THE EFFECT OF DIFFERENT TREATMENT MODALITIES ON THE CALCIFICATION POTENTIAL AND CROSS-LINKING STABILITY OF BOVINE PERICARDIUM

JOHANNES JACOBUS VAN DEN HEEVER

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Supervisors: Prof WML Neethling, PhD
Prof D Litthauer, PhD
Prof FE Smit, MMed

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DECLARATION OF INDEPENDENT WORK

I, JOHANNES JACOBUS VAN DEN HEEVER, do hereby declare that this research project submitted to the University of the Free State for the degree MAGISTER MEDICINAE SCIENTIAE: ANATOMY AND CELL MORPHOLOGY, is my own independent work that has not been submitted before to any institution by myself or any other person in fulfillment of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT **DATE**

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OPSOMMING

In die soeke na geskikte produkte om as plaasvervangende materiale tydens chirurgiese prosedures te kan gebruik, is ‘n groot verskeidenheid van verskillende tipes produkte reeds ondersoek. Vir gebruik tydens kardiotorakale prosedures het biologiese weefsels soos varkkleppe en beesperikardium die beste aan die vereistes vir so ‘n produk voldoen. ‘n Verskeidenheid sintetiese materiale word egter ook vir hierdie doel gebruik.

Biologiese produkte moet aan ‘n lang lys van vereistes voldoen, voordat dit suksesvol en veilig as plaasvervangende materiaal gebruik kan word. Die produk moet onder andere stabiel teen biologiese (ensimatiese) afbraak wees, maklik steriliseerbaar wees, minimale immuunreaksie van die ontvanger ontlok en genoegsame meganiese sterkte en weefselstabiliteit na prosessering behou. Verder moet die produk ook nie maklik verkalk nie, nie toksies of kankerverwekkend wees nie en maklike hantering toelaat. Verskeie chemikalieë en metodes is reeds ondersoek om die mees geskikte materiaal wat aan al hierdie vereistes voldoen, te lewer. Die chemiese reagens wat die meeste van hierdie vereistes aan biologiese weefsels kon toevoeg nadat hulle daarmee gefikseer is, is gluteraldehied.

Ten spyte van die voortgesette wêreldwye gebruik van gluteraldehied-gefikseerde beesperikardium, bly die kalsifikasie en weefseldegenerasie na 10-12 jaar na inplantering steeds ‘n groot problem. Die hoofdoel van hierdie studie was om addisionele biochemiese behandelingsmetodes wat vir die fiksering en berging van die weefsel gebruik kan word, te identifiseer. Hierdie metodes moet die kalsifikasiepotensiaal van die perikardiale weefsel aansienlik verlaag, maar terselfdertyd nie die fisiese eienskappe en kwaliteit daarvan nadelig beïnvloed nie.

Numeriese en kategoriese data is tydens die studie versamel. Gluteraldehied-gefikseerde perikardiale weefsel is deurgaans as kontrole gebruik om die uitkomste van al die parameters wat vir die ander behandelingsmetodes bepaal is, mee te vergelyk.

Tydens die eerste fase van die studie is weefsel op vier verskillende metodes (gluteraldehied, aluminium, glikosaminoglikane en Glycar) behandel en gefikseer. Die weefselmonsters is hierna vir agt weke subkutaan in rotte ingeplant en die effektiwiteit van die behandelings is vergelyk ten opsigte van ekstraeërbare vog- en kalsiuminhoud. Kontrole- en aluminiumbehandelde weefsel het uitermatig verkalk, en daar is op grond hiervan besluit om aluminium as behandelingsmetode te staak. Weefsel wat met glikosaminoglikane (GAG) gefikseer is het belowende resultate getoon en baie goed met kommersiële Glycar-weefsel vergelyk, en daar is besluit om verdere ondersoek hierna in te stel.

In die volgende fase van die studie is weefsel wat met vyf verskillende konsentrasies GAG behandel is, vergelyk met GA-gefikseerde en Glycarweefsel ten opsigte van meganiese eienskappe (tensiele sterkte) en stabiliteit van die kruisbindings (protein denaturasie temperatuur). Die tensiele sterkte van weefsel wat met 0.01M GAG behandel is, was vergelykbaar met die ander twee metodes, terwyl die stabiliteit van die kruisbindings ook bo die aanvaarbare minimum standaard van 80^{o}C was. Op grond van hierdie resultate is 0.01M GAG geïdentifiseer as die optimale GAG-konsentrasie om vir behandeling van weefsel vir verdere inplantings in rotte te gebruik.

In die finale fase van die studie is weefsel wat met 0.0025M, 0.01M en 0.2M GAG behandel is, sowel as GA-gefikseerde en Glycar-behandelde weefsel vir agt weke in jong rotte ingeplant. Na herwinning is die weefsel vergelyk ten opsigte van water- en kalsiuminhoud en antigenisiteit, terwyl die omvang van die kruisbindings in die weefsels voor inplantering deur middel van die weerstand teen ensimatiese vertering bepaal is. Weefsel wat met 0.01M GAG behandel is het baie goed

vergelyk met Glycarweefsel ten opsigte van al die parameters, en die GA-gefikseerde kontroleweefsel beduidend oortref.

Ten spyte daarvan dat metaperiodaat addisioneel gebruik is om die bygevoegde GAG in die perikardium te fikseer, is beduidende bewys gevind dat die GAG steeds nie effektief gestabiliseer was nie. GAG het gedurende ‘n lang stoorperiode uit die weefsel geloog, en baie min GAG was na inplantering in die rotte steeds op die oppervlak van die weefsel sigbaar vergeleke met voor inplantasie. Alhoewel behandeling van perikardium met GAG die kalsifikasiepotensiaal in rotte beduidend verlaag het terwyl goeie tensiele sterkte en lae antigenisiteit behou is, sal die doeltreffende stabilisering van GAG eers voldoende aangespreek moet word alvorens hierdie weefsel met vertroue vir kliniese gebruik aangewend kan word.

SUMMARY

In the quest for suitable substitution materials to be used in surgical procedures, a large variety of different kinds of materials have been investigated. In cardiothoracic surgery, biological tissues such as porcine heart valves and bovine pericardium exhibited the most suitable properties for use as substitute material, while a variety of synthetic materials are also being used.

Biological materials must meet a lengthy list of requirements, before it can be successfully and safely employed as substitution material. Amongst others, it needs to be stable against biological breakdown, easily sterilizable, express minimal immunogenicity, maintain mechanical strength and tissue stability, resist calcification, be non-carcinogenic and non-toxic and permit easy handling. Numerous chemicals and methodologies have been investigated in order to produce the most suitable materials attaining these properties. Glutaraldehyde has emerged as the chemical agent rendering most of these requirements to tissues following fixation and cross-linking with it.

Despite the continued use of GA-fixed bovine pericardium worldwide, calcification and tissue degradation after 10-12 years post-implant remains a big problem. The main objective of this study was to try and identify additional biochemical treatment/s which can be employed in the fixation and storage of bovine pericardium, that will minimize the calcification potential of the tissue significantly without compromising the physical properties or the quality of the tissue.

GA-fixed pericardial tissue was used as the control, and the outcomes of all the parameters for the other tissue treatments were compared against it. Numerical and categorical data were collected.

In the first phase of study, four different methods of tissue treatment were compared for extractable calcium and water contents following 8 weeks implantation of treated samples in the subcutaneous rat model. Aluminium as treatment model was discarded due to the severe calcification of the implants. Results of tissue treated with GAGs were promising and compared favorably with commercial Glycar-treated tissue, and this prompted more detailed investigation.

In the next phase of the study, mechanical properties (tensile strength) and cross-linking stability (thermal denaturation temperatures) of tissues treated with different concentrations of GAGs were compared with GA and Glycar-treated tissue. Treatment with a GAG concentration of 0.01M yielded tissue with comparable tensile strength and thermal denaturation temperatures above the minimum benchmark. This concentration was identified as the optimal GAG concentration to be investigated in subcutaneous rat implant studies.

In the final phase, treated pericardial samples were implanted into weanling rats for 8 weeks and evaluated on the calcification potential, water content, antigenicity and extent of cross-linking of the collagen in the tissues. Tissue treated with 0.01M GAG compared favorably with the commercial Glycar patches regarding all of these parameters, outperforming GA-fixed control tissue significantly.

Significant evidence was however found that added GAGs were still not effectively stabilized despite adding metaperiodate as fixative. GAGs leached out of tissue following an extended storage period. Only a limited amount of GAGs was visible on the outer surface of the explants compared to the layer of GAGs superficially bound to the tissue before implantation. Despite decreasing the tissue calcification substantially while maintaining good mechanical strength and low antigenicity, stabilization of the GAGs in treated tissues will have to be adequately addressed before clinical application of such tissues can be approved.

KEY WORDS: Calcification; cross-linking; collagen; pericardium; subcutaneous implants; glutaraldehyde; glycosaminoglycans; tensile strength.

ABBREVIATIONS

&	And
±	About
[ ]	Concentration
^{o}C	degrees Celcius
=	equals
<	less than
>	more than
≥	more than or equal to
%	percentage
n	number of samples analysed
p	significance
cm	centimeters
^{o}C/min	degrees Celcius per minute
ΔH	entalphy of denaturation
g	gram
IU	international units
MPa	megapascal
µg	micrograms

µl	microliters
mg	milligrams
mg/kg	milligrams per kilogram
ml	milliliters
mm/min	millimeters per minute
mm/s	millimeters per second
mM	millimolars
M	molar
N	Newton
rpm	revolutions per minute
U/ml	units per milliliters
Al^{3+}	aluminium ions
$AlCl_3$	aluminium chloride
AOA	amino-oleic acid
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
COOH	carboxylated
DM	dry mass
DMSO	sodium-dodecyl-sulphate dimethylsulphate
DPPA	diphenylphosphorylazide
DSC	differential scanning calorimetry

EDAC	ethyldimethylaminopropyl carbodiimide
e.g.	example
Fe^{3+}	ferrous ions
GA	glutaraldehyde
GAG(s)	glycosaminoglycan(s)
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GCP	good clinical practice
H_2O_2	hydrogen peroxide
H&E	hematoxylin and eosin
$NaBH_4$	sodium borohydride
PBS	phosphate-buffered saline
PDS	polydioxanone
s.c.	subcutaneous
SO_3H	sulphated
TA	thermal analysis
T_d	thermal denaturation temperature
T_{max}	maximum temperature
T_p	transition peak temperature
VSD	ventricular septal defect

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CHAPTER 1

INTRODUCTION

A variety of different materials have been assessed as substitution materials during reconstructive surgical procedures, with variable success. The first example thereof is found in the field of neurosurgery where, in 1893, Beach used a gold foil to prevent meningocerebral adhesions during dural reconstruction. In 1895 Abbe used a rubber laminate for the reconstruction of a dural defect. Since then, various collagenous tissues or artificial materials have been used to reconstruct dura mater, including fascia lata, pericranium, temporal fascia, amnioplastin allantoic membrane, tantalum plate, cargile membrane, lyophilized dura, gelfoam, fibrin film, polyethylene, vicryl, silicone-coated dacron, teflon and vynyon-N.

Differences in criteria for an ideal material have contributed to the lengthy list of potential substitute materials, although today, most users agree that the ideal material must be inert, non-toxic, noncarcinogenic, impermeable to liquids, able to hold sutures, prevent meningeal adhesions or infections, be handled and sterilized easily and also be inexpensive. Bovine pericardium has lately been identified as the material which satisfies most of the criteria and seems to have suitable properties for use as a substitute material (Baharuddin, 2002).

In the field of cardiac and thoracic surgery, the quest for suitable substitute materials has also been going on for many years and still continues. In the late 1950's and early 1960's cardiac surgeons began utilizing aortic and pulmonary homografts for the treatment of valvular disease and the repair of congenital malformations. Pioneers like Murray, Beall, Kerwin, Bigelow, Ross, Barratt-Boyes and O'Brien were the first to implant aortic homografts or segments thereof to correct diseased aortic valves, while in 1961 Lower and colleagues at Stanford were the first to transplant a pulmonary valve to the mitral position in dogs.

Pillsbury and Shumway soon thereafter transplanted the first autologous pulmonary valve in the aortic position in dogs, and in 1967 Ross did the first similar transplants in humans (Hampton, 2003).

The surgical repair, reconstruction or closure of different cardiac vessels and structures following cardiac surgery often requires the use of a replacement material of either biological or synthetic origin. In the resection and patch or prosthetic reconstruction of the pulmonary artery and superior vena cava, for example, the use of biological materials such as autologous or bovine pericardium, azygos vein and saphenous vein, have achieved greater acceptance than synthetic materials. This was mainly due to improved biocompatibility and a lower risk of infection and thrombolysis, and it also costs less than synthetic materials (D'Andrilli, 2005).

The clinical use of bovine pericardium for the construction of an artificial heart valve was first reported in 1971 by Ionescu, and it was since then used worldwide to treat various congenital cardiac defects (Neuhauser & Oldenburg, 2003). Researchers are however still trying to produce the most optimal tissue that would yield the best results regarding low calcification potential, low antigenicity, durability and maximum strength. Pericardium fixed and stored in glutaraldehyde has been used clinically for many years with good results at our institution, but severe calcification remained a big concern (Neethling, 1996). In this study, we aim to provide sufficient evidence that an alternative biochemical treatment of the tissue before storage in glutaraldehyde (GA) will yield substantial improvement regarding in vivo calcification, without sacrificing any of the required and proven properties, and that it would be safe to use as substitute material during cardiothoracic procedures.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORY

There is an ongoing search for suitable biomaterials that can be used for the repair and replacement of various soft body tissues such as tendons, skin, vascular grafts and heart valves. These biomaterials need to be both versatile and compatible with human tissues, and increased interest is shown in the use of collagen and collagen-containing tissues in medical devices and for transplants. Collagen, in the form of fibers, represents the single most abundant animal protein in mammals. The general properties of collagen include the high strength of the fibers, low extensibility, minimal antigenicity, suitability as a substrate for cell growth, and controllable stability by chemical or physical cross-linking which in combination make this protein an interesting biomaterial (Zeeman, 1998).

Collagen-rich materials such as heart valves, vascular grafts and bovine pericardium which are frequently used as bioprosthetic implants in cardiac surgical procedures, are subjected to degradation immediately following the death of the donor (animal or human). Degradation of the material needs to be arrested as soon as possible in order to prolong the original structural and mechanical integrity of the tissue.

The three polypeptide chains of a collagen molecule are arranged in a trihelical configuration, ending in a non-helical carboxyl terminal at one end and an amino terminal at the other end. The non-helical ends are believed to contribute to most of the antigenic properties of collagen, which also need to be removed or at least neutralized (Khor, 1997). A wide range of chemical treatments and modifications of collagenous tissues, known as cross-linking methods, have been researched and used for this purpose. One of the

earliest chemical modifications of collagen to use it as a biomaterial, is associated with leather tanning (Zeeman, 1998).

2.2 CROSS-LINKING OF COLLAGENOUS MATERIALS

Cross-linking methods concentrate on creating new additional chemical bonds between the collagen molecules, which reinforce the tissue to give a tough and strong but non-viable material. These methods are designed to maintain the original shape and character of the tissue, such as flexibility and mechanical properties as much as possible (Khor, 1997). Cross-linked material should also be biocompatible, have a low tendency to calcify *in vivo*, and be stable towards enzymatic degradation. Reaction conditions such as the reagent concentration, reaction time, the pH of the solution and temperature at which the reaction takes place, all have an influence on the cross-linking rate and density of cross-links formed (Zeeman, 1998).

The chemical agent that has been predominantly used and investigated for the treatment of collagenous tissues is glutaraldehyde (GA), while other chemicals used include formaldehyde, epoxy compounds, acyl-azide, carbodiimide and poly (glycidil methacrylate-butyl acrylate).

Jorge-Herrero and colleagues also used diphenylphosphorylazide (DPPA) and ethyldimethylaminopropyl carbodiimide (EDAC) as alternative chemicals, which acted by activation of the carboxyl groups, which then permits their cross-linking to amino groups. Mixed results towards the different parameters measured, were obtained. Pericardium treated with EDAC showed much less resistance to collagenase degradation than DPPA-treated tissue, but cross-linking with GA alone provided much greater protection. The degree of calcification of tissue implanted subcutaneously in rats for 60 days was however considerably lower for EDAC-treated tissue compared to GA and DPPA-treated tissue (Jorge-Herrero, 1999).

2.2.1 Glutaraldehyde as Cross-linking Agent

Glutaraldehyde was first applied successfully for bioprostheses in the late 1960s by Carpentier (Zeeman, 1998). Glutaraldehyde has been mainly assessed and most frequently used for the treatment of collagenous tissues, since it is less expensive, readily available and highly soluble in aqueous solutions (Jayakrishnan & Jameela, 1996). Materials cross-linked with GA result in the highest degree of cross-linking when compared with other known methods (Khor, 1997). Tissue valves are constructed from porcine aortic valves or bovine pericardium, and are treated with glutaraldehyde (GA) to introduce cross-links that stabilize the valvular structural proteins and make them more durable. Despite extensive studies of the reaction mechanisms during cross-linking, it remains very complex and still not completely understood (Zeeman, 1998).

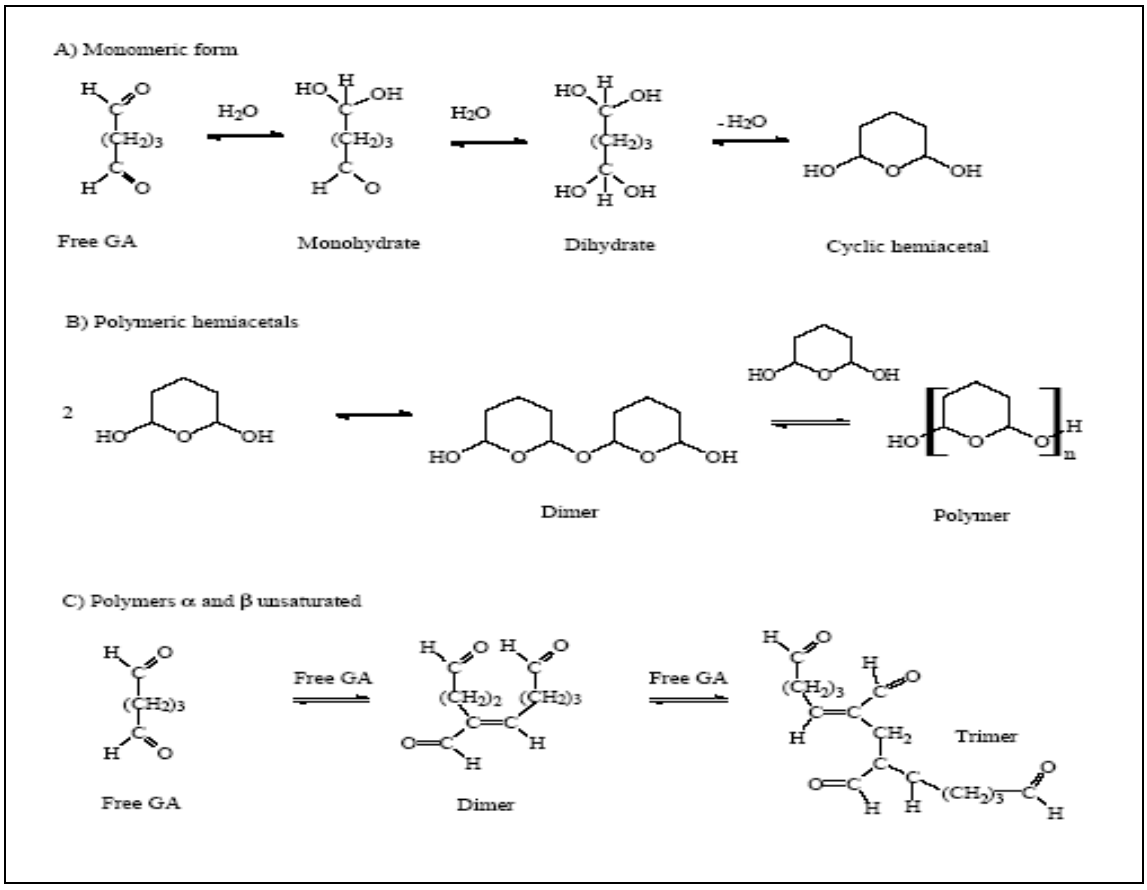


Figure 1 Possible structures of glutaraldehyde in aqueous solutions. (Adapted from http://doc.utwente.nl/9101/1/t000000b.pdf)

Aqueous solutions of GA contain a mixture of free aldehyde and mono- and dihydrated GA, as well as monomeric and polymeric hemiacetals. Because of the ease of hydration and cyclization, the concentration of free, monomeric aldehydes in concentrated, commercial solutions is usually low (Zeeman, 1998). GA solutions may contain various products resulting from aldol condensation during storage and cyclic GA oligomers having a trioxane structure have been described. Because of this complexity of the reaction solutions, many reactions can occur during cross-linking (Olde Damink, 1995).

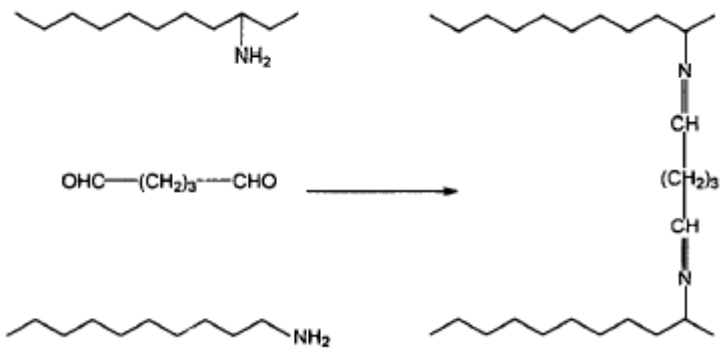


Figure 2 Diagrammatic representation of monomeric glutaraldehyde reacting with amino groups on collagen to form cross-links. (Adapted from www.elsevier.com/wps/find/journaldescription.cws_home/30392/

Cheung and co-workers (Cheung, 1985) suggested that the penetration of GA molecules into dense tissue such as pericardium is slow, and that primarily the outer surfaces of the fibers are fixed. In addition, a polymeric network is created which hinders further cross-linking. It is presumed that GA cross-link in an inter- and intramolecular fashion by the formation of covalent bonds, which can occur in two ways: 1) In general, aldehyde groups react with the amine groups of lysine or hydroxylysine residues of the collagen, yielding a Schiff base (stabilized imino bond), or 2) an aldol condensation is formed between two adjacent aldehydes. The Schiff base linkage is not a very stable bond, but can be stabilized by a reduction reaction, whereas the aldol condensation product is stable (Jayakrishnan & Jameela, 1996).

2.2.2 Pretreatment before Glutaraldehyde Cross-linking

Various other methods apply the pretreatment of the collagenous tissue with a potential anti-calcification agent before the final cross-linking with a glutaraldehyde solution is performed. In 1991, Golomb and Ezra hypothesized that an impaired balance between positively and negatively charged amino acids was created due to the reaction with lysine and hydroxylysine tissue-collagen residues, which exposed affinity sites to Ca^{++}-ions and resulted in calcification. In order to perform positive charge modification of the tissue to prevent their propensity to calcify, they covalently bound protamine sulphate, a polybasic peptide, via formaldehyde to the collagen tissue, followed by glutaraldehyde cross-linking. The tissue exhibited stability towards shrinkage temperature and resistance to collagenase digestion and was less permeable to calcium ions (Golomb & Ezra, 1991).

Chloroform/methanol, sodium-dodecyl-sulphate dimethylsulphate (DMSO) and especially ethanol were also used as pretreatments to extract the majority of acidic phospholipids and cholesterol out of the valve cusp tissue before glutaraldehyde cross-linking (Garcia Paez, 2001). The ethanol pretreatment causes a permanent alteration in collagen conformation, affects cuspal interactions with water and lipids, enhances cuspal resistance to collagenase (Schoen & Levy, 2005) and does not affect the cuspal glutaraldehyde content (Vyavahare, 1998).

Pretreatment of tissues with trivalent metal ions such as aluminium (Al^{3+}) and iron (Fe^{3+}) in order to inhibit the growth of hydroxyapatite crystals which will eventually lead to calcification of the tissue, was also investigated. Promising results were shown in blocking the calcification of aortic walls due to the irreversible binding to elastic fibers, but binding to the collagen in cusp tissue was unstable and the ions leached out into the circulation, with resultant calcification of the collagen (Levy, 2003; Ogle, 2003).

The pretreatment of pericardium with iron(III)citrate reduced calcification in the subcutaneous rat model, as did acyl azide activation of carboxyl and amide

groups. Chondroitin sulphate had no significant effect, while cyanamide treatment was mainly effective in combination with iron(III)citrate. With all these treatments, postfixation with GA had no significant effect on the calcification rate (Bernacca, 1992).

2.2.3 Cross-linking Followed by Chemical Treatments

Various research groups also looked at treating the tissue after cross-linking with glutaraldehyde, in order to minimize or inhibit tissue calcification. Amino-oleic acid (AOA) bonds covalently with bioprosthetic tissue through an amino linkage to residual aldehyde functional groups and inhibits calcium flux into bioprosthetic valve cusps. The AOA is effective in mitigating cusp but not aortic wall calcification in rat subdermal and cardiovascular implants (Chen, 1994).

Chanda and colleagues bounded heparin covalently with GA-treated porcine pericardium, through an intermediate surface-bound substrate containing amino groups. The substrate (0.1% chitosan + 0.015% gentamicin sulphate in deionized water) were coupled with free aldehyde groups of the GA, and the partially degraded heparin then coupled with animated surfaces of the pericardium by reduction with sodium borohydride. The hypothesis is that the coupling of heparin with chitosan-gentamicin-treated grafts fills the intertropocollagen spaces, blocks the potential calcium binding sites and modifies charges, and thus makes the prostheses impermeable to host plasma calcium (Chanda, 1997).

Similar results were reported by Lee and co-workers, who also concluded that the durability of heparin-treated tissue increased significantly when compared with fresh tissue and GA-treated tissue, it has greater resistance to enzymatic digestion, is non-cytotoxic, and the calcium content deposited *in vivo* on heparinized tissue was much less than the calcium deposited on GA-treated tissue (Lee, 2000).

2.3 ROLE OF GLUTARALDEHYDE IN CALCIFICATION

Tissue calcification is regarded as the major mechanism of bioprosthetic implants, and there are currently four proposed theories whereby the calcification of GA-fixed animal valvular prostheses can best be explained: 1) glutaraldehyde fixation; 2) organic matrix composition; 3) mechanical stress, and 4) cell injury theories (Kim, 1999).

1) Glutaraldehyde molecules which are retained in GA-fixed valvular prostheses are probably responsible for the tissue's calcification in rat implants, compared to fresh valves which only provoke inflammation but do not calcify (Levy, 1983). There also appears to be a quantitative relationship between the amount of GA and the calcific deposits. Using a higher concentration of GA did however contradict these findings by diminishing the tissue calcification in rat implants (Zilla, 1997).
2) Collagen, which forms part of the structural proteins in the extracellular matrix, has been implicated as a nucleation site of apatite crystal formation in GA-fixed valve prostheses. Osteocalcin and osteopontin have been isolated from such calcified prosthetic tissue, which suggest that they may play a role in calcification. Other noncollagenous proteins like phosphoproteins have also been implicated in prosthetic valve tissue calcification (Kim,1999).
3) Calcific deposits have been found selectively in the areas of increased mechanical stress of transplanted bioprosthetic heart valves (Thubrikar, 1983), and it is postulated that the movement of plasma contents into the stressed areas might play a role in the calcification. Continuous movement of the valve might also allow calcified particles to migrate and accumulate in the stressed areas (Kim, 1999).
4) Fixation of valve prostheses with GA causes injury to fibroblast cells, making the cell membrane more permeable to calcium and phosphate ions. The concomitant elevation in the influx of these two ions have been implicated to be the underlying mechanism of prosthetic tissue calcification (Kim, 1999).

Although biological tissues treated with GA showed good haemodynamic performance and a low antigenicity (Jayakrishnan & Jameela, 1996), with good tensile strength and pliability, it is now known that the durability of these tissues is not as good as expected (Khor, 1997). GA-treated materials like porcine heart valves calcify to a large extent, and this might be due to the cross-linking process (Zeeman, 1998).

Aldehyde fixation appears to be a prerequisite for bioprosthetic valve calcification. Animal studies have shown that nonfixed valves provoke inflammation reactions, but do not calcify after subcutaneous implantation in rats, and processed but non-GA-fixed human allografts show much less calcification than aldehyde-fixed valves (Levy, 1983). Gong and co-workers demonstrated that bovine pericardial tissue treated with glycerol and then fixed and stored in GA or formaldehyde or a combination thereof, calcified significantly more in the subcutaneous rat model than tissue treated with glycerol alone. They concluded that the presence of free aldehyde groups following cross-linking and storage in GA and/or formaldehyde, plays an important role in the calcification of bioprosthetic valve tissue (Gong, 1991).

Liao and co-workers also demonstrated a direct relationship between the length of time that fresh bovine pericardial patches were exposed to a certain concentration of GA-solution, and the degree of calcification observed following 45 days of subcutaneous implantation in rats. Calcification was found in all autograft, allograft and xenograft implants that were exposed to GA for 15 minutes, and this increased proportionally with increased fixation times. A minimum fixation period of 15 minutes for autologous pericardium was required in order to preserve the basic tissue stability and strength and to reduce the antigenicity of bovine pericardium drastically, but it should not exceed 60 minutes as excessive calcification may result (Liao, 1995).

Depolymerization of polymeric GA cross-links has also been reported, which in turn releases monomeric and highly cytotoxic GA into the recipient of the prosthesis (Zeeman, 1998). Residual GA may also leach out of fixed valves and induce injury to surrounding tissue, which might promote mineralization

(Giachelli, 1999). Residual GA remaining in the bioprostheses, as well as unstable GA polymers retained in the interstitial spaces of the cross-linked tissue, have been implicated for inflammatory reactions, cytotoxicity, calcification and lack of endothelialization. Sufficient care should therefore be taken to remove the unreacted GA present in bioprostheses before implantation, to reduce the cytotoxicity. Thorough washing of the bioprostheses and storage in a solution free of aldehydes (eg. saline or propylene oxide) would eliminate at least the primary cytotoxic effect of glutaraldehyde (Jayakrishnan & Jameela, 1996).

The devitalization of prosthetic valves and tissues with aldehydes has been proposed to alter membrane permeability and the influx of calcium ions. These alterations result in high concentrations of calcium being in contact with high phosphate levels in membrane-bound intracellular compartments, which might react with one another to form calcium phosphates which could precipitate (Giachelli, 1999).

Enhanced fixation of bioprosthetic tissues, with high concentrations of GA compared with the low concentrations that have up to now been used during commercial valve fixation, resulted in a significant reduction in calcification of leaflets, bovine pericardium and aortic wall tissue after 6 weeks in the subcutaneous rat model. Additional amine cross-linking with treatment with L-lysine at high temperature (37^{o}C) and acidic pH reduced calcification significantly, while the further extraction/detoxification of GA by using high-volume urazole solutions followed by sodium borohydride ($NaBH_4$) reduction gave the optimal reduction in calcification of all the tissues (Weissenstein, 2000).

Similar results were reported by Neethling and co-workers, who have managed to significantly reduce the calcification of GA-fixed porcine cusp and aortic wall tissues in the subcutaneous rat model. Freshly harvested tissue was fixed with 0.625% GA, lipids extracted with a short-chain alcohol, residual GA removed and modified by a combination of amine incorporation and carboxyl binding at a high temperature (45^{o}C) and low pH of 4.5 ±0.15, tissue

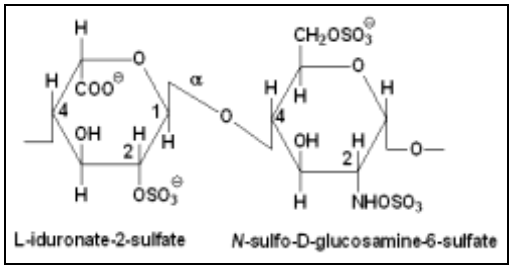
elasticity restored by polymerization of the incorporated GA moieties in the tissue by increased temperature, and stored in 0.25% buffered GA at 4^{o}C (Neethling, 2006).

2.4 GLYCOSAMINOGLYCANS (GAG)

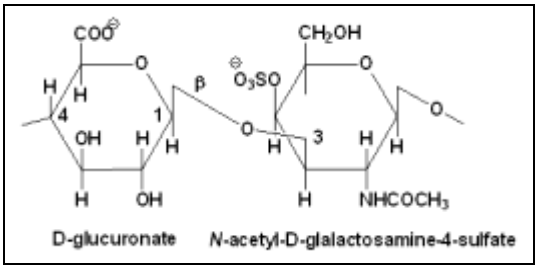
The extracellular matrix is largely comprised of complex polysaccharides, which were historically considered to be inert materials that hydrated the cells and contributed to the structural scaffolds. Recently developed sophisticated analytical techniques have brought about dramatic new insights into the numerous biological roles of these complex polysaccharides. The most abundant heteropolysaccharides (a class of these polysaccharides) in the body are the glycosaminoglycans (GAG), which bind with a variety of proteins and signaling molecules in the cellular environment and modulate their activity, thus impinging on fundamental biological processes (Raman, 2005).

2.4.1 Chemical Structure

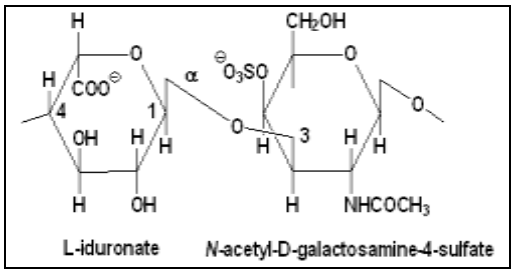
GAG molecules are long unbranched (linear) acidic polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either of two modified sugars, namely N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc), and an uronic acid such as glucuronate or iduronate.



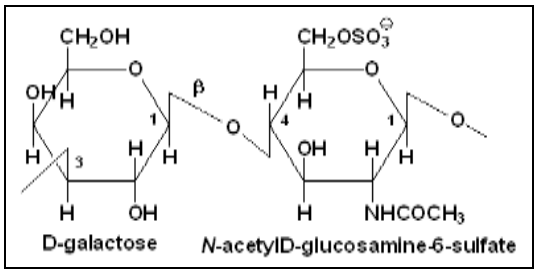
Heparin & Heparan sulphates



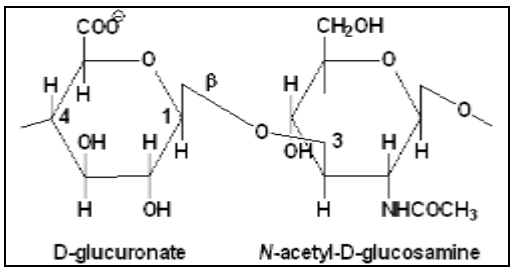
Chondroitin 4- and 6-sulphates



Dermatan sulphates



Keratan sulphates



Hyaluronates

Figure 3 Diagrammatic illustrations of the GAGs of physiological significance. (Adapted from http://web.indstate.edu/thcme/mwking/extracellularmatrix.html)

GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. GAGs are located primarily on the surface of cells or in the extracellular matrix. Along with the high viscosity of GAG comes low compressibility, which makes these molecules ideal for a lubricating fluid in the joints. At the same time their rigidity provides structural integrity to cells, and provides passageways between cells, allowing for cell migration. The specific GAGs of physiological significance, and each with its own predominant disaccharide component, include: 1) heparin & heparan sulphates; 2) chondroitin 4- & 6-sulphates; 3) dermatan sulphate; 4) keratan sulphates, and 5) hyaluronic acid (King, 2004).

2.4.2 Localization

Heparins are more sulphated than the heparan sulphates, and is a component of intracellular granules of mast cells lining the arteries of the lungs, liver and skin, while heparan sulphates contain more highly acetylated glucosamine than heparin and is found in basement membranes and components of cell surfaces.

The chondroitin sulphates are the most abundant GAG, and found in cartilage, bone and heart valves.

Dermatan sulphates are found in skin, blood vessels and heart valves, while keratan sulphates are found in cornea, bone and cartilage, aggregated with chondroitin sulphates.

Hyaluronate is unique among the GAG in that it does not contain any sulphate and is not found covalently attached to proteins as a proteoglycan. Hyaluronic acid polymers are very large and can displace a large volume of water, which makes them excellent lubricants and shock absorbers, localized in synovial fluid, vitreous humour and the extracellular matrix of loose connective tissue (King, 2004).

2.4.3 Biological Role of Glycosaminoglycans

The GAG chains of both the cell surface and secreted proteoglycans are in the presence of various proteins such as growth factors, cytokines, morphogens and enzymes (proteases and protease inhibitors) inside the extracellular environment. GAGs play a critical role in assembling protein-protein complexes such as growth factor-receptor or enzyme-inhibitor on the cell surface and in the extracellular matrix that are directly involved in initiating cell signaling events or inhibiting biochemical pathways. Extracellular GAGs can also potentially sequester proteins and enzymes and present them to the appropriate site for activation. Thus, for a given high-affinity GAG-protein interaction, the positioning of the protein-binding oligosaccharide motifs along

the GAG chain determines whether an active signaling complex is assembled at the cell surface or an inactive complex is sequestered in the matrix (Raman, 2005).

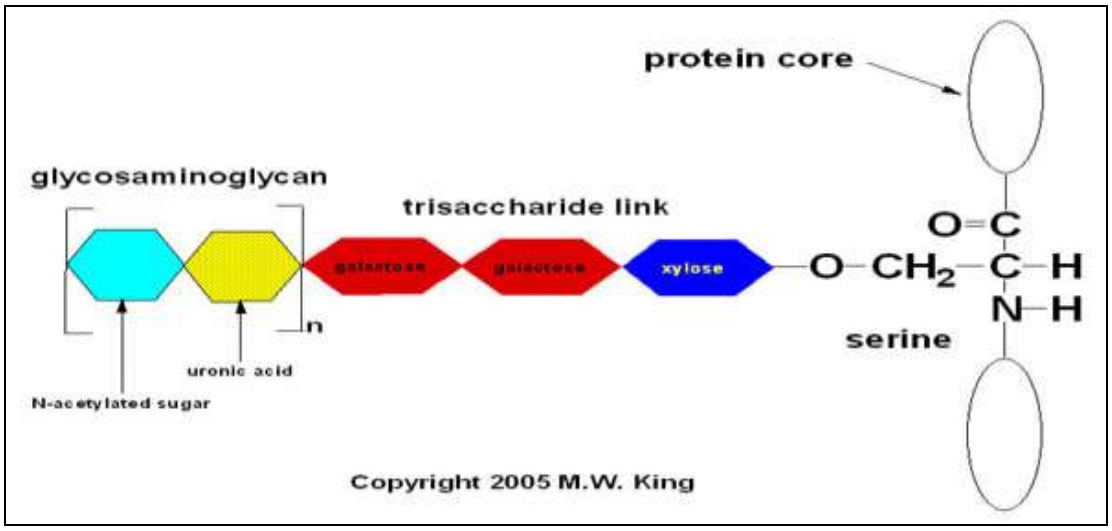


Figure 4 Structure of the GAG linkage to protein in proteoglycans. (Adapted from http://web.indstate.edu/thcme/mwking/extracellularmatrix.html)

However, high-affinity GAG-protein interactions are not the only biologically significant interactions. GAGs have been shown to play important roles in maintaining morphogen gradients across a cell or tissue, which have been implicated in developmental processes. Maintaining a gradient in the concentration of growth factors or morphogens would involve graded affinities between different GAG sequences with the given protein. Thus, the nature of GAG-protein interactions coupled with their sequence diversity enables GAGs to “fine tune” the activity of proteins (Raman, 2005).

Various studies have provided direct evidence of the biological roles of GAGs, but it remains important to understand these roles from the standpoint of structure-function relationships of GAG-protein interactions. Delineating the physiological context of GAG-protein interactions to truly define structure-function relationships *in vivo* remains a challenging task (Raman, 2005).

In the native heart valve cusps, the proteoglycan molecules in the middle spongiosa layer are capable of absorbing a large amount of water within the tissue matrix, because of their high concentration of negative charges and their inherent hydrophylicity. These highly hydrated GAGs then act as a lubricating layer and allow shearing between the two outer layers, the fibrosa and the ventricularis, during valve function. By absorbing compressive forces, they might also help to reduce buckling of the leaflets during flexion, which has been attributed to the mechanical failure of bioprosthetic heart valve leaflets (Lovekamp, 2006). Some researchers also speculate that the presence of negatively charged GAG molecules within the extracellular matrix of cuspal tissue may reduce calcification by chelating calcium ions, thereby preventing hydroxyapatite nucleation (Lovekamp & Vyavahare, 2001).

Proteoglycans are formed by sulphated GAGs that are covalently linked to proteins. The proteoglycans in pericardium are mainly composed of dermatan sulphate and chondroitin sulphate, and they contribute to tissue hydration and tissue elasticity, and may also participate in the interaction with other extracellular matrix components. The addition of high concentrations of chondroitin 4-sulphate to collagen gels inhibits the formation of hydroxyapatite crystals and thus the initiation of the calcification process. The selective extraction of proteoglycans in pericardium also results in a greater accumulation of calcium salts than in unextracted tissue, as well as a reduction in hydrothermal stability (Jorge-Herrero, 2005).

2.4.4 GAG and Cross-linking with Glutaraldehyde

The cross-linking of xenograft implants constructed from either porcine valve tissue or bovine pericardium with GA, results in a tightly linked matrix of proteins, with the majority of proteins being collagen. This cross-linking reduces the immunologic reaction from the recipient, and serves to improve the durability and resistance to enzymatic degradation of the implants *in vivo*. However, the cuspal extracellular matrix components, such as elastin and GAGs (hyaluronic acid and dermatan sulphate), lack free amine functionalities which are necessary to react with GA during conventional cross-linking, and

are therefore not effectively stabilized. Furthermore, unlike other GAG molecules, hyaluronic acid is not linked to a protein (Lovekamp, 2006). The devitalized nature of bioprosthetic heart valves following GA treatment also prevents any cell-mediated remodeling of the extracellular matrix that would otherwise help to maintain the GAG concentrations (Lovekamp & Vyavahare, 2001).

The effect of all the above-mentioned has been a decrease in the levels of GAG molecules from GA-cross-linked porcine aortic cusps of bioprosthetic valves that were retrieved at reoperation. Research has also proved that GAGs leached out of the spongiosa layer under *in vitro* cyclic fatigue, and a reduction in cuspal GAG concentrations have been shown in rheumatic and aged valves, making them highly prone to failure. This decrease in the levels of GAG molecules resulted in a significant reduction in cuspal stiffness, making the bioprosthetic valves vulnerable to material failure (Vyavahare, 1999).

When bovine pericardial tissue was pre-treated with a sodium metaperiodate solution as fixative for the proteoglycans before the final cross-linking with GA was performed, a 1.4-fold increase in the total extractable proteoglycan content of the pericardium was achieved. By adding exogenous chondroitin 4-sulphate to the periodate fixation, a more than 4-fold increase in the total proteoglycan content can be obtained. However, a final fixation with GA is in all cases still required to confer enough mechanical resistance to the implant by inducing covalent cross-links between the collagen molecules (Arenaz, 2004).

Improved stabilization of GAGs by applying additional cross-linking strategies to chemically link GAGs to major components of the extracellular matrix of valve tissue, namely Type 1 collagen and hyaluronic acid, would result in improved preservation of the valve structure and improved mechanical properties, leading to less degeneration during its function (Lovekamp & Vyavahare, 2001).

2.5 METHODS FOR EVALUATION OF CROSS-LINKED BIOMATERIALS

2.5.1 Cross-link Stability

2.5.1.1 Enzyme Degradation Resistance

One of the methods that can be used to quantitatively determine the extent of tissue cross-linking is by collagenase digestion studies. Samples of all the different treated and untreated pericardial patches (or valve leaflets, etc.) are dried and weighed. Collagenase is suspended in a solution of Tris-HCl buffer + $CaCl_2$ at pH 7.4, approximately 1.2 ml of this solution is added for each gram of dried tissue per sample and allowed to react for 24 hours at 37^{o}C. After this period the samples are centrifuged for 5 minutes at 12 000 rpm and the majority of the liquid discarded. Insoluble residues of tissue are again dried completely and weighed. Dry weights of the undigested samples are compared with those obtained before the enzymatic digestion, and the percentage of tissue loss is calculated (Lovekamp & Vyavahare, 2001).

A similar method was described by Neethling and colleagues, using a pronase solution consisting of 100mg pronase E and 100mg calcium chloride dissolved in 200ml HEPES buffer solution containing 0.1M glycine. The resistance to pronase digestion was determined by the mass of remaining tissue following digestion, expressed as a percentage of the predigested dried tissue weight (Neethling, 2004).

2.5.1.2 Thermal Denaturation Temperature (T_d)

Another method to assess the extent and stability of cross-links of different collagenous materials, is by determining the temperature at which denaturation of the triple-helix structure occurs. This temperature is referred to as the thermal denaturation temperature (T_d) or shrinkage temperature. When collagen is heated in the hydrated state, the

material will denature at a specific temperature, resulting in the shrinkage of the material to about one-third of its original length. This shrinkage, which takes place within a narrow temperature range of 2-3^{o}C, is the macroscopical manifestation of the transformation of the triple-helices to random coils. Enhanced cross-linking of collagen by the introduction of covalent bonds will increase the stability of the helix and thus increase the denaturation temperature of the materials (Zeeman, 1998).

A somewhat older technique of measuring the shrinkage temperature of tissue samples is by attaching strips of tissue from each sample to an isometric force transducer, interfaced with a data acquisition system and a desktop personal computer. Samples are kept in constant extension with a load of 90±5g and immersed in an open, temperature-controlled waterbath filled with 0.9% saline. The temperature of the bath is gradually increased by ±1.5^{o}C/min from 25^{o}C to 95^{o}C. The shrinkage temperature is indicated as a sharp deflection point from constant extension when the collagenous material is denaturated (Neethling, 2004).

Probably the most accurate technique available to determine the thermal denaturation temperature of collagenous tissue, is by application of a differential scanning calorimeter. Differential scanning calorimetry (DSC) is a technique which is part of a group of techniques called Thermal Analysis (TA). TA is based upon the detection of changes in the heat content (enthalpy) or the specific heat of a sample with temperature (Friedli G-L, 1996).

As thermal energy is supplied to the sample its enthalpy increases and its temperature rises by an amount determined, for a given energy input, by the specific heat of the sample. The specific heat of a material changes slowly with temperature in a particular physical state, but alters discontinuously at a change of state (Friedli G-L, 1996).

As well as increasing the sample temperature, the supply of thermal energy may induce physical or chemical processes in the sample, e.g. melting or decomposition, accompanied by a change in enthalpy, the latent heat of fusion, heat of reaction etc. Such enthalpic changes may be detected by thermal analysis and related to the processes occurring in the sample (Friedli G-L, 1996).

In DSC, the measuring principle is to compare the rate of heat flow to the sample and to an inert material which are heated or cooled at the same rate. Changes in the sample that is associated with absorption or evolution of heat cause a change in the differential heat flow which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic. For proteins, the thermally induced process detectable by DSC is the structural melting or unfolding of the molecule. The transition of protein from a native to a denatured conformation is accompanied by the rupture of inter- and intra-molecular bonds, and the process has to occur in a cooperative manner to be discerned by DSC (Ma and Harwalkar, 1991).

Analysis of a DSC thermogram enables the determination of two important parameters: denaturation temperature (T_d) (also called transition temperature peak (T_p) or maximum (T_{max}) temperature), and enthalpy of denaturation (ΔH). The denaturation temperatures are measures of the thermal stability of proteins, although they are influenced by the heating rate (Ruegg, 1977) and protein concentration (Wright, 1984).

For determination of the thermal denaturation temperature of collagenous tissue with DSC, a small tissue sample is placed in a hermetically sealed pan and subjected to thermal analysis. The temperature is raised at a rate of 10^{o}C/min from 25^{o}C to 110^{o}C, and the temperature of thermal denaturation for each sample is recorded as a peak maximum (Lovekamp & Vyavahare, 2001).

2.5.1.3 Tensile Strength

One of the most common testing methods, tensile testing, is used to determine the mechanical behaviour of a sample while an axial stretching load is applied. These types of tests may be performed under ambient or controlled (heating and cooling) conditions to determine the tensile properties of a material.

Tensile testing is performed on a variety of materials which includes industrial products like plastics, paper, rubber etc., and for the determination of tissue strength in the medical field (Kofidis, 2002). Tensile testing is used to determine the maximum load (tensile strength) that material or a product can withstand. Tensile testing may be based on a load value or elongation value.

Cross-linking of bovine pericardium with GA or a poly-epoxy compound resulted in an increase in the extensibility (elongation at break) and a reduction in stress relaxation (because of the presence of interfibrillar cross-links), and a twofold increase in ultimate tensile strength.

2.6 ANIMAL MODELS

2.6.1 The Rat Subcutaneous Implant Model

The calcification potential of different treated bioprosthetic tissues can be evaluated in a rat subcutaneous implantation model (Schoen & Levy, 1999).

An incision is made through the skin on either the abdominal or dorsal side of the animal and the washed tissue samples are inserted into small subcutaneous pockets created underneath the skin. Samples are secured to the muscle wall with a very fine suture at both ends, and the incision is closed with sutures. Samples are retrieved after a predetermined period (at least 8

weeks) and histologically examined for calcification, and the calcium content is quantitatively determined.

2.7 HISTOLOGICAL EXAMINATIONS

2.7.1 Hematoxylin & Eosin Staining (H&E)

The hematoxylin and eosin stain is probably the most widely used histological stain. Its popularity is based on its ability to demonstrate an enormous number of different tissue structures, its widespread applicability to tissues from different sites, it can be prepared in different ways, and its comparative simplicity. Essentially, the hematoxylin component stains the cell nuclei blue-black, with good intra-nuclear detail, while eosin stains cell cytoplasm and most connective tissue fibres in varying shades and intensities of pink, orange and red. However, hematoxylin has many more uses than in the hematoxylin and eosin combination (Bancroft and Stevens, 1990).

2.7.2 Von Kossa Staining

The classic method for the demonstration of calcium and certain other salts in tissues was developed by von Kossa in 1901. The tissue sections are treated with a silver nitrate solution; the calcium is reduced by a strong light and replaced with silver deposits, which are visualized as metallic silver (Bancroft & Stevens, 1990).

2.7.3 Alcian Blue Staining

This staining method is specifically used when looking for the presence of acid mucopolysaccharides in the tissue samples. Different amounts of magnesium chloride are added to the alcian blue solution, which yield solutions with different electrolyte molarities. These in turn are used to distinguish between different types of mucopolysaccharides as the magnesium ions are competing with the alcian blue for binding sites on the

mucopolysaccharides. As the concentration of magnesium ions increases, more binding sites are blocked from access to alcian blue. An electrolyte concentration of [0.06M] will stain acid mucopolysaccharides blue; [0.2-0.3M] will stain sulphated acid mucopolysaccharides blue; [0.5-0.6M] will stain strongly sulphated acid mucopolysaccharides blue; [0.7-0.8M] will stain heparin, heparan sulphate and keratan sulphate blue, and [0.9M] will stain only keratan sulphate blue. If desired, a counterstain with neutral red can also be included, which will stain cell nuclei red (Bancroft and Stevens, 1990).

CHAPTER 3

AIM and OBJECTIVES

3.1 RELEVANCE OF THE STUDY

Biological tissues such as porcine aortic valves or bovine pericardium have been successfully used since 1960 for the manufacturing of tissue valve prostheses as substitutes during heart valve replacement procedures. These tissues are treated with various chemicals in order to arrest and defer the degradation process, prolong the original structural and mechanical integrity and remove or at least neutralize the antigenic properties attributed to these materials. In addition, these treatments also strive to reinforce the tissue by creating new additional chemical bonds between the collagen molecules, ensuring a tough and strong but non-viable material that maintains the original shape and properties of the tissue. One of the chemicals most widely used in this regard, is glutaraldehyde.

The long-term success of GA-treated tissues is limited by the tendency of such devitalized tissues to undergo degeneration, primarily calcification and/or structural breakdown. The factors and mechanisms responsible for the induction and the enhancement of calcium phosphate crystal formation and growth seem to be multifactorial and are not fully understood. Both cross-linking with GA and the presence of foreign proteins and cells in the tissue appear to play an important role in this process (Zeeman, 1998).

3.1.1 Aim

Locally-produced glutaraldehyde-fixed bovine pericardial patches have been successfully used for many years at our and other institutions in cardiac repair procedures such as VSD's, pulmonary outflow tract reconstructions and ventricle aneurism repairs. However, re-operations a few years later have shown that these patches do calcify severely. Therefore, the main aim of this

study is to try and identify additional biochemical treatment/s which can be employed in the fixation and storage process of bovine pericardium, that will minimize the calcification potential of the tissue significantly without compromising the physical properties or the quality of the tissue.

3.1.2 Objective

Pericardial patches are routinely used as substitute material in a wide variety of surgical procedures, but tend to calcify at variable rates due to different methods of treatment. The most optimal patch would be one that remains strong and durable, has a low antigenicity and a reduced calcification potential, especially in cardiovascular applications. The objective of this study is therefore to prove that pretreating the pericardium with glycosaminoglycans (GAG) before fixation and storage in glutaraldehyde, will give a material that will remain pliable and calcify less than the GA-fixed patches that are currently used in our institution, without compromising any of the mentioned requirements.

CHAPTER 4

METHODOLOGY

4.1 STUDY LOCATION

The research study was conducted at the University of the Free State, and involved the departments of Cardiothoracic Surgery, Anatomical Pathology, Chemistry and the Large Animal Unit, as well as the department of Mechanical Engineering at the Central University of Technology.

4.2 STUDY DESIGN

The study was designed as an experimental study.

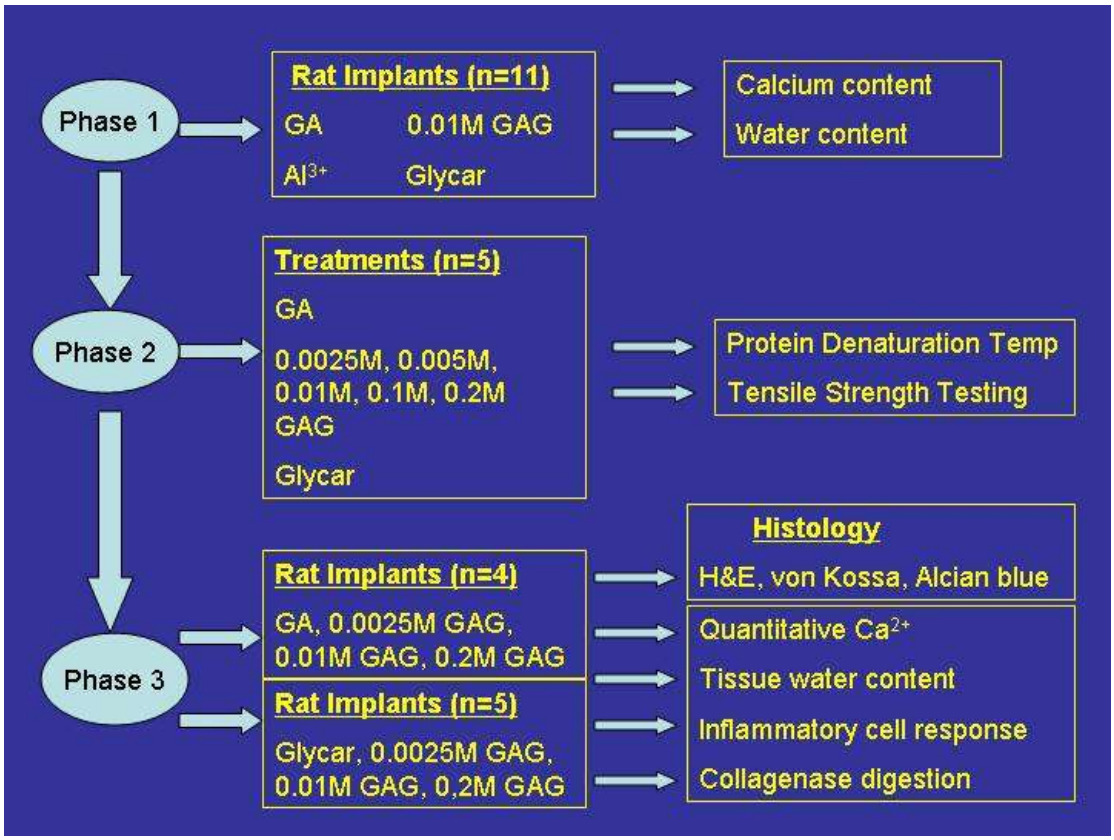


Figure 5 Diagrammatic illustration of the study layout

4.2.1 Study Layout – Phase 1

4.2.1.1 Tissue Treatment

Eleven bovine pericardial sacs were obtained from freshly slaughtered animals at the local abattoir and transported on ice to the laboratory. The pericardiums were manually cleaned of most fat and adventitial tissue while being washed in copious amounts of cold (4^{o}C) Plasmalyte B solution (Adcock Ingram, Johannesburg, South Africa). Within 4-6 hours after collection, 3 samples (3cm X 4cm) were cut from each of the pericardiums (n=33) and divided into four groups according to the following chemical treatments and cross-linking methods:

1) Fixation in 0.625% phosphate-buffered glutaraldehyde (Merck Chemicals, Johannesburg, South Africa), pH 7.4 for 24hrs, and stored in a similar solution (used as control).
2) Samples were treated with a 0.1M $AlCl_3$-solution (Merck Chemicals, Johannesburg, South Africa) at a pH of 3.0 in 0.625% glutaraldehyde for 4 hours while constantly being stirred. Thereafter tissue samples were stored in a 0.625% GA-solution (pH = 7.4) until implantation. Aluminium chloride was chosen because of its high water solubility, and its proven inhibitory effect on calcification of porcine valve leaflets in previous studies (Neethling, 1992).
3) Fixation in 0.01M sodium metaperiodate + 0.5% chondroitin sulphate (Merck Chemicals, Johannesburg, South Africa) in distilled water under constant shaking at 4^{o}C for 24 hours. After fixation the samples were washed thoroughly for 2 cycles of 30 minutes with 4^{o}C phosphate-buffered saline (PBS) (Highveld Biological, Johannesburg, South Africa), and stored in 0.625% GA until implantation or further examinations (Arenaz, 2004).
4) Pericardial tissue samples (n=11) from Glycar, South Africa were used in group four. Preparation involves the cleaning of

the pericardium immediately after harvesting, followed by initial fixation (tanning) in 0,625% GA for at least 72 hours, starting in the abattoir. This ensures the minimization of the ischemic period, and the GA are changed twice during this period. The pericardium to be used in the production of strips is selected and cut into the designated sizes and sterilized in formaldehyde for 48 hours. Excess unreacted aldehyde groups are removed from the solution by washing with saline, and the pericardium is then treated with a high concentration of a liquid polyol, namely propylene glycol, for 7-14 days at room temperature. This results in the “capping” of residual free aldehydes by forming a ring adduct of the aldehyde, and diols yielding 5-6-membered ring adducts seem more beneficial. The samples were finally stored in 2% propylene oxide in sterile water. Sterilization and packaging are performed under environmental control in class 100 Clean Room conditions (Frater, 1997).

4.2.1.2 Study Population (Phase 1)

In the first phase of the study, samples of the control GA, aluminium-treated, 0.01M GAG-treated and Glycar patches were implanted subcutaneously on the back of 11 (n=11) albino Wistar rats and retrieved after 8 weeks for further analysis.

Juvenile male Wistar albino rats with a mass of 100-150 grams, obtained from the Experimental Animal Unit, University of the Free State, Bloemfontein, were used for all subcutaneous implants. All animals were anaesthetised with Ketamine (45mg/kg s.c.) (Centaur Labs, Isando, South Africa) and Medetomidine (0.3mg/kg s.c.) (Pfizer Laboratories, Johannesburg, South Africa) for 45-60 minutes, shaved dorsally and a midline incision of ±3cm made through the skin.

One pre-cut pericardial sample (0.8cm X 1.5cm) from each of the chosen four treatments was rinsed for 15 minutes in sterile 0.9% saline (Adcock Ingram, Johannesburg, South Africa) before implantation. These tissues were inserted subcutaneously into separate pockets made on the back (2 on each side) of the animal and secured with two 6/0 Prolene sutures (Johnson & Johnson, Johannesburg, South Africa). The incision was closed with a continuous 5/0 PDS absorbable suture (Johnson & Johnson, Johannesburg, South Africa) and the anaesthetic reversed with Antisedan (0.2-0.4mg/kg s.c.) (Novartis, Kempton Park, South Africa).

Analgesic (Buprenorphine, 0.01-0.05 mg/kg s.c. 8-12 hourly / 4 days) (Schering-Plough, Isando, South Africa) was administered post-operatively in compliance with the National Code for the Handling of Animals for Research, Training, Diagnosis and Testing of Agents and Related Substances in South Africa, and as approved by the Ethics Committee of the UFS.

On completion of the 8 weeks implantation period, all the animals were sacrificed by means of an overdose CO_2-inhalation, and specimens retrieved for further analysis.

4.2.1.3 Subject Identification

Each rat received a subcutaneously implanted micro-chip with a specific identification number, for easy identification at the time of tissue retrieval.

4.2.1.4 Animal Medication

Drug / Compound	**Route / Dosage / Frequency**
Ketamine (anaesthetic)	45mg/kg sc
Medetomidine	0.3mg/kg sc
Antisedan(anaesthetic reverse)	0.2-0.4mg/kg sc
Buprenorphine (analgesic)	0.01-0.05mg/kg sc. 8-12 hourly

4.2.2 Study Layout - Phase 2

4.2.2.1 Tissue Treatments

Five bovine pericardial sacs were obtained from freshly slaughtered animals at the local abattoir and transported on ice to the laboratory. The pericardiums were manually cleaned of most fat and adventitial tissue while being washed in copious amounts of cold 4^{o}C Plasmalyte B solution. Within 4-6 hours after collection, 6 samples (3cm X 4cm) were cut from each of the pericardiums (n=30) and subjected to the following chemical treatments and cross-linking methods:

1) Fixation in 0.625% phosphate-buffered glutaraldehyde, pH 7.4 at 4^{o}C for 24hrs, and stored in a similar solution at 4^{o}C (used as control) (n=5, one from each sac).
2) Fixation in 0.0025M (n=5), 0.005M (n=5), 0.01M (n=5), 0.1M (n=5) and 0.2M (n=5) sodium metaperiodate + 0.5% chondroitin sulphate (Merck Chemicals, Johannesburg, South Africa) in distilled water under constant shaking at 4^{o}C for 24 hours. After fixation the samples were washed thoroughly for 2 cycles of 30 minutes with phosphate-buffered saline (PBS) at 4^{o}C , and finally stored in 0.625% GA at 4^{o}C until the protein denaturation temperature of each one was determined (n=25).
3) Five samples from individual Glycar patches were also taken and their thermal denaturation temperatures determined (n=5).

4.2.2.2 Protein Denaturation Temperature Determination

A sample from each of the five different pericardiums treated with the five different GAG concentrations (n=25) and the glutaraldehyde (control, n=5), as well as from the five Glycar patch samples (n=5) were used for the protein denaturation temperature determinations.

A small tissue sample was placed in a hermetically sealed pan of a differential scanning calorimeter (Mettler Toledo, DSC 822e, Microsep, Johannesburg, South Africa) and subjected to thermal analysis. The temperature was raised at a rate of 10^{o}C/min from 25^{o}C to 95^{o}C, and the temperature of thermal denaturation for each sample was electronically recorded as a peak maximum (Lovekamp & Vyavahare, 2001).

4.2.2.3 Tensile Strength Testing

Mechanical properties of tissue can be examined by a tensile strength testing machine, in which the tissue sample is fixed by clamps at both ends and gradually stretched (0.1mm/s) by applying constant tension on the two ends, and the data recorded on a personal computer (Thubrikar, 1983).

Six pericardial samples (9cm X 3cm) were cut from each pericardium treated with the different GAG concentrations (n=30), the GA-treated pericardium (control, n=6) and the Glycar patches (n=6), inserted between the clamps of the tensile strength tester (Lloyds LS100 Plus, IMP, Johannesburg, South Africa) and then stretched at a rate of 10mm/min until the breaking point was reached.

Figure 6 The Lloyds LS100 twin column tensile strength tester used for tensile strength testing of different pericardial samples

4.2.3 Study Layout - Phase 3

4.2.3.1 Study Population

In the third phase of the study, samples of pericardial patches treated with the highest (0.2M), lowest (0.0025M) and optimal (0.01M) concentrations of GAGs together with a control patch (GA) were implanted subcutaneously into four rats (n=4) and retrieved after 8 weeks for further analysis.

Samples of pericardial patches treated with the highest (0.2M), lowest (0.0025M) and optimal (0.01M) concentrations of GAGs, together with a sample from the commercial Glycar patch, were also implanted in the

same way into another 5 rats (n=5) and retrieved after 8 weeks for further analysis.

4.2.3.2 Subject Identification

Each rat received a subcutaneously implanted micro-chip with a specific identification number, for easy identification at the time of tissue retrieval.

4.3 SAFETY VARIABLES

The research project was evaluated by a panel consisting of researchers and clinicians from different disciplines. It was confirmed that performing the surgical procedures and chemical treatments of the tissues will hold no safety consequences for researchers or animals. All the surgical procedures on the rats were performed by a trained medical scientist, and overseen by the animal laboratory personnel and a private veterinarian. The study would have been discontinued prematurely if the researcher or any of the study supervisors felt that any unethical events have occurred.

4.4 SPECIAL INVESTIGATIONS:

4.4.1 Histological Procedures

4.4.1.1 Hematoxylin and Eosin Staining

All the retrieved tissue samples were embedded in paraffin wax (Siemens, Johannesburg, South Africa) before sectioning. Slides were deparaffinated by dipping it 6-8 times in two changes of xylene (Labotec, Johannesburg, South Africa), two changes of absolute alcohol (Merck, Johannesburg, South Africa) and one change each of 96% and 70% alcohol. Sections were rehydrated by 6-8 dips in distilled

water, slightly overstained with Mayer's hematoxylin (Merck, Johannesburg, South Africa) for 10 minutes, and the excess stain removed by 6-8 dips in running tap water. Sections were blued in Scott's tap water (Merck, Johannesburg, South Africa) and dipped in running tap water and for a few seconds (6-8 dips) in 70% alcohol. Sections were stained in 0.2% alcoholic eosin (Merck, Johannesburg, South Africa) solution for 1 minute, dehydrated by 6-8 dips in first 96% alcohol, then two changes of 100% alcohol and finally two changes of xylene. A drop of Entellan (Merck, Johannesburg, South Africa) was placed on the slide with a glass rod, a coverslip gently placed on top and left to dry. The cell nuclei stained blue and the cell cytoplasm and most connective tissue fibers stained in shades of pink, orange and red (Bancroft & Stevens, 1990).

4.4.1.2 von Kossa Staining

Pericardial samples retrieved after implantation were set in standard paraffin wax blocks, and sectioned. Sections were dewaxed by two changes each in xylene and absolute alcohol, rinsed in first 96% alcohol and then 70% alcohol and then rehydrated by rinsing them several times in distilled water. Sections were placed in a 1.5% silver nitrate solution (Merck, Johannesburg, South Africa) under strong light for 30 minutes, before being washed in at least three changes of distilled water. The stain was reduced with 0.5% hydroquinone (Merck, Johannesburg, South Africa) for 5 minutes and again well rinsed in distilled water, treated with 3% sodium thiosulphate (Merck, Johannesburg, South Africa) for 5 minutes and washed in running tap water. Sections were counterstained with 1% nuclear fast red (Merck, Johannesburg, South Africa) for 5 minutes and rinsed in distilled water, dehydrated by 6-8 dips in first 96% alcohol and then two changes of absolute alcohol, cleared in 2 changes of xylene and mounted with Entellan. The calcium deposits in the tissue stained black and nuclei stained red (Bancroft & Stevens, 1990).

4.4.1.3 Alcian Blue Staining

Pericardial samples retrieved after implantation were set in standard paraffin wax blocks, and sectioned. Sections were dewaxed with xylene and ethanol and hydrated in running tap water. Sections were stained in a solution of 1% alcian blue 8GX (Merck, Johannesburg, South Africa) in 3% acetic acid (Merck, Johannesburg, South Africa) at pH 2.5 for 30 minutes and well rinsed in distilled water. Sections were then counterstained in 0.5% nuclear fast red for 5 minutes and again rinsed in distilled water. Finally, sections were dehydrated with graded concentrations (96%, 100%, 100%) of alcohol, cleared in two changes of xylene and mounted with Entellan. Carboxylated (COOH) acid mucopolysaccharides stained strongly blue and sulphated (SO_3H) acid mucopolysaccharides stained weakly blue (Bancroft & Stevens, 1990).

4.4.1.4 Gomori Trichrome Staining

Explanted tissue samples (n=36) from the rats were stained with Gomori trichrome and assessed by light microscopy for evidence of possible infiltration of host immune cells, which served as an indicator of the host immune response towards the implanted tissue. Explanted tissue samples were embedded in paraffin blocks and sectioned. Sections were dewaxed by two changes each in xylene and absolute alcohol and rehydrated with distilled water. Sections were stained with Mayer's hematoxylin for 5 minutes and rinsed in running tap water. Differentiation of the nuclei staining was achieved in 1% acid alcohol for 10 seconds, followed by rinsing in running tap water. Sections were allowed to turn blue in Scott's tap water for 30 seconds, rinsed again in running tap water and stained in the Trichrome solution for 10 minutes. This solution consists of: chromotrope 2R (0.6g), light green (SF yellow) (0.3g), phosphotungstic acid (0.6g), glacial acetic acid (1ml) (Merck, Johannesburg, South Africa) and distilled water (100ml), with a pH of 3.4. The stain was quickly rinsed off with running tap water, the sections dehydrated with graded alcohol solutions, cleared in two

changes of xylene and mounted with Entellan. The nuclei stained blue, the cytoplasm and muscle fibers red and the collagen green (Bancroft & Stevens, 1990).

4.4.2 Quantitative Calcium Analysis

The quantitative calcium analyses was performed by the Eco-Analytica Laboratory, School for Environmental Sciences & Development, Northwest University, Potchefstroom, South Africa.

Explanted samples were dried in a temperature controlled incubator (Scientific, Series 100, Lasec, Johannesburg, South Africa) at 45^{o}C for 48 hours, weighed, hydrolyzed in 1ml 50% nitric acid (Protea Laboratories, Johannesburg, South Africa) + 50µl hydrogen peroxide (H_2O_2) (Diagnostic Media Products, Sandringham, South Africa) / dry sample at 90^{o}C for 30-40 minutes, and the extractable calcium content was determined by atomic absorption spectrophotometry (Agilent ICP-MS 7500c, Chemetrix, Midrand, South Africa) and expressed as µg calcium per mg tissue (dry weight).

4.4.3 Collagenase Digestion

Samples of all the different GAG treated (n=30) and untreated (control, n=6) pericardial patches were dried in a temperature controlled incubator at 70^{o}C for 24 hours and weighed. Collagenase enzyme (Sigma-Aldrich, Johannesburg, South Africa) was suspended at a concentration of 440U/ml in a solution of 50mM Tris-HCl buffer (Sigma-Aldrich, Johannesburg, South Africa) and 0.36mM $CaCl_2$ at a pH of 7.4. Approximately 1.2 ml of this solution was added to each of the dried tissue samples and allowed to react for 24 hours at 37^{o}C under constant shaking. After this period the samples were centrifuged for 5 minutes at 4000 rpm and most of the liquid was discarded. Insoluble residues of tissue were again dried completely and weighed. Dry weights of the undigested samples were compared with those obtained before the enzymatic digestion, and the tissue loss was calculated and expressed as a percentage of the dry weight.

4.4.4 Tissue Water Content

The water content of the different treated pericardial samples (5 x [GAG] + GA + Glycar) after implantation into the rats (n=36) was also determined and compared. Explanted tissue samples were cleaned of excess host tissue, and weighed on a digital balance (Mettler AE 100, Protea Laboratories, Sandton, South Africa). Samples were then dried in a temperature controlled incubator at 45^{o}C for 48 hours, again weighed and the water content calculated as a percentage of the wet weight.

4.4.5 Statistical Analysis

Histological findings were categorised and summarised according to frequencies and percentages. Numeric data was expressed as means and standard deviations or percentiles, depending on the distribution of the data. Comparisons were done by paired t-tests or signed rank tests (numerical variables), or McNemer tests (categorical variables) in the case of paired data. For unpaired data t-tests, Mann-Whitney tests or chi-squered tests were used. Confidence intervals were calculated for differences in means, medians or percentages. Statistical analysis was performed by the Department of Biostatistics, University of the Free State, Bloemfontein.

4.5 ETHICAL ASPECTS AND GOOD CLINICAL PRACTICE

4.5.1 Ethical Clearance

The study protocol was submitted to the Ethics Committee of the University of the Free State dealing with animal research in order to gain ethical approval before the first phase of the study could commence. Approval to continue with the animal studies was provided under Protocol number:

Animal project number 17/05

4.5.2 Good Clinical Practice (GCP) / Quality Assurance

All clinical work conducted under this protocol was subjected to the GCP guidelines (The Principles of the Declaration of Helsinki, GCP, 2004).

The Helsinki declaration's basic principle number 3 states that research should be conducted only by scientifically qualified people and under the supervision of adequately qualified people (World Medical Association, 2002). Therefore, the entire research project was compiled and supervised by internationally recognized researchers in their respective fields of expertise.

CHAPTER 5

RESULTS

5.1 INTRODUCTION

All numerical results are given in tables, while histological results are demonstrated by representative images. Results of phase 1 of the study are presented first, followed by the results of the parameters used to determine the optimal GAG concentration (phase 2). Finally the results of the individual parameters used to determine the cross-linking stability and calcification potential of each treatment modality on the tissue (phase 3) are shown and discussed.

5.2 RESULTS OF PHASE 1

5.2.1 Extractable Calcium and Water Content

Table 5.2.1 indicates the extractable calcium content and water content of pericardial samples treated by four different methods and implanted subcutaneously into male Wistar rats for eight weeks. These results were required in order to determine the feasibility of further investigating GAGs as an alternative fixative to glutaraldehyde.

Table 5.2.1 The extractable calcium and water content of the different groups of pericardial samples (n=11) after 8 weeks in the subcutaneous rat model (Phase 1)

	% Water			μg Ca^{2+}/mg DM		
TREATMENT	**Mean**	**Std Dev**	**Median**	**Mean**	**Std Dev**	**Median**
0.625% GA	35.64	5.49	36.01	178.93	14.97	179.48
Aluminium	46.46	6.37	46.50	143.50	22.96	140.99
0.01M GAG	75.61	1.56	75.90*	9.04	2.08	9.11**
Glycar	60.01	8.44	64.79*	34.35	51.82	0.93**

* Significantly higher ($p<0.0001$) than GA and aluminium

** Significantly lower ($p<0.05$) than GA and aluminium

No significant difference ($p>0.05$) existed between the extractable calcium content (median values) of GA (control) and aluminium-treated or between GAG and Glycar-treated pericardial tissues after explantation. The extractable calcium content (median values) for the GAG and Glycar treatments were however significantly lower ($p<0.05$) compared to the GA (control) and aluminium treatments.

The extractable water content (median values) showed a similar pattern, with GAG-treated and Glycar patches significantly ($p<0.0001$) higher compared to the GA (control) and aluminium treatments.

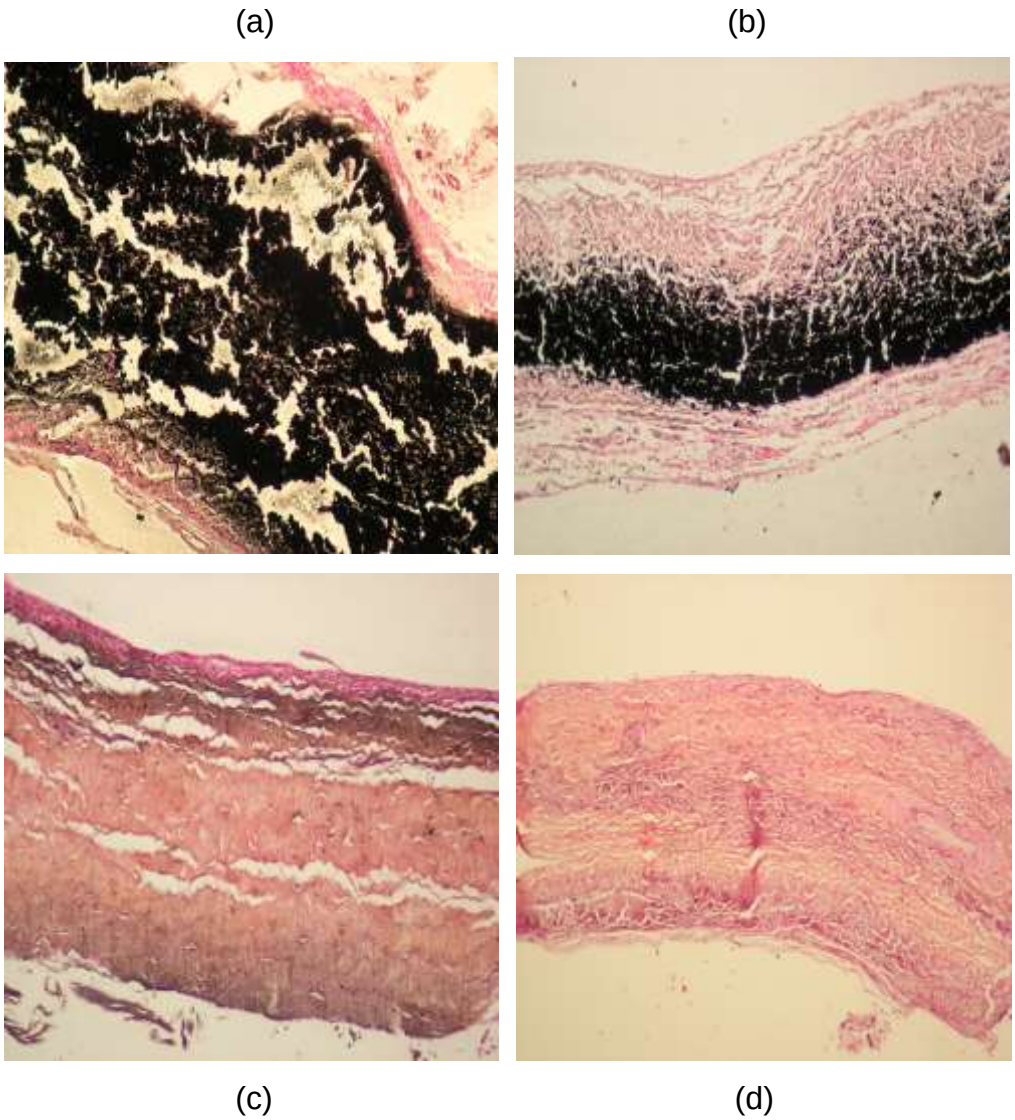


Figure 7 Histological comparison of light microscopy images of the degree of calcification of the explanted pericardial samples treated with (a) GA, (b) aluminium, (c) GAG and (d) Glycar method. (von Kossa, magnification (a)x40, (b,c,d)x30**)**

5.3 RESULTS OF PHASE 2

5.3.1 Thermal Denaturation Temperature

In order to determine the optimal GAGs concentration to be used for cross-linking of bovine pericardial tissue without sacrificing the stability of the triple helix, samples from the same donor tissue were treated with different concentrations of GAGs as previously described. Samples were then heated at a controlled rate in a differential scanning calorimeter (Mettler Toledo, DSC 822e, Microsep, Johannesburg, South Africa) and the peak temperature at which denaturation of the triple helix occurred, was recorded.

Table 5.3.1 Thermal denaturation temperatures (ºC) for differently treated samples from five pericardial sacs and the commercial tissue

	Treatments						
	GA	**0.0025M GAG**	**0.005M GAG**	**0.01M GAG**	**0.1M GAG**	**0.2M GAG**	**Glycar**
Patch 1	86.00	82.50	82.40	82.60	80.00	79.20	87.89
Patch 2	85.64	82.76	82.70	81.85	80.14	79.16	88.66
Patch 3	84.41	81.93	81.84	80.91	78.64	77.24	88.91
Patch 4	85.36	82.88	82.76	82.11	79.51	78.54	87.17
Patch 5	83.39	81.44	80.86	79.70	77.85	76.93	87.15
Mean	**84.96**	**82.30**	**82.11**	**81.43**	**79.23**	**78.21**	**87.90**
Std Dev	1.06	0.61	0.79	1.15	0.97	1.07	0.82
Median	85.36	82.50*	82.40*	81.85*	79.51*	78.54*	87.89**

* Significantly lower ($p<0.0005$) than GA and Glycar
** Significantly higher ($p=0.001$) than GA

Table 5.3.1 shows the peak temperatures at which unfolding of the triple helix protein molecules occurred. There was no statistically significant ($p>0.05$) difference in denaturation temperature between tissues treated with 0.0025M and 0.005M GAGs, but tissues treated with 0.01M, 0.1M and 0.2M GAGs showed a significant ($p<0.05$) decrease in denaturation temperature compared to the 0.0025M and 0.005M GAG-treated tissue.

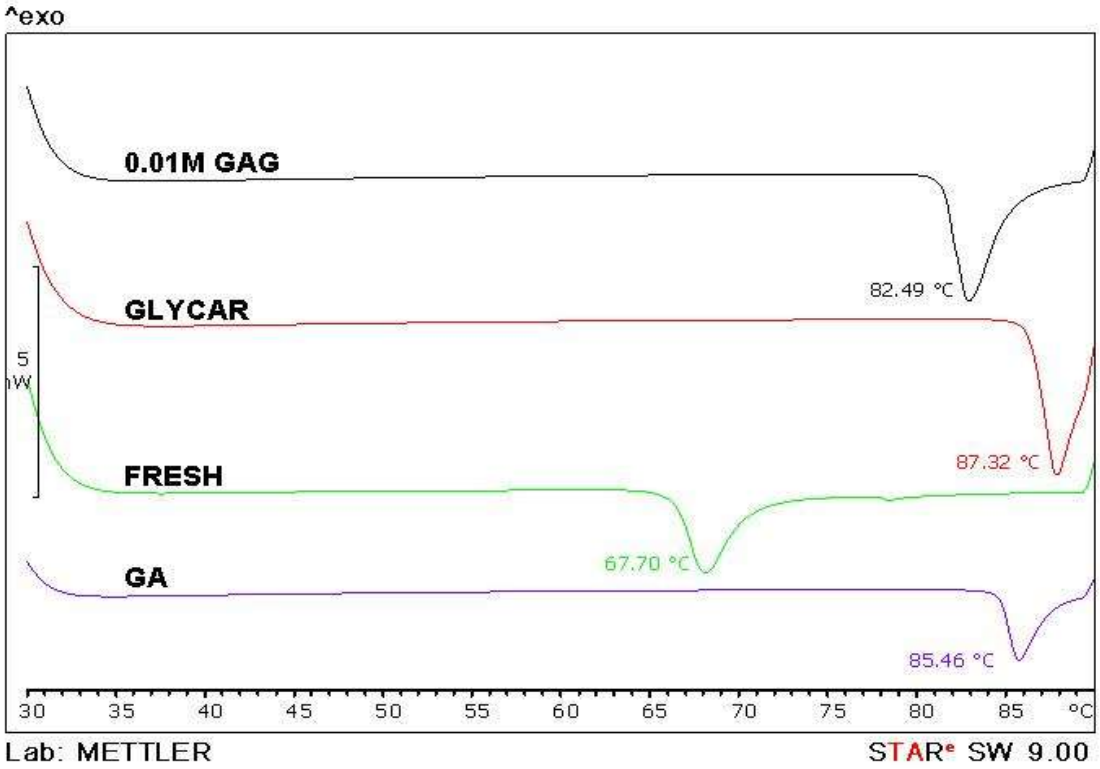


Figure 8 Representative diagram of the cyclic warming data for fresh, GA, 0.01M GAG and Glycar-treated patches as recorded by the differential scanning calorimeter, with the point of protein denaturation clearly indicated

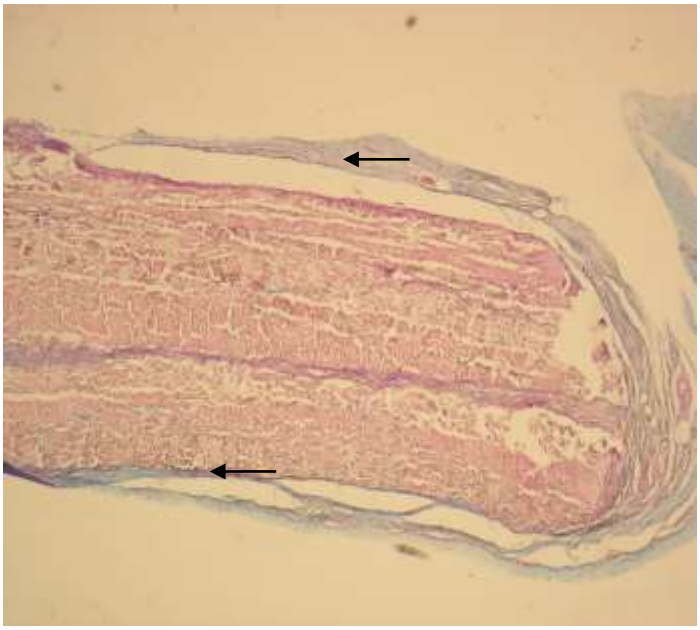


Figure 9 Light microscopy image of a 0.01M GAG-treated implant, demonstrating the superficial GAG (light blue), bound to the outer surface of the pericardium. (Alcian Blue, magnification x4**)**

GA-fixed and Glycar-treated tissue showed a statistically significant ($p \leq 0.0005$) increase in denaturation temperature compared to all the GAG-treated tissues. The denaturation temperature of Glycar patches was significantly ($p=0.001$) higher compared to GA-fixed tissue, while tissues treated with higher GAGs concentrations (0.1M and 0.2M) resulted in a denaturation temperature below the benchmark minimum of 80^{o}C (Lovekamp & Vyavahare, 2001).

Paired t-tests were used to determine confidence intervals between GA and the different GAGs treatments (median values), while Mann-Whitney tests for unpaired data were used when results of Glycar-treated tissue were compared with GA and the GAG treatments (median values).

5.3.2 Tensile Strength

Table 5.3.2 shows the tensile strength at the breaking point of different pericardial tissues treated with (a) GA (control), (b) different concentrations of GAGs and (c) the Glycar method. These results were also used to determine the effect of the use of different concentrations of GAGs on the tensile strength of the tissue.

Table 5.3.2 The tensile strength (MPa) of differently treated pericardial strips from six pericardial sacs and the Glycar patch

	TREATMENT						
SAMPLE	0.625% Glut	0.0025M GAG	0.005M GAG	0.01M GAG	0.1M GAG	0.2M GAG	Glycar
Sample 1	6.40	13.78	9.87	7.91	6.32	7.73	14.73
Sample 2	9.30	10.17	12.85	18.97	6.79	12.20	15.55
Sample 3	7.10	8.94	15.47	13.42	13.09	16.57	15.60
Sample 4	13.57	12.44	18.63	16.26	18.14	20.87	16.52
Sample 5	13.20	16.63	15.66	22.82	6.05	6.69	14.85
Sample 6	10.53	13.98	15.54	13.48	11.07	10.02	15.89
Mean	10.02	12.66	14.67	15.48	10.24	12.35	15.52
Std Dev	3.00	2.78	2.98	5.14	4.82	5.46	0.67
Median	9.92	13.11	15.51*	14.87*	8.93	11.11	15.58*

* Significantly higher ($p<0.05$) than GA

The tensile strength of control (GA) tissue was significantly ($p<0.05$) lower when compared to 0.005M GAG, 0.01M GAG-treated and Glycar tissue, but no significant ($p>0.05$) difference existed between GA and the remaining GAG treatments.

Tissue treated with the lowest (0.0025M) and two highest (0.1M & 0.2M) GAG concentrations appears to have a lower tensile strength compared to 0.005M and 0.01M GAG-treated tissues, but only 0.1M GAG-treated tissue showed a significant ($p<0.05$) decrease in tensile strength when compared to 0.005M GAG-treated tissue.

Glycar-treated tissue demonstrated a significantly ($p=0.0039$) higher tensile strength when compared to GA-fixed control tissue (median values), but no

significant difference (p>0.05) in tensile strength existed between Glycar tissue and any of the GAG treatments (median values).
Signed rank tests were used to determine confidence intervals between paired data (GA and GAG), and Mann-Whitney tests for comparison between unpaired data.

5.4 RESULTS OF PHASE 3

5.4.1 Extractable Calcium and Water Content

Table 5.4.1.1 The calcium and water content of the different pericardial samples after 8 weeks in the subcutaneous rat model

	% Water			μg Ca^{2+}/mg DM		
TREATMENT	**Mean**	**Std Dev**	**Median**	**Mean**	**Std Dev**	**Median**
0.625% GA	35.64	5.49	36.01	107.63	22.66	101.05
0.0025M GAG	74.75	3.92	75.71*	53.87	40.02	52.44**
0.01M GAG	73.14	3.20	75.71*	59.40	44.17	71.25**
0.2M GAG	76.94	5.00	77.12*	25.02	35.50	4.00**
Glycar	76.60	1.16	76.79*	1.56	1.23	0.89**

* Significantly (p<0.0001) higher than GA

**Significantly (p<0.0001) lower than GA

Table 5.4.1.1 shows the calcium and water content for control samples compared to low, high and optimal concentration GAG-treated samples and commercial tissue after 8 weeks in the subcutaneous rat model. All treatments showed a significantly (p<0.0001) higher water content compared to control (GA) tissue. There was no significant difference (p>0.2) between the water content of all the GAG-treated tissues, while the Glycar patch only showed a significantly (p<0.05) increased water content when compared to the 0.01M GAG-treated tissue.

A statistically significant (p<0.0001) decrease in the extractable calcium content of all the GAG-treated and the Glycar-treated patches compared to the control (GA) tissue was shown. When tissue treated with the different

GAGs concentrations were compared with each other as well as with the Glycar-treated tissue, no significant difference ($p>0.06$) was found. Because of the unpaired data, a Mann-Whitney test was used to determine confidence intervals.

Table 5.4.1.2 Frequency of the degree of calcification of the different pericardial samples on histological appearance following von Kossa staining

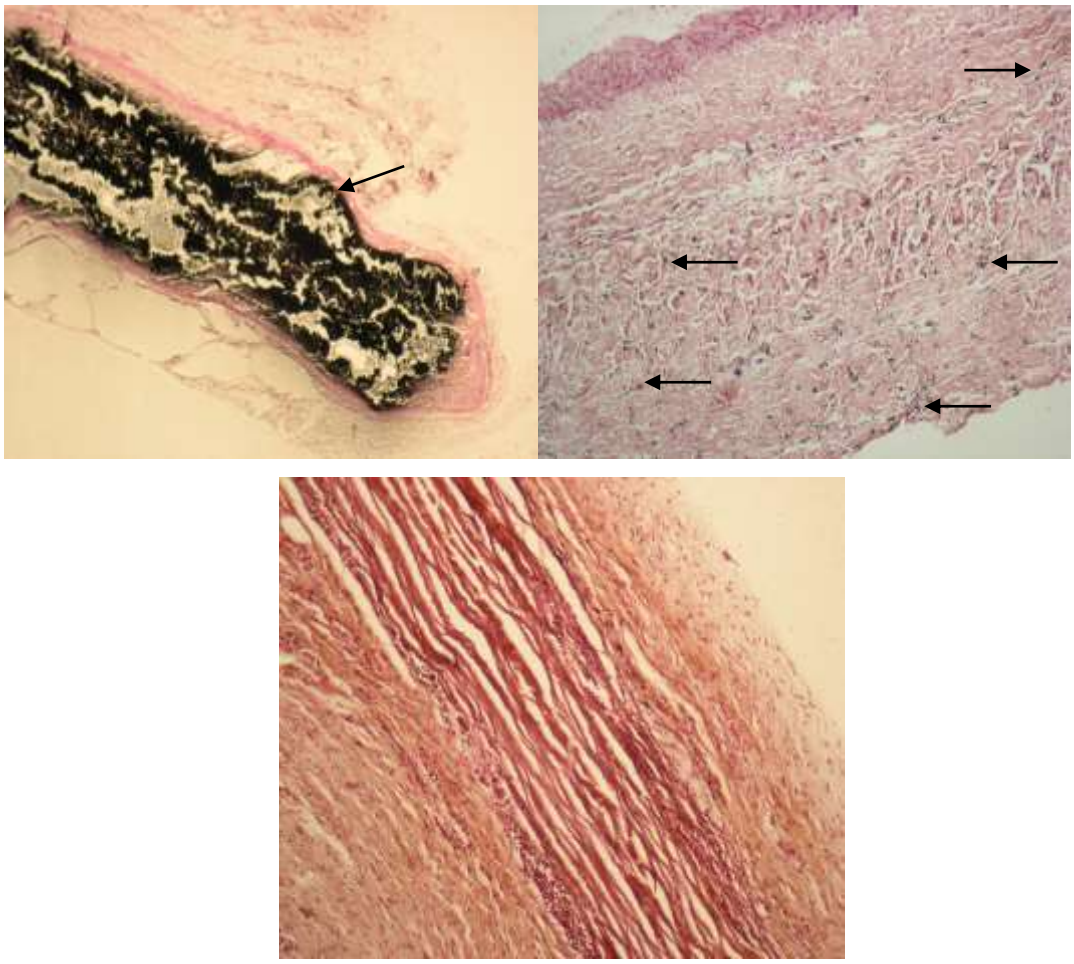
Treatment	**Degree of Calcification**				
	-	**+-**	**+**	**++**	**+++**
0.625% GA	0	0	0	0	4
0.0025M GAG	0	2	1	4	2
0.01M GAG	1	3	0	1	4
0.2M GAG	1	5	2	0	1*
Glycar	5**	0**	0**	0**	0**

* Significantly lower ($p<0.05$) than GA

**Significantly lower ($p<0.02$) than all the other treatments

Table 5.4.1.2 shows the frequency of the degree of calcification of the different pericardial samples after 8 weeks implantation in the subcutaneous rat model, as interpreted on histological appearance with von Kossa staining. Glycar-treated tissue calcified significantly ($p<0.02$) less than all the other tissues, while 0.2M GAG showed significantly ($p<0.05$) less calcification than the control tissue. No significant ($p>0.05$) difference in calcification on histological appearance could be shown between any of the other tissue treatments. Because of the unpaired data, a Fisher's exact test was used to determine the confidence intervals.

(a) (b)



(c)

Figure 10 Histological comparison of light microscopy images of explanted pericardial samples treated with (a) 0.625% GA, (b) 0.01M GAG and (c) Glycar method. (von Kossa, magnification x10**)**

5.4.2 Presence of GAG Post-implant

Table 5.4.2 Frequency of the degree of GAG remaining in the different tissues post-implant, on histological comparison (Alcian blue)

Treatment	Degree of remaining GAG				
	-	+-	+	++	+++
0.625% GA	2	2	0	0	0
0.0025M GAG	3	4	2	0	0
0.01M GAG	4	3	2	0	0
0.2M GAG	6	1	2	0	0
Glycar	1	3	1	0	0

Table 5.4.2 shows the frequency of the degree of GAGs that remained in the different tissues following 8 weeks implantation in the rat subcutaneous model. No significant difference ($p>0.1$) in the presence of glycosaminoglycans was seen between any of the different pericardial samples.

5.4.3 Host Inflammatory Response

A mild to moderate presence of host lymphocytes was seen in the superficial layers of all explants, with mild infiltration into deeper layers only observed in some explants. One sample showed an acute inflammatory response due to infection after partial excavation of the patch by the recipient animal.

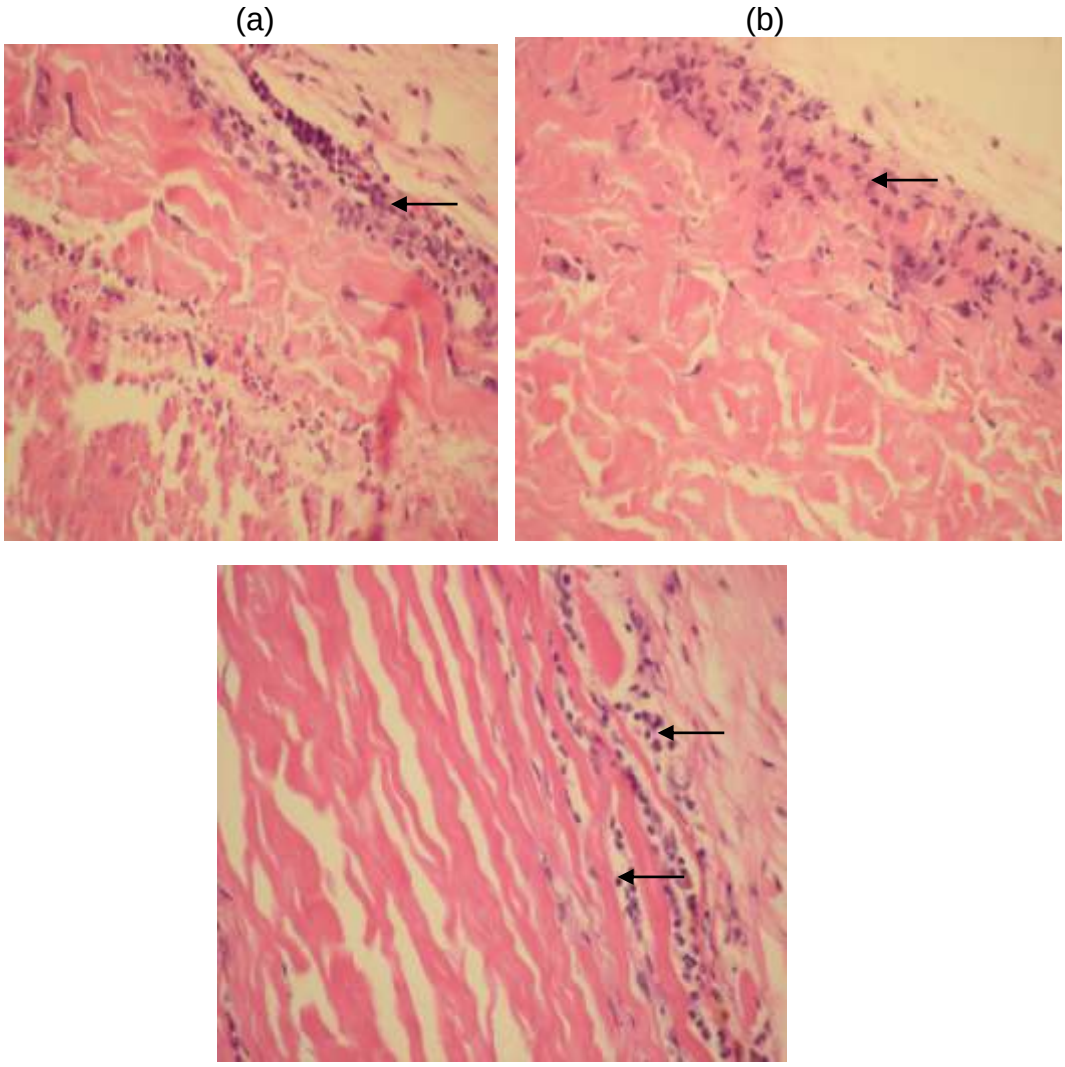


Figure 11 Light microscopy images (a=GA; b=GAG; c=Glycar), demonstrating the presence of host lymphocytes on the surface of all the implants after 8 weeks in the subcutaneous rat model (H/E stain, magnification x40**)**

5.4.4 Enzymatic Resistance

The extent of cross-linking of biological tissues is demonstrated by its resistance to enzymatic digestion. Table 5.4.4 shows the resistance of different pericardial samples, expressed as a percentage of tissue dry weight after digestion with collagenase at 37^{o}C for 24 hours.

Table 5.4.4 Enzymatic degradation of different pericardial tissues, expressed as a percentage of tissue dry weight before digestion by collagenase

	Treatments						
	0.625% Glut	**0.0025M GAG**	**0.005M GAG**	**0.01M GAG**	**0.1M GAG**	**0.2M GAG**	**Glycar**
Sample 1	2.17	1.68	2.48	1.63	1.06	1.02	0.33
Sample 2	2.12	1.89	1.86	2.08	0.67	3.47	0.33
Sample 3	2	2.24	2.13	2.16	0.67	2	0
Sample 4	2.07	1.92	2.07	0.69	2.86	3.03	0.93
Sample 5	0.4	1.77	2.31	0.7	0.8	3.72	0
Mean	1.75	1.9	2.17	1.45	1.21	2.65	0.32*
Std Dev	0.76	0.21	0.24	0.72	0.93	1.12	0.38
Median	2.07	1.89	2.13	1.63	0.8	3.03	0.33*

* Significantly lower ($p<0.05$) than all the other treatments

No significant difference ($p>0.1$) in tissue loss (mean values) was found between the different treatment groups, except for the Glycar tissue, which had significantly ($p<0.05$) less collagen digested when compared to all the other treated tissues. Signed rank tests were used to determine confidence intervals between paired data (GA and GAG), and Mann-Whitney tests for comparison between unpaired data.

CHAPTER 6

DISCUSSION

6.1 INTRODUCTION

Glutaraldehyde has been widely used as cross-linking agent for biological tissues for many years, because of some unique cross-linking and sterilizing properties. It produces materials with the highest degree of cross-linking, excellent haemodynamic performance, increased mechanical strength and a low antigenicity. In spite of these advantages, the durability of GA-treated biological tissues turned out to be suboptimal. Calcification and tissue degradation are major problems, and the detrimental role of GA in the fixation process has largely been blamed for this unsatisfactory outcome.

Many alternative cross-linking methods and chemical compounds have been investigated, each showing different degrees of success regarding calcification reduction, maintaining tissue integrity and mechanical durability. In this study the optimal concentration to be used in pre-treating pericardial tissue with glycosaminoglycans was determined, and its effect on protein stabilization, mechanical strength and calcification potential was compared with glutaraldehyde-fixed tissue.

6.2 DISCUSSION OF DATA FROM PHASE 1

6.2.1 Extractable Water Content

The significantly higher water content of GAG-treated and Glycar-treated patches compared to GA- and Aluminium-GA-treated tissue (Table 5.2.1) can be contributed to the hydrophilic properties present of the former two treatments. The functional carboxyl and sulphonate groups present in the structure of chondroitin sulphate, the GAG used during pretreatment of the pericardium, are highly negatively charged units, which results in a significant

water-binding capacity. An important mechanical function of proteoglycans involves the hydration of the extracellular matrix in valve leaflets while their GAG side chains contribute to a large extent to the tissue hydration and elasticity.

Polyols like the diols propylene glycol ($H(CHOH)_2CH_3$), 1,3-propanediol ($(CH_2OH)_2CH_2$) and 2,3-butylene glycol ($CH_3(CHOH)_2CH_3$), and the triol glycerol ($(CH_2OH)_2CHOH$), which are liquids at room temperature, are preferred for the post-tanning treatment of the pericardial tissue. They show good chemical reactivity and tend to keep the tissue more hydrated and supple when compared to diols that are solid at room temperature. Concentrated polyols (solvent-free) are also preferred, as the presence of solvents may introduce additional functional groups to the solution. These may react with the amino groups of the collagenous tissue, which might hinder the formation of cross-links by GA in the tissue, since the aldehyde tanning mechanisms are reversible (U.S. Patent No. 5476516, 1995).

Proteoglycans and GAGs are important role-players in many vital functions within the body, and are of particular importance in joint lubrication and as shock absorbers in areas where a lot of tissue movement occurs. This will also be true for heart valve tissue, where repeated opening and closing of the leaflets under physiological blood pressure will exert constant strain on the leaflet tissue. Their importance in this regard has been highlighted by the decrease in GAG levels of GA-fixed porcine aortic cusps from bioprosthetic heart valves that were retrieved following clinical implantation. A similar reduction in GAG concentrations and a resultant loss in shock absorbing abilities have also been shown in the leaflets of rheumatic and aged valves, making them highly prone to failure (Lovekamp & Vyavahare, 2001).

6.2.2 Extractable Calcium Content

Bovine pericardial tissue fixed with glutaraldehyde (control) as well as with an aluminium-GA combination showed severe calcification following 8 weeks subdermal implantation in rats (Fig. 7). Aldehyde groups in aqueous GA-

containing reaction solutions react with the amine groups of lysine or hydroxylysine residues of the collagen to form additional cross-links between the fibers. Free aldehyde groups that are retained in the GA-tanned tissue following the reaction of both amino groups with the same aldehyde group of the GA during the cross-linking process, do however occur in equilibrium with the two aldehyde groups of the GA which has reacted with different amino groups (U.S. Patent No. 5476516, 1995). These free aldehyde groups appear to be the prime instigation of calcification, acting as nucleation sites for the formation of calcium hydroxyapatite crystals.

The binding of aluminium ions (Al^{3+}) to membrane-associated phosphate to occupy potential calcium binding sites and thus prevent the onset of calcification of the pericardial tissue, still resulted in severe calcification of the pericardial tissue. This can best be explained by the known reversible binding of aluminium to collagen, as opposed to the permanent alteration of structure and inhibition of calcification when bound to elastin in aortic wall tissue (Levy, 2003). Dissociation of the aluminium from the collagen could cause the formation of aluminium precipitates like carbonates and phosphates, which could serve as nucleation sites for deposits of calcium phosphate and ultimately calcification of the tissue. Because of the demonstrated severe calcification of the Al-GA-treated tissue, a decision was taken to discontinue the further use of aluminium as a pretreatment chemical in the processing of bovine pericardial tissue.

Both the GAG-treated and commercial Glycar patches performed significantly better than the previous two treatments (Table 5.2.1). The binding of GAGs like heparin to collagenous tissues like pericardium, might result in the GAGs filling the intertropocollagen spaces. This would block the potential calcium binding sites and make the tissue impermeable to host plasma calcium (Chanda, 1997). The histological evidence (Fig. 9) that GAGs only bound to the outer surface and did not penetrate into the deeper layers of the pericardial tissue, supports this proposal. Calcification of the implants was not completely mitigated by the GAG treatment, but significantly reduced over the period of the study, probably as a result of limited influx of host calcium.

6.3 DISCUSSION OF DATA FROM PHASE 2

6.3.1 Thermal Denaturation Temperature

Cross-linking of collagenous tissues increases the stability of the triple helix by forming additional chemical bonds between the molecules, thus increasing the denaturation temperature of the material. GA is recognized as the chemical that currently gives the highest degree of stable cross-linking to biological tissue, and this is reflected in the denaturation temperature results (Fig. 8), which is much higher than for fresh pericardium (66±2^{o}C).

Treatment of pericardium with five different GAGs concentrations before final fixation with GA, showed a significant decrease in thermal denaturation temperatures compared to GA-fixed and Glycar-treated tissues (Table 5.3.1). This indicated a very high degree of cross-linking of collagen achieved by the GA and Glycar treatments. Histology of GAG-treated pericardium (Fig. 9) before implantation demonstrated that GAGs were only bound superficially to the outer surface of the tissue, and no deep penetration took place. Treatment with lower GAG concentrations (0.0025M & 0.005M) resulted in a limited amount of GAG-collagen cross-links being formed. This allowed opportunity for GA to form the majority of cross-links during the final fixation treatment, resulting in the higher denaturation temperatures. Tissues treated with high (0.1M & 0.2M) concentrations of GAGs yielded denaturation temperatures below the accepted benchmark of 80^{o}C, indicating limited cross-links formed by GA fixation following GAG fixation. This corresponds to a previous study which suggested that high concentrations of GA promote rapid cross-linking of the tissue surface during fixation, generating a barrier that impedes or prevents further diffusion of GA into the tissue bulk (Cheung*, 1985)*.

Treatment with 0.01M GAG revealed a significantly lower denaturation temperature than tissues treated with 0.0025M and 0.005M GAG, but it was still above the acceptable minimum of 80^{o}C. This was indicative of an adequate degree of stable cross-linking, and therefore the 0.01M GAG treatment was identified as the optimal concentration for further use.

The thickness of the layer of GAGs bound to the outer surface of the pericardium for different GAG concentrations was not investigated. A difference in tissue thickness might affect the rate of GA penetration and cross-linking formation during the final fixation phase, which would ultimately result in different thermal denaturation temperatures. Fisher and colleagues did however demonstrate that the penetration of GA into bovine pericardium and the resultant cross-linking was uniform, even after only 2 hours of fixation (Fisher, 1987).

Glycar-treated tissue showed superior resistance to thermal denaturation, indicating that the cross-links introduced by tanning with GA were adequately maintained. Limiting the ischemic time between harvesting of the tissue to initial fixation in 0,625% GA to introduce the collagen cross-links will be crucial in order to minimize the degradation of the tissue before fixation.

The presence of solvents in polyol solutions will introduce additional functional groups, which may react with the amino groups of the collagen. This will reduce the formation of cross-links by the aldehyde groups of GA. Using concentrated propylene glycol and other polyols instead of solvent-containing solutions to ‘cap’ the free aldehyde groups in the tissue will also minimize the possibility of impeding in the development of cross-links in the GA-tanned tissue, which might reduce the tissue strength.

6.3.2 Tensile Strength

Contrary to the high thermal denaturation temperatures achieved with GA fixation and Glycar-treated tissues compared to GAG treatments, the tensile strength of GA-treated pericardium was significantly ($p=0.03$) lower than that of tissue treated with 0.005M and 0.01M GAGs and Glycar-treated tissue (Table 5.3.2). Cross-linking of fresh bovine pericardium with GA resulted in an increase in tensile strength due to the increase in the stiffness (reduction in stress relaxation) of the tissue as a result of added interfibrillar cross-links, as well as an increase in the elongation of the tissue at break (Lee, 1994). The substantial increase in extractable water content of the GAG-treated and

Glycar tissue compared to the GA-fixed tissue also had a positive effect on the mechanical properties of the tissue by maintaining good elasticity and suppleness. The treatment of Glycar patches with concentrated propylene glycol following GA-fixation, ensured that the tissue maintained a high water content and remained supple, compared to GA-fixed tissue which was much dryer and more rigid.

This, together with the adequate cross-linking of the pericardium by GA during processing as proven by DSC, resulted in the increased tensile strength of tissue treated with the two GAG concentrations and the Glycar patches. This is supported by Maestro and colleagues, arguing that the higher water content may facilitate the reorganization of collagen fibers within the tissue when tensile forces are exerted, improving the visco-elastic properties of the pericardium (Maestro, 2006).

The more pronounced influence of GA-induced cross-links formed in the 0.0025M GAG-treated tissue compared to higher GAG concentrations, resulted in a tensile strength comparable to GA-fixed tissue. The presence of chondroitin sulphate was shown to decrease the tensile strength of cross-linked collagenous matrices, due to interfibrillar slippage of collagen (Pieper, 1999). This, in combination with the limited GA cross-links formed, might explain why the tensile strength of the 0.1M and 0.2M GAG treatments was not increased significantly compared to the GA-fixed tissue.

6.4 DISCUSSION OF DATA FROM PHASE 3

6.4.1 Extractable Water Content

Because of the small size of the recipient animals, only four tissue samples (3 GAG concentrations + GA or Glycar) were implanted per animal. Unpaired t-tests were used to compare the extractable water content of the explants. Similar results to that earlier discussed for phase 1 of the study, were obtained. The hydrophilic nature of the GAG-treated tissue, because of their

highly negatively charged units, was responsible for the significant increase in the water content compared to GA-fixed tissue. GAG-treated and Glycar-treated patches displayed similar water contents, which would be indicative of a hydrophilic nature of the Glycar treatment on pericardial tissue as well (Table 5.4.1.1.

6.4.2 Extractable Calcium Content

The potential effect of the barrier formed by GAGs on the outer surface of the pericardium was discussed earlier. Although sufficient cross-linking with GA fixation was achieved to give the tissue adequate strength and enzymatic resistance, the penetration of GA following GAG treatment might also have been slowed down. Depending on amongst others the concentration and temperature at fixation, the penetration of GA into dense tissue like pericardium can be very slow (Khor, 1997), and complete stabilization of collagen fibers with GA might take up to one month (Chanda, 1997). This could result in incomplete cross-linking of the deeper collagen layers, presenting binding sites for calcium and serving as nucleation sites for calcification. This is supported by the fairly evenly dispersed sites of calcification in the inner layers of the GAG explants, as seen at histological evaluation (Fig. 10b).

Implants were only done for a period of 8 weeks, after which the surface-bound GAG were completely lost into the surrounding host tissue in approximately 50% of the explants. Significant cuspal calcification of bioprosthetic mitral valve implants in the circulatory system of sheep requires an implant period of 150 days or more, with no significant calcium accumulation shown at 90 days (Levy, 2003). If the GAG-treated implants in this study were implanted for a longer period of time (e.g. 120 days), it might have resulted in more severe calcification of the tissue due to further loss of GAGs into the host tissue and a reduced protective effect by the GAG treatment.

Blocking of the free aldehyde groups in GA-treated bovine pericardium with a liquid polyol like propylene glycol (Glycar patch), showed a substantial decrease in the aldehyde-induced calcification of the tissue following subcutaneous implantation in rats. Treatment of GA-tanned biological tissues with di-substituted diols like the various propane diols and 2,3-butylene glycol rather than tri-substituted triols such as glycerol, proved to be more stable and effective in blocking the free aldehyde groups and thus minimizing the calcification of the tissue even further (U.S. Patent No. 5476516, 1995).

6.4.3 Presence of GAG Post-implant

Explanted tissue samples were prepared for histological examination and stained with alcian blue for detection of glycosaminoglycans. An arbitrary interpretation of the sections was done by an independent anatomical pathologist. A scale of no GAGs (-), very mild (+-), mild (+), moderate (++) or severe (+++) presence of GAGs was employed to interpret the results (Table 5.4.2).

In almost half of the explants no GAGs could be seen on the outer surface of the tissue, with only a mild presence in the remaining tissues, and with no GAGs present in the deeper layers. A mild presence of GAGs was also seen on the outer surface of some of the GA-fixed and Glycar-treated patches. The origin of these GAGs must have been from the host animal, as these tissues were not subjected to any treatment with GAGs before implantation. Surrounding host tissue indicated a moderate presence of GAGs which was evenly dispersed. Absence of GAGs from the outer surface of GAG-treated implants indicates a leaching out of the GAGs into the surrounding host tissue, but no correlation with the calcification of the tissue could be made. Conventional GA cross-linking of bioprosthetic heart valves does not stabilize the GAGs present, and the gradual loss of these GAGs might make these implants prone to calcification and tissue failure (Lovekamp & Vyavahare, 2001).

More than 80% of GAGs were lost from GA-fixed bioprosthetic heart valves after ten million cycles in an accelerated fatigue tester, thereby affecting the mechanical properties of the tissue. More than 90% of cuspal GAGs were lost after 5 months of implantation in a sheep circulatory model, similar to the level of GAG losses from implanted bioprosthetic heart valves reported in a clinical retrieval study (Simionescu, 2003).

Histology done with alcian blue staining on the remaining GAG-treated pericardial samples, stored in 0.625% GA for several months after the rat implantations were done, revealed the complete absence of GAGs (results not shown). This correlates with the finding by other authors that GAGs continues to be lost from bioprosthetic heart valve leaflet tissue following storage in a 0.2% GA solution. The method as to how these high molecular weight GAGs leach out of the tissue is still unknown (Lovekamp, 2006).

6.4.4 Immunology

Although the different treatments employed were not designed to influence or minimize their antigenicity, the outcomes were evaluated histologically. All the explanted tissue samples from all the different treatments demonstrated a mild to moderate presence of lymphocytes in the superficial layers, and in some cases mild infiltration into the deeper collagen layers (Fig. 11). This correlates with a normal immune response from the recipient to a foreign devitalized substance being introduced into the body.

All implants in this study demonstrated a similar degree of host immune cell response to the different implants, but the degree of calcification differed completely. This correlates with previous studies reporting that in subcutaneous xenograft implants in rats, neither nonspecific inflammation nor specific immunologic responses appear to favor or cause bioprosthetic tissue to calcify (Schoen & Levy, 2005). Liao and colleagues also reported that there was no relation between the host immune reaction to differently treated pericardial implants and their calcification. The degree of calcification

increased with prolonged GA fixation times, and not because of their antigenicity (Liao, 1995).

Manji and colleagues demonstrated that despite the fact that fixation of live collagenous tissues with GA reduced the antigenicity of bioprosthetic valves, there is inflammation to and rejection of GA-fixed homografts and xenografts implanted intravascularly in a young animal model. Xenografts showed significantly more inflammation compared to homografts, and it was more than what could be accounted for by GA-fixation alone. The rejection was found to be mediated by both macrophages and T-cells, and the amount of macrophage infiltrate correlated significantly with the degree of calcification of the graft. Macrophages attack the graft first, because they do not require specific antigen recognition and then, through the release of cytokines and other chemo-attractants, T-cells are recruited for further attack (Manji, 2006). This is in sharp contrast to findings from xenograft subcutaneous implants, and the influence of the host immune response to bioprosthetic valve calcification will hold serious implications for future valve development.

Free aldehyde groups remain present in GA-treated bioprosthetic implants, being bound to the collagen and unable to be removed from the tissue by washing in saline. These free aldehyde groups are believed to be responsible for the initiation of a local inflammatory response from the host tissue, with the subsequent calcification of the implant. According to Frater and co-workers, the polyol treated pericardium (Glycar) evokes a minimal inflammatory response from the host, with scattered lymphocytes as the dominant inflammatory cell type (Frater, 1997). This indicates that residual unbound aldehyde groups which activates the host immune response are still present in the Glycar tissue, but that the response is to a large extent reduced by the ‘capping’ of the free aldehyde groups with propylene glycol.

6.4.5 Enzymatic Digestion

Collagenase can cleave both soluble and insoluble collagen. However, because of the triple-helix specificity, it cannot cleave the nonhelical telopeptide regions of insoluble collagen, which is the intra- and intermolecular cross-link region. The resistance of bovine pericardium to collagenase digestion therefore indirectly reveals the degree of cross-linking of collagen (Jee, 2003).

The comparative resistance of the GAG-treated and GA tissues to enzymatic digestion thus confirms the adequate degree of cross-linking obtained by pretreating pericardium with GAGs, without sacrificing thermal stability or tensile strength. Despite a reduction in thermal stability of the GAG treatment compared to the control tissue, the thermal denaturation temperature could still be maintained above the acceptable minimum requirements.

Glycar-treated tissue demonstrated significantly higher resistance to enzymatic digestion when compared to all the other treatments (Table 5.4.4). This, combined with its low calcification potential, high degree of cross-linking stability and tensile strength, makes this tissue a superior substitute material for use in cardiac and other reconstruction procedures.

CHAPTER 7

CONCLUSION

The known detrimental influence of GA towards the calcification of biological tissue was once again highlighted, as was the reversible binding of aluminium to collagen, leading to severe calcification of pericardial tissues treated with these methods. Binding sulphated glycosaminoglycans to proteins in pericardial tissue to form extra proteoglycans before the final fixation with glutaraldehyde, resulted in a significant reduction in calcification of the tissue, while maintaining good structural integrity, mechanical properties and low antigenicity.

Fixation of the proteoglycans by using a 0.01M sodium metaperiodate concentration in the presence of chondroitin sulphate, proved to be the optimal GAG concentration to be used. Lower concentrations appear to have a reduced tensile strength (not significant), while higher concentrations yielded a significant decrease in tensile strength and denaturation temperature.

Glycosaminoglycans leaching out of treated tissues will hold a major challenge for the future clinical use of tissues treated by this method. Even though these implants were only done in the subcutaneous rat model where they remained static for 8 weeks, evidence for the leaching out of the GAG into the surrounding host tissue was found. Permanent cross-linking and fixation of the GAGs in order to produce stable and durable bioprostheses will be required before they can be used safely and with confidence, especially in a pulsatile haemodynamic system.

The chemical composition of the storage medium for bioprostheses and biological tissue transplants plays an important role in the longterm performance of these substitutes. Minimum requirements of these storage media would include suitable sterilization of the finished product during

storage, without increasing its calcification potential. Agents free of potentially harmful aldehyde groups in solution will be preferable, and propylene oxide as employed in the Glycar-treated patches provides a good alternative. Tissues stored in propyl- and methylhydroxybenzoate were shown to be less toxic than GA storage, while treatment with glycine was also reported to reduce the toxicity to some extent (Jayakrishnan & Jameela, 1996).

Long-term storage of bioprostheses in glutaraldehyde might have some effect on the calcification rate of the tissue, but it will not alter its ultimate fate. Factors like the storage time, concentration of the solution and treatment conditions does affect the chemical stability of the GA cross-links, and unstable GA polymers present in the interstitial spaces could make the solution more cytotoxic and thus contribute to the calcification response. Long-term storage of bioprosthetic tissue for several years delays its calcification, but eventually it calcifies to the same extent as tissue which was stored or exposed to GA for a shorter period of time (Jorge-Herrero, 2005).

The finding that GAGs were only bound to the outer surface of the pericardial tissue was particularly important. This makes it prone to damaging of the layer during handling when implanted, which can accelerate the tissue's calcification and degradation. Future fixation strategies will also require the deeper penetration of the GAG into the tissue layers before final fixation with GA, which will minimize the detrimental effect that GA cross-linking has on the calcification of the tissue. More effective methods of stabilizing the cross-links formed by GAG-fixation will also be required, in order to prevent the GAG from leaching out of the implant into the host tissue.

The positive effect of treating aldehyde-fixed pericardial tissue with a polyol in order to reduce the detrimental effect of the remaining free aldehyde groups in the implant, was again proven. 'Capping' the free aldehydes resulted in significantly less calcification, improved suppleness and tensile strength, low immunogenicity and superior resistance to enzymatic degradation, and this method will hopefully improve the future outcomes of clinically implanted bioprostheses substantially.

Determinants of the calcification of bioprosthetic tissue implants has been proven to be multifactorial. Host metabolism, possible damage to the tissue structure, chemical changes in the material as a result of preservation and cross-linking techniques and mechanical stresses exerted in a pulsatile system may all contribute to the calcification of the tissue. Tissue treatment will require enhanced cross-linking stability, removal of residual GA and inactivation of free aldehyde groups, reduction in lipid content and restoration of tissue elasticity (Neethling, 2004). Optimal management of all these factors will be required in order to enhance the longevity of bioprosthetic implants.

CHAPTER 8

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