A STUDY OF THE EFFECTS OF WARM ISCHAEMIC TIMES ON HARVESTED HOMOGRAFTS

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DECLARATION OF INDEPENDENT WORK

I, DREYER BESTER, do hereby declare that this research project submitted to the Central University of Technology for the degree MAGISTER TECHNOLOGIAE CLINICAL TECHNOLOGY is my own independent work that has not been submitted to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

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SIGNATURE OF STUDENT **DATE**

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ABBREVIATIONS / ACRONYMS / SYMBOLS

A	Cross Sectional Area
AHV	Allograft Heart Valve
Al	Aluminium
AoH	Aorta Homograft
AR	Aortic Regurgitation
BM	Basement Membrane
BMI	Body Mass Index
CIT	Cold Ischaemic Time
cm	Centimetres
CO_2	Carbon Dioxide
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
e	Strain
E	Young's Modulus
EM	Equal Mean
et al.	et alii (and others)
etc.	Etcetera
F	Applied Force / Tensile Force
g	Gram
h	Hour
H&E	Haematoxylin and Eosin Stain
hrs	Hours
IT	Ischaemic Time
IU	International Units
kg	Kilogram
kV	Kilovolt
L_0	Initial Unstressed Length
Lbs/in^2	Ponds per Square Inch
M199	Medium 199
m^2	Square Meter

mg	Milligrams
mg/hour	Milligrams per Hour
min	Minute
ml	Millilitres
mm	Millimetre
mm/min	Millimetres per minute
mm/s	Millimetres per Second
mmol	Millimole
MPa	Mega Pascal
N	Newton / Load
N/mm^2	Newton per Square Millimetres
N_2	Nitrogen
P120	Sanding Paper Grid Size
Pa	Pascal
psi	Ponds per Square Inch
PUH	Pulmonary Homograft
S	Stress
SEM	Scanning Electron Microscopy
Td	Denaturation Temperature
TDT	Thermal Denaturation Temperature
Tmax	Transition Temperature Maximum
Tp	Transition Temperature Peak
TS	Tensile Strength
UFS	University of the Free State
vs.	Verus
W/g	Watt per Gram
WIT	Warm Ischaemic Time
ΔL	Change in Length Due to Stress
ΔH	Enthalpy of Denaturation
σ	Tensile Stress
&	And
°C	Degrees Celsius
°C/min	Degrees Celsius per Minute
=	Equals
<	Less Than
>	More Than
≤	Less-Than or Equal TO
≥	Greater-Than or Equal To
%	Percentage

n	Number of Samples Analysed
p	Statistical Significance
X	Times / Magnification

IMPORTANT DEFINITIONS

ALLOGRAFT	A homograft between allogenic individuals
ANTIBIOTIC STERILIZED HOMOGRAFT	Antibiotic-sterilized valves stored at 4°C in nutrient media are considered to be non-viable valves. (Yacoub & Kittle, 1970)
AUTOLYSIS	In this study autolysis was defined as necrotic cells that showed increased eosinophilia attributed in part to loss of the normal basophilia imparted by the RNA in the cytoplasm and in part to the increased binding of eosin to denatured intracytoplasmic protein It was deemed to be wither present or absent in the specimens examined and no grading system was applied.
COLD ISCHAEMIC TIME	This study defines cold ischaemic time as the ischaemic time period during which the intact sheep carcasses were maintained at room temperature of 23^0C for 2-3 hours after death, during which time the stomachs were removed, before being cooled to 4^0C.
CRITERIA	A standard on which a judgment or decision may be based, or a characterizing mark or trait.
CRYOPRESERVED HOMOGRAFTS	Cryopreserved valves are valves sterilized in antibiotic solution and subsequently cryopreserved (O'Brien *et al*., 1987).
DIFFERENTIAL SCANNING CALORIMETRY	Differential scanning calorimetry (DSC) means the measurement of the change of the difference in the heat flow rate to the sample and to a reference sample while they are subjected to a controlled temperature program (Höhne, Hemminger and Flammersheim, 2003)
ENDOTHELIUM	An epithelium of mesoblastic origin compose of a single layer of thin flattened cells that lines internal body cavities (as the serous cavities or the interior of the heart).
HARVESTING	To remove or extract (as living cells, tissues, or organs) from culture or from a living or recently deceased body especially for transplanting.
HEAMATOXYLIN AND EOSIN STAIN	Probably the most generally useful staining method for tissues, nuclei are stained a deep blue-black with haematoxylin, and cytoplasm is stained pink after counterstaining with eosin, usually in water (Bancroft and Stevens, 1982).
HERMETICALLY	Being air tight or impervious to air.
HOMOGRAFT	A graft of tissue from a donor of the same species as the recipient.

HOMOGRAFT VIABILITY	Viability of a homograft refers to survival of endothelial cells and fibroblasts that retain their ability to replicate and regenerate extracellular matrix elements (Barili *et al*., 2007). Cryopreserved valves are valves sterilized in antibiotic solution and subsequently cryopreserved. They are considered viable if cryopreserved within 4 days of procurement (O'Brien *et al*., 1987).
HOMOVITAL HOMOGRAFT	These homografts are harvested under sterile conditions, are storage in an antibiotic solution at 4^0C and are not frozen prior to implantation (Yacoub *et al*., 1995).
IN VITRO	Outside the living body and in an artificial environment.
IN VIVO	In the living body of a plant or animal.
ISCHAEMIC TIME	Ischaemic time is defined as the time interval between donor death and valve procurement (Angell *et al*., 1989; O'Brien *et al*., 1995). It is sometimes referred to as harvesting time. This study further qualifies ischaemic time as either cold or warm.
SCANNING ELECTRON MICROSCOPE	An electron microscope in which a beam of focused electrons moves across the object with the secondary electrons produced by the object and the electrons scattered by the object being collected to form a three-dimensional image on a display screen.
TENSILE STRENGTH	The greatest longitudinal stress a substance can bear without tearing apart.
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 (Ma and Harwalkar, 1991).
WARM ISCHAEMIC TIME	This study defines warm ischaemic time as the ischaemic time period during which the intact sheep carcasses were maintained at room temperature of 23^0C for 6 hours after death, before being cooled to 4^0C. Stomachs were not removed.
YOUNG'S MODULUS	The modulus of elasticity in tension, also known as Young's modulus *E*, is the ratio of stress to strain on the loading plane along the loading direction (Pukacki, *et al*., 2000).

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OPSOMMING

In hierdie studie word homografts gedefinieer as aorta-, pulmonale- of mitraal-weefselkleppe wat geoes word van kloppende hart donors of kadawers (Anastasiadi *et al*., 2004). Die konsep van homograft lewensvatbaarheid is sentraal in hierdie studie sowel as in die kliniese toepassing van homografts in die praktyk. Lewensvatbaarheid word gedefinieer as die oorlewing van endoteelselle in die transplantaat en sluit die potensiaal in vir seldeling en -hernuwing van die ekstasellulêre matriks elemente (Barili *et al*., 2007). O'Brien en medewerkers het die verwantskap tussen lewensvatbaarheid en langtermyn uithouvermoë of werkverrigting van homografts reeds in 1987 vasgestel (O'Brien *et al*., 1987).

Verskeie faktore bepaal homograft lewensvatbaarheid, waarvan isgemiese tyd vanaf donor dood tot homograft oesting die belangrikste is. Die meeste homograft banke vereis dat die kleppe binne 24 uur vanaf die donor se dood geoes moet word, maar hierdie stelling is uiters kontroversieël (Livi *et al*., 1987). Die tydsinterval van 24 uur word voorgekryf om endoteelselle en fibroblaste se lewensvatbaarheid te verseker wat weer 'n daadwerklike impak het op die langtermyn uithouvermoë van die klep (Angell *et al*., 1989; O'Brien *et al*., 1995). Ondanks die limiet op isgemiese/oestingstyd, verleng talle homograft banke die isgemiese tyd weens kriopreservering. Kleppe wat gekriopreserveer word binne 96 uur word beskou as lewensvatbaar (O' Brien *et al*., 1987).

Wheatley en McGregor (1977) het gevind dat vars homografts geoes van honde (Homovital) vinniger degenereer en kalsifiseer as die kontroles en het voorgestel dat die moontlike rede toegeskryf kan word aan 'n transplantaat-gasheer immuun reaksie. Bogenoemde stelling is teenstrydig met die bevindings van Yacoub en medewerkers,

en toon dat ‘n oestingstyd minder as 24 uur nie noodwendig langetermyn uithouvermoë verseker nie (Yacoub *et al*., 1995).

Aangesien die beskikbaarheid van homografts ‘n internasionale probleem is kan die verlenging van die voorgekrewe 24 uur oestingtyd voordelig wees vir kadawer-gebaseerde programme.

Tydens die studie is twee groepe skaap homografts genaamd Groep B en Groep C met ‘n kontrole groep (Groep A) vergelyk. Die skaapharte in Groep A is binne 6 uur vanaf dood geoes. Die karkasse in Groep B is afgekoel na 4ºC en slegs vir 2-3 ure blootgestel aan kamertemperatuur (23^{0}C). Groep B vorm gevolglik die koue isgemiese groep. Die karkasse in Groep C is blootgestel aan kamertemperatuur (23ºC) vir ‘n periode van 6 uur waarna die karkasse vir ‘n tydperk van 3 dae stadig afgekoel is na 4ºC. Groep C vorm dus die warm isgemiese groep.

Tensiele sterkte en differiensiële skandeer kalorimetrie tegnieke het geen verskil in weefselsterkte tussen die groepe gedemonstreer nie. Kriopreservering- en sterilisasieprosesse het ook nie bygedra tot ‘n afname in sterkte nie.

Groep B (koue isgemiese groep) het met histologiese ondersoeke geen outolise getoon in enige van die kleppe nie. Die outolise wat wel voorgekom het in die 48 en 72 uur weefsel van Groep C (warm isgemiese groep) het geen effek op die weefselsterkte gehad nie. Soortgelyk, die vermindering van endoteelselle oor tyd het in beide Groep B en C tot en met 72 uur geen invloed getoon op weefselsterkte nie.

Om hierdie redes bleik dit aanvaarbaar te wees om die oestingstyd van 24 uur te verleng.

Hierdie resultate sal egter deur verdere proefdierstudies gestaaf moet word aangesien *in vivo* transplantaat-gasheerinteraksie resultate teenstrydig met bogenoemde bevindinge kan wees.

In die inplanterings weefsel mag meganismes van degenerasie belangriker wees in die *in vivo* omgewing as bloot net absolute weefselsterkte en -integriteit.

SUMMARY

In this study, homografts are aortic, pulmonary or mitral tissue allografts harvested from either beating heart donors or cadavers (Anastasiadis *et al*., 2004). The concept of homograft viability is central to both this study and the clinical application of homografts in practice. Viability refers to the survival of endothelial cells in the implant and includes the potential for cellular replication and renewal of extracellular matrix elements (Barili *et al*., 2007). The relationship between viability and long term durability or performance of homografts was established by O'Brien and co-workers (O'Brien *et al*., 1987).

Homograft viability is determined by many factors, the most topical of which is the ischaemic time from death of the donor to harvesting of the homograft. Most homograft banks require that valves should be harvested within 24 hours of the donor's death but this requirement is controversial (Livi et al., 1987). The time interval of 24 hours seems to ensure viability of endothelial cells and fibroblasts which has an impact on long-term valve durability (Angell *et al*., 1989; O'Brien *et al*., 1995). Notwithstanding the limit on ischaemic/harvesting time, many homograft banks prolong the ischaemic time as a result of cryopreservation. Valves cryopreserved within 96 hours are regarded as viable (O' Brien *et al*., 1987).

Wheatley and McGregor (1977) noted that fresh canine homografts (Homovital) degenerated and calcified quicker than controls and suggested that this might be due to a graft-host immune interaction. This appears to contradict the findings of Yacoub and co-workers, and shows that a harvesting time of less than 24 hours does not necessarily guarantee long-term durability (Yacoub *et al*., 1995).

As homograft availability is an international problem, extending harvesting time beyond 24 hours might benefit cadaver donor based programs.

This study compared two groups of sheep homografts, namely Group B and Group C, with a control group of sheep obtained within 6 hours of death, namely Group A. Group B carcasses were cooled to 4^0C and were exposed to room temperature at 23^0C for only 2-3 hours. This was therefore the cold ischaemic group. Group C carcasses were kept a room temperature of 23^0C for 6 hours prior to being cooled slowly over a period of 3 days to achieve a temperature of 4^0C. This was therefore the warm ischaemic group.

Using tensile strength and DSC techniques, no difference in tissue strength could be demonstrated between the groups. No reduction in strength could be demonstrated as a result of the cryopreservation and sterilisation process

Histological examination did not show autolysis in any of the valves in Group B (cold ischaemic group). The autolysis observed in the 48 and 72 hour tissue of Group C (warm Ischaemic time) was not sufficient to affect tissue strength. Similarly, a reduction of endothelial cells over time in both Group B and Group C did not influence tissue strength up to 72 hours.

It therefore seems to be acceptable to extend harvesting time beyond 24 hours, based on these investigations.

Further animal studies need to be performed to substantiate these results, as the *in vivo* graft host interactions might produce results that are not consistent with these findings.

Mechanisms of degeneration might be more important in the *in vivo* situation than absolute strength and integrity of the implanted tissue.

CHAPTER 1
INTRODUCTION

Use of homografts in cardiac surgery is well established but availability remains the main limiting factor in their clinical application.

Homografts are procured from two sources, namely, beating heart donors and cadavers. The factors that limit availability of solid organ transplants from beating heart donors apply equally to homografts. In addition, cadaver homograft procurement is limited by the ischaemic time between cardiac arrest and organ harvest.

Current practice regarding allograft harvesting from either source, attempts to confine the harvest to within 12 to 24 hours of brain death or cardiac arrest (O'Brien, Johnston, Stafford, Gardner, Pohlner, McGiffin, Brosnan, and Duffy, 1988; Livi, Abdulla, Parker, Olsen and Ross, 1987). Although widely accepted, this time limitation has not been established scientifically. Several publications suggest that longer harvest times might be acceptable.

In fact there are a number of articles that actually suggest that it would be acceptable to extend harvesting times.

Armiger and associates (Arminger, 1995) have suggested that the ischaemic interval could be extended up to 30 hours after death. Messier and colleagues (Messier, Domkowski, Aly, Abd-Elfattah, Crescenzo, Wallace and Hopkins, 1992) demonstrated high levels of adenine nucleotide pools in cryopreserved allografts after prolonged ischaemia. Kadoba and associates (Kadoba, Arminger, Sawatari and Jonas, 1991) confirmed that a 48-hour delay from donor to graft harvest did not have a significant effect on conduit function.

Periods of ischaemia are unavoidable during homograft processing. Such periods occur between cessation of the donor heartbeat and harvesting (cadaveric recovery) and during the preservation in tissue storage solution and the sterilization process that follows. A period of up to 4 days between death and final cryopreservation is commensurate with homograft viability (O'Brien, Stafford, Gardner, Pohlner and McGiffin, 1987). Viability refers to the survival of endothelial cells in the implant and includes the potential for cellular replication and renewal of extracellular matrix elements (Barili, Dainese, Cheema, Dell'Antonio, Topkara, Rossoni, Guarino, Micheli, Doglioni, Biglioli and Polvani, 2007). The circumstances of death, post-mortem temperature changes, Body Mass Index's (BMI's) of the donors and immediate changes in tissue quality and its impact on objective tissue deterioration over time, have been insufficiently studied. Standardization of time related harvesting protocols which are currently internationally accepted, are based on observational studies rather than controlled scientific data such as that of O'Brien *et al*., (1987).

The fact that acceptable long-term results were obtained in the fresh albumin stored homografts, stored at 4°C for up to 90 days (Goffin, De Gouveia, Szombathelyi, Toussaint and Gruys, 1997), implies that ischaemic time between death, harvesting and implantation is well tolerated. The time after death, before harvesting, forms a fraction of the time in this setting. What needs to be addressed is what happens to the homograft tissue in the body after death before harvesting, and how this impacts on homograft performance.

Most heart valve banks worldwide require that donor tissue be harvested up to and less than 24 hours after death. This can limit the availability of valves dramatically in cadaver donors requiring medico-legal autopsies, an important donor pool in some countries like South Africa, because of time and logistical implications. The mean time from death to harvesting in Bloemfontein is 33 hours.

Ideally, objective criteria must be developed in order to predict the quality of the homograft tissue before processing and implantation. This will enable the surgeon to apply additional selection criteria other than time, to select homografts for processing (cryopreservation) with a greater degree of confidence as to their ultimate *in vivo* performance.

The purpose of the study was:

1. To evaluate tissue degeneration post mortem in a sheep model in a controlled environment using 24 h, 48 h and 72 h groups, using objective criteria including histology (H&E) Scanning Electron Microscopy (SEM), Tensile Strength and Differential Scanning Calorimetry (thermal denaturation temperature)(T_d).

2. To evaluate the impact of different ischaemic temperatures upon tissue degeneration between "warm" and "cold" ischaemic time groups in the *in vitro* sheep homograft model.

3. An attempt to establish a relationship between the quality of tissue as assessed histologically (e.g. signs of autolysis) and presence of endothelium (SEM) on the one hand, and tissue strength, (tensile strength and DSC) on the other.

4. An attempt was made to establish whether a relationship existed between the aorta, pulmonary wall and mitral valve, aorta and pulmonary valve leaflets, in order to predict quality of implanted tissue by using non implanted tissue as reference tissue.

Hopefully this study would provide objective data (post cryopreservation) on sheep homografts that would demonstrate the quality, or the lack thereof, of tissue harvested up to 72 hours after death using the described methods.

Lu and co-workers in 1998 concluded that a 30 – 60 hour delay did not have significant metabolic effects on the cardiac leaflets (Lu, Chang, Hsu, Hwang, Chong, Wu, Yang and Hsing-Wen, 1998). This suggests that it may be possible to safely extend the permissible ischaemic periods after organ harvest, which could have an important impact on homgraft availability in South Africa.

CHAPTER 2
LITERATURE REVIEW

2.1 BACKGROUND

In this study, the term homograft refers to aortic, pulmonary or mitral tissue allografts harvested from beating heart donors or cadavers (Anastasiadis, Kambouroglou and Spanos, 2004).

The history of using homologous cardiac valves and great vessel valve-containing conduits dates back more than 30 years. Animal studies reported by Lam *et al.,* (1952) formed the basis for other investigators to use the concept in a different and more successful way (Lam, Aram and Mennell, 1952). In 1956, Gordon Murray's pioneering work in Toronto demonstrated that homologous aortic valve segments could be transplanted into not only the descending aorta but also the mitral position (Murray, 1956). After experimenting with this technique in dogs, Murray successfully applied the concept in two patients with aortic regurgitation and later in one with mitral valve disease.

It was not until 1962, however, that the aortic homograft (Figure 2.1.1) was inserted in the native or sub coronary position of the heart (Ross, 1962). This was made possible by the development of the heart-lung machine, which opened the door for all types of intra-cardiac corrective and palliative procedures. With technical help from Gunning and Duran, Donald Ross performed this procedure for the first time at Guy's Hospital in London on July 24, 1962 (Ross, 1962). Barratt-Boyes at the Green Lane Hospital in New Zealand independently developed the same concept for sub-coronary replacement of the aortic valve with a

homograft aortic valve and began performing it clinically in August 1962 (Barret-Boyes, 1965).

In the absence of mechanical alternatives, it is understandable that these early pioneers turned to the homograft valve. They felt it would be difficult to improve on the efficient design characteristics of the human aortic valve (Ross, 1962). In the normal heart it provides no obstructive gradient at rest or with exercise and provides central blood flow with minimal turbulence during left ventricular ejection. It also has an absolutely regurgitation-free natural closure mechanism due to the design of the sinuses of Valsalva.

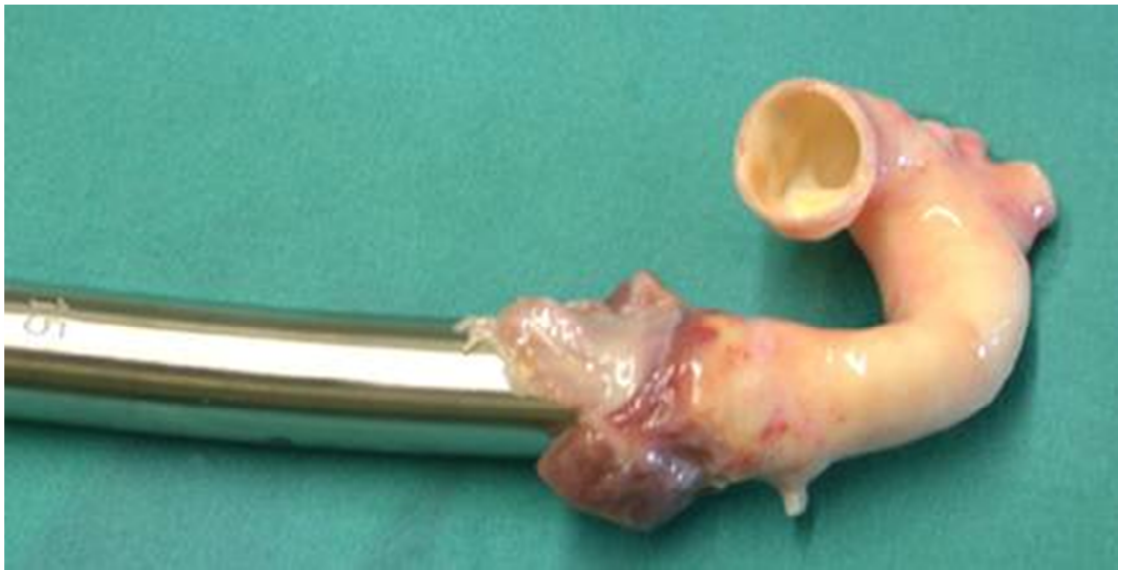


Figure 2.1.1 Aortic Homograft Conduit

The technical difficulty of inserting a competent homograft valve was a greater surgical challenge than the insertion of a corresponding mechanical prosthetic device. However, within 3.5 years of experience, Ross had implanted 110 aortic homografts with progressively decreasing operative risk and improved technical performance (Ross, 1962).

At this point in time the aortic homograft was well established due to its low gradient, low risk of thromboembolic events, high resistance to endocarditis and

acceptable long-term results when compared with alternative bioprosthetic and mechanical valves (Barret-Boyes, Roche, Subramanian, Pemberton and Whitlock, 1987; O'Brien, Stafford, Gardner, Pohlner, McGiffin, Johnston, Brosnan and Duffy, 1987).

Prompted by the limited availability of donor valves, emphasis was placed on pulmonary valve homografts (Figure 2.1.2) as aortic valve substitutes in the early 1990's (Koolbergen, Hazekamp, de Heer, van Hoorn, Huysmans, Bruijn and Dion, 2002). *In vitro* biomechanical testing supported this argument and showed that the pulmonary valve was capable of withstanding the higher pressure of the systemic circulation. It was also reported that the pulmonary homograft wall tissue calcified less than the aortic homograft wall tissue, and the pulmonary autograft in the aortic position had excellent long term results. However, acute cusp rupture of the pulmonary homograft implanted in the aortic position was reported in 1994 (Koolbergen *et al.,* 2002).

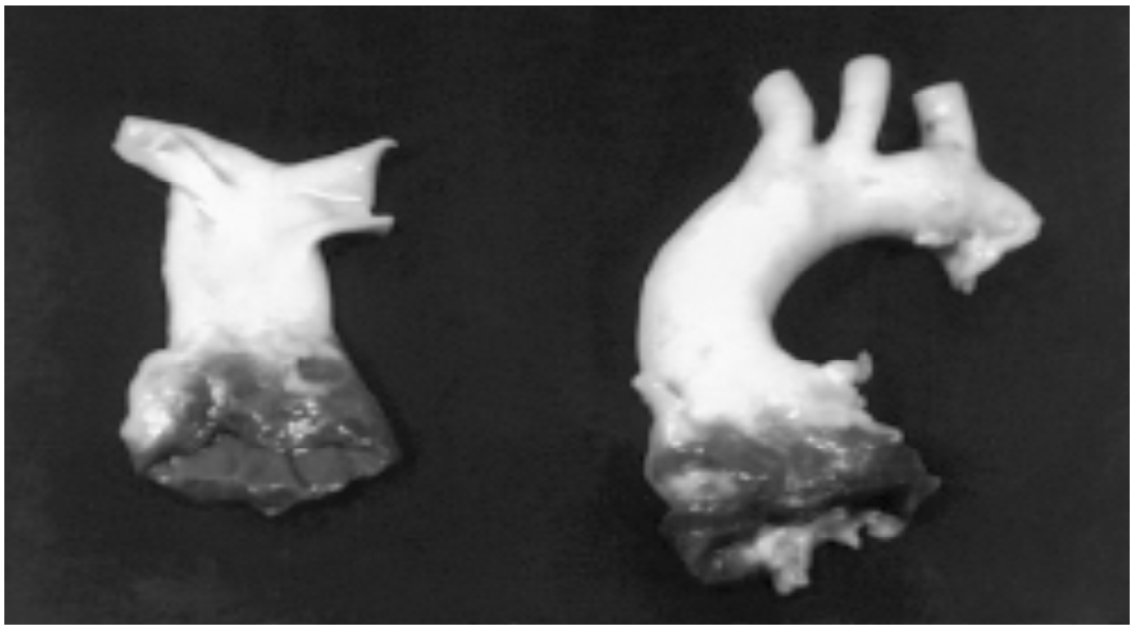


Figure 2.1.2 **Aortic and Pulmonary Homograft Conduits** (adapted from Verghese, Padmaja, Sindhu, Elizabeth, Lesley and Cherian, 2004).

2.2 PROCUREMENT OF HOMOGRAFTS

2.2.1 Donors

Homografts can be harvested from non-beating hearts or beating hearts:

2.2.1.1 Non-beating heart donors

Historically, fresh cadavers have been the source of various types of human tissues. The European practice of obtaining tissues from the hospital morgue is well known. Non-beating heart donors are deceased hospital patients or forensic cases.

The postmortem delay must be less than 24 hours with an exposed warm ischaemic time of less than 6 hours (Goffin, Grandmougin and Van Hoeck, 1996).

2.2.1.2 Beating heart donors

The maximum delay after brain death must not exceed 18 hours with no warm ischaemic time. The operating room is currently the preferred site of harvesting, because it maximizes the sterility of tissue procurement.

2.2.2 Inclusion and Exclusion Criteria for Homograft Donation

2.2.2.1 Donor screening

Donor screening is an important element of the procurement process (O'Brien *et al*., 1987). Any history or laboratory evidence of communicable disease makes transplantation of the donor's organs or tissues inadvisable. Heart valves obtained from donors of approximately 55 years of age or less are generally suitable, provided there is no history

of diabetes, hyperlipidemia, or hypertension. Donors following cardiac or great vessel trauma or those with severe mediastinal adhesions may be excluded as potential donors.

Laboratory data includes serologic tests for syphilis, hepatitis, and human immunodeficiency virus (HIV). Blood cultures and autopsy findings are obtained, and blood typing is also performed. Full term infants and young children are the only suitable donors of small diameter grafts needed for cardiac reconstructive surgery in infants.

Other exclusion criteria include clinical evidence of Marfan's syndrome, history of collagen or immune complex diseases, dementia or neurological degenerative disease of any form, irradiation to the thorax and a history of consuming toxic substances.

2.2.2.2 Human Decomposition after Death

Human decomposition or autolysis begins approximately 4 minutes after death. As cells of the body are deprived of oxygen, blood carbon dioxide levels will increase which will decrease pH as waste accumulates which causes cell poisoning. Simultaneously, cellular enzymes (lipases, proteases, amylases, etc) begin to dissolve cells from the inside out, eventually causing them to rupture, releasing nutrient-rich fluids.

Autolysis usually does not become visually apparent for a few days. Meanwhile, as the body acclimates to ambient temperature (*algor mortis*), blood settles in the body causing skin discoloration (*livor mortis*) and cellular cytoplasm gells due to increased acidity (*rigor mortis*). After enough cells have ruptured the process of putrefaction begins (Vass, 2001).

Putrefaction is the destruction of soft tissue by micro-organisms (bacteria, fungi and protozoa) and results in the catabolism of tissue into gases, liquids and simple molecules. Although decomposition is a complicated process, it is primarily dependent on temperature and to a lesser extend moisture. The rate of decay can be affected by variables of different natures concerning the corpse itself (intrinsic factors) and the external environment. Among the intrinsic factors are age (slower in fetuses and newborns), constitution (obese corpses decompose more rapidly due to the greater amount of liquid in the tissues whose succulence favours the development and dissemination of bacteria), cause of death (early and rapid putrefaction occurs in wasted away persons, those suffering from septic infections, in death from asphyxia because blood fluidity promoting bacterial diffusion), integrity of corpse (cuts in the skin are an easy way in for external bacteria and Diptera). Among the extrinsic factors, the most important is temperature (temperatures ranging between 25 and 35°C are optimal for bacterial development) followed by ventilation and humidity. Clothing can slow down postmortem body cooling and favour the onset of the putrefaction process (Henssge, Knight, Krompecher, Madea and Nokes, 1995).

2.2.2.3 Warm Ischaemic Time (WIT)

Homograft durability has clinically been related to cellular "viability" (Angell, Oury, Lamberti and Koziol, 1989; O'Brien, Stafford, Gardner, 1995). Warm ischaemic time or the harvesting interval, is defined as the time it takes from death to the actual harvesting of the valve and has been recognized as the main determinant of cell survival. However, there is a great controversy regarding an acceptable warm ischaemic period. For cardiac valves the graft harvesting time varies from 2 to 72 hours. Some investigators believe that the use of valves harvested more than 24 hours after death is unacceptable because the valve leaflet cells are non-viable.

Endothelial cell viability plays a role in homograft valve durability but the extent is unclear. It has been suggested that endothelial cell retention might be a factor for homograft valve resistance to the degeneration process. The resistance of endothelial cells to warm ischaemic time (WIT) is still debatable, but vascular endothelium is thought to have a lower resistance to ischaemic injury. Yankah and Hertzer (1987) reported a 24% survival rate of endothelium after a 2 hour exposure to room temperature, while other experiments suggested the destruction of the endothelial layer between 24 to 48 hours postmortem. A study performed on rat models indicated that the endothelium was viable for at least 40 hours postmortem (Yankah and Hertzer, 1987).

Current practice regarding the harvesting and cryopreservation of homograft valves are based on the premise that the cell matrix (the smooth muscle cells and fibroblasts) viability enhances valve durability. Recently, a more precise definition of fibroblast ischaemic damage was presented (Crescenzo, Hilbert, Barrik, Corcoran, St. Louis, Messier, Ferrans, Wallace and Hopkins, 1992). It was demonstrated that an ischaemic time-dependent progression in fibroblast damage, in the first 12 hours of warm ischaemic time, reversible damage predominated, while a marked increase of irreversible damage after 12 hours was reported. Fibroblast response to warm ischaemic times, correlate well with the morphometric findings, (St Louis, Corcoran, Rajan, Conte, Wolfinbarger, Hu, Wang, Hilbert, Analouei, and Hopkins, 1991) in functional studies, and also the mechanisms of damage due to the depletion of high-energy phosphate intermediates (Messier *et al*., 1992).

Time spent on sterilization and processing before cryopreservation differs widely between institutions. Sterilization and incubation methods vary from 6 hours to 48 hours at 37 °C to longer periods (48-72 hours) at 4 °C. This extends the warm ischaemic time for different reasons and times. Clearly the component between death and harvesting and between

harvesting and cryopreservation are two distinct times and its separate effects should be separately addressed, although it has never been seen this way. Limited data is available concerning the effects of warm ischaemic times on valvular grafts. In order to secure a stable clinical supply of allografts, the time limit of exposure to warm ischaemia must be clarified (St Louis et al., 1991).

2.3 AVAILIBILITY OF HOMOGRAFTS

A more significant problem is the availability of homografts. Logistically, it is rare that a patient with just the right size valve dies at just the right time to make that valve available to the patient who needs it the following morning. The technique of cryopreservation has been most important in providing banks for cardiac valves.

The availability of aortic homografts is limited and therefore their use is restricted to special indications. Prompted by the limited availability of donor valves, emphasis was placed on pulmonary valve homografts as aortic valve substitutes in the early 1990's (Koolbergen *et al.,* 2002). An argument to support this policy was that *in vitro* biomechanical testing had shown that the pulmonary valve is able to withstand the higher pressures in the systemic circulation (Groczynski, Trenker, Anisimowicz, Gutkowski, Drapella, Kwiatkowska and Dobke, 1982). In addition, calcification of the pulmonary homograft wall had been reported to be less than that of the aortic homograft wall tissue (Allen, Shoji, Fujimura, Gordon, Thomas, Brockbank and Disteche, 1991). Therefore, availability was addressed by the procurement of both aortic and pulmonary homografts.

2.3.1 Factors that Influence the Amount of Available Homografts

2.3.1.1 Ethical Considerations

Advances in medical technology and practices and the success of organ transplantation over the past two decades vastly increased the demand for organ donors. However, a worldwide shortage of donor organs is experienced by the health care community and the organ procurement organizations. The most problematic factor is the non-consent provided by families of suitable potential donors (West and Burr, 2002).

In the South African scenario, consent is not the only hurdle to overcome; strong cultural beliefs limit the amount of potential donors dramatically. Cultural beliefs are part of people's values and may be difficult to change.

2.3.1.2 Microbial Contamination

The allograft heart valve is harvested from a brain-dead or postmortem donor, processed in an antimicrobial disinfection solution, cryopreserved, and stored until required. Tissue samples obtained for sterility testing are cultured for bacteria and fungi at multiple stages during harvesting and processing to rule out microbial contamination of the valve (Wain, Pearce, Riddell and Ross, 1977).

Allograft heart valves are more prone to microbial contamination than fungal contamination due to unsterile harvesting conditions. Common contaminants found before disinfection, consist of gastrointestinal and skin flora, including coliforms, viridans group streptococci, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus species*. Pathogens that cause early onset of allograft valve endocarditis include *Staphylococcus*

and *Streptococcus* species (Gall, Smith, Willmette, Wong and O'Brien, 1995). Sources of contamination of a valve allograft include the donor, the environment during harvesting and processing, and the operating room during implantation.

Varying contamination rates measured during allograft heart valve harvesting and processing may reflect different methods of harvesting, disinfection, and cryopreservation. In order to meet the demand for valve allografts, supply has been increased through harvest from deceased in addition to brain-dead donors. Microbial contamination is quite common at the time of harvesting, particularly in postmortem procurement. In one series 54% of postmortem heart valves retrieved in open mortuary areas were contaminated at collection; 31 of 642 valves were discarded because of contamination, the minority of contaminating organisms were fungal based (6 of 642) (Gall, Smith, Willmette and O'Brien, 1998).

Disinfection of valve allografts through the use of various antimicrobial combinations was first described in the 1960s (Gall *et al.,* 1995). Since that time, antimicrobial agents used for disinfection have been further modified to improve efficacy and valve viability, increasing the supply of usable allografts.

The establishment of "homograft banks" eventually addressed the problem of availability. Although valve banks employ different disinfection protocols, it is obvious that a valve yielding positive microbial culture post sterilization will be discarded and considered unfit for transplantation.

2.3.1.3 Histocompatibility (HLA)

Histocompatibility control or homograft cross-matching is currently performed in certain centers to avoid graft degeneration caused by the

recipient's immune response. However, this appears to be unnecessary as valvular endothelium lacks expression of carbohydrate antigens (Kadner *et al.,* 2001).

The effect of histocompatibility on the long term results of homografts is the subject of ongoing research (Bechtel, Bartlets, Schmidtke, Skibba, Müller-Steinhardt, Klüter and Siever, 2001).

2.4 PRESERVATION OF HOMOGRAFTS

The availability of homografts was addressed by shifting the emphasis to the improvement and development of more advanced preservation techniques.

2.4.1 Classification of Homografts

Homograft valves are classified as homovital, antibiotic sterilized and cryopreserved valves based upon the method of preservation:

2.4.1.1 Homovital Valves

Homovital valves are untreated valves, harvested under sterile conditions usually from the recipient at the time of the heart transplantation and kept in nutrient media they are considered viable if implanted within 3 days (Yacoub, Rasmi, Sundt, Lund, Boyland, Radley-Smith, Khaghani and Mitchell, 1995).

The early sterilization and storage methods included the use of chlorhexidine, formaldehyde, propiolactone, ethylene oxide and γ-irradiation.

2.4.1.2 Antibiotic Sterilized Valves

Antibiotic-sterilized valves stored at 4° C in nutrient media are considered to be non-viable valves.

Since 1968 homograft valves have been sterilized using antibiotics and stored in culture media at 4° C (Yacoub and Kittle, 1970). The short- and mid-term clinical results with antibiotic-sterilized valves were superior to those with chemical sterilization and similar to those with untreated fresh homograft valves (Barret-Boyes, Roche and Whitlock, 1977). Antibiotic-sterilized valves stored at 4° C are considered to have a finite storage time of about 6-8 weeks.

These grafts were all nonviable and had a high risk of cusp rupture and valve calcification and an unpredictable development of graft failure (Beach, Bowman, Kaiser, Parodi and Malm, 1972).

2.4.1.3 Cryopreserved Valves

Cryopreserved valves are valves sterilized in antibiotic solution and subsequently cryopreserved. They are considered viable if cryopreserved within 4 days of procurement (O'Brien *et al*., 1987).

The development of cryopreservation led to the resurgence of homografts. This allowed for the procurement of quality tissue for implantation both in distance and time, and allowed the development of so-called "valve Banks".

The basic concepts of homograft valve cryopreservation were initially reported by Ross in 1972 who freeze-dried, or rapidly froze sheep valves in dimethylsulfoxide (DMSO) (Savage, Jones, Thompson and Ross, 1972). Mark O'Brien in Brisbane, Australia, developed a standardized technique

of valve cryopreservation involving controlled-rate freezing after short-term antibiotic sterilization (O'Brien *et al*., 1987). This made the concept of long-term storage of viable heart valves possible and available on a widespread basis. The O'Brien technique was imported by Kirklin in Birmingham in 1981 (Kirklin, Smith, Novick, Naftel, Kirklin, Pacifico, Nanda, Helmcke and Bourge, 1993) and was further developed by a commercial cryopreservation center for the processing and nationwide redistribution of homograft cardiac valves. However, the development of valve storage by cryopreservation in liquid nitrogen in the 1970s has significantly extended storage time, probably indefinitely (Mermet, Buch and Angell, 1970). This made the availability of high-quality homograft tissue a reality.

Cryopreservation is the standard preservation technique currently in use.

2.4.2 The Effects of Preservation Techniques on Homograft Viability and Integrity

While major centers were working on the technical problems of homograft aortic valve replacement, it became apparent that preservation techniques were not only destroying the viability and cellular architecture of the homograft valve but they were also substantially altering their matrix and ground substance. Valve durability may be related to the presence of viable graft cells or the structural integrity of the collagen and elastic matrix (or both), although this issue remains extremely controversial (Gonzalez_Lavin, Spotnitz, Mackenzie, Gu, Gadi, Gullo, Boyd and Graf, 1990).

Some authors (McNally and Brockbank, 1992) have suggested that several tissue processing variables such as ischaemic time, antibiotic disinfection, cryopreservation, and thawing methods all affect viability. In spite of improved cryopreservation techniques, significant morphologic and metabolic changes may lead to implant failure.

Viability of the homograft valve is determined by the viability of fibroblasts present in the valve leaflets (O'Brien *et al*., 1987) and there is evidence that long-term durability may be determined, at least partially by viability of the valve at the time of implantation (McGiffin, O'Brien, Stafford, Gardner and Pohlner, 1988). The criteria for valve viability is when fibroblasts could be cultured from the leaflet, when living cells were seen histologically, and when the whole valve had a glucose utilization exceeding 16mg/dl/24 hour (O'Brien *et al*., 1987).

Cryopreserved homografts have shown favorable clinical results after implantation that seems to be attributable to viable fibroblasts present in the valve (O'Brien *et al*., 1988). Viable fibroblasts synthesize the main components of the extracellular matrix: collagen, elastin, reticulin, and mucopolysaccharides; therefore the longevity of the implantation is likely to be related to the viability of fibroblasts in the implanted valve (Van der Kamp and Nauta, 1979).

However, cryopreservation can damage cells and thereby can affect cell viability. The process of cryopreservation is relatively complex involving many variables; harvesting (warm and cold ischaemic times), sterilization (antibiotics, including antifungal media for 24 hours), freezing (fluid shifts and ice crystal formation), storage and thawing. Each of these steps involves the potential for cellular injury (Gall *et al.,* 1995).

There is a great deal of controversy regarding the presence of viable fibroblasts within the allograft leaflet matrix at the time of implantation. Evidence suggests that cryopreserved valves viable at the time of cryopreservation have a much lower level of structural deterioration than nonviable valves. Yacoub *et al.,* (1995) also observed that valves with a larger degree of fibroblast viability had an improved long-term durability. Cellular viability is also affected by multiple combinations of antibiotics with or without fungal drugs to obtain a sterile graft for implantation. It

has been observed that cryopreservation after a period of antibiotic sterilization significantly reduces viability and the use of antifungal drugs reduces this viability even further (Goffin *et al*., 1996).

On the other hand Wheatley and McGregor (1977), importantly showed that there is a pronounced immunological response to homovital valves, suggesting that intact viable endothelium evokes a normal host-graft reaction that leads to immune mediated early deterioration of allografts. The results of this study indicated that pre-implantation viability results in gross valve distortion and shrinkage with consequent loss of function. Nonviable valves, in contrast, showed minimal alteration in valve dimensions with retention of normal function. These findings have considerable implications in the preparation and clinical use of allograft heart valves (Wheatley and McGregor, 1977).

This finding suggests that a certain degree of endothelial destruction might be beneficial for graft survival. These findings also suggested that very fresh homovital valves might require immunosuppressant therapy to enhance long-term graft survival.

Wheatley's findings suggest that there is a degree of autolysis or process related damage to endothelium, that benefits graft survival. Presumably this correlates with retention of the tissue scaffold of the graft (Wheatley and McGregor, 1977).

Cryopreservation remains the most commonly used method for valvular preservation and storage.

2.4.3 Methods for Evaluating Homograft Viability and Integrity

Homografts can be examined for their viability (with dye exclusion method during light microscopy), for their ultra structural morphology (by

transmission electron microscopy), for their cellular detail (by hematoxylin and eosin staining) and for their endothelial function (with pharmacological analysis) (Pompilio, Polvani, Antona, Rossoni, Guarino, Porqueddu, Buche, Biglioli and Sala, 1996). However the two most widely used techniques are transmission electron microscopy and the hematoxylin and eosin stain.

2.4.3.1 Electron Microscopy (EM)

Electron microscopy is used to assess the extent of cellular damage. A set of criteria proposed by Crescenzo and colleagues are followed to evaluate cellular damage: that is cytoplasmic oedema, dilation of endoplasmic reticulum, mitochondrial swelling, (as signs of reversible cellular injury), and mitochondrial flocculent densities, karyolysis, and disrupted plasma membrane (signs of irreversible cell injury) (Crezscenzo *et al*., 1992).

2.4.3.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) proved to be a valuable tool for investigating biological surfaces. In the vascular wall and the cardiac valves, it is especially suitable for detection of fine changes in endothelial covering and underlying layers (Figure 2.4.1 Heart valve X140 magnification; Figure 2.4.2 heart valve x1200 magnification) (Feng, van Hove, Mohan, Walter and Herman, 1993; Hammon, O'Sullivan, Oury and Fosburg, 1974; Páral, Ferko, Měřička, 2000).

Both sides of the pulmonary and aorta valve leaflets are covered by endothelial cells, arranged in the circumference of the valve mechanism. The pattern of the endothelial cells and the arrangement of the collagen fibers may serve as an indicator for the organizing leaflet structure. Scanning electron microscopy is regarded as a valuable tool

to detect changes in the endothelial covering, basement membrane covering and supporting collagen layers of the valve leaflets and arterial wall (Figure 2.4.3)(Krs, Burkert, Slizova, Kobylka and Spatenka, 2006).

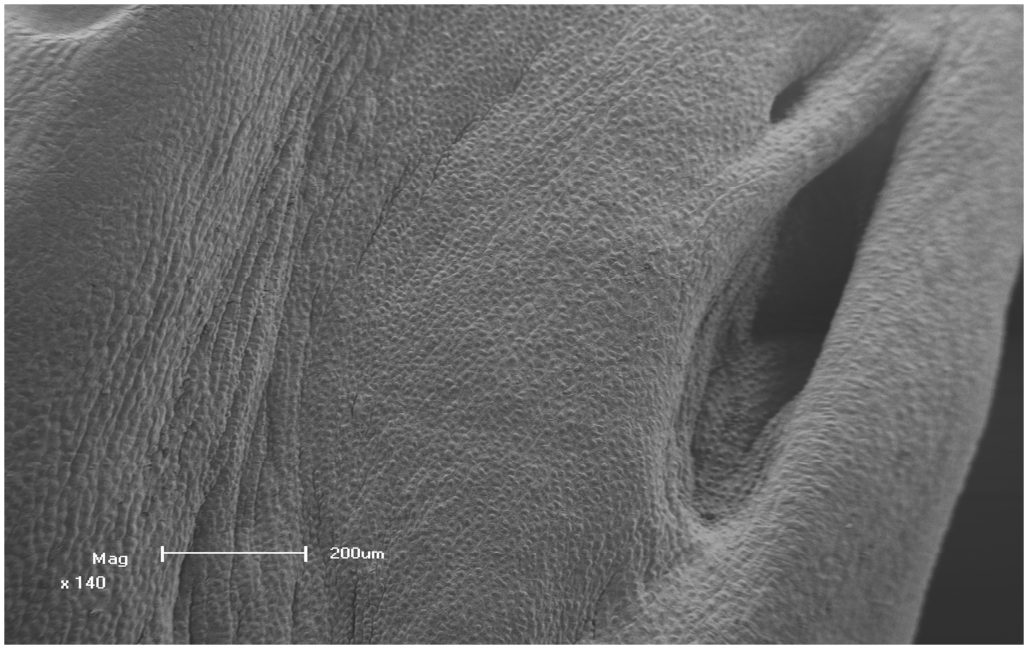


Figure 2.4.1 Aortic valve leaflet (x140 magnification)

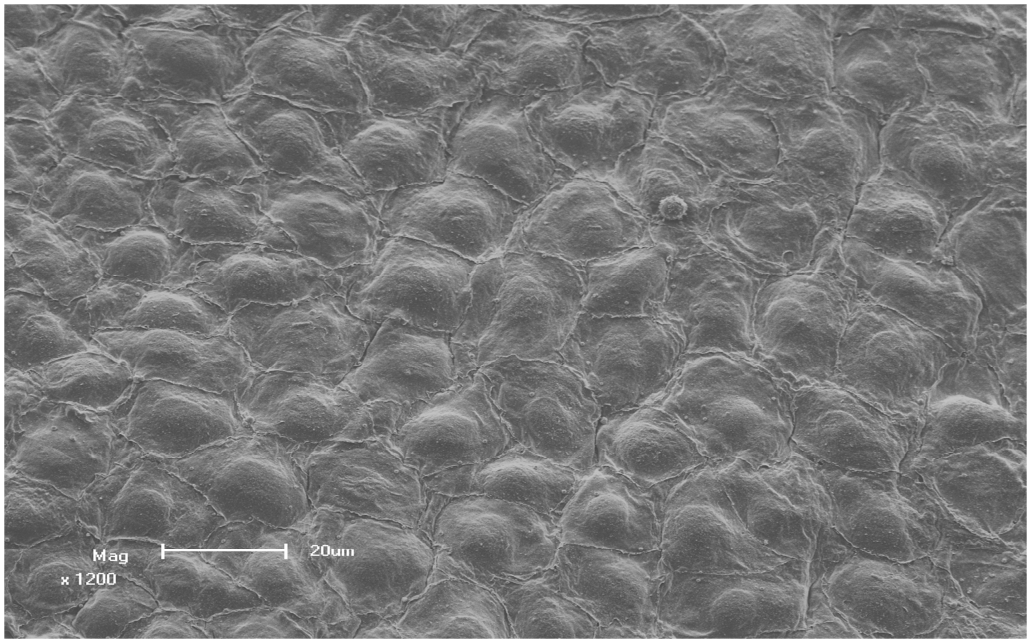
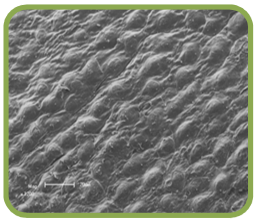
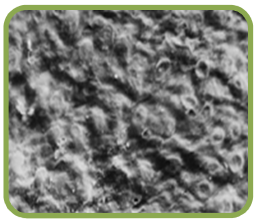


Figure 2.4.2 Aortic valve leaflet (x1200 magnification)



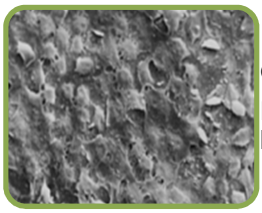
CATEGORY VI

Morphological intact endothelium – putative physiological changes are not reflected in the superficial morphology of endothelial cells



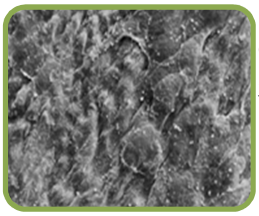
CATEGORY V

Confluent endothelium with structural inhomogenity – irregularities in the form of individual cells and changes of their membranes are detectable.



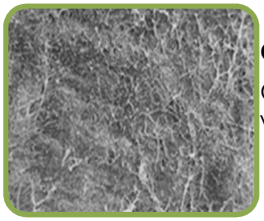
CATEGORY IV

Disruption of intercellular contacts – continuity of endothelial coverage is lost, endotheliocytes shrink while still adhering to the basal membrane.



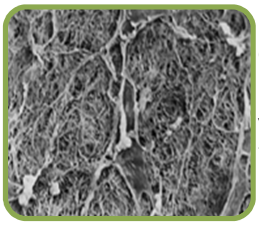
CATEGORY III

Separation of endothelial cells – endotheliocytes separate from the basal lamina. Initially they protrude by their intercellular edges into the lumen; later it is possible to observe patchy stripping of endothelium.



CATEGORY II

Complete loss of endothelium – denudation of the endothelial covering with the basal lamina exposed.



CATEGORY I

Damage of subendothelial layers – the valvular surface is covered only with remnants of basal membrane, the fiber structure of the lamina fibrosa and the lamina ventricularis may be dissolved.

Figure 2.4.3 **Criteria Scanning Electron Microscopy** (adapted from Krs *et al.*, 2006)

2.4.3.3 Haematoxylin and Eosin Staining (H&E)

The haematoxylin and eosin stain is probably the most widely used histological stain. It is essential for recognizing various types and morphologic changes that form the basis of contemporary cancer diagnosis. Its popularity is based in its ability to demonstrate clearly an enormous number of different tissue structures like cytoplasmic, nuclei and extracellular matrix features (Gamble, 2008).

Haematoxylin has a deep blue colour and stains nucleic acids where eosin stains proteins non-specifically (Figure 2.4.4). In biological tissue the colour indicators for specific structures are:

Table 2.4.1 Haematoxylin and Eosin staining characteristics (Gamble, 2008)

Tissue structure	H&E staining colours
Nuclei	Blue
Cytoplasm	Varying degrees of pink
Extracellular matrix	Varying degrees of pink

Considerable detail is revealed in well-fixed cells. The nuclei show varying degrees of cell-type- and cancer-type specific patterns (heterochromatin consideration) that are diagnostically very significant. Nucleoli are stained by eosin. When there is an abundance of polyribosomes the cytoplasm will have a distinct blue cast.

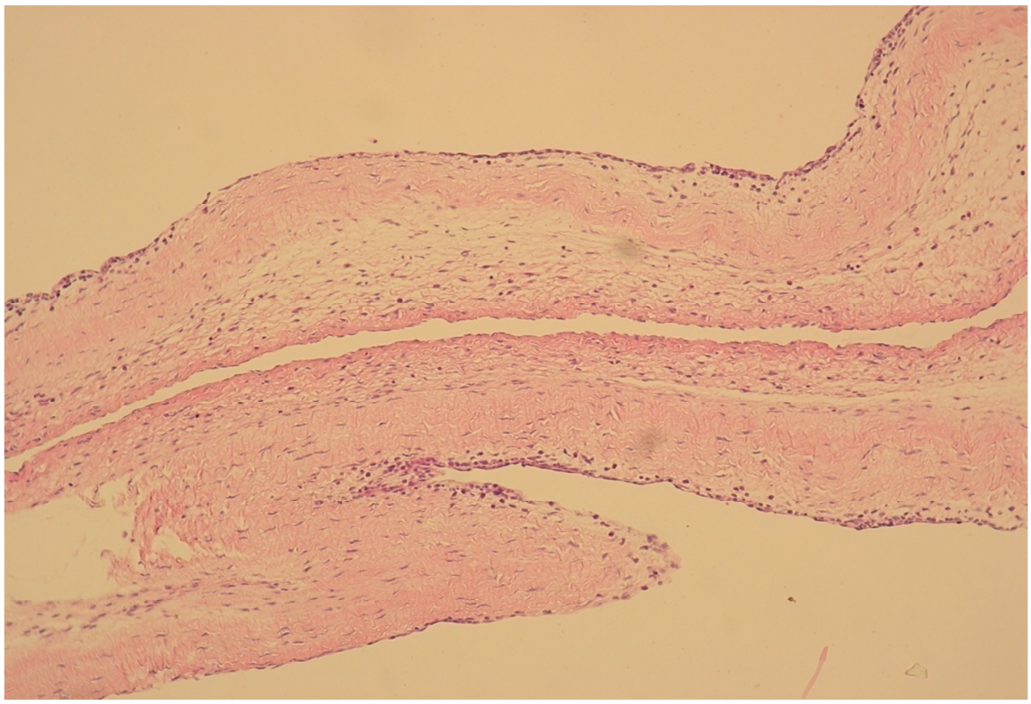


Figure 2.4.4 **Aortic valve leaflet** (H&E stained – 40x magnification)

Therefore, the stain discloses abundant structural information, with specific functional implications. On the other hand a limitation of Hematoxylin staining is that it is incompatible with immunofluorescence (Gamble, 2008).

2.4.3.4 Glucose Utilization Test

According to O' Brien (1987) the viability can be assessed with a glucose utilization test. A homograft is considered viable when the whole valve has a glucose utilization exceeding 16mg/24 hour.

2.4.3.5 Tensile strength

One of the most common testing methods called tensile testing is used to determine the 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, papers, rubbers etc and for the determination of tissue strength in the medical field (Akhyari, Fedak, Weisel, Lee, Verma, Mickle and Li, 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 (Figure 2.4.5).

Mechanical properties of tissue can be investigated by using a tensile testing machine, where the tissue sample is fixed between clamps at both ends. The fixed tissue is gradually pulled apart (0.1mm/s) by applying constant pressure on the two ends, and the data recorded on a personal computer (Thubrikar, Deck, Aouad and Nolan, 1983).

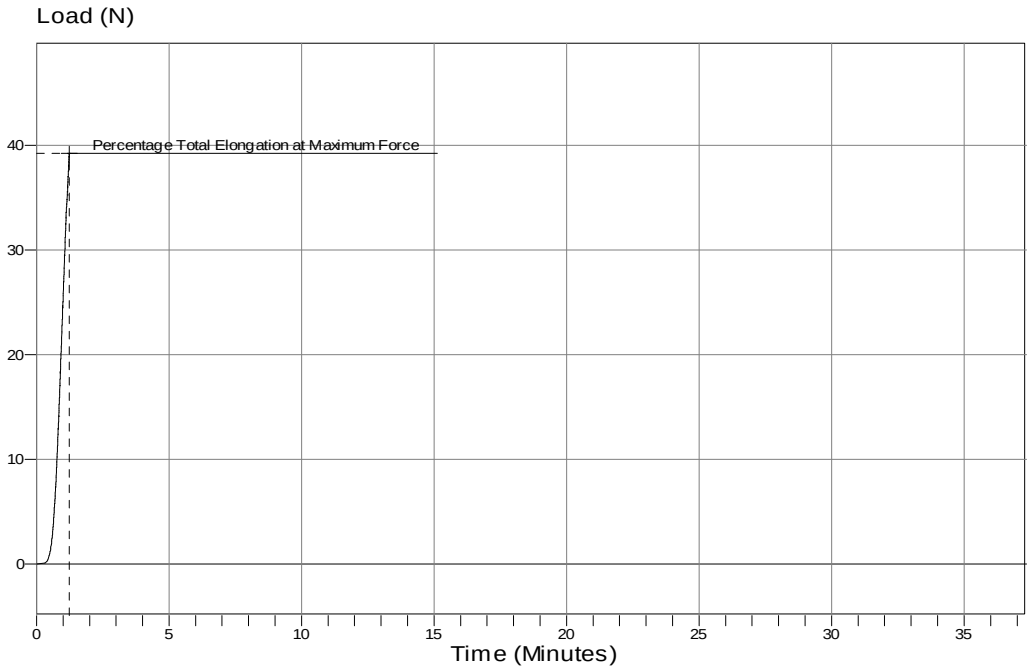


Figure 2.4.5 Tensile strength curve (Load (N) / Time (minutes)

2.4.3.5.1 Tensile stress and strain

By measuring the average force exerted per unit area, stress can be measured. The total internal forces acting in a body, as a reaction to an external applied force gives a measurement for stress. The unit for stress is Pascal (Pa) which translates to one Newton (N) force applied per square meter (m^2) area (Sasaki and Odajima, 1996).

The deformation of a material by the action of stress is called strain. Strain is expressed as the change in shape or size of an object, by measuring the difference between two points, one in the unchanged state before deformation and one in the changed state after deformation gives us a measure of strain (Figure 2.4.6).

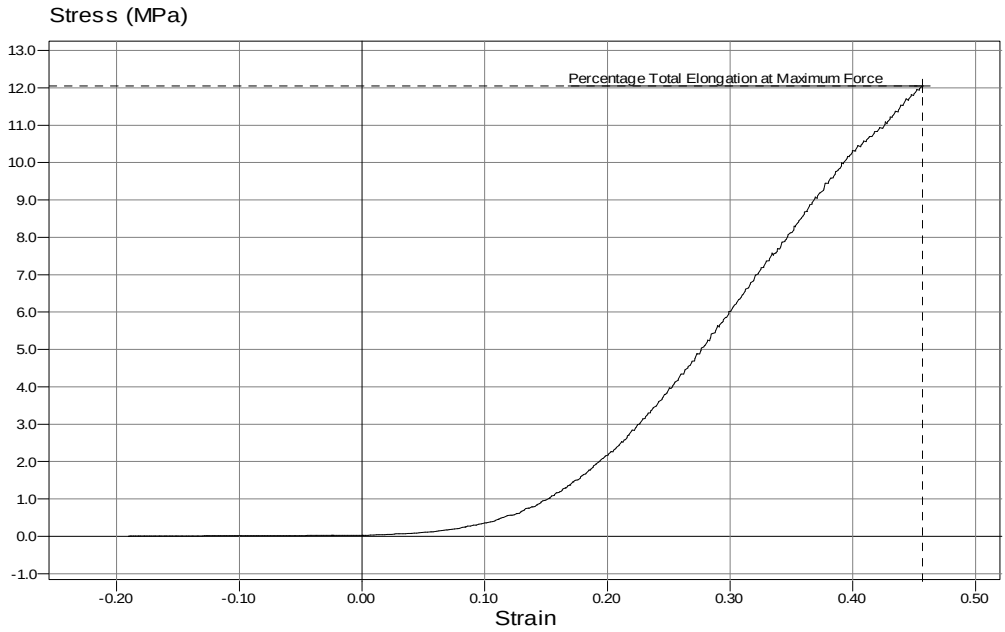


Figure 2.4.6 Stress-Strain curve

2.4.3.5.2 Young's Modulus

Modulus of elasticity (or Young's Modulus) is a measurement of the rate of change of strain as a function of stress (Figure 2.4.7). It represents the

slope of the straight-line portion of a stress-strain curve. With respect to tensile testing, it may be referred to as tensile modulus. This method of testing is used to determine a sample's behavior under an axial stretching load. Common tensile test results include elastic limit, tensile strength, yield point, yield strength, elongation, and Young's modulus. Young's Modulus is reported commonly as N/mm^2 (lbs/in^2), MPa (psi).

Therefore, Young's modulus can be determined from the slope of a stress/strain curve that was created from a tensile strength test of a piece of material (Figure 2.4.8).

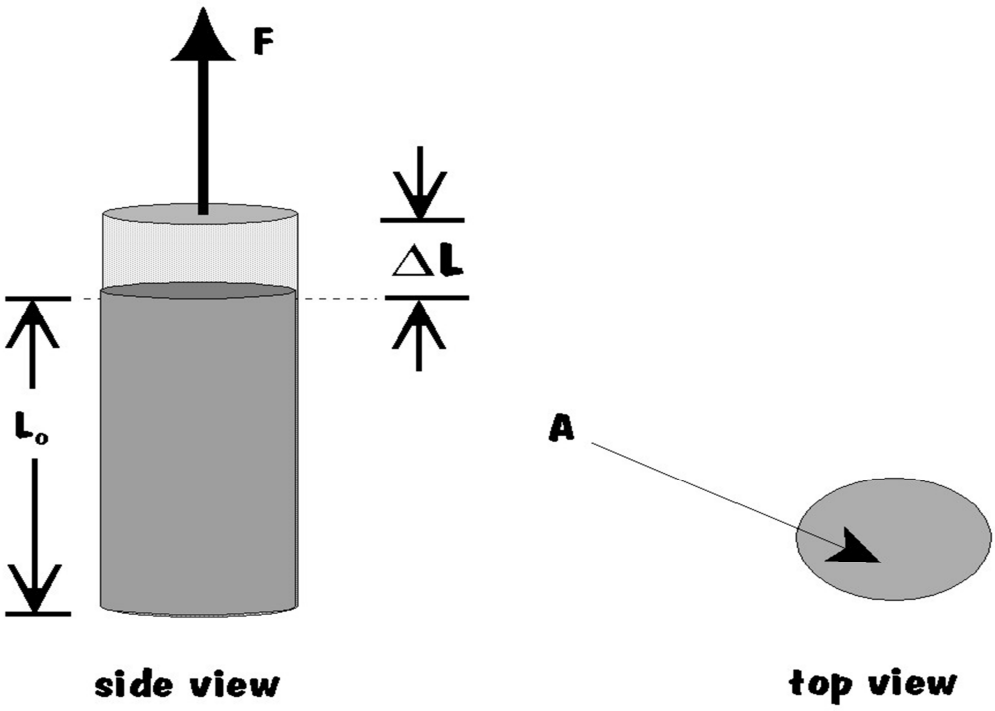


Figure 2.4.7 Diagrammatic illustration of tensile stress (F) applied to tissue sample.

A) Quantities for defining Young's Modulus (Pukacki *et al.*, 2000).

- The applied force **(F)**
- The cross sectional area **(A)**
- The Initial, unstressed length (L_0)
- The change in length due to stress **(ΔL)**

B) Define stress and strain

Stress **(S)** is the force per unit area

$$S = \frac{F}{A}$$

Stain **(e)** is the fractional change in length

$$e = \frac{\Delta L}{L_0}$$

Young's modulus (E) is defined as the ration of stress to strain

$$E = \frac{\text{Stress}}{\text{Strain}} = \frac{S}{e} = \frac{F/A}{\Delta L/L_0}$$

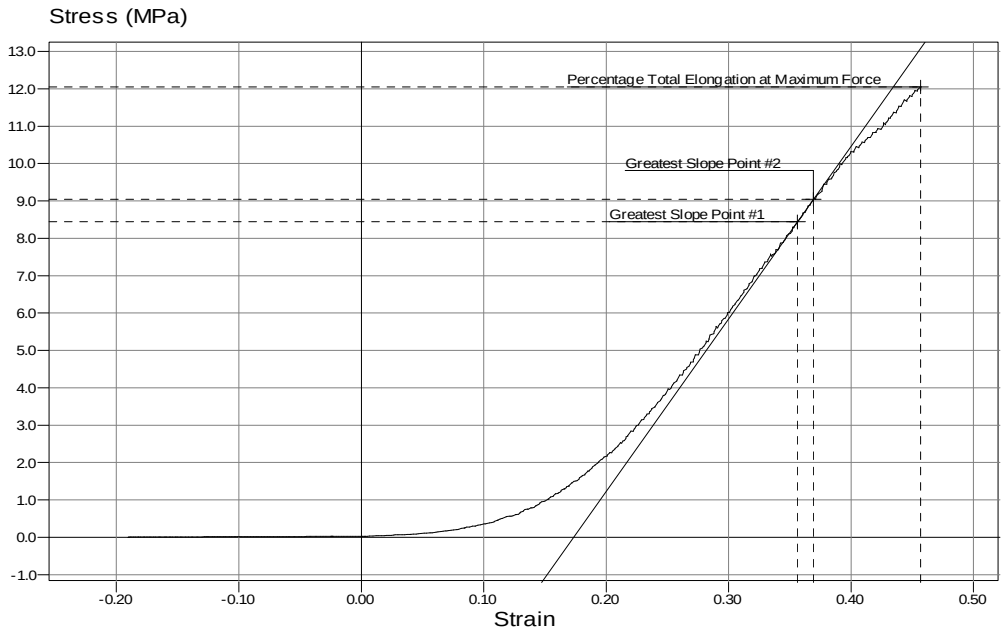


Figure 2.4.8 **Stress-strain curve** (determining Young's Modulus)

2.4.3.6 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is a technique which is part of a group of techniques called Thermal Analysis (TA) (Ma and Harwalkar, 1991). TA is based upon the detection of changes in the heat content (enthalpy) or the specific heat of a sample with temperature. 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.

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 and the heat of the reaction. Such enthalpy changes may be detected by thermal analysis and related to the processes occurring in the sample.

In DCS, the measuring principle is to compare the rate of heat flow to the sample and to an inert material which is 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 below the peak is directly proportional to the enthalpy 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: transition temperature peak (T_p) or maximum (T_{max}) or denaturation (T_d) 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, Moor and Blanc, 1977) and protein concentration (Wright, 1984).

2.5 APPLICATIONS OF HOMOGRAFTS

Despite the technical problems with valve availability, sterility, and preservation, homograft assessment continued through the 1960s. This was because of the difficulties encountered with alternative artificial valves which include thromboembolism, hemorrhage, infection, hemolysis, mechanical failure, and impaired hemodynamic function (Stelzer and Elkins, 1989). The homograft valve had an excellent beginning in the aortic position, but its use in the mitral position was delayed. The particular challenges of the mitral valve led to the development of various support structures upon which homograft valves could be mounted for use in either the semilunar or the atrioventricular position. In 1967 Angell and associates at Stanford demonstrated the use of homografts for

double valve replacement (Angell *et al*., 1968). The first patient in their series underwent aortic valve replacement with an unstented, fresh homograft valve and mitral valve replacement with a stented fresh aortic homograft. Shortly thereafter, in July 1967, they performed their first triple valve replacement with aortic homografts, commenting that the heart sounds were indistinguishable from those of a normal heart. In 1968 Ionescu reported in 63 patients the use of these mounted valves which had been sterilized and preserved with a 4% buffered formaldehyde solution (Ionescu, Wooler, Whitaker, Smith, Taylor and Hargreaves, 1968).

The fresh aortic homograft became the preferred replacement in either isolated or combined valve disease (Stinson, Angell and Shumway, 1968). During a period of 14 months, the Stanford group implanted homograft valves in a series of 93 patients including 6 multiple valve replacements. The early results were encouraging and led to the bold conclusion that if valve supply is adequate, sterility is guaranteed, and proper cusp orientation and support are assured, the ideal valve replacement is available in the fresh aortic homograft (Angell *et al*., 1968).

Because of their superior hemodynamics and excellent durability, especially in younger patients, the homograft valve has been established as an excellent aortic valve replacement prosthesis. With the use of homograft valves there is no need for the patient to commit to lifelong anticoagulation treatment (Ross, 1962).

2.5.1 Indications

- ☑ Females of reproductive age
- ☑ Any patient with bacterial endocarditis affecting the aortic valve
- ☑ Younger patients with a small aortic root
- ☑ Patients with a bleeding diathesis
- ☑ Patients in whom anticoagulation is contra-indicated

- ☑ Homograft valves are less expensive (Langley, Livesey, Tsang, Barron, Lamb, Ross and Monro, 1996).

2.5.2 Contra-Indications

- ☒ Patients with moderate to severe hypertension
- ☒ Patients with dilated or distorted aortic root
- ☒ Patients with associated coronary disease
- ☒ Patients with poor left ventricular function (Langley *et al*., 1996).

2.5.3 Clinical Applications

2.5.3.1 Adult Cardiac Surgery

- Aortic valve replacement with homograft valves
- Pulmonary autograft aortic valve replacement
- Homograft replacement of atrioventricular valves
- Aortic root replacement using homograft conduit (Fontan, Choussat, Deville, Doutremepuich, Coupilaud and Vosa, 1984).

Figure 2.5.1 **Surgical repair using various components of the aortic homograft (A)** Mitral valve repair, **(B)** Aortic root replacement **(C)** Mycotic aneurysm (adapted from Frank, Mavroudis, Backer and Rocchini, 1998).

2.5.3.2 Pediatric Cardiac Surgery (Congenital Defects)

- Right ventricular outflow tract reconstruction

Figure 2.5.2 **Ross-Konno procedure in neonates** (The left ventricular outflow tract obstruction is reconstructed using the infundibular free wall of the aorta autograft to directly close the ventriclular septal defect. The neo-aortic annulus was not reinforced, so do not limit growth. The right ventricular outflow tract was reconstructed with a right ventricle pulmonary artery valve conduit (adapted from Lacour-Gayet, Sauer, Ntalakoura, Müller, Razek, Weil and Haun, 2004).

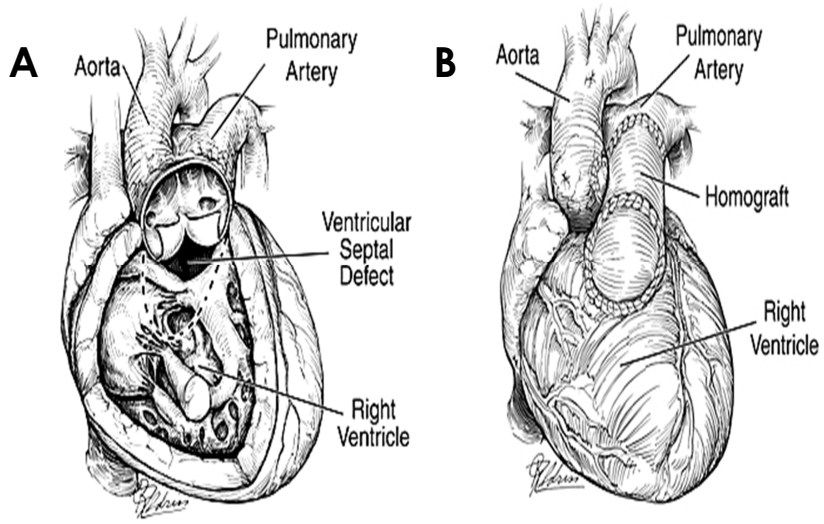


Figure 2.5.3 **A) Truncus Arteriosus defect B) Surgical repair of a Truncus Arteriosus defect using the Rastelli procedure** (adapted from Wallace, Rastelli, Ongley, Titus and McGoon, 1969)

- Pulmonary valve replacement
- Truncus Arteriosus
- Rastelli Procedure
- Hypoplastic left heart
- Fontan procedure (Fontan *et al*., 1984).

2.5.4 Surgical Complications associated with Homograft Implantation

Two of the major modes of homograft failure in aorta implants have been aortic regurgitation (AR) and valve calcification.

The AR is associated with technical factors, such as incorrect homograft sizing or valve distortion due to the scalloped technique. Late AR is most commonly linked to commissural misalignment, cusp distortion and subsequent cusp prolapse because of root enlargement. The cusp deterioration is less common and occurs later, with leaflet thickening and occasional cusp calcification (Angell *et al*., 1989).

Although homograft wall calcification is common severe aortic valvular stenosis is uncommon. In a study conducted by Kirklin and associates (1993), 112 patients with available data, 77% had a mean greater than 10mm Hg, and 94% had a mean gradient of less than 20 mm Hg (Kirklin, Blacstone, Maehara, Pacifico, Kirklin, Pollock and Stewart, 1987). Leaflet failure causing severe stenosis is relatively unusual but may occur with extensive leaflet calcification.

Some other causes of outflow obstruction can be linked to subvalvular obstruction unrelated to the homograft or, rarely, partial dehiscence of the valve (Kirklin *et al*., 1993).

2.5.5 Advantages and Disadvantages

To date, no valve is perfect; each type has specific advantages and disadvantages. Therefore, the choice of valve must be individualized to the patient and the disease process.

2.5.5.1 Advantages

- No need for anticoagulation (absence of thromboembolic events)
- Absence of haemolysis
- Lack of ring/cuff of graft support (minimizing transvalvular gradient compared to the stented prosthetic valves
- Very good haemodynamic performances
- Higher resistance to endocarditis
- Attractive in children because of growth of the valve with somatic growth (Anastasiadis *et al*., 2004).

2.5.5.2 Disadvantages

- Ethical considerations (consent)
- More demanding implantation techniques
- Difficult to access from a "Homograft Bank" (availability)
- Processing techniques results in acellular grafts, which in time degenerates and calcifies (5-15 years)
- Cost implications (1400 US dollars a piece for ad hoc preparation with an open market value that averages 5000 US dollars each).

In general the logical use of homografts in adult cardiac surgery when indicated with proper surgical technique revealed very good post-operative results and an excellent quality of life for the patient.

2.6 THE ANIMAL MODEL FOR HOMOGRAFT RESEARCH

Pulse duplicator studies of new prostheses and subcutaneous implantation of new materials in small animals are commonly used methods in cardiac research. Although these methods have become more sophisticated, the ultimate test remains the successful valve implantation in a medium-sized animal. High cost of animal experimentation necessitates the careful selection of the most applicable animal model and application of the most appropriate surgical techniques during this kind of research. The dog has been the traditional model for this type of research, but because of the strong opposition of the antivivisectionist lobby, other medium-sized animals have been used.

Sheep were selected as the animal of choice because they have similar valve anatomy to humans, are readily available, and their care is simple, ensuring low cost and ease of handling. They are also available in a wide selection of body weights and sizes (Ali, Kumar, Bjornstad and Duran, 1996).

On the other hand a study performed on rat models indicated that the endothelium was viable for at least 40 hours postmortem (Yankah and Hertzer, 1987).

2.7 RELEVANCE OF THE STUDY

Homograft valves are susceptible to calcification and structural degeneration in the long term. The pathophysiological mechanism is not fully understood. Immunologic phenomena may contribute to the degeneration process (Mitchell, Jonas and Schoen, 1995). It has been suggested that the presence of some viable endothelial cells at the implantation time may have a delaying effect in the calcification process and also might be a factor in the long term survival of cryopreserved homograft valves.

There has been extensive evaluation on the viability effects of storage of porcine heart valves in nutrient rich media at 4°C or in liquid nitrogen at different temperatures. There is a great deal of variability in the World regarding time dependent damage of valvular endothelial cells after periods of warm ischaemia (Pompilio, Polvani, Rossoni, Porqueddu, Berti, Barajon, Petruccoili, Guarino, Aguggini, Biglioli and Sala, 1997).

Worldwide there is great diversity regarding the optimum harvesting time of homograft valves. Most heart valve banks require that donor tissue be harvested up to 24 hrs after death, and a few will extend the harvesting time to an absolute maximum of 30 - 36 hours. Protocols worldwide also prefer the harvesting of homografts from a brain-dead beating heart donor within a theatre. This limits the donor pool to valves explanted from transplant recipients or valves from hearts not suited for transplant surgery.

Cadaver donor pools form the backbone of the present supply to homograft banks in South Africa. These donors are also quite often identified at the state mortuary. The average time from death to harvesting in the University of the Free State homograft program is 33 hours because of restrictions imposed by medico-legal autopsies therefore exceeding the suggested 24 hour limit. Strict adherence to this time limit impact negatively on our homograft processing ability.

Despite the suggested harvest time of less than 24 hours, all programs extend ischaemic time due to the incubation, sterilization and processing before cryopreservation, which normally takes place up to 4 days for viable valves. Fresh albumin stored non viable homografts also have shown acceptable longterm results (Goffin *et al.*, 1997).

The effect of ischaemic time in the intact cadaver donor before harvesting and its impact on the outcomes of homograft quality and function therefore needs to be clearly defined.

This study attempted to produce relevant scientific data to develop objective criteria for accepting homografts for processing and cryopreservation. This might lead to the safe extension of harvesting times and an increase in the homograft donor pool.

2.7.1 Aim

To determine whether the current contraints regarding cadaver homograft harvest time can be extended.

2.7.2 Objectives

- To determine the degree of autolysis by histological evaluation of the pulmonary and aortic valve leaflets and arterial walls (H&E and SEM).

- To investigate whether a constant relationship in tensile strength and thermal denaturation temperature (T_d) exists between the aorta, pulmonary and mitral valves as well as aortic and pulmonary arterial walls and whether mitral valve or pulmonary or aortic wall samples can have reliable predictive outcomes value in harvested valves.

- To establish the relationship between ischaemic time, tensile strength and thermal denaturation temperature (T_d) values after cryopreservation.

CHAPTER 3
METHODOLOGY

3.1 STUDY LOCATION

The research study was conducted at the University of the Free State (UFS), and involved the departments of Cardiothoracic Surgery, Anatomical Pathology and the Large Animal Unit.

3.2 STUDY POPULATION

3.2.1 The number of subjects

Fresh sheep hearts (n=5) were obtained from the local abattoir to standardize baseline values for thermal denaturation temperature (T_d), H&E and SEM analyses. An additional 15 hearts were used for the standardization of tensile strength and stress-strain studies due to the high level of variance.

Thirty sheep were sacrificed to investigate *in vitro* the effects of ischaemic times on homograft heart valves.

3.2.2 Sample population and identification

Dorper sheep of 6 – 9 months of age, weighing between18 kg and 25 kg were used as animal models.

3.2.2.1 Group A

Five sheep hearts were harvested from the abattoir and transported on ice to the tissue laboratory. The pulmonary, aorta and mitral leaflet and arterial vessel walls were dissected from the hearts. These hearts served as a baseline to define normal reference ranges. The laboratory tests conducted on these samples included tensile strength (AoH wall and leaflet, PuH wall and leaflet, mitral leaflet), thermal denaturation temperature (T_d) (AOH wall and leaflet, PuH wall and leaflet, mitral leaflet), H&E (AOH wall and leaflet, PuH wall and leaflet, mitral leaflet) and scanning electron microscopy (AOH wall and leaflet, PuH wall and leaflet, mitral leaflet). An additional 15 hearts were used to establish baseline values for tensile strength.

3.2.2.2 Group B

Fifteen sheep were sacrificed to study the *in vitro* effects of cold ischaemic time at 24 h, 48 h and 72 h intervals on homograft heart valves. The sheep were sacrificed under supervision of a veterinarian by injecting an overdose of potassium chloride (20mmol/sheep) (Adcock Ingram, Johannesburg, South Africa) intravenously. The 15 sheep carcasses remained at room temperature (23°C) for 2-3 h to mimic the conditions preceding a deceased person's transportation to the mortuary. The carcass was skinned and the stomach removed. The stomach was removed to exclude the effect of the ongoing intestinal fermentation process which increases the body temperature and subsequently the rate of autolysis. The temperature in the heart was recorded by inserting a temperature probe in the thorax next to the heart. The temperature was recorded till the carcass reached 4°C or for a period 72 h. After 2-3 h the sheep was transported to a fridge (4°C) at the Department Animal Wild Life and Grassland Sciences, UFS. After a period of 24 h, sheep (n=5) were surgically opened and the hearts removed for harvesting of both the aorta

and pulmonary homografts. The same procedure was repeated with two groups of five sheep each at 48 h and 72 h post mortem.
Harvested tissues were fixed for histological (H&E) and, SEM evaluation as well as thermal denaturation temperature (T_d) testing. Tensile strength and stress / strain evaluation were performed on the selected tissue samples respectively.

3.2.2.3 Group C

Fifteen sheep were sacrificed to study the *in vitro* effect of warm ischaemic time on harvested homografts at 24 h, 48 h and 72 h time intervals. The sheep were sacrificed under supervision of a veterinarian by injecting an overdose of potassium chloride (20mmol/sheep) (Adcock Ingram, Johannesburg, South Africa) intravenously. The carcasses remained at room temperature (23°C) for 6 h to mimic the conditions preceding a deceased person's transportation to the mortuary. The stomach was not removed and the carcasses were not skinned to study the effect of the ongoing intestinal fermentation process which increases the body temperature and subsequently the rate of autolysis. The temperature in the heart was recorded by inserting a temperature probe in the thorax next to the heart. The temperature was recorded till the carcass reached 4°C or for a period 72 h. After 6 h the sheep was transported to a fridge (4°C) at the Department Animal Wild Life and Grassland Sciences, UFS. After a period of 24 h, sheep (n=5) were surgically opened and the hearts removed for harvesting of both the aorta and pulmonary homografts. The same procedure was repeated with two groups of five sheep each at 48 h and 72 h post mortem.

After the hearts were surgically removed the remaining carcasses were burned (as per standard practice) by the Large Animal Unit.

Harvested tissues were fixed for histological (H&E) and SEM evaluation, as well as thermal denaturation temperature (T_d) testing. Tensile strength and stress / strain evaluation were performed on the selected tissue samples respectively.

3.3 STUDY DESIGN

The project design was an observational cohort analytical study.

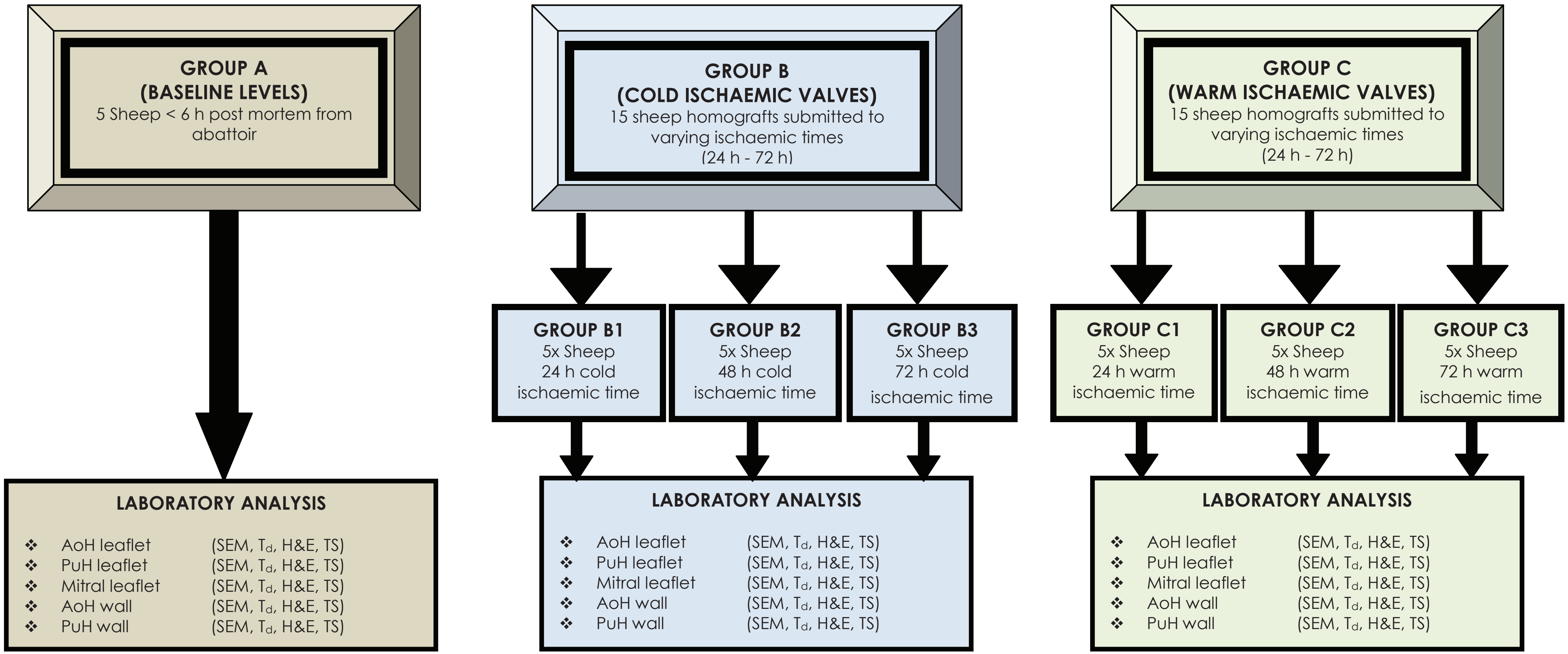


Figure 3.3.1 Study design (**Group A** – Baseline levels; **Group B** – Cold Ischaemic Times; **Group C** – Warm Ischaemic Times); Aorta (AoH); Pulmonary (PuH); Scanning Electron Microscopy (SEM); Haematoxylin and Eosin stain (H&E); Thermal Denaturation Temperature (T_d); Tensile Strength (TS)

3.4 SPECIAL INVESTIGATIONS

3.4.1 Harvesting of Homografts

This procedure was performed on Group B (n=15) and Group C (n=15)

3.4.1.1 Procurement of Homografts

- The thorax was opened at the 4th intercostal space.
- The heart was isolated and the pericardium was loosened from the sternum.
- The heart with the pericardium and as much length of the aorta and pulmonary artery were dissected out, and placed in two plastic bags and transported on ice.
- The thorax was closed with a suture to prevent spillage of blood.

3.4.1.2 Transportation

- The hearts were transported to the tissue laboratory in an insulated cold box at 4°C.

3.4.1.3 Trimming Procedure

- The heart or block of tissue was first washed in Ringers Lactate (IntraMed, Johannesburg, South Africa) to remove all traces of blood clots.
- The aortic valve with the proximal leaflet of the mitral valve and the pulmonary valve were dissected out.
- The valves were kept moist during dissection by irrigation with 500ml Ringers Lactate (Intramed, Johannesburg, South Africa).
- The coronary arteries were ligated and then cut.

- The aortic root was trimmed at a level 1cm below the lowest point of the aortic leaflets.
- A moistened finger was placed through the valve from below with the myocardium resting against the finger, which could be trimmed with the scissors to reduce the external diameter of the aortic root mass.
- The thickness of the myocardium was reduced to approximately 5 mm.
- The pulmonary valve was dissected in the same manner as the aortic valve and conduit.

3.4.1.4 Antibiotic Sterilization

- Each valve was immersed in approximately 200ml of M199 with Earle's Base (Highveld Biological (Pty) Ltd., Johannesburg, South Africa).
- The following antibiotics were added:
  - 2.5mg Fungizone (Bristol-Meyers Squibb, Johannesburg, South Africa)
  - 25mg Amikacin ((Fresenius) (Bodene (Pty) Limited trading as Intramed), Johannesburg, South Africa)
  - 50mg Cefoxitin (Sabax, Johannesburg, South Africa)
  - 50mg Piperacillin (Sabax, Johannesburg, South Africa)
- The valves were incubated for 24h at 4°C.

3.4.1.5 Cryopreservation Procedure

- After 24h of sterilization, the aortic and pulmonary valves were prepared for cryopreservation under sterile conditions.
- The Cryoson BV-9 Biological Freezer was calibrated by running Program 4 (Table 3.4.1).

Table 3.4.1 Calibrating program of the Cryoson BV-9 Biological Freezer

Program 4			
Segment No.	**Final Temperature ^{0}C**	**Runtime Segment min**	**Speed ^{0}C/min**
1	20.0	2.0	0.0
2	20.0	4.0	0.0
3	15.0	2.0	-2.5
4	15.0	4.0	0.0
5	10.0	2.0	-2.5
6	10.0	4.0	0.0
7	5.0	2.0	-2.5
8	5.0	5.0	0.0

- Two hundred millilitres of sterile Medium 199 with Earl's base were poured into a sterile bottle and 22ml dimethyl sulphoxide (DMSO) were added to the medium.
- The aortic valve was placed in an aluminium-lined cryobag.
- 100 ml of M199 with 10% DMSO was added.
- The aluminium-lined bag was de-aired and sealed.
- The same procedure was repeated for the pulmonary valve.

Note As DMSO is toxic above 10°C, the packed valves were kept in a fridge or on ice till they were cryopreserved.

- Both the valve packages were placed in the controlled rate freezer (Cryoson BV-9 Biological Freezer) which would freeze the valve at approximately -1°C/min. The freezer started with Program 22 (Table 3.4.2) followed by Programme 23 (Table 3.4.2) and 24 (Table 3.4.3) in succession.

Table 3.4.2 DMSO freezing (Program 22)

Program 22			
Segment No.	**Final Temperature ^{0}C**	**Runtime Segment min**	**Speed ^{0}C/min**
1	5.0	4.0	-3.7
2	5.0	15.0	0.0
3	-15.0	1.0	-20.0
4	-15.0	15.0	0.0
5	-35.0	7.0	-2.8
6	-35.0	1.0	0.0
7	-100.0	2.0	-32.5

Table 3.4.3 DMSO freezing (Program 23)

Program 23			
Segment No.	**Final Temperature ^{0}C**	**Runtime Segment min**	**Speed ^{0}C/min**
1	-30.0	5.0	-10.0
2	-35.0	1.0	-5.0
3	-35.0	7.0	-0.0

Table 3.4.4 DMSO freezing (Program 24)

Program 24			
Segment No.	**Final Temperature ^{0}C**	**Runtime Segment min**	**Speed ^{0}C/min**
1	-60.0	30.0	-2.6
2	-80.0	10.0	-2.0
3	-140.0	12.0	-5.0
4	-140.0	2.0	0.0

- After the freezing cycle, the valves were stored in the vapor phase of liquid nitrogen in a liquid nitrogen container (Cryoson) between 120°C-160°C.

3.5 LABORATORY INVESTIGATIONS

3.5.1 Haematoxylin and Eosin Stain (H&E)

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 over stained with Mayer's haematoxylin (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 cover slip gently placed on top and left to dry.

Table 3.5.1 Results H&E stain (Gamble, M. 2008)

STRUCTURE	COLOR
Collagen	Pale Pink
Muscle	Deep Pink
Acidophillic cytoplasm	Red
Basophilic Cytoplasm	Purple
Nuclei	Blue
Erythrocytes	Cherry Red

3.5.2 Scanning Electron Microscopy

All valves were fixed in 2.5% glutaraldehyde (Merck, Johannesburg, South Africa) overnight with a minimum of 8 hours exposure time.

Each valve cusp was divided into two specimens, sized about 3 x 6 mm. The surface area of each part was examined. Specimens of the same size were cut from the aortic and pulmonary wall, below the sinotubular junction, and scanned from the vascular site. The valve cusps were then subjected to the following:

- Rinsed in 0.1M Sorenson's phosphate buffer for 2x10 minutes
- Post-fixed with 1% Palade's osmium tetroxide (Wirsam BDH Gurr®) for 1½ hour.
- Dehydrated with a graded alcohol series (70%, 95%, and 100%) for 2 x 10 minutes.
- The samples were dried in the critical point dryer (Bio-Rad, Palaron E3100), where all the alcohol was replaced by liquid CO_2 (Affrox, Bloemfontein, South Africa) by rinsing the samples with liquid CO_2

(4x 1minute rinsing) at 10°C with a waiting time of 10 minutes between each rinse.

- The samples were heated to 37°C (CO_2 critical evaporation point) to evaporate all the CO_2.
- The processed specimens were mounted on aluminium pin stubs (SPI supplies, Rick Loveland & Associates cc, Halfway House, South Africa), coated with gold, and stored in a air tight container until studied and photographed by SEM (Shimadzu SSX 550) (Figure 3.5.1) on scanning mode operating at:

-	Accelerating voltage:	5kV
-	Probe size:	150nm
-	Working distance:	±15mm
-	Magnification:	X140
		X1200

Figure 3.5.1 Scanning Electron Microscopy (Shimadzu SSX 550)

3.5.2.1 Criteria for evaluating Scanning Electron Microscopy

A 3 category scoring system was adapted from the 6 categories described by Krs and associates (2006) to define endothelial integrity and to evaluate the quality of the surface area. Figure 3.5.2 illustrates the 3 categories.

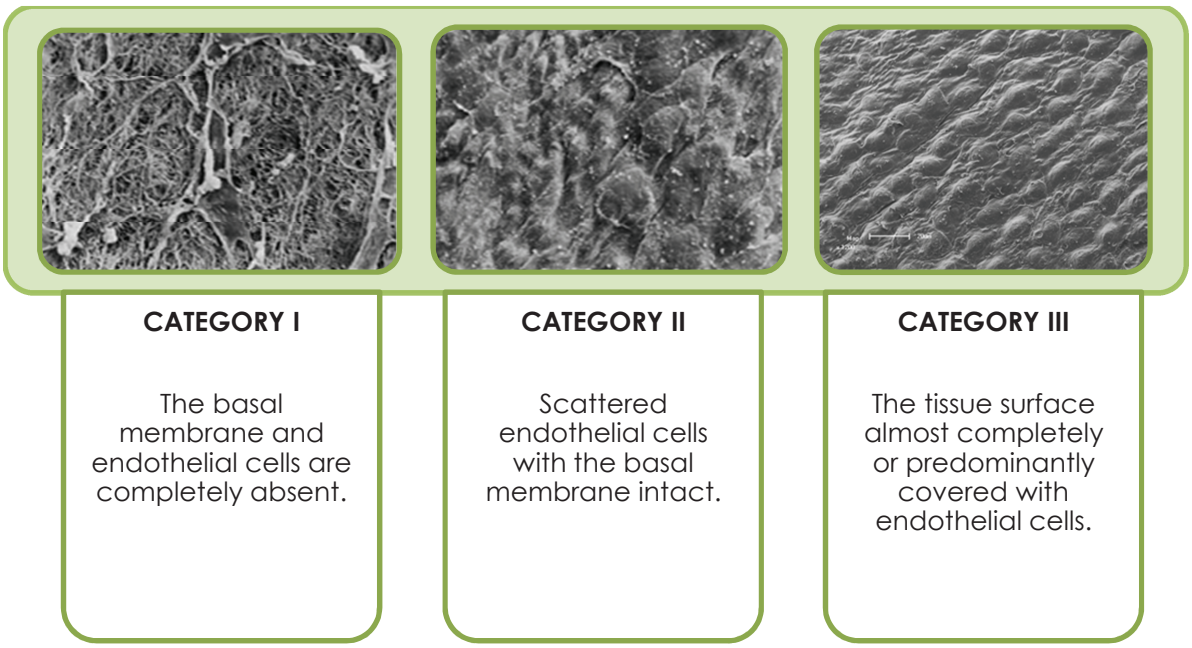


Figure 3.5.2 Modified Scanning Electron Microscopy Criteria

3.5.3 Tensile Strength

Mechanical properties of tissue samples were examined by a tensile strength testing apparatus (Figure 3.5.3), 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).

Figure 3.5.3 Lloyds LS100 Plus Tensile strength tester

- The following tissue samples were taken:
  - ❖ the right-coronary leaflet of each Aorta valve,
  - ❖ a segmental ring of the aortic wall (7-8mm above the leaflet commissure),
  - ❖ the largest leaflet of each pulmonary valve,
  - ❖ a segmental ring of pulmonary wall (7-8mm above the leaflet commissure) and
  - ❖ part of the mitral leaflet.
- The valve leaflets were cut in the circumferential direction.
- The thickness, height and width were measured with the Mitutoyo Digimatic digital calliper (Model CD-6'CX with rated accuracy of 0.02mm).
- Each strip was tested on a universal testing machine (Lloyds LS100 Plus, IMP, Johannesburg, South Africa).

- The Lloyds machine consisted of a moveable crosshead which applied a fixed rate of deformation, a load cell for detecting the forces within the specimen and a chart recorder which recorded these variables.
- The tissue sample was mounted at a nominal gauge length of 6mm and held between two manually tightened steel grips lined with no. P120 waterproof sandpaper to prevent spillage.
- The upper grip was attached to the crosshead load cell (50N).
- The rate of extension was kept constant with all the leaflet samples: preload/stress 0.1N, preload/stress speed 5mm/min, extension rate 3mm/min.
- The ring samples were tested by clamping two 5mm hexagonal keys in the vice-grip claps. The ring was placed over the hexagonal keys and extended.
- The rate of extension for the aortic and pulmonary rings: preload/stress 0.1N, preload/stress speed 10mm/min, extension rate 10mm/min.

The following parameters were measured and calculated:

A) Tensile strength/stress

$$\sigma = \frac{F}{A}$$

σ = tensile stress

F = Tensile force

A = Cross-sectional area

B) Young's Modulus

Young's modulus (E) is defined as the ration of stress to strain

$$E = \frac{\text{Stress (S)}}{\text{Strain (e)}}$$

3.5.4 Thermal Denaturation Temperature (T_d)

Samples from the aorta, pulmonary and mitral valves leaflets and artery walls (Group A (n=5), Group B (n=15), Group C (n=15)) were analyzed to establish denaturation temperature (T_d).

A small tissue sample was placed in a hermetically sealed pan of a differential scanning calorimeter (Mettler Toledo, DSC 822e, Microsep, Johannesburg, South Africa)(Figure 3.5.3) and subjected to thermal analysis. The temperature was raised at a rate of 10°C/min from 25°C to 95°C, and the temperature of thermal denaturation for each sample was electronically recorded as a peak maximum (Lovekamp & Vyavahare, 2001).

Figure 3.5.4 Mettler Toledo, Differentail Scanning Calorimetry 822e

3.6 ETHICAL ASPECTS AND GOOD CLINICAL PRACTICE

3.6.1 Ethical Clearance

The study protocol was submitted to the Ethical Committee of the University of the Free State in to gain ethical approval before the study commenced. The Ethics Committee approved the animal studies under the Protocol number: **Animal experiment number 11/06.**

3.6.2 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.

3.6.3 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 sheep 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.

CHAPTER 4
RESULTS

4.1 INTRODUCTION

All numerical results are presented in table format. Histology (H&E and Scanning Electron Microscopy), thermal denaturation temperature (T_d), tensile strength and Young's modulus are demonstrated by representative images and graphs.

Results are categorised according to the ischaemic times and temperatures. Group A (control group – < 6 h Ischaemic Time at 4°C), Group B (Cold Ischaemic Time – 24 h, 48 h, 72 h at 4°C), and Group C (Warm Ischaemic Time – 24 h, 48 h, 72 h at room temperature (23°C) for 6 h then transported to fridge at 4°C for 18 h, 42 h and 66 h). The samples were assessed in the following sequence: a) the influence of ischaemic time on valvular strength and morphology assessed by thermal denaturation temperature (T_d), Tensile Strength (TS), Scanning Electron Microscopy (SEM) and light microscopy (H&E).

Statistical data analyses include Fisher's Exact Test (electron microscopy and histology), T-test (thermal denaturation temperature (T_d) and tensile strength) and the Mann Whitney test (Young's modulus).

4.2 THE INFLUENCE OF ISCHAEMIC TIME ON HARVESTED VALVULAR HOMOGRAFTS

4.2.1 Temperature

Figure 4.2.1 indicates the mean temperature decline of warm and cold ischaemic groups from death till homograft procurement. After euthanasia, the carcasses

(Group B) were placed in a temperature controlled cold room at 4°C. A temperature probe was placed inside the thoracic cavity next to the heart to continuously measure cooling of l the carcasses to 4°C. Group C was exposed to room temperature (23°C) for six hours before the carcasses were transported to the cold room and monitored until it reached 4˚C.

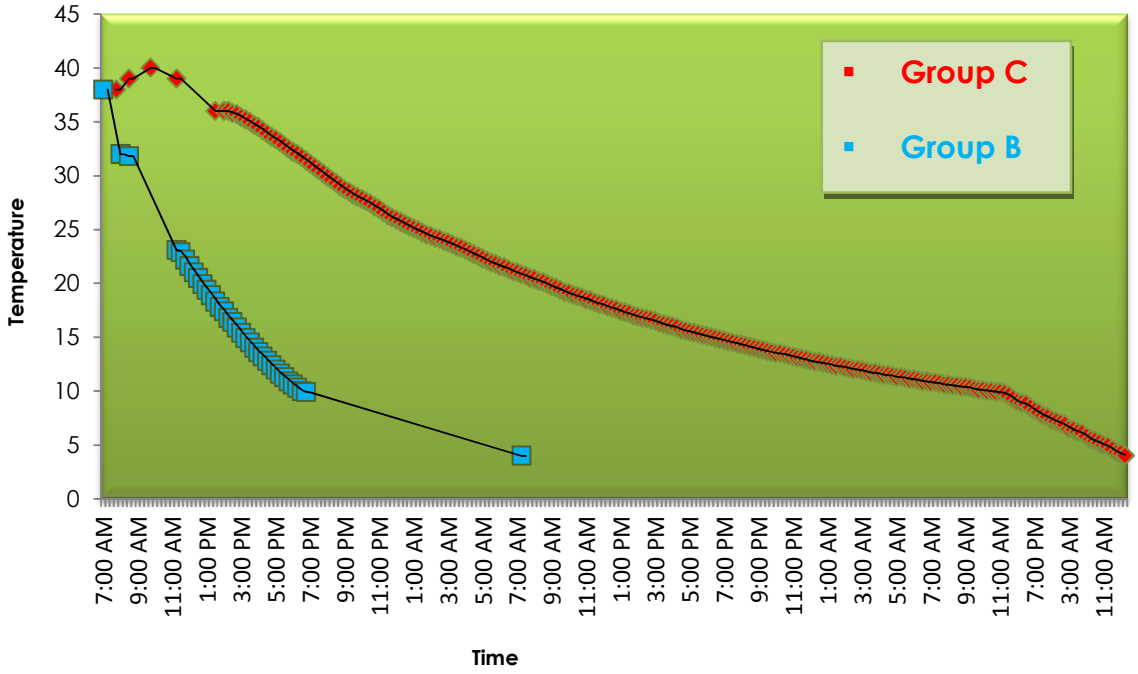


Figure 4.2.1 **Mean temperature decline of sheep carcasses after euthanasia** (Group B (blue) (STD 0.65˚C) and Group C (red) (STD 0.43 ˚C).

4.2.2 Thermal Denaturation Temperature (T_d)

The DSC technique allows for the derivation of the heat capacity of proteins as a function of temperature [thermal denaturation temperature (T_d) measured in degrees of Celsius (°C)]. Figure 4.2.2 demonstrates the peak temperature at which the triple helix protein molecules unfold during DSC of the pulmonary leaflets in Group A, B, and C.

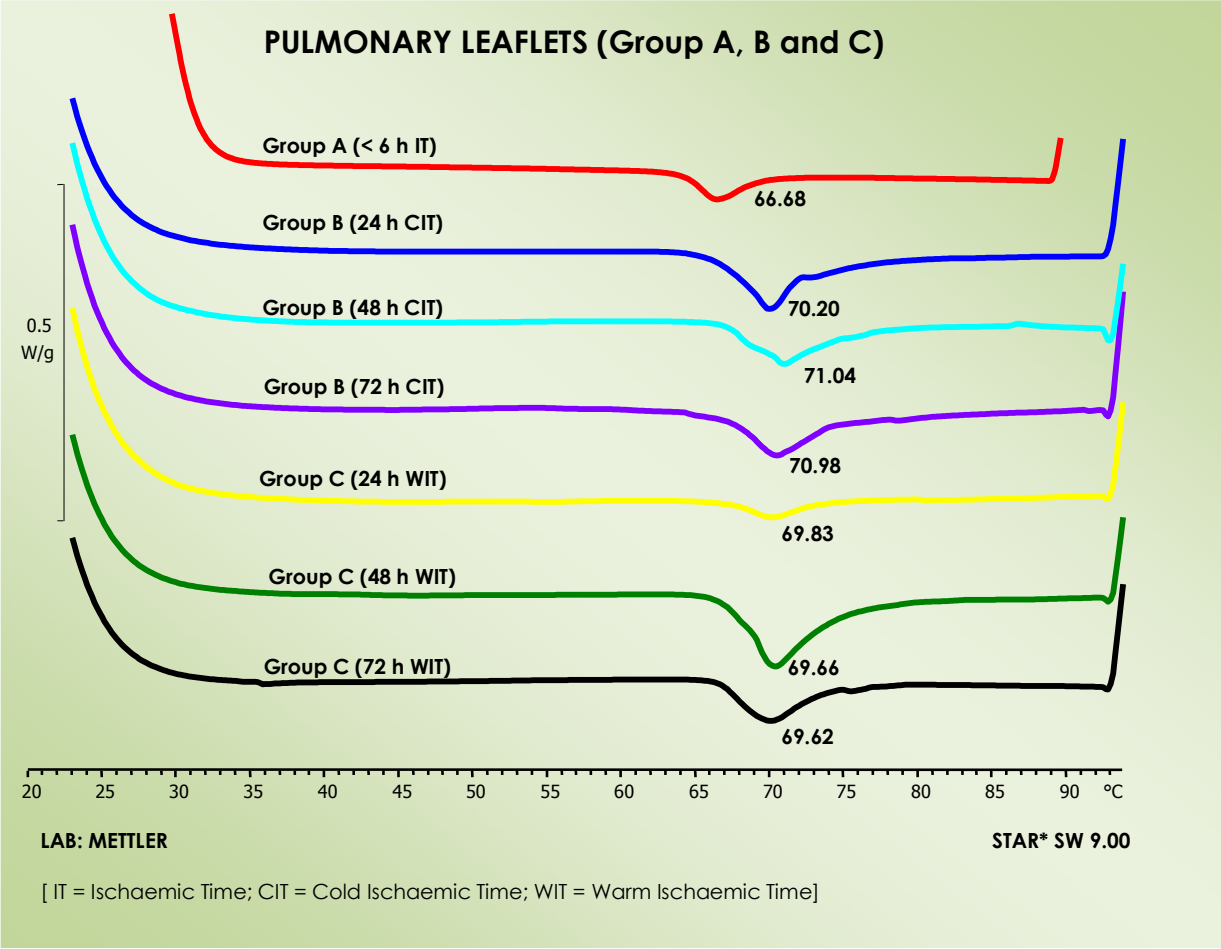


Figure 4.2.2 Example of thermal denaturation temperature (T_d), graphs for pulmonary leaflets

Table 4.2.2 represents the thermal denaturation temperature (T_d) results for Group A (< 6 h Ischaemic Time), Group B (24 h, 48 h, 72 h Cold Ischaemic Time) and Group C (24 h, 48 h, 72 h Warm Ischaemic Time). Statistical significant differences of group and inter-group comparisons in relation to varying harvesting times are discussed under 4.2.7.

Table 4.2.2 Thermal denaturation temperature (T_d) for Group A, B and C

	GROUP A	GROUP B			GROUP C		
n	5	5	5	5	5	5	5
TIME	< 6 h	24 h	48 h	72 h	24 h	48 h	72 h
AORTIC LEAFLET							
MEAN	66.95	70.16	69.83	70.61	70.70	69.22	69.58
STD	0.42	0.83	0.47	0.85	1.31	0.88	1.12
MINIMUM	66.46	69.2	69.39	69.85	69.55	67.98	68.67
MAXIMUM	67.38	71.47	70.62	71.73	72.94	70.12	71.5
MEDIAN	66.95	70.04	69.7	70.14	70.33	69.67	69.17
PULMONARY LEAFLET							
MEAN	67.16	70.25	71.25	71.01	70.74	69.60	69.63
STD	1.31	0.76	1.17	0.36	0.80	0.76	0.40
MINIMUM	66.1	69.24	69.65	70.53	69.95	68.28	69.28
MAXIMUM	69.35	71.38	72.7	71.41	71.61	70.15	70.32
MEDIAN	66.68	70.2	71.04	70.98	70.48	69.91	69.49
MITRAL LEAFLET							
MEAN	68.32	71.19	69.99	71.28	71.76	70.35	69.08
STD	0.92	1.02	0.63	0.65	3.27	1.46	0.71
MINIMUM	67.46	69.62	69.41	70.68	69.47	68.55	68.48
MAXIMUM	69.74	72.31	70.71	72	77.39	72.59	70.3
MEDIAN	68.33	71.21	69.68	70.93	70.55	70.03	68.93
AORTIC WALL							
MEAN	69.68	70.40	69.96	70.81	70.93	70.36	69.95
STD	0.49	0.29	0.55	0.94	0.87	0.46	0.75
MINIMUM	69.15	70.07	69.16	69.38	69.83	69.69	69.21
MAXIMUM	70.39	70.79	70.71	71.63	72.21	70.83	71.05
MEDIAN	69.47	70.45	69.94	70.96	70.85	70.31	69.65
PULMONARY WALL							
MEAN	70.57	70.57	71.19	72.44	71.12	71.36	70.02
STD	1.03	1.12	1.13	2.85	1.15	1.01	0.59
MINIMUM	69.43	68.8	69.75	70.78	69.53	70.81	69.47
MAXIMUM	71.95	71.49	72.75	77.5	72.58	73.15	70.94
MEDIAN	70.3	71.15	70.9	71.16	71.21	70.91	70.03

[GROUP A = Control Group (< 6 h Ischaemic Time); GROUP B = Cold Ischaemic Time (24 h, 48 h, 72 h); GROUP C = Warm Ischaemic Time (24 h, 48 h, 72 h); T_d = Thermal Denaturation Temperature (°C)]

4.2.3 Tensile Strength

Tensile strength testing was performed to determine the breakage point measured in MPa of the valve leaflets and the arterial wall tissue (Figure 4.2.3a). Tensile strength

testing lends itself to greater variance due to operational difficulties (small sample size and slippage of valve leaflets (Figure 4.2.3b).

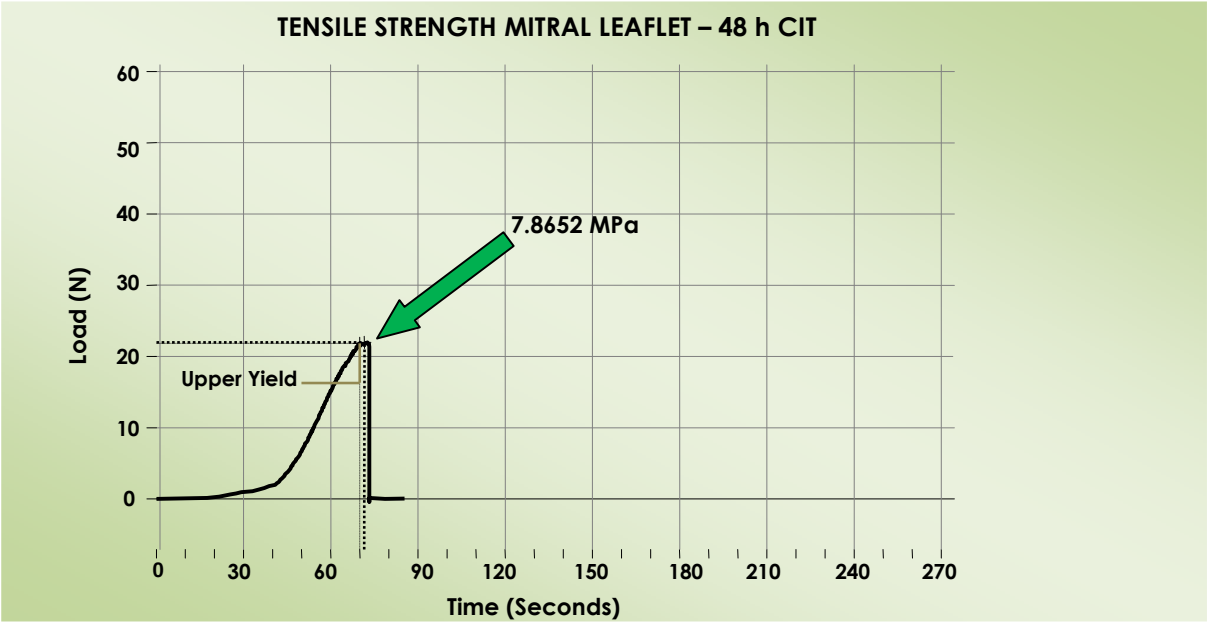


Figure 4.2.3(a) **Example of Tensile Strength testing performed on a mitral valve leaflet** (CIT = Cold Ischaemic Time; MPa = Mega Pascal; N = Newton; h = hour).

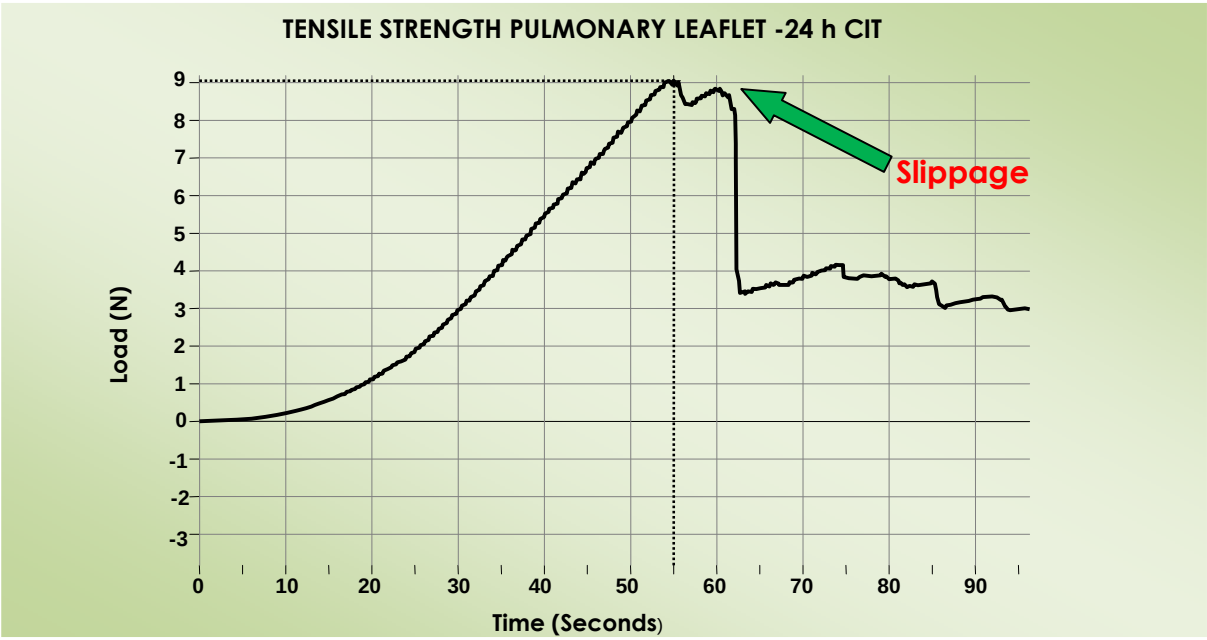


Figure 4.2.3(b) **Example of operational difficulty** (slippage of leaflet in mechanical grip during testing)

Table 4.2.3 provides a statistical summary of the tensile strength test performed on Group A, B and C. In Group A twenty samples were analysed to minimise the degree

of statistical variance. Statistical significant differences of group and inter-group comparisons in relation to varying harvesting times are discussed under 4.2.7.

Table 4.2.3 Tensile Strength (MPa) for Group's A, B and C

	GROUP A	GROUP B			GROUP C		
n	20	5	5	5	5	5	5
TIME	< 6 h	24 h	48 h	72 h	24 h	48 h	72 h
AORTIC LEAFLET							
MEAN	1.45	2.66	4.56	3.82	3.72	5.51	4.45
STD	0.75	0.72	0.63	2.03	1.06	1.14	1.82
MINIMUM	0.19	1.74	4.05	1.14	2.05	3.89	1.85
MAXIMUM	2.67	3.54	5.59	6.78	4.72	6.55	6.43
MEDIAN	1.35	2.45	4.50	3.67	4.02	5.82	4.40
PULMONARY LEAFLET							
MEAN	1.24	2.57	2.93	3.03	4.10	4.52	3.17
STD	0.79	1.07	0.50	1.02	0.86	0.84	1.73
MINIMUM	0.25	1.54	2.45	1.44	2.94	3.28	0.55
MAXIMUM	3.73	4.11	3.65	4.09	4.80	5.28	4.97
MEDIAN	1.01	2.22	2.93	3.04	4.65	5.00	3.29
MITRAL LEAFLET							
MEAN	2.27	4.94	5.84	4.94	11.80	7.11	8.79
STD	1.54	4.40	3.58	4.40	1.64	3.70	2.39
MINIMUM	0.29	1.13	1.18	1.13	9.99	3.09	7.12
MAXIMUM	6.13	12.44	9.36	12.44	13.98	12.05	12.77
MEDIAN	2.25	3.82	7.87	3.82	12.13	7.29	7.69
AORTIC WALL							
MEAN	1.34	1.09	1.36	1.33	1.28	1.49	1.22
STD	0.29	0.24	0.39	0.47	0.34	0.30	0.14
MINIMUM	0.80	0.69	0.82	0.74	0.98	1.26	1.06
MAXIMUM	1.87	1.29	1.75	1.94	1.85	2.03	1.45
MEDIAN	1.30	1.13	1.31	1.33	1.18	1.40	1.21
PULMONARY WALL							
MEAN	1.04	1.03	0.95	1.16	1.18	1.41	0.87
STD	0.36	0.27	0.22	0.34	0.41	0.32	0.22
MINIMUM	0.55	0.78	0.64	0.78	0.59	1.07	0.65
MAXIMUM	2.26	1.46	1.15	1.68	1.68	1.80	1.11
MEDIAN	1.00	0.96	1.01	1.18	1.19	1.35	0.82

[GROUP A = Control Group (< 6 h Ischaemic Time); GROUP B = Cold Ischaemic Time (24 h, 48 h, 72 h); GROUP C = Warm Ischaemic Time (24 h, 48 h, 72 h); MPa=Mega Pascal]

4.2.4 Young's Modulus

The stiffness of a material represents the ability of materials to resist deformation. Stiffness is commonly characterized by the slope of the linear region of a stress-strain curve, also referred to as Young's Modulus (Julien *et al*., 1997).

Figure 4.2.4 indicates a stress-strain curve of a mitral valve leaflet (48 h cold ischaemic times) from which the Young's modulus was calculated. The results of the calculated Young's modulus are displayed in terms of the mean, standard deviation, median, maximum and minimum in Table 4.2.4 for Group A, B and C. Statistical significant differences of group and inter-group comparisons in relation to varying harvesting times are discussed under 4.2.7.

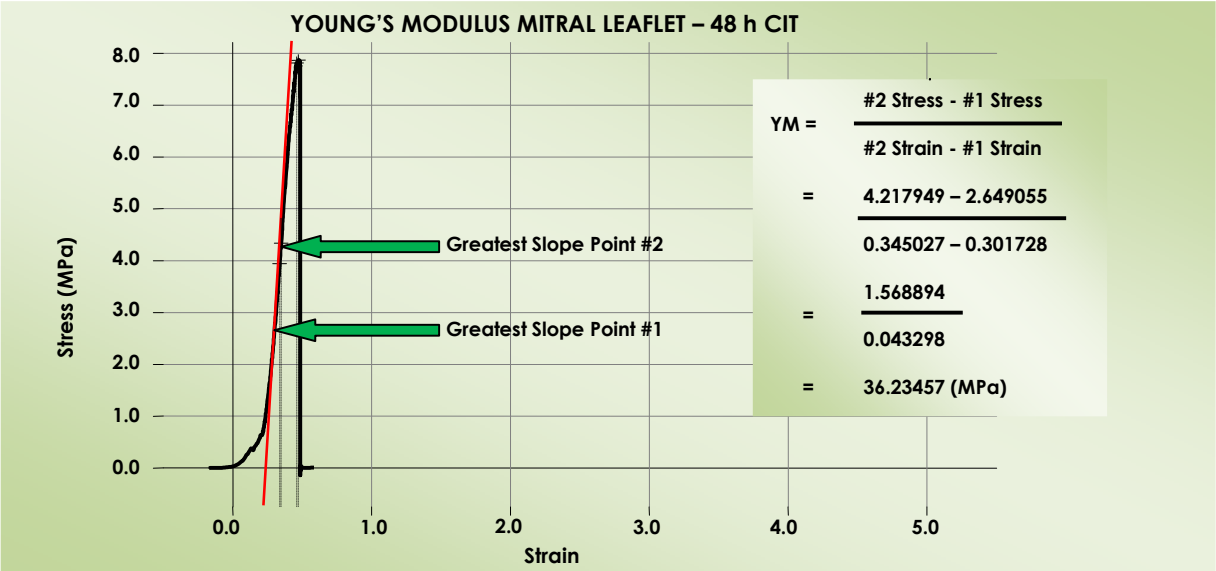


Figure 4.2.4 Young's Modulus calculated from the slope of the linear region of the Stress Stain curve for a mitral valvular leaflet after 48 h CIT (CIT=Cold Ischaemic Time; MPa=Mega Pascal; h = hour).

Table 4.2.4 Young's Modulus (MPa) calculated for Group A, B and C

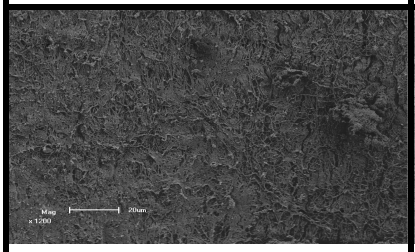
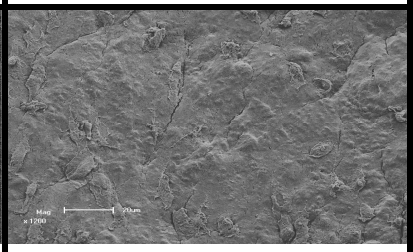
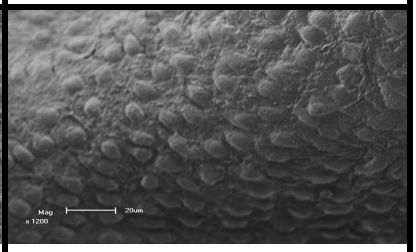
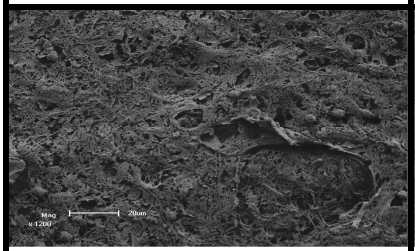
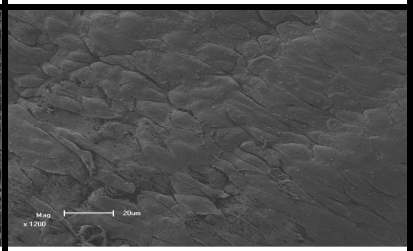
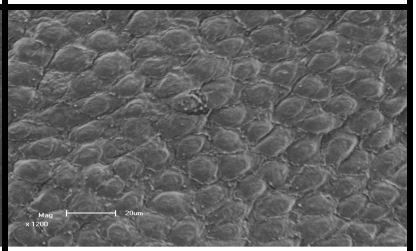
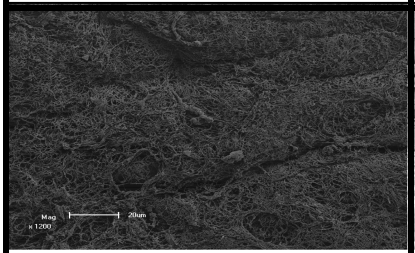
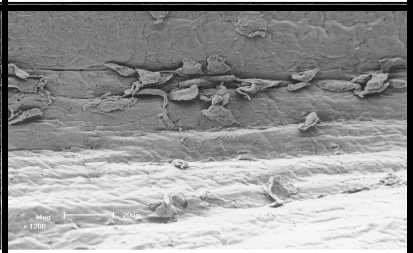
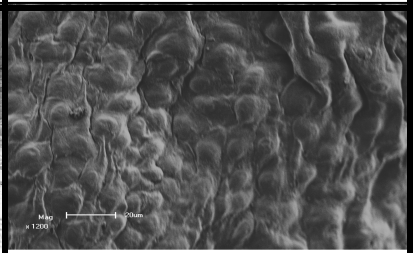
	GROUP A	GROUP B			GROUP C		
n	20	5	5	5	5	5	5
TIME	< 6 h	24 h	48 h	72 h	24 h	48 h	72 h
AORTIC LEAFLET							
MEAN	5.42	10.29	18.21	14.98	15.07	20.16	14.78
STD	4.33	3.72	7.93	7.50	5.41	4.77	3.88
MINIMUM	0.68	4.12	9.47	5.37	7.06	13.82	9.15
MAXIMUM	13.67	13.69	27.74	26.34	20.38	26.74	18.51
MEDIAN	3.77	10.58	19.44	14.39	17.89	20.24	14.18
PULMONARY LEAFLET							
MEAN	5.68	9.93	9.25	10.73	14.60	11.68	10.49
STD	4.57	2.63	5.02	4.33	3.18	3.70	5.62
MINIMUM	0.84	7.34	2.82	4.21	9.94	7.58	1.83
MAXIMUM	18.42	14.01	15.62	13.95	17.55	16.89	17.34
MEDIAN	4.78	8.85	10.78	13.37	16.38	10.92	10.86
MITRAL LEAFLET							
MEAN	7.79	19.44	24.52	33.42	47.47	25.06	30.09
STD	7.27	15.95	21.04	14.98	2.68	10.41	4.48
MINIMUM	0.39	3.08	1.62	15.62	43.83	15.35	25.71
MAXIMUM	30.96	45.78	45.65	47.91	49.98	42.00	36.29
MEDIAN	6.25	15.43	36.23	37.65	48.45	24.10	28.04
AORTIC WALL							
MEAN	3.19	3.43	4.11	4.40	3.62	4.31	4.80
STD	0.65	0.95	1.24	1.37	0.77	0.48	0.51
MINIMUM	2.12	2.17	2.49	2.44	2.95	3.57	4.28
MAXIMUM	4.11	4.45	5.41	6.04	4.80	4.85	5.53
MEDIAN	3.38	3.23	4.54	4.84	3.38	4.31	4.76
PULMONARY WALL							
MEAN	2.06	3.21	3.26	3.81	3.72	5.04	2.56
STD	0.77	0.90	0.93	1.31	1.26	0.78	0.75
MINIMUM	1.17	2.31	2.05	2.65	1.93	4.41	1.75
MAXIMUM	4.45	4.62	4.25	5.88	5.00	6.26	3.60
MEDIAN	2.00	2.90	3.32	3.68	3.68	4.59	2.35

[GROUP A = Control Group (< 6 h Ischaemic Time); GROUP B = Cold Ischaemic Time (24 h, 48 h, 72 h); GROUP C = Warm Ischaemic Time (24 h, 48 h, 72 h); MPa=Mega Pascal]

4.2.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to evaluate the integrity and quality of the endothelial surface of the valvular leaflets and arterial walls. A special scoring system was adopted and modified to categorize the samples in three categories (I-III) (Table 4.2.5.1).

Table 4.2.5.1 Summary of SEM scoring system – Category I, II, and III

CATEGORY I	CATEGORY II	CATEGORY III
The basal membrane and endothelial cells are completely absent	Scattered endothelial cells with the basal membrane intact.	Tissue surface almost completely or predominantly covered with endothelial cells.
		
Pulmonary Leaflet – 72 h CIT	Pulmonary Leaflet – 24 h CIT	Pulmonary Leaflet – < 6 h IT
		
Aortic Wall – 48 h WIT	Pulmonary Wall – < 6 h IT	Aortic Leaflet – < 6 h IT
		
Aortic Leaflet – 48 h WIT	Aortic Leaflet – 24 h WIT	Mitral Leaflet – < 6 h IT

[CIT = Cold Ischaemic Time; WIT = Warm Ischaemic Time; IT = Ischaemic Time; h = hour]

The SEM results are displayed in a frequency table (Table 4.2.5.2). Each sample was represented by 4-5 images taken at different sites to provide an overall perspective on the quality of the surface area in each sample. Statistically significant differences of group and inter-group comparisons in relation to varying harvesting times are discussed under 4.2.7.

Table 4.2.5.2 Scanning electron microscopy categorized according to Category I-III for Group's A, B and C

		GROUP A							GROUP B							GROUP C						
			Category I		Category II		Category III			Category I		Category II		Category III			Category I		Category II		Category III	
	Score	Total	n	%	n	%	n	%	Total	n	%	n	%	n	%	Total	n	%	n	%	n	%
AORTIC LEAFLET	< 6 h	5	0	0.0	1	20.0	4	80.0														
	24 h								24	1	4.2	23	95.8	0	0.0	25	2	8.0	21	84.0	2	8.0
	48 h								25	5	20.0	20	80.0	0	0.0	25	25	100	0	0.0	0	0.0
	72 h								23	4	17.4	17	73.9	2	8.70	25	10	40.0	14	56.0	1	4.0
PULMONARY LEAFLET	< 6 h	5	0	0.0	0	0.0	5	100														
	24 h								24	3	12.5	9	37.5	12	50.0	25	17	68.0	5	20.0	3	12.0
	48 h								23	5	21.7	14	60.9	4	17.4	25	15	60.0	7	28.0	3	12.0
	72 h								22	4	18.2	9	40.9	9	40.9	25	9	36.0	10	40.0	6	24.0
MITRAL LEAFLET	< 6 h	5	0	0.0	4	80.0	1	20.0														
	24 h								25	15	60.0	10	40.0	0	0.0	25	20	80.0	5	20.0	0	0.0
	48 h								23	14	60.9	9	39.1	0	0.0	23	22	95.7	1	4.3	0	0.0
	72 h								23	16	69.6	7	30.4	0	0.0	25	21	84.0	4	16.0	0	0.0
AORTIC WALL	< 6 h	15	1	6.65	13	86.7	1	6.65														
	24 h								25	24	96.0	1	4.0	0	0.0	25	25	100	0	0.0	0	0.0
	48 h								24	24	100	0	0.0	0	0.0	25	25	100	0	0.0	0	0.0
	72 h								23	23	100	0	0.0	0	0.0	25	25	100	0	0.0	0	0.0
PULMONARY WALL	< 6 h	15	2	13.3	13	86.7	0	0.0														
	24 h								25	24	96.0	1	4.0	0	0.0	25	25	100	0	0.0	0	0.0
	48 h								23	23	100	0	0.0	0	0.0	25	25	100	0	0.0	0	0.0
	72 h								23	23	100	0	0.0	0	0.0	25	25	100	0	0.0	0	0.0

[GROUP A = Control Group (<6 h Ischaemic Time); GROUP B = Cold Ischaemic Time (24 h, 48 h, 72 h); GROUP C = Warm Ischaemic Time (24 h, 48 h, 72 h)]

4.2.6 Haematoxylin and Eosin stain (H&E)

The H&E histological stain was used to evaluate changes in the cellular composition and tissue architecture. The samples were graded as either being normal (Figure 4.2.6a) or demonstrating the presence of autolysis (Figure 4.2.6b).

Autolysis were defined as necrotic cells that showed increased eosinophilia attributed in part to loss of the normal basophilia imparted by the RNA in the cytoplasm and in part to the increased binding of eosin to denatured intracytoplasmic proteins. The cells may have a more glassy homogeneous appearance than that of normal cells, mainly as a result of the loss of glycogen particles. When enzymes have digested the cytoplasmic organelles, the cytoplasm becomes vacuolated and appears moth-eaten. Finally, calcification of the dead cells may occur (Kumar, Abbas, Fausto, Robbins and Cotran, 2005.)

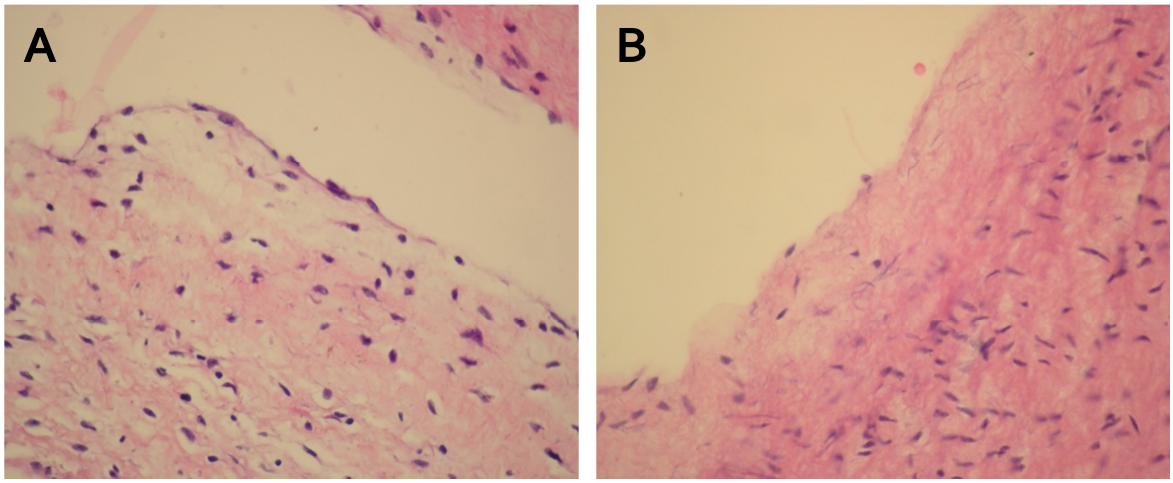


Figure 4.2.6 **Haematoxylin and Eosin stain performed on (a) normal valve leaflets and (b) autolytic valve leaflets**

The H&E results are displayed in a frequency table (Table 4.2.6) for Group's A, B and C. Statistical significant differences of group and inter-group comparisons in relation to varying harvesting times are discussed under 4.2.7.

Table 4.2.6 H&E categorized as normal or for the presence of autolysis for Groups A, B and C

		GROUP A					GROUP B					GROUP C				
			Normal		Autolysis			Normal		Autolysis			Normal		Autolysis	
	Score	Total	n	%	n	%	Total	n	%	n	%	Total	n	%	n	%
AORTIC LEAFLET	< 6 h	5	5	100	0	0.0										
	24 h						5	5	100	0	0.0	5	5	100	0	0.0
	48 h						5	5	100	0	0.0	5	4	80.0	1	20.0
	72 h						5	5	100	0	0.0	5	0	0.0	5	100
PULMONARY LEAFLET	< 6 h	5	5	100	0	0.0										
	24 h						5	5	100	0	0.0	5	5	100	0	0.0
	48 h						5	5	100	0	0.0	5	2	40.0	3	60.0
	72 h						5	5	100	0	0.0	5	0	0.0	5	100
MITRAL LEAFLET	< 6 h	5	5	100	0	0.0										
	24 h						5	5	100	0	0.0	5	5	100	0	0.0
	48 h						5	5	100	0	0.0	5	3	60.0	2	40.0
	72 h						5	5	100	0	0.0	5	0	0.0	5	100
AORTIC WALL	< 6 h	5	5	100	0	0.0										
	24 h						5	5	100	0	0.0	5	5	100	0	0.0
	48 h						5	5	100	0	0.0	5	5	100	0	0.0
	72 h						5	5	100	0	0.0	5	0	0.0	5	100
PULMONARY WALL	< 6 h	5	5	100	0	0.0										
	24 h						5	5	100	0	0.0	5	5	100	0	0.0
	48 h						5	5	100	0	0.0	5	1	20.0	4	80.0
	72 h						5	5	100	0	0.0	5	0	0.0	5	100

[GROUP A = Control Group (< 6 h Ischaemic Time); GROUP B = Cold Ischaemic Time (24 h, 48 h, 72 h); GROUP C = Warm Ischaemic Time (24 h, 48 h, 72 h)]

4.2.7 Inter-group and group comparisons in relation to varying harvested ischaemic times

4.2.7.1 Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time)

The only statistically significant difference ($p < 0.05$) was found between the thermal denaturation temperature of mitral valve leaflet at 48 h and 72 h (Table 4.2.7.1). Tensile strength, Young's Modulus, SEM and H&E did not reveal any statistically significant differences ($p > 0.05$) at 24 h, 48 h and 72 h.

Table 4.2.7.1 Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time)

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM II		H & E	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	24 vs. 48 vs. 72 h	0.282	24 vs. 48 vs. 72 h	0.105	24 vs. 48 vs. 72 h	0.209	24 vs. 48 vs. 72 h	0.106	24 vs. 48 vs. 72 h	EM
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-
PULMONARY LEAFLET	24 vs. 48 vs. 72 h	0.184	24 vs. 48 vs. 72 h	0.702	24 vs. 48 vs. 72 h	0.853	24 vs. 48 vs. 72 h	0.198	24 vs. 48 vs. 72 h	EM
	24 vs. 48	-	24 vs. 48	-	24 vs. 48	-	24 vs. 48	-	24 vs. 48	-
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-
MITRAL LAEFLET	24 vs. 48 vs. 72 h	**0.045**	24 vs. 48 vs. 72 h	0.351	24 vs. 48 vs. 72 h	0.466	24 vs. 48 vs. 72 h	0.754	24 vs. 48 vs. 72 h	EM
	24 vs. 48 h	0.055	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-
	24 vs. 72 h	0.872	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-
	48 vs. 72 h	**0.013**	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-
AORTIC WALL	24 vs. 48 vs. 72 h	0.158	24 vs. 48 vs. 72 h	0.487	24 vs. 48 vs. 72 h	0.447	24 vs. 48 vs. 72 h	1.000	24 vs. 48 vs. 72 h	EM
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-
PULMONARY WALL	24 vs. 48 vs. 72 h	0.314	24 vs. 48 vs. 72 h	0.516	24 vs. 48 vs. 72 h	0.625	24 vs. 48 vs. 72 h	1.000	24 vs. 48 vs. 72 h	EM
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-

p<0.05 = statistically significant; p>0.05 = not statistically significant

(T_d = Thermal Denaturation Temperature (°C); IT = Ischaemic Time (h); SEM = Scanning Electron Microscopy; H&E = Haematoxylin and Eosin; h =hours; EM = Equal Mean; - No Values)

4.2.7.2 Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

Table 4.2.7.2 represents a summary of the inter-group relationship between 24 h, 48 h and 72 h. The thermal denaturation temperature showed a statistically significant difference ($p < 0.05$) between pulmonary valve leaflets at 24 h and 72 h.

The Young's Modulus calculated from the stress-stain curve for mitral valve leaflets indicated a statistically significant difference ($p < 0.05$) between 24 h and 48 h, as well as between 24 h and 72 h. The aortic wall showed a significant difference ($p < 0.05$) between tissue at 24 h and 72 h. Pulmonary wall tissue showed a significant difference ($p < 0.05$) between tissues at 48 h and 72 h.

H&E results showed a statistically significant difference ($p < 0.05$) between all sample tested (mitral, aortic and pulmonary valve leaflets between 24 h and 48 h, 24 h and 72 h, 48 h and 72 h; pulmonary and aortic wall between 24 h and 48 h, 24 h and 72 h, 48 h and 72 h.

Table 4.2.7.2 Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM		H & E	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	24 vs. 48 vs. 72 h	0.137	24 vs. 48 vs. 72 h	0.162	24 vs. 48 vs. 72 h	0.172	24 vs. 48 vs. 72 h	**p<0.001**	24 vs. 48 vs. 72 h	**0.006**
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	1.000
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	**0.008**
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	**0.048**
PULMONARY LEAFLET	24 vs. 48 vs. 72 h	**0.034**	24 vs. 48 vs. 72 h	0.238	24 vs. 48 vs. 72 h	0.333	24 vs. 48 vs. 72 h	0.222	24 vs. 48 vs. 72 h	**0.009**
	24 vs. 48 h	0.051	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	0.167
	24 vs. 72 h	**0.025**	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	**0.008**
	48 vs. 72 h	0.944	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	0.444
MITRAL LAEFLET	24 vs. 48 vs. 72 h	0.175	24 vs. 48 vs. 72 h	0.052	24 vs. 48 vs. 72 h	**0.001**	24 vs. 48 vs. 72 h	0.320	24 vs. 48 vs. 72 h	**0.009**
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	**0.009**	24 vs. 48 h	-	24 vs. 48 h	0.444
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	**0.009**	24 vs. 72 h	-	24 vs. 72 h	**0.008**
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	0.175	48 vs. 72 h	-	48 vs. 72 h	0.167
AORTIC WALL	24 vs. 48 vs. 72 h	0.134	24 vs. 48 vs. 72 h	0.294	24 vs. 48 vs. 72 h	**0.028**	24 vs. 48 vs. 72 h	EM	24 vs. 48 vs. 72 h	**0.001**
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	0.117	24 vs. 48 h	-	24 vs. 48 h	-
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	**0.047**	24 vs. 72 h	-	24 vs. 72 h	**0.008**
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	0.175	48 vs. 72 h	-	48 vs. 72 h	**0.008**
PULMONARY WALL	24 vs. 48 vs. 72 h	0.098	24 vs. 48 vs. 72 h	0.063	24 vs. 48 vs. 72 h	**0.005**	24 vs. 48 vs. 72 h	EM	24 vs. 48 vs. 72 h	**0.006**
	24 vs. 48 h	-	24 vs. 48 h	-	24 vs. 48 h	0.175	24 vs. 48 h	-	24 vs. 48 h	**0.048**
	24 vs. 72 h	-	24 vs. 72 h	-	24 vs. 72 h	0.117	24 vs. 72 h	-	24 vs. 72 h	**0.008**
	48 vs. 72 h	-	48 vs. 72 h	-	48 vs. 72 h	**0.009**	48 vs. 72 h	-	48 vs. 72 h	1.000

p < 0.05 = statistically significant; p > 0.05 = not statistically significant

(T_d = Thermal Denaturation Temperature (°C); IT = Ischaemic Time (h); SEM = Scanning Electron Microscopy; H&E = Haematoxylin and Eosin; h =hours; – = No Values; EM = Equal Mean)

4.2.7.3 Group A (< 6 h Ischaemic Time) vs. Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time)

The general impression of Table 4.2.7.3 is that a statistically significant difference exists in the majority of the tests performed with the exception of H& E (none of the samples showed autolysis).

Table 4.2.7.3 Group A (< 6 h Ischaemic Time) vs. Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time)

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM		H&E	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	24 h	p<0.001	24 h	0.004	24 h	0.035	24 h	p<0.001	24 h	EM
	48 h	p<0.001	48 h	p<0.001	48 h	0.004	48 h	p<0.001	48 h	EM
	72 h	p<0.001	72 h	0.058	72 h	0.005	72 h	0.006	72 h	EM
PULMONARY LEAFLET	24 h	0.002	24 h	0.005	24 h	0.025	24 h	0.179	24 h	EM
	48 h	0.001	48 h	p<0.001	48 h	0.103	48 h	0.003	48 h	EM
	72 h	0.002	72 h	0.014	72 h	0.036	72 h	0.074	72 h	EM
MITRAL LAEFLET	24 h	0.002	24 h	0.250	24 h	0.035	24 h	0.007	24 h	EM
	48 h	0.010	48 h	0.089	48 h	0.277	48 h	0.007	48 h	EM
	72 h	p<0.001	72 h	0.053	72 h	0.001	72 h	0.003	72 h	EM
AORTIC WALL	24 h	0.022	24 h	0.081	24 h	0.054	24 h	p<0.001	24 h	EM
	48 h	0.425	48 h	0.913	48 h	0.118	48 h	p<0.001	48 h	EM
	72 h	0.044	72 h	0.936	72 h	0.067	72 h	p<0.001	72 h	EM
PULMONARY WALL	24 h	1.000	24 h	0.933	24 h	0.007	24 h	p<0.001	24 h	EM
	48 h	0.390	48 h	0.606	48 h	0.015	48 h	p<0.001	48 h	EM
	72 h	0.226	72 h	0.519	72 h	0.004	72 h	p<0.001	72 h	EM

p<0.05 = statistically significant; p>0.05 = no statistically significant

(T_d = Thermal Denaturation Temperature (°C); IT = Ischaemic Time (h); SEM = Scanning Electron Microscopy; H&E = Haematoxylin and Eosin; h =hours; EM = Equal Mean)

4.2.7.4 Group A (< 6 h Ischaemic Time) vs. Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

Table 4.2.7.4 summarizes the statistical differences (p-values) between Group A and C. Major statistical differences were present between samples of Group A and samples of Group B.

Table 4.2.7.4 Group A (< 6 h Ischaemic Time) vs. Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM		H & E	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	24 h	0.002	24 h	p<0.001	24 h	0.007	24 h	0.005	24 h	EM
	48 h	0.001	48 h	p<0.001	48 h	0.001	48 h	p<0.001	48 h	1.000
	72 h	0.004	72 h	0.020	72 h	0.003	72 h	0.001	72 h	0.008
PULMONARY LEAFLET	24 h	0.001	24 h	p<0.001	24 h	0.004	24 h	0.001	24 h	EM
	48 h	0.007	48 h	p<0.001	48 h	0.015	48 h	0.001	48 h	0.167
	72 h	0.011	72 h	0.067	72 h	0.103	72 h	0.006	72 h	0.008
MITRAL LAEFLET	24 h	0.077	24 h	p<0.001	24 h	0.001	24 h	0.002	24 h	EM
	48 h	0.030	48 h	0.041	48 h	0.003	48 h	p<0.001	48 h	0.444
	72 h	0.186	72 h	p<0.001	72 h	0.001	72 h	0.001	72 h	0.008
AORTIC WALL	24 h	0.023	24 h	0.689	24 h	0.308	24 h	p<0.001	24 h	EM
	48 h	0.054	48 h	0.300	48 h	0.004	48 h	p<0.001	48 h	EM
	72 h	0.516	72 h	0.382	72 h	0.001	72 h	p<0.001	72 h	0.008
PULMONARY WALL	24 h	0.452	24 h	0.468	24 h	0.015	24 h	p<0.001	24 h	EM
	48 h	0.259	48 h	0.054	48 h	0.001	48 h	p<0.001	48 h	0.048
	72 h	0.329	72 h	0.310	72 h	0.154	72 h	p<0.001	72 h	0.008

p < 0.05 = statistically significant; p > 0.05 = not statistically significant (T_d = Thermal Denaturation Temperature (°C); IT = Ischaemic Time (h); SEM = Scanning Electron Microscopy; H&E = Hematoxylin and Eosin; h =hours; EM = Equal Mean)

4.2.7.5 Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time) vs. Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

Table 4.2.7.5 represents a statistical summary between group B and Group C. The thermal denaturation temperature (T_d) showed a statistically significant difference ($p < 0.05$) at 48 h (CIT & WIT), and 72 h (CIT & WIT) for pulmonary valve leaflets and 72 h (CIT & WIT) for mitral valve leaflets.

For the tensile strength tests, statistically significant differences ($p < 0.05$) were evident 24 h (CIT & WIT) and 48 h (CIT & WIT) for pulmonary valve leaflets; 24 h (CIT & WIT) for mitral valve leaflets; 48 h (CIT & WIT) for the aortic wall and 48 h (CIT & WIT) for the pulmonary wall.

The Young's Modulus calculated from the stress-stain curve indicated a statistically significant difference ($p < 0.05$) at 24 h (CIT & WIT) for pulmonary valve leaflets; 24 h (CIT & WIT) for mitral valve leaflets and 48 h (CIT & WIT) for the pulmonary wall.

SEM results demonstrated statistically significant differences ($p < 0.05$) were found for the SEM results at the 48 h (CIT & WIT) for aortic valve leaflets; 24 h (CIT & WIT) and 48 h (CIT & WIT) for pulmonary valve leaflets and 48 h (CIT & WIT) for mitral valve leaflets.

All the samples in Group C showed the presence of autolysis after 72 h ischaemic time. H&E histology results showed a statistically significant difference ($p < 0.05$) among the samples tested for the mitral, aortic and pulmonary valve leaflets at 72 h (CIT & WIT) as well as samples tested for pulmonary wall tissue at 48 h and 72 h (CIT & WIT) and aortic wall tissue at 72 h (CIT & WIT).

Table 4.2.7.5 Group B (24 h vs. 48 h vs. 72 h Cold Ischaemic Time) vs. Group C (24 h vs. 48 h vs. 72 h Warm Ischaemic Time)

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM		H&E	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	24 h	0.462	24 h	0.101	24 h	0.143	24 h	0.509	24 h	EM
	48 h	0.210	48 h	0.140	48 h	0.651	48 h	**0.000**	48 h	1.000
	72 h	0.141	72 h	0.623	72 h	0.959	72 h	0.260	72 h	**0.008**
PULMONARY LEAFLET	24 h	0.357	24 h	**0.037**	24 h	**0.035**	24 h	**0.000**	24 h	EM
	48 h	**0.030**	48 h	**0.007**	48 h	0.409	48 h	**0.024**	48 h	EM
	72 h	**p<0.00**	72 h	0.883	72 h	0.941	72 h	0.302	72 h	**0.008**
MITRAL LEAFLET	24 h	0.724	24 h	**0.022**	24 h	**0.016**	24 h	0.123	24 h	EM
	48 h	0.621	48 h	0.596	48 h	0.960	48 h	**0.004**	48 h	0.444
	72 h	**0.001**	72 h	0.902	72 h	0.655	72 h	0.235	72 h	**0.008**
AORTIC WALL	24 h	0.251	24 h	0.506	24 h	0.746	24 h	1.000	24 h	EM
	48 h	0.244	48 h	**0.030**	48 h	0.754	48 h	1.000	48 h	0.167
	72 h	0.147	72 h	0.141	72 h	0.569	72 h	1.000	72 h	**0.008**
PULMONARY WALL	24 h	0.469	24 h	0.506	24 h	0.483	24 h	1.000	24 h	EM
	48 h	0.814	48 h	**0.030**	48 h	**0.011**	48 h	1.000	48 h	**0.048**
	72 h	0.131	72 h	0.141	72 h	0.101	72 h	1.000	72 h	**0.008**

p<0.05 = statistically significant; p>0.05 = no statistically significant; p =1.00 = Equal mean

(T_d = Thermal Denaturation Temperature; IT = Ischaemic Time; SEM = Scanning Electron Microscopy; H&E = Haematoxylin and Eosin; h =hours; EM= Equal Mean)

4.3 THE QUALITY OF TISSUE MORPHOLOGY OF HARVESTED VALVULAR HOMOGRAFTS

After evaluating the influence of harvesting time all the results were re-classified according to their morphological quality (SEM categorization I-III). The SEM categorization was summarized in Table 4.3.1.

Table 4.3.1 SEM categorization criteria

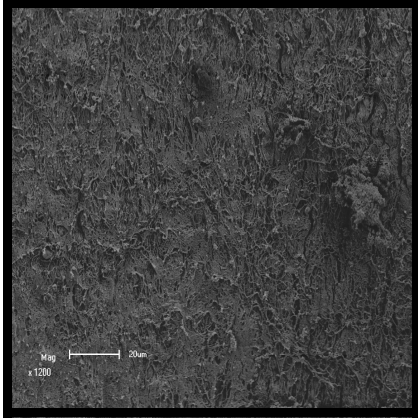
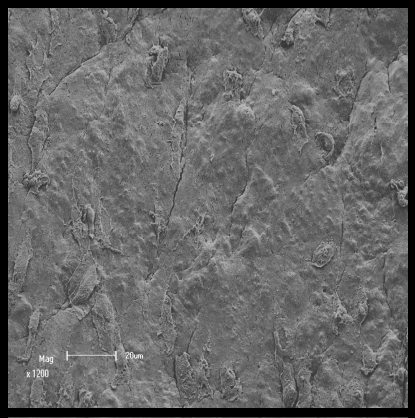
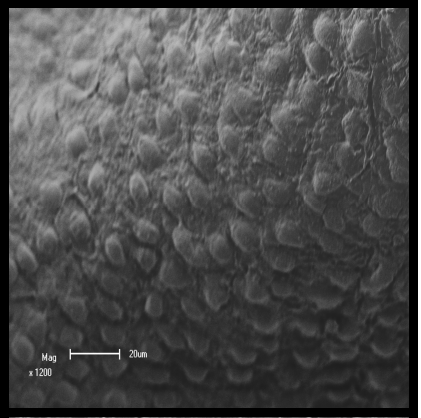
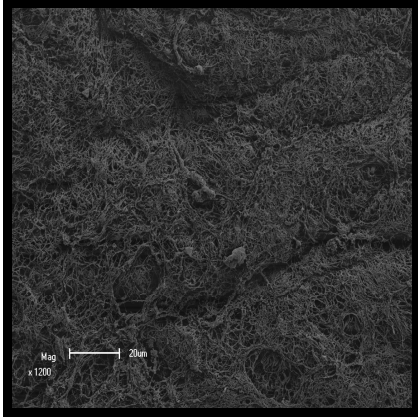
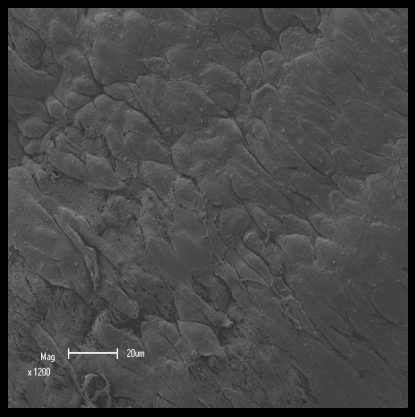
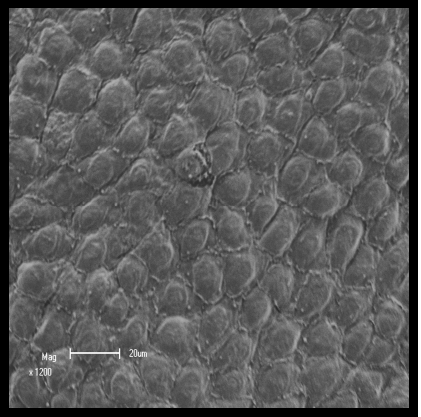
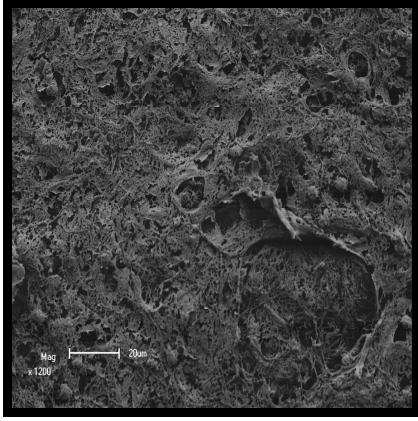
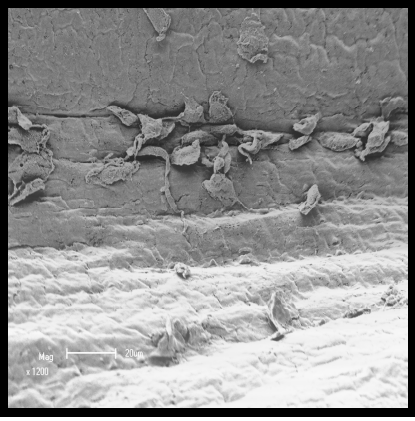
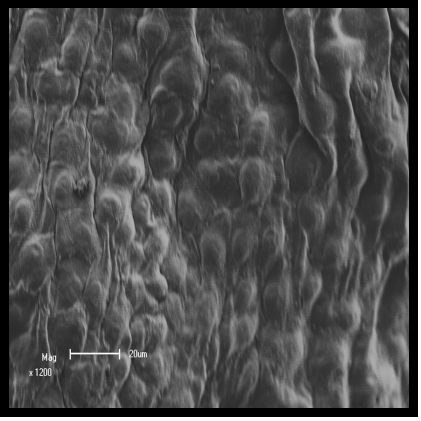
Category I	Category II	Category III
The basal membrane and endothelial cells are completely absent	Scattered endothelial cells and the basal membrane are still intact.	The tissue surface is almost completely endothelialyzed.
		
		
		

Table 4.3.2 represents the morphological distribution of the samples according to the SEM categorization I-III in a frequency table.

Table 4.3.2 Morphological distribution according to SEM categorization I-III

	Hour	Category I		Category II		Category III	
		n	%	n	%	n	%
AORTIC LEAFLET							
GROUP A	< 6 h	0	0.00	1	2.86	4	11.43
	24 h	0	0.00	5	14.29	0	0.00
GROUP B	48 h	1	2.86	4	11.43	0	0.00
	72 h	1	2.86	4	11.43	0	0.00
	24 h	0	0.00	5	14.29	0	0.00
GROUP C	48 h	1	2.86	4	11.43	0	0.00
	72 h	2	5.71	3	8.57	0	0.00
TOTAL		**5**	**14.29**	**26**	**74.29**	**4**	**11.43**
PULMONARY LEAFLET							
GROUP A	< 6 h	0	0.00	0	0.00	5	14.29
	24 h	0	0.00	2	5.71	3	8.57
GROUP B	48 h	1	2.86	3	8.57	1	2.86
	72 h	1	2.86	2	5.71	2	5.71
	24 h	3	8.57	1	2.86	1	2.86
GROUP C	48 h	3	8.57	1	2.86	1	2.86
	72 h	1	2.86	3	8.57	1	2.86
TOTAL		**9**	**25.71**	**12**	**34.29**	**14**	**40.00**
MITRAL LEAFLET							
GROUP A	< 6 h	0	0.00	4	11.43	1	2.86
	24 h	3	8.57	2	5.71	0	0.00
GROUP B	48 h	3	8.57	2	5.71	0	0.00
	72 h	4	11.43	1	2.86	0	0.00
	24 h	4	11.43	1	2.86	0	0.00
GROUP C	48 h	5	14.29	0	0.00	0	0.00
	72 h	5	14.29	0	0.00	0	0.00
TOTAL		**24**	**68.57**	**10**	**28.57**	**1**	**2.86**
AORTIC WALL							
GROUP A	< 6 h	0	0.00	5	14.29	0	0.00
	24 h	5	14.29	0	0.00	0	0.00
GROUP B	48 h	5	14.29	0	0.00	0	0.00
	72 h	5	14.29	0	0.00	0	0.00
	24 h	5	14.29	0	0.00	0	0.00
GROUP C	48 h	5	14.29	0	0.00	0	0.00
	72 h	5	14.29	0	0.00	0	0.00
TOTAL		**30**	**85.71**	**5**	**14.29**	**0**	**0.00**
PULMONARY WALL							
GROUP A	< 6 h	0	0.00	5	14.29	0	0.00
	24 h	5	14.29	0	0.00	0	0.00
GROUP B	48 h	5	14.29	0	0.00	0	0.00
	72 h	5	14.29	0	0.00	0	0.00
	24 h	5	14.29	0	0.00	0	0.00
GROUP C	48 h	5	14.29	0	0.00	0	0.00
	72 h	5	14.29	0	0.00	0	0.00
TOTAL		**30**	**85.71**	**5**	**14.29**	**0**	**0.00**

[n = sample size; % = percentage]

Table 4.3.3 represents statistical data of thermal denaturation temperature, tensile strength and Young's modulus, performed on samples categorized according to SEM classification I-III.

Table 4.3.3 T_d, Tensile strength and Young's Modulus according to SEM Categorization I-III

	T_d			TENSILE STRENGTH			YOUNG'S MODULUS		
CATEGORY	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
AORTIC LEAFLET									
n	5	26	4	5	26	4	5	26	4
MEAN	70.05	69.88	67.04	3.86	4.04	1.24	13.82	15.39	2.81
SD	0.87	1.23	0.43	2.34	1.49	0.50	8.66	6.18	1.78
MINIMUM	69.17	66.61	66.46	1.14	0.70	0.63	5.37	1.94	1.15
MAXIMUM	71.50	72.94	67.38	6.43	6.78	1.79	26.74	27.74	5.23
MEDIAN	69.87	69.85	67.16	4.05	4.05	1.26	9.47	14.29	2.43
PULMONARY LEAFLET									
n	9	12	14	9	12	14	9	12	14
MEAN	70.17	70.26	69.55	3.45	3.23	2.52	11.28	11.30	7.72
SD	0.99	0.95	2.09	1.43	0.93	1.78	4.63	3.42	5.82
MINIMUM	68.28	69.24	66.10	0.55	1.78	0.52	1.83	5.76	1.14
MAXIMUM	71.57	72.70	72.04	5.02	4.80	5.28	16.89	17.55	17.34
MEDIAN	70.08	70.17	70.13	3.41	3.09	2.19	11.29	10.82	6.27
MITRAL LEAFLET									
n	24	10	1	24	10	1	24	10	1
MEAN	70.66	69.54	68.53	8.02	5.25	0.42	30.81	17.44	1.09
SD	1.87	1.43		3.75	5.42		12.83	21.32	
MINIMUM	68.48	67.46		1.13	0.45		3.08	0.93	
MAXIMUM	77.39	71.74		15.18	13.98		49.57	49.98	
MEDIAN	70.57	69.65		7.90	2.53		30.65	5.32	
AOTRIC WALL									
n	30	5	-	30	5	-	30	5	-
MEAN	70.40	69.68		1.30	1.27		4.11	3.16	
SD	0.73	0.49		0.33	0.19		0.98	0.86	
MINIMUM	69.16	69.15		0.69	1.05		2.17	2.12	
MAXIMUM	72.21	70.39		2.03	1.49		6.04	3.97	
MEDIAN	70.40	69.47		1.27	1.32		4.31	3.49	
PULMONARY WALL									
n	30	5	-	30	5	-	30	5	-
MEAN	71.12	70.57		1.10	1.11		3.60	2.41	
SD	1.55	1.03		0.33	0.34		1.20	0.67	
MINIMUM	68.80	69.43		0.59	0.71		1.75	1.46	
MAXIMUM	77.50	71.95		1.80	1.62		6.26	3.21	
MEDIAN	70.93	70.30		1.09	1.11		3.56	2.43	

[n=sample size; SD = standard deviation; T_d = Thermal denaturation Temperature; - No Values]

Table 4.3.4 is a frequency table for H&E results based on SEM categorization I-III.

Table 4.3.4 H&E categorized as normal or autolysis for SEM categorization I-III

		NORMAL		AUTOLYSIS	
CATEGORY	**n**	**n-x**	**%**	**x**	**%**
AORTIC LEAFLET					
I	**5**	3	60.0	2	40.0
II	**26**	22	84.6	4	15.4
III	**4**	4	100.0	0	0.0
Total	**35**	**29**	**82.9**	**6**	**17.1**
PULMONARY LEAFLET					
I	**9**	6	66.7	3	33.3
II	**12**	9	75.0	3	25.0
III	**14**	12	85.7	2	14.3
Total	**35**	**27**	**77.1**	**8**	**22.9**
MITRAL LEAFLET					
I	**24**	17	70.8	7	29.2
II	**10**	10	100.0	0	0.0
III	**1**	1	100.0	0	0.0
Total	**35**	**28**	**80.0**	**7**	**20.0**
AORTIC WALL					
I	**30**	25	83.3	5	16.7
II	**5**	5	100.0	0	0.0
III	**0**	0	0.0	0	0.0
Total	**35**	**30**	**85.7**	**5**	**14.3**
PULMONARY WALL					
I	**30**	21	70.0	9	30.0
II	**5**	5	100.0	0	0.0
III	**0**	0	0.0	0	0.0
Total	**35**	**26**	**74.3**	**9**	**25.7**

[n=sample size; x=autolysis; % = percentage]

Table 4.3.5 is a summary of the statistical analysis of inter-group relationships between categories I - III. The valve leaflets (aortic, pulmonary and mitral) were distributed throughout the three SEM categories. The artic wall tissue only met the criteria of Category I and II. Aortic valve leaflets showed a statistically significant difference ($p<0.05$) between category I, II and III for DSC, Tensile strength and Young's Modulus.

4.3.1 Morphology according to SEM category I, II, III

Table 4.3.5 provides a summary of the statistical analysis of the morphological classification of harvested valvular homograft tissue according to SEM category I, II and III.

Thermal denaturation temperature (T_d) showed a statistical significant difference ($p < 0.05$) for aortic valve leaflets between categories I & III as well as between II & III, and also between categories I & II of the aorta wall.

Statistical analysis of the tensile strength indicated a significant difference ($p < 0.05$) in categories II & III of the aortic leaflet.

Young's modulus as calculated from the stress strain curve indicated a statistical significant difference ($p < 0.05$) between categories I & III as well II & III of the aortic valve leaflet. A significant difference ($p < 0.05$) was also shown between categories I & II of the aorta and pulmonary wall respectively.

No statistical significant difference ($p < 0.05$) was found within the H&E results.

Table 4.3.5 Category I vs. II vs. III

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		H & E	
	Category	p-value	Category	p-value	Category	p-value	Category	p-value
AORTIC LEAFLET	I vs. II vs. III	p<0.001	I vs. II vs. III	0.008	I vs. II vs. III	0.008	I vs. II vs. III	0.309
	I vs. II	0.780	I vs. II	0.821	I vs. II	0.452	I vs. II	-
	I vs. III	p<0.001	I vs. III	0.067	I vs. III	0.014	I vs. III	-
	II vs. III	p<0.001	II vs. III	0.001	II vs. III	0.003	II vs. III	-
PULMONARY LEAFLET	I vs. II vs. III	0.445	I vs. II vs. III	0.265	I vs. II vs. III	0.212	I vs. II vs. III	0.607
	I vs. II	-	I vs. II	-	I vs. II	-	I vs. II	-
	I vs. III	-	I vs. III	-	I vs. III	-	I vs. III	-
	II vs. III	-	II vs. III	-	II vs. III	-	II vs. III	-
MITRAL LAEFLET	I vs. II vs. III	0.161	I vs. II vs. III	0.083	I vs. II vs. III	0.050	I vs. II vs. III	0.128
	I vs. II	-	I vs. II	-	I vs. II	-	I vs. II	-
	I vs. III	-	I vs. III	-	I vs. III	-	I vs. III	-
	II vs. III	-	II vs. III	-	II vs. III	-	II vs. III	-
AORTIC WALL	I vs. II vs. III	-	I vs. II vs. III	-	I vs. II vs. III	-	I vs. II vs. III	-
	I vs. II	0.042	I vs. II	0.854	I vs. II	0.048	I vs. II	1.000
	I vs. III	-	I vs. III	-	I vs. III	-	I vs. III	-
	II vs. III	-	II vs. III	-	II vs. III	-	II vs. III	-
PULMONARY WALL	I vs. II vs. III	-	I vs. II vs. III	-	I vs. II vs. III	-	I vs. II vs. III	-
	I vs. II	0.458	I vs. II	0.943	I vs. II	0.048	I vs. II	0.297
	I vs. III	-	I vs. III	-	I vs. III	-	I vs. III	-
	II vs. III	-	II vs. III	-	II vs. III	-	II vs. III	-

p<0.05 = statistically significant; p>0.05 = no statistically significant

(T_d= Thermal Denaturation Temperature; IT = Ischaemic Time; SEM = Scanning Electron Microscopy; H&E = Haematoxylin and Eosin; h =hours; EM = Equal Mean; - = No Value)

4.4 THE QUALITY OF TISSUE HISTOLOGY OF HARVESTED VALVULAR HOMOGRAFTS

The H&E histological stain was used to evaluate changes in cellular composition and tissue architecture. The samples were graded as either being normal or autolytic, demonstrating the presence of autolysis.

Table 4.4.1 represents the statistical data of the thermal denaturation temperature (T_d), tensile strength and Young's modulus preformed on the samples categorized according to H&E classification as either normal or autolytic.

Table 4.4.1 T_d, Tensile strength and Young's Modulus according to H & E Classification (Normal or Autolysis)

H & E CLASSIFICATION	T_d		TENSILE STRENGTH		YOUNG'S MODULUS	
	NORMAL	AUTOLYSIS	NORMAL	AUTOLYSIS	NORMAL	AUTOLYSIS
AORTIC LEAFLET						
n	29	6	29	6	29	6
MEAN	69.58	69.60	3.47	4.79	13.33	15.69
SD	1.53	1.00	1.69	1.83	7.79	4.12
MINIMUM	66.46	68.67	0.63	1.85	1.15	9.15
MAXIMUM	72.94	71.50	6.78	6.49	27.74	20.24
MEDIAN	69.85	69.37	3.67	5.13	13.19	16.28
PULMONARY LEAFLET						
n	27	8	27	8	27	8
MEAN	70.07	69.56	2.74	3.89	9.30	11.75
SD	1.69	0.63	1.32	1.65	4.98	4.86
MINIMUM	66.10	68.28	0.52	0.55	1.14	1.83
MAXIMUM	72.70	70.32	4.80	5.28	17.55	17.34
MEDIAN	70.34	69.56	2.94	4.69	9.27	11.72
MITRAL LEAFLET						
n	28	7	28	7	28	7
MEAN	70.42	69.71	6.62	8.57	25.17	30.05
SD	1.89	1.44	4.75	3.06	18.54	7.85
MINIMUM	67.46	68.48	0.42	3.98	0.93	17.94
MAXIMUM	77.39	72.59	15.18	12.77	49.98	42.00
MEDIAN	70.57	68.99	6.00	7.69	21.95	28.04
AORTIC WALL						
n	30	5	30	5	30	5
MEAN	70.35	69.95	1.30	1.22	3.84	4.80
SD	0.74	0.75	0.33	0.14	1.02	0.51
MINIMUM	69.15	69.21	0.69	1.06	2.12	4.28
MAXIMUM	72.21	71.05	2.03	1.45	6.04	5.53
MEDIAN	70.35	69.65	1.29	1.21	3.89	4.76
PULMONARY WALL						
n	26	9	26	9	26	9
MEAN	71.17	70.67	1.09	1.14	3.33	3.71
SD	1.60	1.11	0.30	0.41	1.09	1.56
MINIMUM	68.80	69.47	0.59	0.65	1.46	1.75
MAXIMUM	77.50	73.15	1.68	1.80	5.88	6.26
MEDIAN	71.05	70.81	1.08	1.11	3.18	3.60

[n=sample size; SD = standard deviation; T_d Thermal Denaturation Temperature]

Table 4.4.2 SEM categorization I-III according to H & E classification (Normal or Autolysis)

H&E Classification		Category I		Category II		Category III	
	n	n	%	n	%	n	%
AORTIC LEAFLET							
NORMAL	29	3	(10.3)	22	(75.9)	4	(13.8)
AUTOLYSIS	6	2	(33.3)	4	(66.7)	0	(0.0)
TOTAL	35	**5**	**(14.3)**	**26**	**(74.3)**	**4**	**(11.4)**
PULMONARY LEAFLET							
NORMAL	27	6	(22.2)	9	(33.3)	12	(44.4)
AUTOLYSIS	8	3	(37.5)	3	(37.5)	2	(25.0)
TOTAL	35	**9**	**(25.7)**	**12**	**(34.3)**	**14**	**(40.0)**
AORTIC WALL							
NORMAL	30	25	(83.3)	5	(16.7)	0	(0.0)
AUTOLYSIS	5	5	(100.0)	0	(0.0)	0	(0.0)
TOTAL	35	**30**	**(85.7)**	**5**	**(14.3)**	**0**	**(0.0)**
PULMONARY WALL							
NORMAL	26	21	(80.8)	5	(19.2)	0	(0.0)
AUTOLYSIS	9	9	(100.0)	0	(0.0)	0	(0.0)
TOTAL	35	**30**	**(85.7)**	**5**	**(14.3)**	**0**	**(0.0)**
MITRAL LEAFLET							
NORMAL	28	17	(60.7)	10	(35.7)	1	(3.6)
AUTOLYSIS	7	7	(100.0)	0	(0.0)	0	(0.0)
TOTAL	35	**24**	**(68.6)**	**10**	**(28.6)**	**1**	**(2.9)**

[n=sample size; %= percentages]

4.4.1 THE HISTOLOGY QUALITY OF HARVESTED VALVULAR HOMOGRAFTS

A summary of the histological quality of the harvested valvular homografts is displayed in Table 4.4.3. The only statistically significant differences ($p < 0.05$) were found in the pulmonary leaflets (tensile strength) and aorta wall (young's modulus) normal vs. autolysis. The other test results did not reveal any statistical significant differences ($p > 0.05$) between normal and autolytic tissue.

Table 4.4.3 Normal vs. Autolysis

	T_d		TENSILE STRENGTH		YOUNG'S MODULUS		SEM	
	IT	p-value	IT	p-value	IT	p-value	IT	p-value
AORTIC LEAFLET	Normal vs. Autolysis	0.969	Normal vs. Autolysis	0.095	Normal vs. Autolysis	0.381	Normal vs. Autolysis	0.309
PULMONARY LEAFLET	Normal vs. Autolysis	0.418	Normal vs. Autolysis	**0.049**	Normal vs. Autolysis	0.239	Normal vs. Autolysis	0.607
MITRAL LEAFLET	Normal vs. Autolysis	0.363	Normal vs. Autolysis	0.313	Normal vs. Autolysis	0.592	Normal vs. Autolysis	0.128
AORTIC LEAFLET	Normal vs. Autolysis	0.263	Normal vs. Autolysis	0.599	Normal vs. Autolysis	**0.043**	Normal vs. Autolysis	1.000
PULMONARY LEAFLET	Normal vs. Autolysis	0.394	Normal vs. Autolysis	0.655	Normal vs. Autolysis	0.624	Normal vs. Autolysis	0.297

$p<0.05$ = statistically significant; $p>0.05$ = not statistically significant

(T_d = Thermal Denaturation Temperature; SEM = Scanning Electron Microscopy)

CHAPTER 5
DISCUSSION

5.1 INTRODUCTION

Despite the establishment of homograft banks through the development of cryopreservation, demand for homografts exceeds the supply. The shortage may in part be due to the harvesting protocols popularised by O'Brien *et al*., (1988) and Livi *et al.,* (1987) who promoted a harvest time of less than 24 hours (O'Brien *et al*., 1988; Livi *et al.,* 1987). The time limit of 24 hours was thought necessary to ensure cellular viability, particularly of endothelial cells and fibroblasts, which in turn was thought to influence long-term valve stability (Angell et al., 1989; O'Brien et al., 1995). However, other series demonstrated that acceptable results could be achieved with post mortem harvest times of up to 60 hours (Armiger 1995, Messier *et al*., 1992, Lu *et al*., 1998).

Prior to 1995, harvested homograft valves were stored in an albumin and antibiotic solution for periods of up to 90 hours (Langley *et al*., 1996). The incidence of re-operation for aortic valve homograft failure in the aortic position at 10 years was 12.1% (Langley *et al*., 1996).

The Homovital homograft concept was introduced by Yacoub and co-workers in 1995, (Yacoub *et al*., 1995). This series implanted homografts with storage times of 3 hours to 60 days. Although most of these implants had storage times of less than 48 hours, the authors did not comment on the implants with very long storage times. Other authors (Yankah and Hertzer, 1987) have shown that only 24% of the endothelium survives a storage time at room temperature of 2 hours, with complete destruction of the endothelium noted after storage times between 24 hours and 48

hours. It appears that the anticipated absence of endothelium in homografts with storage times greater than 48 hours would contradict the claim that the grafts implanted in the Yacoub series (Yacoub *et al.,* 1995) were in fact Homovital by definintion.

O'Brien *et al.,* 1987 stated that optimal viability of a homograft could only be ensured if harvest times were not longer than 24 hours and moreover, that this time period should ideally be less than 6 hours. Furthermore, the homograft should be cryopreserved within 4 days of harvesting (O'Brien *et al*., 1987; Langley *et al*., 1996). Post mortem autolysis of human tissue begins approximately 4 minutes after death but these changes are not macroscopically detectable for several days (Vass, 2001). This direct relationship between progressive autolysis and ischaemic time, dictates the acceptable limit of ischaemic time from death to cryopreservation (Moriyama, Utoh, Hagiwara, Kunitomo, Katsuhide and Kitamura, 2001). Importantly, O'Brien *et al.,* has shown that homograft degeneration is delayed if it contains viable fibroblasts and endothelial cells at the time of implantation (O'Brien *et al.,* 1987*)*.

Notwithstanding the limitation on ischaemic/harvesting time, many homograft banks prolong the ischaemic time. The ischaemic time is prolonged because of the pre-cryopreservation processes to which the valves are subjected, namely dissection, incubation and sterilisation. This prolongation of ischaemic time can be as long as 96 hours (Gall *et al.,* 1998).

The insistence that harvest time must be limited to 24 hours is refuted in the above publications. However, this 24 hour limitation might still apply, if homograft tissue in the cadaver was compromised by any adverse event.

Another important aspect of this discussion is that harvest time of less than 24 hours does not necessarily guarantee viable fibroblasts and endothelial cells at implantation. Therefore, it appears that Ischaemic time is not the only factor that influences homograft viability. Although numerous studies have demonstrated that cryopreservation maintains tissue integrity, there is evidence that the number of

viable cells decline during the pre-implantation processes (Gall *et al.,* 1998). The likely reasons for this decline are possibly related to the handling, freezing and thawing processes used (Mirabet, Carda, Solves, Novella-Maestre, Carbonell-Uberos, Caffarena, Hornero, Montero and Roig, 2008).

On the other hand, importantly, Wheatley and co-workers have raised important questions about the presence of excessive viable endothelium cells during implantation. In their study, large numbers of viable cells (fresh homografts) lead to immune reactions, which accelerate the calcification of these valves (Wheatley and McGregor, 1977).

The question might arise: how many viable cells are necessary during implantation to ensure optimal stability and how many endothelium cells should be lost before the immune response is blunted?

Is time the only factor promoting tissue autolysis? Numerous other factors contribute to the development of tissue autolysis. The circumstances surrounding death, post-mortem temperature changes (tempo of cooling down), body mass index (BMI) etc., and are all factors that may influence the tempo of degenerative processes as is well described by (Vass, 2001).

A study was therefore designed to establish the impact on tissue strength for periods up to 72 hours on sheep homografts. The homografts were prepared in two groups, where the one group was allowed to remain at warmer temperatures and the other group was cooled intentionally. The impact of ischaemic time under these conditions was evaluated by documenting the histological appearance as well as the condition of the endothelium using SEM.

These groups were then evaluated as regards tissue strength using objective measurements (Tensile strength and thermal denaturation temperatures).

5.2 Results:

To determine the impact of ischaemic time and temperature, the study focused on three groups: (a) the control group (Group A) which contained homograft valves subjected to < 6 hours ischaemic time, stored at 4ºC; (b) the cold ischaemic group (Group B) subjected to 24 hours, 48 hours and 72 hours ischaemic times, stored at 4ºC, after which the valves were processed and cryopreserved; (c) the warm ischaemic group (Group C) subjected to 24 hours, 48 hours and 72 hours ischaemic times, stored for 6 hours at room temperature (23ºC), followed by 18 hours, 42 hours and 66 hours at 4ºC, after which the valves were processed and cryopreserved. The actual temperatures reached are reported in figure 4.2.1.

To achieve the above-mentioned goals, the following tests were selected to evaluate tissue integrity and quality in the different subsets.

- **Tissue strength:**
  - Thermal denaturation temperature,
  - Tensile strength
  - Young's Modulus

  Morphology
  - Scanning Electron Microscopy (SEM)
  - H&E histological stain

5.3 Group A (Control group)

SEM demonstrated endothelium on all aortic and pulmonary valve structures, although some of the structures showed signs of endothelium damage. The aorta and pulmonary walls lost nearly all their endothelium, but the basement membrane was still clearly visible. Morphologically, however, it was clear that the degenerative processes began sooner in the aorta than in the pulmonary valve scaffolding (SEM evaluation).

H&E colouring showed no autolysis, and all the samples were classified as normal.

DSC indicated little variation as well as a constant denaturation temperature.

Tensile strength was very difficult to determine as a result of the small size of the valvular samples. This caused sample slippage but the same problem was encountered by Patwardhan when evaluating the stress-strain curves (a common problem when testing small biological samples which influence the ability to grip the sample). As a result, the valve tensile strength could only be determined in one direction (uniaxial), although both directions (biaxial) would be preferred. The former method, however, produces acceptable results (Patwardhan and Vaideeswar, 2004).

The results showed a wide variation in the tensile strength. A larger number of samples were used, but the variation remained a problem unique to this test.

Young's modulus was performed as an alternative test to determine whether more reliable results could be obtained with regard to tensile strength. Since Young's modulus is inferred from the tensile strength graph, the "slippage" however also resulted in wide variations.

There was no statistically significant difference between the respective strengths of the aortic and pulmonary valve structures and the aorta and pulmonary walls.

5.4 Ischaemic time

5.4.1 Group B

Group B constituted the "cold ischaemic" group.

Fifteen carcasses were divided into three groups of five each and exposed to 24 hours, 48 hours and 72 hours ischaemic time respectively. During the ischaemic time, the carcasses were kept in a refrigerator at 4ºC. It took the carcasses 24 hours to reach 4˚C core temperature. Afterwards, they were processed, sterilised and frozen. The valves were thawed and then underwent a battery of tests.

SEM demonstrated a reduction in endothelium over time. The majority of the aortic valve leaflets fell in Category II (scattered endothelial cells and intact basal membrane), 24 h (95.8%), 48 h (80%) and 72 h (73.9%). The pulmonary valve leaflets showed that at 24 h (50%) in category III, 48 h (60.9%) in Category II and 72 h (40.9%) in Category II and (40.9%) in Category III). Therefore, both the aortic and pulmonary valves showed endothelial cells up to 72 h.

H&E colouring showed no visible histological signs of early autolysis after 72 h.

No statistically significant reduction in tensile strength could be demonstrated between the three ischaemic time intervals. Tensile strength was not lower than that of the control group in any of the groups.

DSC confirmed the results with regard to tensile strength. Once again, the tissue strength remained constant with respect to the control group and group B despite the prolonged ischaemic times and cryopreservation techniques.

5.4.2 Group C

In group C (warm ischaemic group), 15 carcasses were stored, first at room temperature (23˚C) for 6 hours, and afterwards at 4ºC. The carcasses were also divided into three groups of five each, involving 24

hours, 48 hours and 72 hours ischaemic time respectively. After the groups were subjected to the applicable ischaemic times and cryopreservation techniques, samples of valve and wall tissue underwent five tests.

SEM indicated that a progressive decrease in endothelium coverage and viability took place with a concurrent increase in ischaemic time. Although endothelium declined significantly, some valves had an endothelium presence after 72 hours worth mentioning. The aortic valves leaflets after 24 h (84%) fell in Category II (scattered endothelial cells and intact basal membrane), after 48 h (100%) in Category I (Basal membrane and endothelial cells completely absent), and 72 h (56%) in category II (scattered endothelial cells and intact basal membrane). The pulmonary valve leaflets showed that at 24 h and 48 h (68% and 60%) respectively fell in Category I, and 72 h (40%) in Category II and (36%) in Category I.

H&E colouring showed visible signs of autolysis in the .aortic valve leaflets (20%), pulmonary valve leaflets (60%), mitral valve leaflets (40%) and pulmonary wall (80%) of the 48 h group. Of the 72 h group (100%) of the valve leaflets and artery wall samples showed the presence of autolysis. These results were not unexpected since the tissue had already shown macroscopic signs of degenerative change. The tissue showed changes in colour, accompanied by an unpleasant odour.

No statistically significant reduction in tensile strength could be demonstrated between the three groups and there were no differences between the three ischaemic time intervals. Tensile strength was not lower than that of the control group, and comparable to the results obtained from the cold ischaemic group.

Once again, the results of the DSC analysis confirmed the measurements/conclusions of the tensile strength tests. The strength of

the tissue did not decrease as a result of prolonged ischaemic times, and apparently, even at higher temperatures, tissue strength was retained. Even in the presence of autolysis, the thermal denaturation temperature results were not lower than that of the control group (T_d mean = 69.60 °C for group C (autolytic samples) vs. T_d mean = 66.95 °C for Group A (control group).

5.5 SEM Classification

In order to analyse tissue using the retention of endothelium as a benchmark for possible viability of tissue and its impact on tissue strength, samples were divided into three groups, irrespective of the ischaemic time or temperature that it was exposed to.

No statistically significant differences were found between the three groups with regard to DSC, tensile strength, Young's modulus and H&E.

CHAPTER 6
CONCLUSION

6.1 GENERAL CONCLUSION

The fresh tissue in group A showed morphologically good confluent endothelium coverage of the valve scaffolding, although the aortic and pulmonary walls lost a great deal of their endothelium. No autolysis was observed and a bench mark was set for tissue strength using Tensile strength, Young's modules and thermal denaturation temperature (T_d) tests.

Compared to the baseline (Group A), the cold ischaemic group (Group B) showed no increased signs of autolysis up to 72 hours. The endothelium coverage decreased with increased ischaemic time and in the 72 hour group 91.3% of aortic and 59.1% of pulmonary valvular tissue had no or incomplete endothelium cover.

No reduction of tensile strength or in Young's Modules could be demonstrated and thermal denaturation temperature showed no decrease in strength up to 72 hours compared to the control group.

These results, surprisingly, hold true for Group C (warm ischaemic time). In Group C some valves demonstrated early autolysis, but still retained tissue strength as demonstrated by tensile strength and thermal denaturation temperature evaluation.

This study demonstrates that tensile strength as well as basic morphology (H&E stain) of valvular tissue is retained up to 72 hours.

There is a reduction of endothelium cover over time, but this does not have a significant impact on the physical properties of the homograft tissue according to

the *in vitro* tests we applied. It therefore remains to be demonstrated in an *in vivo* model, what, if any impact, ischaemic time has on the functioning and degeneration of homografts.

We could not demonstrate a reliable relationship between pulmonary and aortic wall data that would provide predictive methods.

Cryopreservation and tissue processing had no deleterious effect on tissue strength.

6.2 FUTURE RECOMMENDATIONS

In vivo studies of animals are proposed to further investigate the outcomes of the various ischaemic groups in a sheep model.

Degeneration of homografts should be studied in an attempt to qualify and quantify underlying mechanisms.

An attempt should be made to explain the biological impact or lack thereof, of increased ischaemic time on *in vivo* behaviour of homografts.

Host tissue interaction might be important in understanding the final outcome of homografts *in vivo*.

6.3 LIMITATIONS

The study was limited by:

- The size of tissue samples (valvular leaflets) caused technical difficulties especially when performing tensile strenght testing
- Analysis requires a high level of skills (Scanning Electron Microscopy)
- Financial constraints limited sample pool

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Appendix A

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Me / Ms H Strauss 2006-10-06

MR D BESTER
C/O MR H VAN DEN HEEVER
DEPT OF CARDIOTHORACIC SURGERY
FACULTY OF HEALTH SCIENCES
UFS

Dear Mr Bester

ANINMAL EXPERIMENT NR 11/06
PROJECT TITLE: HISTOLOGICAL COMPARISON OF THE EFFECTS OF WARM ISCHEMIC TIMES ON HARVESTED HOMOGRAFTS

We hereby wish to inform you that the above-mentioned protocol was approved by the Control Committee for Animal Experimentation

ANIMAL	AMOUNT	EXPIRY DATE
Sheep	15	31 MARCH 2007

A report regarding these projects have to be submitted after completion

REMARKS: *None*

Regards

H. Strauss

For DIRECTOR: MEDICINE ADMINISTRATION
Cc Dr FJ Potgieter, Unit for Animal Experimentation, UFS

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2007-06-12

MNR D BESTER
P/A MNR H VAN DEN HEEVER
DEPT KARDIOTORAKSCHIRURGIE
FAKULTEIT GESONDHEIDSWETENSKAPPE

Geagte Mnr Bester

PROEFDIEREKSPERIMENT NO 11/06
PROJEKTITEL: „HISTOLOGICAL COMPARISON OF THE EFFECTS OF WARM ISCHEMIC TIMES ON HARVESTED HOMOGRAFTS."

Graag wens ons u mee te deel dat u aansoek om hernuwing van die bogenoemde diereproef deur die Dagbestuur van die Beheerkomitee vir Proefdiereksperimente oorweeg en goedgekeur is.

DIERSOORT	AANTAL	VERVALDATUM
Dorper Skape	15	September 2007

'n Verslag met betrekking tot hierdie studie moet na afhandeling ingedien word.

KOMMENTAAR:

- *Studie kan herhaal word in „sarsies" van n=3*
- *Reël asseblief net afspraak voordat genadedood toegepas word dat daar wel op daardie stadium voldoende ruimte is in die koelkamers van die Dept Vee-, Wild- en Weidingkunde.*

Vriendelike groete

H Strauss

n. VOORSITTER: BEHEERKOMITEE VIR PROEFDIEREKSPERIMENTE
aa Me Tokkie Vivier, Proefdiereenheid, UV