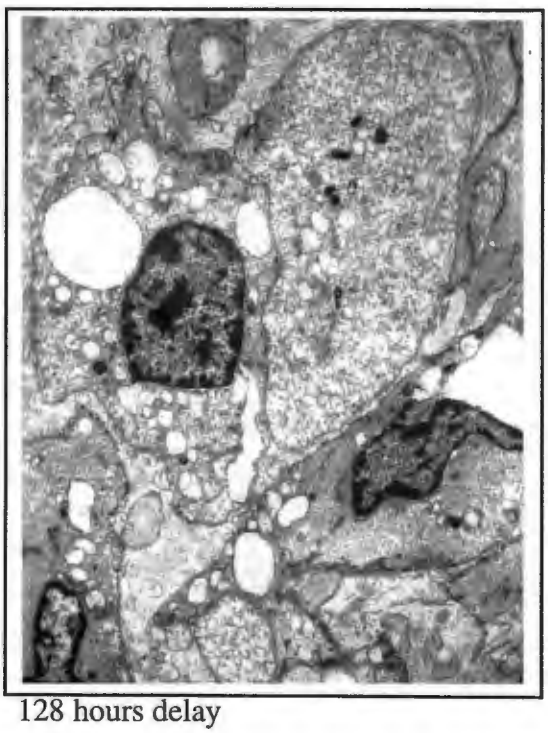


Immediate fixation



30 min delay

4 hours delay



128 hours delay

Fig. 18 Influence of delayed fixation on tissue damage. Well preserved myocytes with only mild signs of extracellular edema in immediately fixed tissue. As early as after 30 minutes of storage at 4°C, intracellular and perinuclear vacuoles and intercellular waterspaces are found. After 4 hours of storage at 4°C, the tissue damage is approximately the same as after 30 minutes. Aortic wall tissue fixed after 128 hours of cold storage shows completely flocculated, disintegrated cytoplasm, partly disintegrated cell membranes and huge intracellular vacuoles.

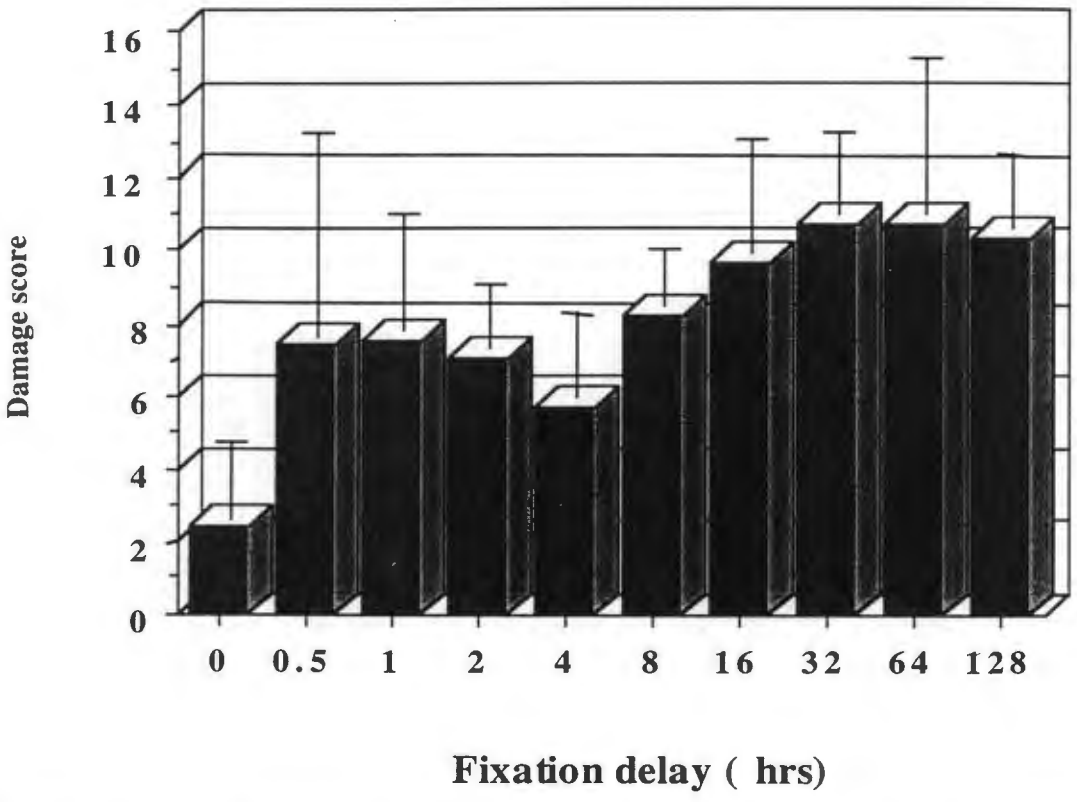


Fig. 19 Damage score of aortic wall tissue fixed after cold storage at 4°C. It is obvious that the major tissue damage occurs during the initial 30 minutes($p>0.05$). A second significant deterioration takes place between 4 hours and 16 hours of storage($p< 0.05$).

Table. 3 Standard deviation and p value of damage scores of tissues fixed after different periods of cold storage (4°C) in buffer.

	Mean	SD	P value									
			0 min	30min	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr	64 hr	128hr
0 min	2.33	2.07		0.026	0.003	0.001	0.003	0.002	0.000	0.001	0.002	0.001
30min	7.40	5.54	0.026		0.323	0.196	0.133	0.284	0.419	0.292	0.286	0.026
1hr	7.5	3.18	0.003	0.323		0.357	0.155	0.371	0.114	0.108	0.135	0.128
2 hr	7	1.79	0.001	0.196	0.357		0.148	0.100	0.044	0.023	0.069	0.027
4 hr	5.67	2.33	0.003	0.133	0.155	0.148		0.076	0.017	0.009	0.007	0.010
8 hr	8.17	1.57	0.002	0.284	0.371	0.100	0.076		0.129	0.060	0.124	0.021
16 hr	9.67	3.08	0.000	0.419	0.114	0.044	0.017	0.129		0.290	0.027	0.293
32 hr	10.67	2.25	0.001	0.292	0.108	0.023	0.009	0.060	0.290		0.500	0.410
64 hr	10.67	4.27	0.002	0.286	0.135	0.069	0.007	0.124	0.027	0.500		0.410
128hr	10.33	1.97	0.001	0.026	0.128	0.027	0.010	0.021	0.293	0.410	0.410	

3 IN VIVO TESTING OF OPTIMIZED TISSUE

3.1 CALCIUM ANALYSIS

Rat subcutaneous implants (Fig. 20)

The tissue calcium levels for aortic discs explanted from rats after 12 weeks are shown in Figure 12. Discs fixed according to a commercial regimen (48 hour delay with 0.2% GA), irrespective of enhancement with L-lysine, showed the highest level of tissue calcium in this model (162.17±7.56μg/mg, n=6 and 120.67±4.82μg/mg, n=6; Groups I and V). There was an inverse correlation between tissue calcification levels and the concentration of glutaraldehyde used during fixation with the 3.0% GA fixed tissue exhibiting the lowest level of tissue calcium (90.33±2.55μg/mg, n=6 and 25.83±3.76μg/mg, n=6; Groups IV and VIII). Overall, increasing glutaraldehyde concentrations had a significant beneficial effect ($p<0.0001$, two factor analysis of variance) on calcification of tissue irrespective of enhancement with L-lysine. Enhancement of fixation with L-lysine further reduced tissue calcium levels at all glutaraldehyde concentrations ($p<0.0005$, two factor analysis of variance) with a significant synergistic effect ($p<0.0001$, two factor analysis of variance) being exhibited between increasing glutaraldehyde concentrations and L-lysine enhancement . Only in tissue fixed with 3.0% GA, however, was intergroup calcification significantly reduced by L-lysine ($p<0.001$, Scheffè).

Baboon iliac implants (Fig. 21)

Figure 21 shows calcium levels for aortic tubes implanted in the iliac position in baboons and explanted after six weeks. Group I (commercial regimen) showed the highest level of calcification (11.18±6.60μg/mg, n=6) which correlated well with image analysis of histological sections of tissue using the von Kossa stain (Fig. 22 .r=0.957). Immediate fixation appeared to substantially decrease calcification of tissue (Group II, 1.51±0.66μg/mg, n=5) although this did again not reach statistical significance. Fixation with 3.0% glutaraldehyde best protected the tissue against calcification (Group IV, 0.87±0.13μg/mg, n=4) compared to lower concentrations, with L-lysine enhancement (Group VIII, 0.31±0.04μg/mg, n=6) further reducing calcification although this was not statistically significant.

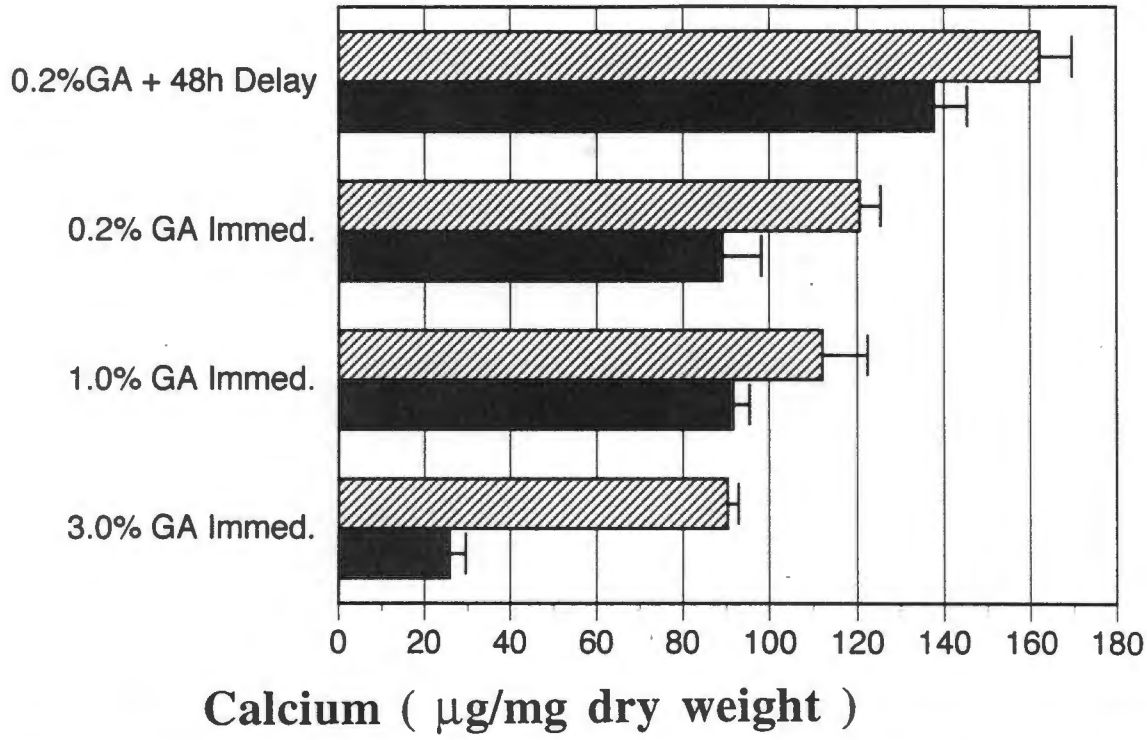


Fig. 20 Tissue calcium levels of porcine aortic discs following 12 weeks of subcutaneous implantation in the rat. Hatched bars represent standard fixation in glutaraldehyde (groups I to IV) and solid bars represent additional enhanced fixation with L-lysine (groups V to VIII).

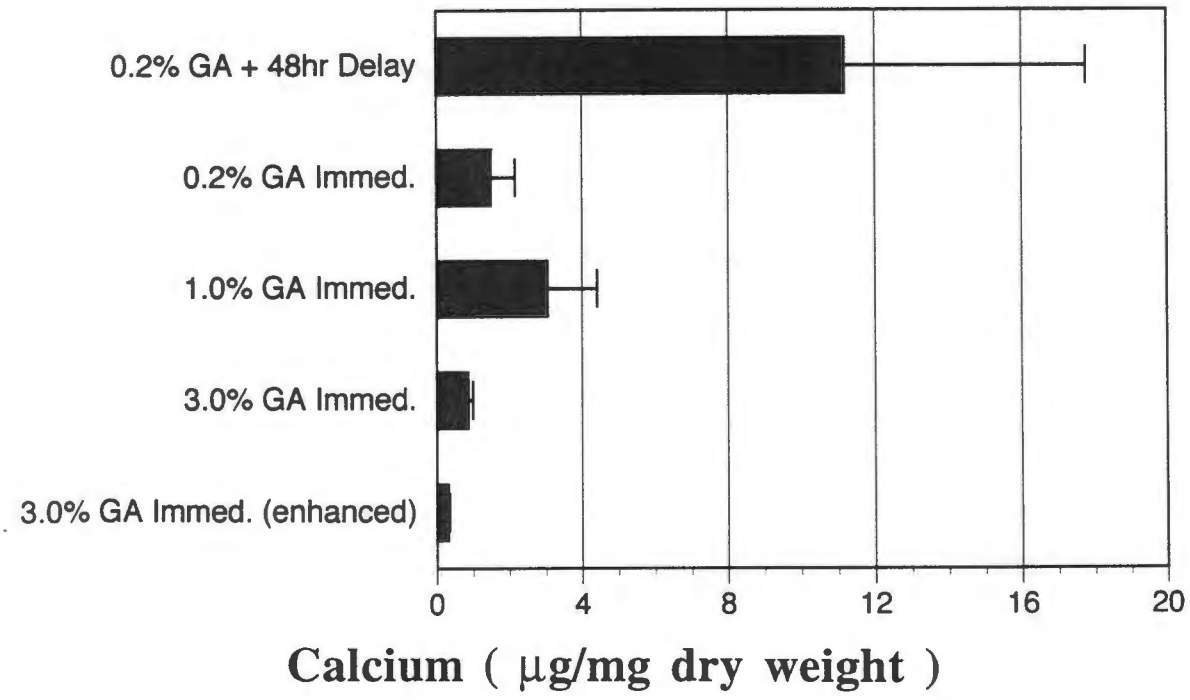


Fig. 21 Tissue calcium levels of porcine aortic tube grafts following six weeks of implantation in the iliac position in baboons.

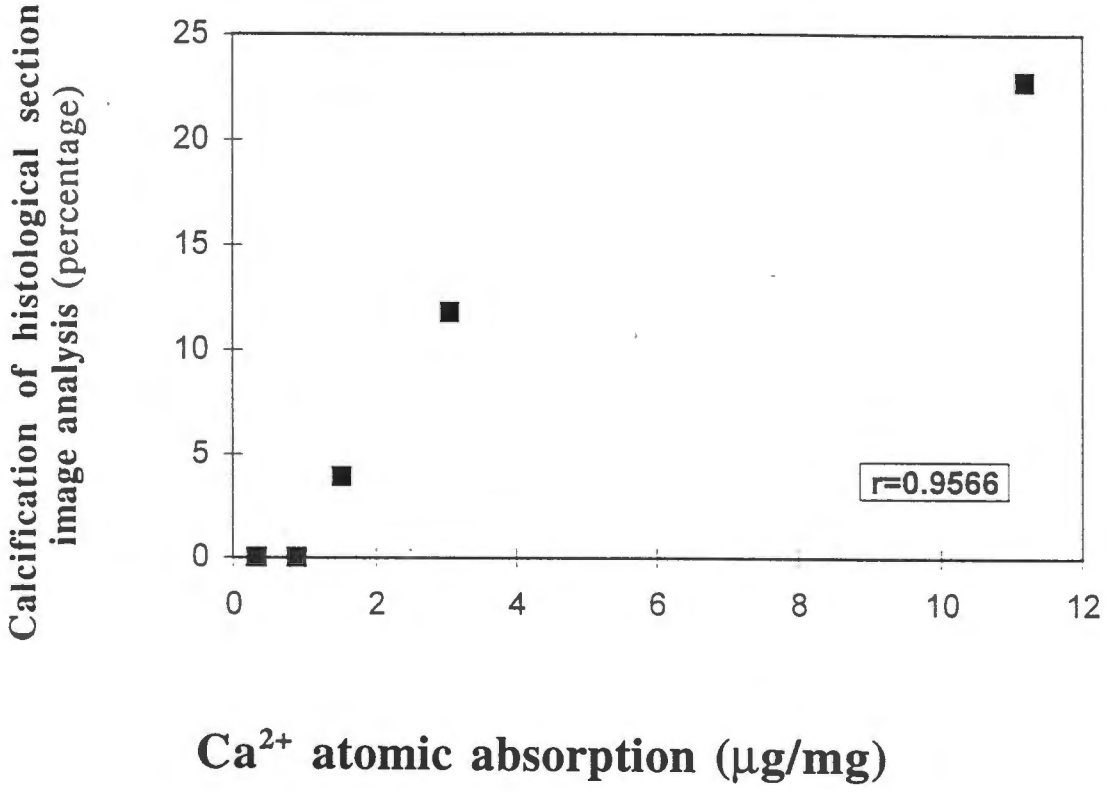


Fig. 22 Image analysis of histologically detectable calcium deposits in explants of bioprosthetic aortic wall tissue (Baboon). There is a significant correlation between the semi-quantitative morphologic Ca^{++} detection and the quantitative atomic absorption method.

3.2 MORPHOLOGICAL ANALYSIS

Calcification was much more distinct in the rat samples than in the baboon explants. In the von Kossa stains the heavily calcified groups (particularly Group I) showed relatively diffuse calcification on rat explants (Fig. 23 a) with a more layered structure on the baboon specimens (Fig. 23 c). There was, however, no clear pattern concerning these layers. In the least calcified groups (particularly group VIII) there was hardly any positive staining in the baboon samples (Fig. 23 d) whereas a more surface-related staining was found in the rat explants (Fig. 23 b).

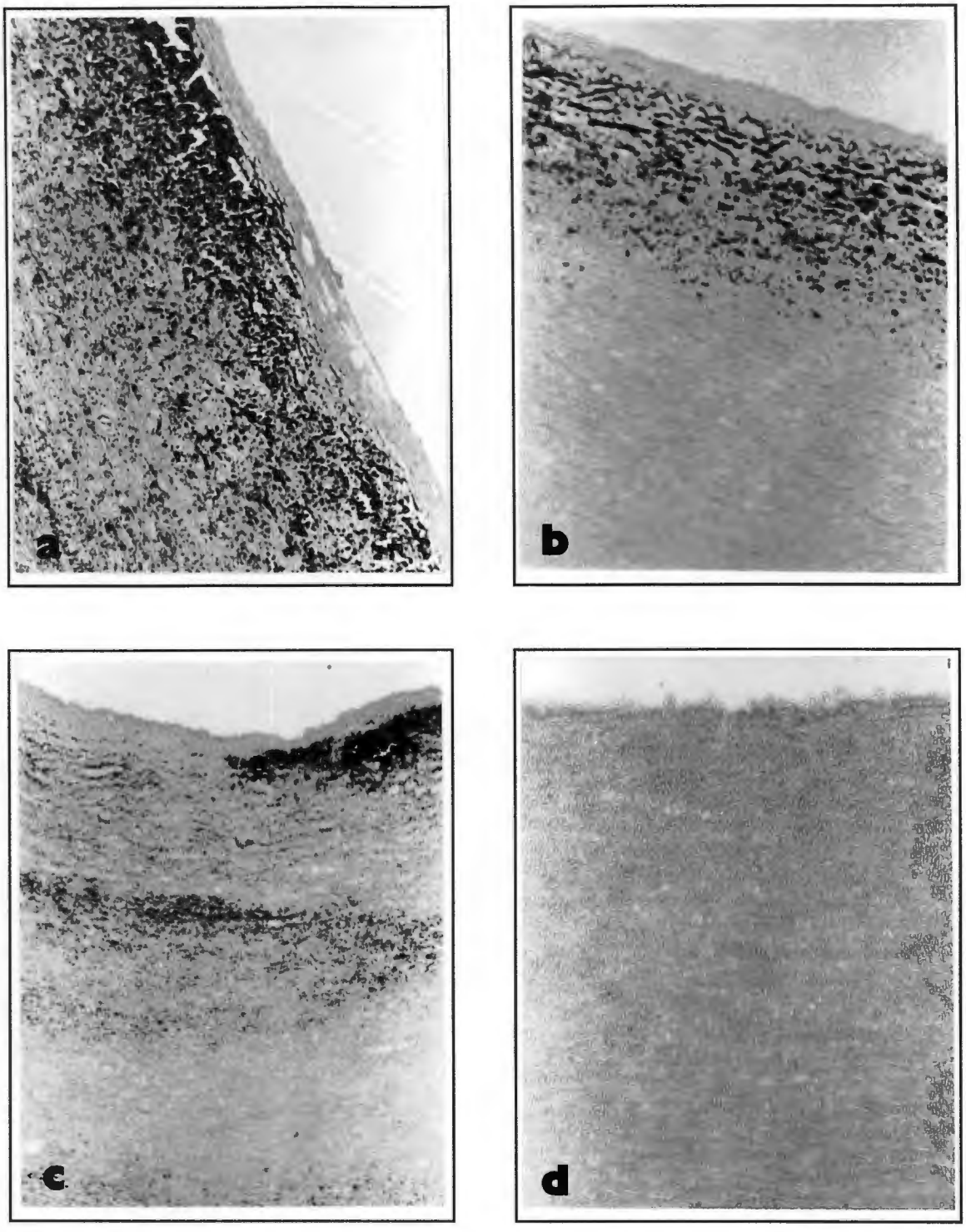


Fig. 23 Aortic wall tissue fixed in low concentrations of glutaraldehyde (0.2%) after 48 hours of storage on ice: diffuse presence of mineral deposits with a mild preference for the surface regions in the rat (Fig 23 a) and a more layered patten in baboon (fig 23 c) Immediate fixation in higher concentrations of glutaraldehyde(3%) together with the introduction of additional cross-links: moderate presence of calcium deposits in areas close to the surface in rats (Fig. 23 b) and hardly any positive staining in baboons (Fig.23 d).

In transmission electron microscopy it was a uniform finding that elastin and to a lesser degree, collagen was affected by calcification. In elastin structures mineralization did not occur diffusely but rather in the form of huge intra-lamellar conglomerates which showed a dense outer layer and a much looser core (Fig. 24 c). In the less calcified groups these mineralised areas looked "greyer" all together whereas, in groups with high calcium levels, they had a distinctly denser appearance (Fig. 24 d). The main difference between the heavily calcified samples and the less calcified ones was the mineralization of the cellular component of the tissue. While huge calcium depots were primarily associated with membrane structures of cells and their debris in the strongly calcified specimens (Fig. 24 a, b, d, e), the better preserved cells of the less calcified specimens were hardly affected (Fig. 24 f).

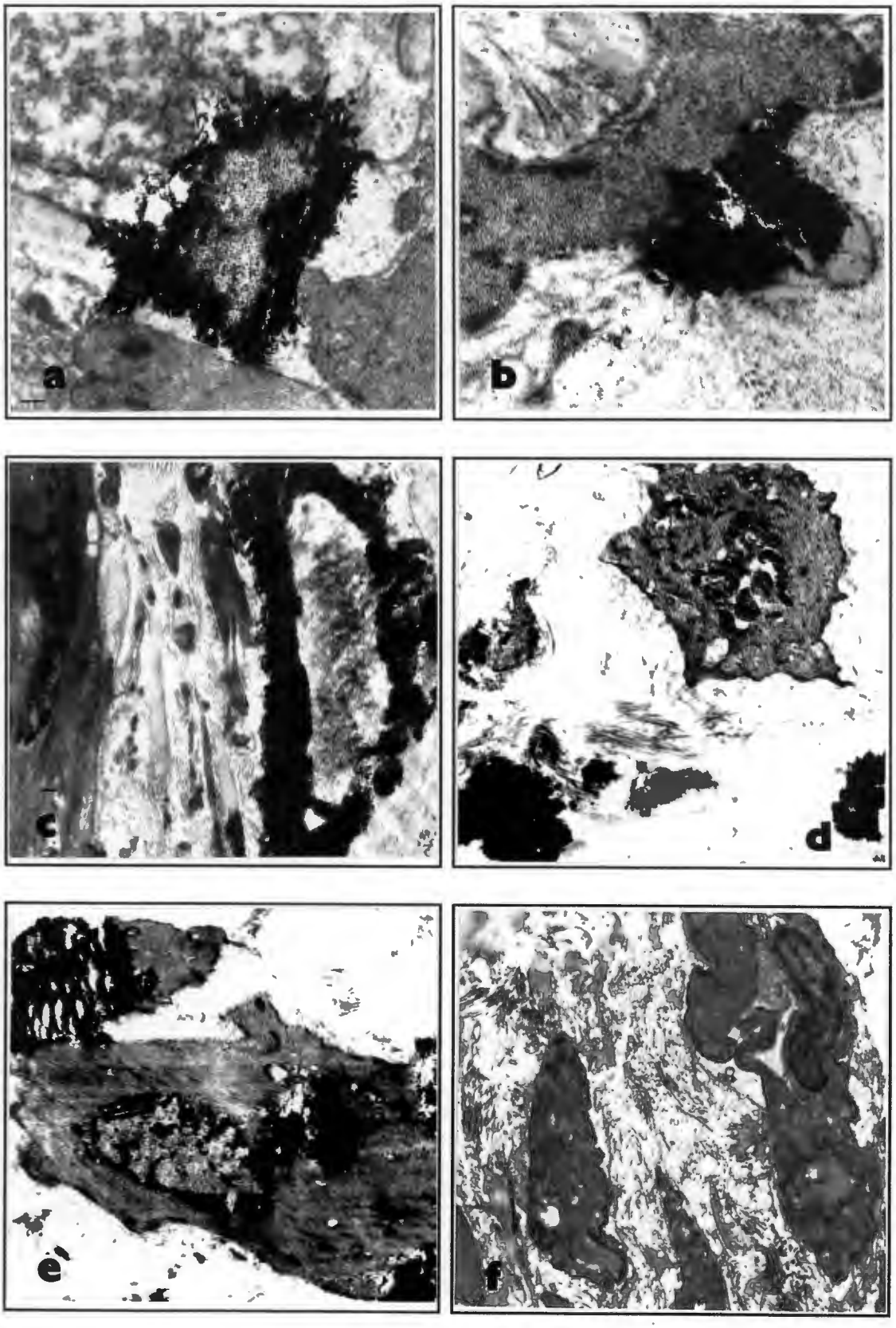

a
b
c
d
e
f

Fig. 24 *Delayed fixation (48 hours) of aortic wall tissue in 0.2% glutaraldehyde*

(Fig 24 a/rat) Mineral deposition associated with the shadow of the surface of a dissolving cell. Note the typical flocculated remnant of the cytoplasm on the right (Fig. 24 b /rat). Dense calcium particles associated with badly preserved cell membrane (Fig. 24 d /rat). Scattered calcium granule representing mitochondria inside a damaged cell remnant. Two large mineralised areas inside an elastin lamella and moderate calcification between collagen fibres (Fig. 24 e /baboon). Heavy calcium deposits extending from the cellular and nuclear membrane

Immediate fixation of aortic wall tissue in 3.0% glutaraldehyde plus introduction of additional crosslinks.

Small-granular mineral deposits in collagen-rich space between cells and elastin and huge mineral agglomerate inside elastin with a "hard " outer surface and a "soft" core.
(Fig 24/rat). Well preserved Ultrastructural morphology of the tissue with mild signs of degeneration in form of small intracellular vacuoles. Cellular elements in tissue fixed with high concentrations of gluteraldehyde were mostly free of calcification (Fig. 24/baboon).

D. DISCUSSION

This thesis successfully established a link between the ultrastructural preservation of porcine bioprosthetic tissue and its propensity to calcify after implantation. Our work was initiated by the observation that tissue heart valves which were implanted for particularly long periods of time without failing and had to be explanted for unrelated reasons, showed an astonishing degree of ultrastructural integrity. For this reason, we first wanted to demonstrate that all contemporary commercial tissue heart valves show a high degree of pre-implantation tissue damage. In the second step we attempted to improve the fixation conditions to a degree that ultrastructural integrity was preserved. Finally, we needed to demonstrate that such well preserved ultra-structure does result in a significantly reduced calcific degeneration.

The comparison of the four most widely used contemporary stentless bioprosthetic heart valves showed a frightening degree of tissue damage in all of them. Although there were minor differences between the four valve types, the damage score did not differ significantly amongst them. The most striking findings were an almost unanimous loss of cellular integrity due to fragmented cell membranes and hugely swollen vacuoles intracellularly. Very often this disintegration of cells led to the leaking of intracellular organelle debris into the extracellular space. Apart from vacuoles, the most commonly found deviation

from normal cellular ultra-structure was the enormous swelling of mitochondria. Extracellularly, the loss of ground substance led to huge water spaces between the cells and the extracellular structures. Only the Biocor prostheses showed in one case pre-implantation calcification. This is an interesting finding because such calcification of native tissue has not been reported in any bioprosthetic study before. However, it is easy to imagine that native aortic wall calcification can be found in porcine tissue. Pigs are known to have an equal propensity for arterial sclerotic- and vascular-degenerative diseases as humans. Moreover, most of the contemporary commercial heart valves come from North America whereas Biocor is being produced in Brasilia. Different feeding patterns and nutritional aspects may well affect the resistance of native tissue towards degenerative calcification.

A systematic analysis of the variants of the fixation process enabled us to develop a fixation procedure which almost optimally preserves the ultrastructural integrity of the bioprosthetic tissue. The three most crucial aspects we investigated with regard to perfect fixation were the delay between slaughter and fixation, the temperature at which the fixation process is performed and the concentration of the crosslinking agent. Of these three variables, the delay between slaughter and tissue fixation was the most important determinant of ultrastructural integrity followed by fixation temperature and glutaraldehyde concentration. On a 21 point damage score, the difference between the best and the worst treatments was 8.3 for delayed fixation, 5.5 for fixation temperature and 4.8 for glutaraldehyde concentrations.

The influence of glutaraldehyde concentrations on tissue damage was least dramatic. A non-linear correlation between fixative concentration and ultrastructural tissue preservation drew a cut-off point between good and poor fixation at 1% glutaraldehyde. The commercially used concentrations of 0.2% to 0.65% all resulted in a similar degree of tissue decay (5.8 to 7.3) as did the concentrations of 1% and higher (2.5 to 3.8) show an equally well preserved ultrastructure. This non-linear change of crosslinking efficiency from very bad below 1% to relatively good above 1% means that particularly the concentration of the fixative would offer a certain flexibility when eventually addressing other issues of fixation. On the one hand there is a point close to 4% where excessive tissue hardening occurs[22]. On the other hand glutaraldehyde treated tissue was shown to retain significant antigenicity when 0.2% was used[36]. It is a well known phenomenon for pathologists that immunohistochemistry does not work from a certain concentration of fixative onwards. At the same time it was also shown that no inflammatory reactions to implants were seen when tissue was crosslinked with high concentrations of 2.5% glutaraldehyde [37].

The second most important determinant of tissue damage was fixation temperature. By raising the crosslinking temperature, a faster glutaraldehyde penetration and tissue reaction are opposed by an accelerated autolytic process. It turned out that at a low temperature of 4°C autolysis was sufficiently suppressed allowing optimal tissue preservation in spite of slower glutaraldehyde kinetics. Interestingly, this relationship between

autolysis and crosslinking could not be linearly extrapolated because the tissue damage was worse at 22 degrees than at 37 degrees. It seems that at room temperature the more than 40% faster tissue penetration of glutaraldehyde[12] is outweighed by accelerated autolysis whereas at 37 degrees further improved tissue penetration [43]. appears to moderately catch up with the progression of autolysis. Although low temperature fixation was used in experimental studies evaluating bioprosthetic tissue[44] others did not specify fixation temperature, making room temperature crosslinking likely. Many other studies used commercial tissue which was fixed at room temperature [6, 33, 40, 45] . Considering the dramatic increase in tissue damage when room temperature-fixation was used in our experiments, it becomes more obvious how the focus on anti-calcification treatments has led to the negligence of the actual fixation in the past.

The most influential factor of tissue-preserving fixation was immediate fixation. Even short delays of the fixation procedure resulted in tissue damage. The most significant damage occurred within the initial 30 minutes after slaughter. Another significant loss of tissue integrity was observed between 4 and 16 hours. After this time tissue morphology remained almost unchanged for the following 4 days. The practical consequence of this observation is that commercial valve production does not even need to attempt to shorten its current transport time of 2 to 4 days but rather take some radical steps like moving the production site onto the premises of an abattoir or into its immediate vicinity. From our experiments it became obvious that aortic wall tissue which physiologically relies on vasa vasorum for nutrient

and oxygen supply does not tolerate ischemia even when kept on ice. The remaining basic damage score of 2.3 for immediate fixation indicates that even the short delay caused by the penetration of glutaraldehyde into the depth of the aortic tissue is poorly tolerated. The wide range of morphological appearance in this ideally fixed group indicates that tissue penetration by the fixative is uneven and may need additional facilitation. Apart from implications for xenograft production - where delayed fixation is known to result in distinctly higher tissue calcification [41] - this observation also puts our perceptions with regard to homografts into a different light: since even cryopreserved homografts spend up to 4 days in antibiotic solutions as part of the sterilisation procedure, autolytic tissue damage has already happened for many valves before they are frozen.

Our reason for having chosen aortic wall tissue rather than pericardium or leaflet tissue was the current trend towards stentless aortic valves. Stentless aortic valves as well as root prostheses have created enthusiasm among the surgical community because of their superior hemodynamic performance and reduced leaflet strain [46] . Unfortunately, the higher propensity of aortic wall tissue towards calcification[47] threatens to defy the advantages of root mechanics which were gained through the new design. This threat is even more serious in view of the realisation that calcific degeneration of the aortic wall is much less amenable to anticalcification treatment [42] than leaflet tissue. Therefore, for even stronger reasons, the actual crosslinking procedure itself may hold the key to an alleviation of tissue degeneration if postfixation treatment is limited in its

success. Unfortunately, the issue of crosslinking of bioprosthetic tissue has been trapped in a predicament of superficial understanding, complexity and prejudice. While superficial understanding and prejudice were inherited from an era in which clinicians rather than basic scientists were dealing with fundamental issues of material chemistry and biology, complexity stems from a situation in which three demands on tissue fixation seem incompatible: crosslinking, biocompatability and mechanical performance. In an attempt to find a compromise, all three areas were inadequately dealt with. For fear of stiffness and tissue toxicity sub-optimal concentrations of crosslinking agents were used. The resulting tissue damage [2, 5, 6] as well as ongoing immunogenicity, [48] alkaline phosphatase activity [45] and protease activity [49] were quietly accepted. At the same time the widely shared verdict on glutaraldehyde - the longest used crosslinking agent for bioprosthetic heart valves - was based on these sub-optimal fixation conditions resulting in a premature search for alternatives. Today we know that long exposure to glutaraldehyde [50] as well as higher concentrations [51] reduce rather than increase tissue calcification. Furthermore, with the ability to extract cytotoxic unbound tissue glutaraldehyde thoroughly after fixation [52, 53] glutaraldehyde may loose its stigma of being the villain behind calcific degeneration. Therefore, its superior tissue penetration [54] and swiftness of the crosslinking reaction make it the logical candidate for optimising tissue fixation. Although alternative fixation methods may eventually be used, glutaraldehyde seems ideal for establishing the standards against which other crosslinking methods should be held. If such standards

were to be established, one would need to deal with each aspect separately in an uncompromising way.

Our in vivo assessment of the influence of better crosslinking efficiency resulted in a surprising realisation. Although immediate fixation had a positive influence on tissue calcification, the most significant difference was achieved through higher concentrations of the crosslinking agent. In view of the long-standing belief that an increasing number of glutaraldehyde crosslinks leads to increasing tissue calcification [5] , it seems justified to revisit certain positions. Considering the fact that the aortic wall is the most troublesome component of contemporary stentless valve and root prosthesis because of its particular resistance against a variety of anticalcification treatments [13] a 84% reduction in calcification prior to any anticalcification treatment is a remarkable achievement. How can one explain such a distinct outcome against the background of opposing previous beliefs? It seems that the many apparently contradictory conclusions which were drawn in the past may well provide us with a holistic explanation for tissue calcification as long as we recognise the multi-factorial nature of this issue. To do so one may need to acknowledge that all five major aspects of calcific degeneration are somehow interrelated: Crosslink configuration [58] and excess aldol content in the tissue [53] autolytic tissue damage,[6, 55, 56] qualitative and quantitative changes of proteoglycan content of the tissue [57] , continual enzyme activity[45,49] and insufficiently suppressed immunogenicity [69]. Any single approach has most likely ignored advantageous effects with regard to all of those aspects.

Therefore, our combined effort, which nevertheless intends to address all problems separately, seems to have the highest prospect of success.

By looking into the issue of aldol condensates the complexity of interpreting calcification studies becomes obvious. When Golomb et al reported a direct correlation between glutaraldehyde incorporation and tissue calcification, they used a low concentration of 0.2% glutaraldehyde and varied the pH during the crosslinking reaction [5] . As Hughes et al pointed out later, by radiolabelling the fixative [43] Golomb had measured the overall glutaraldehyde incorporation into the tissue rather than the actual crosslinks. Since the aldol condensation of glutaraldehyde increases at higher pH values of the fixative, one could speculate that it were rather the polymerised hemi-acetals than the actual stable crosslinks which caused calcification. The removal of these easily degradable labile bonds and the subsequent decrease of tissue calcification [52] with minimal effect on stable crosslinks [57] support this view. The remarkable decrease of calcification after prolonged storage in glutaraldehyde [50] similarly suggests that internal rearrangement of the glutaraldehyde polymer amine complexes [58] rather than the number of crosslinks is partly responsible for the calcification potential of bioprosthetic tissue. Based on these observations the higher concentrations of glutaraldehyde which we used are most likely increasing the propensity for tissue calcification. Nevertheless, this effect seems to be outweighed by the consequences of better fixation. These consequences may well be

the distinctly lower autolytic tissue damage together with the various aspects of higher crosslinking efficacy.

Tissue damage related to the fixation process was the most neglected aspect of bioprosthetic tissue preservation in the past [6, 55,56]. This is somehow surprising because studies of the 1980's reported already that explanted tissue valves which showed a well preserved ultrastructural integrity did not undergo calcific dysfunction, even after long term implantation[6]. By fixing tissue immediately with higher concentrations of glutaraldehyde we had tissue for implantation with a minimal degree of autolytic damage. Previous investigators had already shown a 27% reduction of calcification through immediate fixation, although it was achieved with low concentrations of glutaraldehyde which we know only modestly prevent tissue damage [60] . One of the reasons for lower calcification in better preserved tissue may lie in the fact that glutaraldehyde crosslinking of intact cell membranes seems to "freeze" the membrane bound Calcium ion pump rather than leading to its break down [61, 62] . Tissue integrity would thus prevent the enormous intracellular membrane area which has a high content of calcium-attracting phospholipids from being overwhelmed with the high extracellular calcium concentrations. This explanation is partly supported by our ultrastructural observations. While the standard fixed tissue showed a uniform calcification of cell debris, elastin and collagen, mineralization was primarily found in elastin and to a lesser extent in collagen in samples which were fixed in 3% glutaraldehyde immediately after harvest. These mineralizations were modest and appeared somewhat "softer" with a distinct

peripheral "shell". This confirms previous observations by Levy et al that elastin is a predominant site of calcification in aortic tissue [39] . However, since even the most conscientious approach to tissue preservation still shows a certain degree of ultrastructural damage [59] it was interesting that even those areas which showed some signs of autolytic degradation had a distinctly lower degree of calcification. Therefore, it seems likely that an additional benefit towards reduced calcification is achieved through better crosslinking efficacy. While 30% of the L-Lysine in tissue fixed with low concentration of glutaraldehyde remains uncrosslinked, this proportion can be reduced through higher concentrations [63] . A higher crosslinking efficiency may stabilise the proteoglycan component of the tissue [63] , eliminate continual enzyme activity [45, 49] and sufficiently suppress residual antigenicity [48] . Although proteoglycans are known to be potent inhibitors of mineralization [65] they appear to be partially extracted during standard fixation [66] . The protein backbone of the proteoglycans may well be more amenable to crosslinking by higher glutaraldehyde concentrations. Moreover, inorganic pyrophosphate which is a potent natural inhibitor of calcification [67] and related to the anticalcification agent diphosphonate [68] is labile towards phosphatases. These enzymes as well as tissue metalloproteinases are known to remain active in standard fixed bioprosthetic tissue [45,49] . A true inactivation through better crosslinking may, amongst other tissue components, preserve its protective pyrophosphate content. And finally, antigenicity which was shown to elicit calcification is also sufficiently suppressed by higher glutaraldehyde concentrations [58] . However, since crosslinking is only possible to a certain saturable level probably

limited by the number of optimally spaced amino groups exposed by the collagen Simionescu et al [41] and Nimni et al [69] have suggested enhancing crosslinking by introducing diamines between two glutaraldehyde fixation steps. A significant increase in shrinkage temperature was found after this two-staged approach [41] . In our own experiments we decided to use L-Lysine for this purpose [41] . Since the important first phase of fixation was carried out at 4°C, autolysis was already confined when the second phase of fixation was performed at 37°C. To attribute the effects of enhanced crosslinking to a hidden detoxification procedure through the aminoreagent [52, 57] seems unlikely because the subsequent incubation in high glutaraldehyde concentrations at 37°C certainly results in the repeat-build-up of large amounts of aldol condensates. Nevertheless, a combination of this crosslinking procedure with an extraction process for aldol condensates may well further reduce calcification [52] . We have previously shown that a complete extraction of all cytotoxic residues from aortic tissue is possible allowing prolonged growth of endothelium on its surface [57] .

E. CONCLUSION

The two determining factors that were shown to have the highest impact on tissue preservation are fixation delay and fixative temperature [38] . Optimisation of these factors could be implemented without affecting tissue consistency or biocompatibility. The third factor, concentration of the fixative, had the highst impact on tissue calcification but would need to be determined by a multitude of aspects including complete suppression of immunogenicity as well as inflammatory erosion. While residual glutaraldehyde toxicity can be eliminated through a variety of detoxification procedures, tolerable physical properties would need to be established on a pulse duplicator and wear tester rather than through subjective surgical preference.

In both animal models we used, the highest level of calcification was found in the group of delayed fixation in 0.2% GA. In the rat model there was an inverse correlation between tissue calcification and the concentration of glutaraldehyde, with the 3% GA fixed tissue showing the lowest level of tissue calcium. Overall, increasing GA concentration had a significant beneficial effect on calcification ($p<0.0001$; two factor analysis of variance). Enhancement of crosslinking with L-Lysine further abrogated tissue calcium levels at all GA concentrations ($p<0.0001$; two factor analysis of variance). Although the short term baboon model showed lower calcium levels in the tissue, the trend seen in the rat model was confirmed.

Im summary, our results suggest that, against previous beliefs, immediate fixation at higher glutaraldehyde concentrations reduces the calcification tendency of cross-linked aortic tissue.

They furthermore demonstrate that aortic wall calcification does not need to be the necessary evil of modern stentless valve and root prostheses. By preserving the ultrastructural integrity of the tissue as well as strengthening the crosslinks it is possible to reduce aortic wall calcification by 84%. Considering that even the worst of our groups was fixed at 4°C whereas commercial fixation is mostly done at room temperature, it is likely that the actual reduction of the calcification potential is even higher when compared with commercially fixed tissue. Assuming that a detoxification treatment will further reduce calcification through the removal of acetals and possibly through facilitation of endothelialization, calcific degeneration may well become a negligible threat for bioprosthetic tissue. Furthermore, it seems likely that other detrimental phenomena like ongoing immunogenicity and metalloproteinase activity are better controlled with this treatment.

If, however, alternative fixation methods should eventually prevail, glutaraldehyde will at least retrospectively have helped to establish certain principles which can be applied to any other crosslinking procedure.

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