# The impact of extended harvesting times on tissue integrity of cryopreserved ovine pulmonary homografts

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**(PhD)**

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# Declaration of independent work

I, Dreyer Bester, do hereby declare that this dissertation:

**The impact of extended harvesting times on tissue integrity of cryopreserved ovine pulmonary homografts**

submitted to the University of the Free State for the degree ***Philosophiae Doctor*** is my own independent work and that neither nor any other person in fulfillment of the requirements for the attainment of any qualification has submitted it to any institution.

Signed: _____ Date: _____

**Dreyer Bester**
**Principle Investigator**

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# Statement of compliance

This study was conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice (ICH E6), the Code of Federal Regulations on the Protection of Human Subjects (45 CFR Part 46), and the World Medical Association Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013). All personnel involved in the conduct of this study have completed Good Clinical Practice (GCP) training or has been under the direct supervision of such an accredited researcher.

All animal experiments and surgical procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication 85-23, revised 1996).

# List of abbreviations

| | |
|---|---|
| % | Percentage |
| & | And |
| + | Positive |
| < | Less than |
| = | Equals |
| > | More than |
| ± | Plus minus |
| ≤ | Less-than or equal to |
| ≥ | Greater-than or equal to |
| °C | Degrees celsius |
| °C/min | Degrees celsius per minute |
| µg/mg | Micrograms per milligram |
| µl | Microlitre |
| A | Cross sectional area |
| AHV | Allograft heart valve |
| Al | Aluminium |
| ANOVA | Analysis of variance |
| AoH | Aorta homograft |
| AR | Aortic valve regurgitation |
| BM | Basement membrane |
| BMI | Body mass index |
| CD31 | Platlete endothelial cell adhesion molecule antigen |
| CD34 | Platlete endothelial cell antigen |
| CD4 | T-helper antigen |
| CIT | Cold ischaemic time |
| cm | Centimetres |
| $CO_2$ | Carbon dioxide |
| CPA | Cryopreserved pulmonary homografts |
| DMSO | Dimethyl sulfoxide |
| DSC | Differential scanning calorimetry |

| | |
|---|---|
| e | Strain |
| ECM | Extra cellular matrix |
| EGF | Epidermal growth factor |
| ELAM–1 | Endothelial–leukocyte adhesion molecule 1 |
| EM | Equal mean |
| *et al*. | *Et alia*(and others) |
| *etc.* | *Etcetera* |
| ETOVS | Animal Ethics Committee of the University of the Free State |
| F | Applied force / tensile force |
| FGF | Fibroblast growth factor |
| Fig. | Figure |
| g | Gram |
| g/l | Grams per litre |
| GM–CSF | Granulocyte–macrophage colony–stimulating actor |
| h | Hour |
| H&E | Haematoxylin and eosin stain |
| HB–EGF | Heparin binding epidermal growth factor |
| HLA | Human leukocyte antigen |
| hrs | Hours |
| i.e. | *Id est* (that is) |
| ICAM–1 | Intercellular adhesion molecule 1 |
| IgE | Immunoglobulin E |
| IGF–1 | Insulin–like growth factor |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IL–1 | Interleukin–1 |
| IL–2 | Interleukin–2 |
| IL–6 | Interleukin–6 |
| ISO9000 | International organisation for standardisation |
| IT | Ischaemic time |
| IU | International units |
| kg | Kilogram |
| kU/l | Kilo-units per litre |
| kV | Kilovolt |
| $L_0$ | Initial unstressed length |

| | |
|---|---|
| $Lbs/in^2$ | Pounds per square inch |
| $LN_2$ | Liquid nitrogen |
| Ltd | Limited |
| M199 | Medium 199 |
| $m^2$ | Square metre |
| mg | Milligrams |
| mg/hour | Milligrams per hour |
| mg/kg | Milligrams per kilogram |
| MHz | Mega Hertze |
| min | Minute |
| ml | Millilitres |
| mm | Millimetre |
| mm Hg | Millimetres mercury |
| mm/min | Millimetres per minute |
| mm/s | Millimetres per second |
| mmol | Millimole |
| MPa | Mega Pascal |
| N | Newton / load |
| n | Number of samples analysed |
| $N/mm^2$ | Newton per square millimetres |
| $N_2$ | Nitrogen |
| Nr | Number |
| p | Statistical significance |
| P120 | Sanding paper grid size |
| Pa | Pascal |
| PDGF | Platelet–derived growth factor |
| Ph.D. | Philosophiae Doctor |
| PM | Post mortem |
| psi | Pounds per square inch |
| Pty | Proprietary |
| PUH | Pulmonary homograft |
| RSA | Republic of South Africa |
| RVOT | Right ventricular outflow tract |
| S | Stress |
| SA | South Africa |
| SD | Standard deviation |

| | |
|---|---|
| SEM | Scanning electron microscopy |
| ST | Sino-tubular Junction |
| $T_d$ | Thermal denaturation temperature |
| TDT | Thermal denaturation temperature |
| TGF–β | Transforming growth factor beta |
| Tmax | Transition temperature maximum |
| TNFα | Tumor necrosis factor alpha |
| Tp | Transition temperature peak |
| TS | Tensile strength |
| UFS | University of the Free State |
| UK | United Kingdom |
| USA | United States of America |
| VCAM –1 | Vascular cell adhesion molecule 1 |
| VK | von Kossa stain |
| vs. | Versus |
| vWF | von Willebrand factor |
| W/g | Watt per gram |
| WIT | Warm ischaemic time |
| X | Times / Magnification |
| YM | Young's modulus |
| ΔH | Enthalpy of denaturation |
| ΔL | Change in length due to stress |
| σ | Tensile stress |

# Focal definitions

| | |
|---|---|
| **Allograft** | A homograft between allogenic individuals. |
| **Antibiotic sterilised homograft** | Antibiotic-sterilised valves stored at 4°C in nutrient media are considered to be nonviable 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 either 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°C for two to three hours after death, during which time the stomachs were removed, before being cooled to 4°C. |
| **Criteria** | A standard on which a judgment or decision may be based, or a characterising mark or trait. |
| **Cryopreserved homografts** | Cryopreserved valves are valves sterilised in antibiotic solution and subsequently cryopreserved (O'Brien *et al*., 1987). |
| **Differential scanning calorimetry (DSC)** | 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 programme (Höhne *et al*., 2003). |
| **Endothelium** | Endothelial cells of mesoblastic origin composed 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, especially for transplanting. |
| **Heamatoxylin and Eosin stain (H&E)** | 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 interstitial cells such as fibroblasts that retain their ability to replicate and regenerate extracellular matrix elements (Barili *et al*., 2007). Cryopreserved valves are valves sterilised in antibiotic solution and subsequently cryopreserved. They are considered viable if cryopreserved within four days of procurement (O'Brien *et al*., 1987). |
| **Homovital homograft** | These homografts are harvested under sterile conditions, are stored in an antibiotic solution at 4°C 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, animal or human. |
| **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. |
| **Scanning electron microscope (SEM)** | 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 (TS)** | 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). |
| **Young's modulus (YM)** | 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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# Executive summary

The use of aorta valve homografts in cardiac surgery was pioneered by Donald Ross and Barratt-Boyes (Ross, 1962; Barratt-Boyes, 1964) and today, pulmonary valve homografts remain the valved conduit of choice for reconstruction of the right ventricle outflow tract (RVOT) required in the treatment of common congenital cardiac conditions.

Initially, homografts were harvested from cadavers generally within seventy-two hours after death, in a non-sterile environment, and then freshly preserved in a sterile antibiotic medium at 4°C. These homografts were then used within six to eight weeks after procurement (Botes *et al*., 2012).

Cryopreservation was popularised by Marc O'Brien (O'Brien *et al*., 1987), which saw the introduction of the development of homograft banks. It was claimed that these valves retained a degree of viability, which enhances long term durability after implantation. Freshly unprocessed valves that were harvested under sterile conditions from beating heart donors or within hours after death, were implanted (unprocessed) shortly afterwards (Yacoub *et al.*, 1995).

These studies resulted in the demise of cadaver programmes and programmes cryopreserving homografts harvested from beating heart donors, or less than six hours to a maximum of twenty-four hours post mortem became the norm.

On the other hand, it became clear that immune response to viable tissue, especially viable endothelium, resulted in earlier rejection of homografts, especially in children (Yankah *et al*., 1995). Furthermore, long term results of fresh antibiotic sterilised valves stored at 4°C compared to early cryopreservation of viable valves failed to confirm or support earlier expectations and were similar in several studies, notably in that of O'Brien *et al,* in 2001.

In, a number of explant studies it was also concluded that homografts become nonviable and essentially acellular within months of implantation and are essentially nonviable scaffolds (Mitchell *et al*, 1998, Koolbergen *et al,* 2002). The primary role of immunological processes on

homograft survival was therefore questioned. The damaging effect on homograft tissue during the cryopreservation process was also described (Schenke-Layland *et al*., 2006).
Thus, during the last fifty years of homograft banking, cryopreservation remained the technique of choice with various studies suggesting that early post mortem harvesting has a beneficial effect on homograft survival after implantation. This could however not be demonstrated in several long term studies. The deleterious effect of truly viable valves and associated immune processes on homograft survival were also described. In addition, several studies showed that explanted valves were essentially acellular and thus nonviable.

In reality, the time from post mortem cardiectomy or homograft bank receipt before processing and cryopreservation commonly extend to fourty-eight hours as reported in the Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013). This implies that the inevitable cold ischaemic time before cryopreservation is extended to three to four days in a significant percentage of cases anyway. This, and the complexity of issues of homograft viability as well as inconclusive long term advantages of homograft viability in published series, beg the question whether cadaver programmes should not be re-evaluated.

The Bloemfontein homograft bank is an almost exclusively cadaver donor based programme, with average post mortem harvest times exceeding twenty-four hours (mean thirty hours). Unpublished clinical results evaluating outcomes of pulmonary homografts implanted in the RVOT of children less than fourteen years, could not show a difference in freedom from reoperation between homografts harvested more than twenty-four hours post mortem and those harvested less than twenty-four hours post mortem. As the Bloemfontein homograft bank is presently the only homograft bank in South Africa, it embarked on a number of experimental studies in the ovine model in order to validate its practise and by implication, also that of cadaver based programmes.

Four studies are presented evaluating the impact of increased post mortem harvest times and cryopreservation on homograft tissue integrity and *in vivo* performance.

In the first study, the impact of increased post mortem homograft harvest times is described in cryopreserved ovine pulmonary homografts harvested twenty-four hours, fourty-eight hours and seventy-two hours post mortem. In the *in vitro* studies evaluating the morphology and tissue strength before implantation, no differences could be observed between the groups up to seventy-two hours post mortem harvest times. In the *in vivo* study no differences could be discerned in clinical performance, immunological processes, morphology, tissue strength and

calcification after 180 days implantation. It was concluded that post mortem harvest times of pulmonary homografts can safely be extended up to seventy-two hours.
In the second study, the morphology of unprocessed and cryopreserved pulmonary homograft leaflets with post mortem harvest times up to seventy-two hours was described. The impact of cryopreservation on leaflets *per se* was described in a control group as well as in tissue harvested at twenty-four hours, fourty-eight hours and seventy-two hours post mortem. Once again, no impact of extended post mortem harvest times could be perceived, except for increased oedema on TEM in the seventy-two hour group. Picrosirius red staining demonstrated that cryopreservation had a compressing and flattening impact on collagen in all groups. Disruption of collagen was observed on TEM in all cryopreserved groups. It demonstrated that cryopreservation had an immediate impact on tissue morphology and produced more ultrastructural tissue disruption than extending post mortem harvest times.

In the third study, the impact of increased post mortem harvest times was studied *in vitro* comparing unprocessed and cryopreserved leaflets in relation to tissue strength. No difference in strength using tensile strength, Young's modulus and thermal denaturation temperture, could be observed between the control group and the twenty-four hour, fourty-eight hour and seventy-two hour groups in the unprocessed leaflets. In addition, no difference could be discerned between leaflets processed and cryopreserved after twenty-four hours, fourty-eight hours and seventy-two hours post mortem harvesting. Tensile strength was potentially reduced by cryopreservation when compared to unprocessed leaflets, but did not reach statistical significance in all instances.

In the final study, a fourty-eight hour post mortem homograft harvested group was processed and cryopreserved for implantation. This mimicks the clinical circumstances of cadaver programmes.

The objective of this study was to evaluate the stability of homografts' leaflet tissue after two periods of implantation. Control tissue (processed, cryopreserved and thawed) was compared to tissue explanted after two weeks and after 180 days in the ovine model. Despite the disruptive effect of cryopreservation demonstrated by TEM in all groups, the tissue remained stable throughout the period with normal clinical function and minimal calcification at 180 days.

Through these studies conducted in the ovine model in order to provide experimental evidence for the safe extension of cold post mortem harvest times, it was concluded that *in vitro* and *in vivo* studies could not reveal detrimental effects on tissue integrity up to at least fourty-eight

hours and possibly to seventy-two hours post mortem harvesting. The safety of fourty-eight hour post mortem harvested and thereafter cryopreserved pulmonary homografts was specifically studied in order to mimic the human clinical scenario wherein the stability of the homografts was confirmed in two study periods.

It is concluded that these studies provide experimental scientific evidence to increase post mortem homograft harvest times to at least fourty-eight hours. Furthermore, these studies collectively provide experimental support for the re-evaluation of human cadaver homograft donor banks in order to attenuate international homograft shortages.

# Bestuursopsomming

Die gebruik van aorta-homotransplantate in kardiale chirurgie het deur die pogings van die baanbrekers Donald Ross en Barratt-Boyes (Ross, 1962; Barratt-Boyes, 1964) gewildheid bereik en tot vandag toe nog is pulmonale homotransplantate die eerste keuse wanneer dit kom by die gebruik van kleppe vir die rekonstruksie van die regterventrikel-uitvloeikanaal (RVUK) wat noodsaaklik is in die behandeling van algemene kongenitale hart-toestande.

Aanvanklik is homotransplantate oor die algemeen binne twee-en-sewentig uur na afsterwe in ‘n nie-steriele omgewing van kadawers geoes en daarna vars gepreserveer in ‘n steriele antibiotiese middel teen 4°C. Hierdie homotransplantate is dan binne weke vanaf verkryging gebruik.

Kriobewaring is deur Marc O’Brien (O’Brien *et al.*,1987) gewild gemaak en het aanleiding gegee tot die totstandkoming van homotransplantaatbanke. Daar is aangevoer dat hierdie kleppe ‘n mate van lewensvatbaarheid behou, wat hul langtermyn bestendigheid na inplanting verhoog. Vars, onverwerkte kleppe wat onder steriele toestande van kloppende-hart skenkers of kort na afsterwe geoes is, is kort daarna ingeplant (onverwerk) (Yacoub *et al.*, 1995).

Hierdie studies het tot die heengaan van kadawerprogramme gelei en programme wat die kriobewaring van homotransplantate wat geoes is van kloppende-hart skenkers, of minder as ses ure tot ‘n maksimum van vier-en-twintig uur na afsterwe voorstaan, het die norm geword.

Dit het egter ook terselfdertyd duidelik geword dat die immuunreaksie op weefsel-lewensvatbaarheid, veral lewensvatbare endoteel, tot vroeër verwerping van homotransplantate gelei het, veral wat kinders aanbetref (Yankah *et al.*, 1995). Langtermyn resultate van vars, antibioties gesteriliseerde kleppe wat teen 4°C bewaar is en met vroeë kriobewaring van lewensvatbare kleppe vergelyk is, het boonop nie daarin geslaag om aanvanklike verwagtings te bevestig of te ondersteun nie en was soortgelyk in verskeie studies, noemenswaardig dié in 2001 deur O’Brien *et al*.

Uit ‘n hele aantal uitplanting-studies is dit afgelei dat homotransplantate binne maande na inplanting nie-lewensvatbaar en hoofsaaklik asellulêr word, wat dit wesentlik nie-lewensvatbare raamwerke maak (Mitchell *et al.*, 1998, Koolbergen *et al.*, 2002). Die primêre rol van immunologiese prosesse in die oorlewing van homotransplantate is dus bevraagteken. Die skade-effek van kriobewaring op homotransplantaatweefsel gedurende die kriobewaringsproses is ook beskryf (Schenke-Layland *et al.*, 2006).

Gedurende die laaste vyftig jaar van die bestaan van homotransplantaatbanke, het kriobewaring sodoende die gekose tegniek gebly met verskeie studies wat te kenne gegee het dat vroeë *post mortem*–oes ‘n voordelige effek op die oorlewing van homotransplantate na inplanting het. Dit kon egter nie deur verskeie langtermyn studies bewys word nie. Die verwoestende uitwerking van ware lewensvatbare kleppe en geassosieerde immuun-prosesse op die oorlewing van homotransplantate is ook beskryf. Verskeie studies het ook bewys dat uitgeplante kleppe essensieel asellulêr en dus onlewensvatbaar was.

In realiteit is dit heel algemeen vir die tydperk, vanaf ‘n *post mortem*-kardiektomie of ontvangs deur die homotransplantaatbank voor die verwerking en kriobewaring, om na agt-en-veertig uur verleng te word soos gerapporteer is in *the Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013)*. Dit suggereer dat die onafwendbare koue iskemiese tyd wat kriobewaring voorafgaan buitendien met drie tot vier dae verleng word – in ‘n aansienlike aantal gevalle. Dít, tesame met die kompleksiteit van kwessies rondom die lewensvatbaarheid van homotransplantate asook gebrekkige bewyse in publikasiereekse ten opsigte van die langtermyn voordele van die lewensvaatbaarheid van homotransplantate, dwing die vraag na die herevaluering of nie van kadawerprogramme af.

Die homotransplantaatbank in Bloemfontein is ‘n bykans algehele kadawer-gebaseerde program, met gemiddelde *post mortem*-oestye wat vier-en-twintig uur oorskry (gemiddeld dertig uur). Ongepubliseerde kliniese resultate wat die uitkoms van pulmonale homotransplantate in die regterventrikel-uitvloeikanaal van kinders jonger as veertien jaar evalueer, kon nie ‘n verskil in die oorbodigheid van heroperasie toon tussen homotransplantate wat meer as vier-en-twintig uur na afsterwe geoes is en homotransplantate wat minder as vier-en-twintig uur na afsterwe geoes is nie. Aangesien die Bloemfontein homotransplantaatbank huidiglik die enigste homotransplantaatbank in Suid-Afrika is, is ‘n aantal studies in ‘n skaapmodel aangepak om die bank se praktyk, en per implikasie, die kadawergebaseerde program van die bank te staaf.

Vier studies wat die impak van verlengde *post mortem*-oestye en kriobewaring op homotransplantaatweefsel se integriteit en *in vivo*-prestasie evalueer, word hier voorgelê.

Tydens die eerste studie, word die impak van verlengde *post mortem*-oestye beskryf in skape waarvan die kriobewaarde, pulmonale homotransplantate vier-en-twintig uur, agt-en-veertig uur en twee-en-sewentig uur *post mortem* geoes is. In die *in vitro*-studies wat die morfologie en weefselsterkte voor inplanting evalueer, kon geen verskil tussen die groepe waargeneem word nie, tot en met twee-en-sewentig uur *post mortem*-oes. In die *in vivo*-studies kon geen verskil in kliniese werking, immunologiese prosessse, morfologie, weefselsterkte en verkalking na inplanting op 180 dae opgemerk word nie. Daaruit is afgelei dat *post mortem*-oestye van homotransplantate met veiligheid tot en met twee-en-sewentig dae verleng kan word.

In die tweede studie word die morfologie van onbewerkte en kriobewaarde pulmonale homotransplantaatklepsuile met *post mortem*-oestye van tot en met twee-en-sewentig uur beskryf. Die impak van kriobewaring op spesifiek klepsuile is in 'n kontrolegroep beskryf, sowel as in weefsel wat vier-en-twintig uur, agt-en-veertig uur en twee-en-sewentig uur na afsterwe geoes is. Daar kon weereens geen impak deur die verlenging van *post mortem*-oestye waargeneem word nie, behalwe vir 'n toename in edeem op die transmissie elektron mikroskopie (TEM) in die twee-en-sewentig uur-groep. Deur middel van Picrosirius-rooi is daar gedemonstreer dat kriobewaring 'n samepersende en afplattende effek op kollageen in alle groepe het. Kollageenskeuring is op die transmissie elektron mikroskopie van alle kriobewaarde groepe waargeneem. Dit het gedemonstreer dat kriobewaring 'n onmiddellike impak op weefselmorfologie het en dat dit meer ultra-strukturele weefselskeuring as die verlenging van *post mortem*-oestye veroorsaak.

Tydens die derde studie is die impak van verlengde *post mortem*-oestye *in vitro* bestudeer deur onverwerkte en kriobewaarde klepsuile ten opsigte van weefselsterkte te vergelyk. Geen verskil tussen die onbewerkte klepsuile van die kontrolegroep, die vier-en-twintig uur-, agt-en-veertig uur- en twee-en-sewentig uur-groepe ten opsigte van sterkte kon deur middel van rekbaarheidsterktetoeste, Young se moduletoets en termiese denaturering temperatuur waargeneem word nie. Benewens dit, kon geen verskil tussen klepsuile wat na vier-en-twintig uur, agt-en-veertig uur en twee-en-sewentig uur *post mortem* geoes, verwerk en kriobewaar is, onderskei word nie. Wanneer dit met onverwerkte klepsuile vergelyk is, is rekbaarheidsterkte moontlik deur kriobewaring verminder, maar dit was nie in enige van die gevalle statisties beduidend nie.

In die finale studie is ‘n homotransplantaatgroep wat agt-en-veertig uur na afsterwe geoes is bewerk en kriobewaar vir inplantingsdoeleindes. Dit boots spesifiek die kliniese omstandighede van kadawerprogramme na. Die doelwit van hierdie studie was om die stabiliteit van homotransplantaatklepsuilweefsel na twee tydperke van inplanting te evalueer. Kontroleweefsel (verwerk, kriobewaar en ontdooi) is met weefsel wat na twee weke en 180 dae in die skaapmodel uitgeplant is vergelyk. Ten spyte van die skeuringseffek van kriobewaring wat deur transmissie–elektronmikroskopie in alle groepe gedemonstreer is, het die weefsel teen 180 dae regdeur die tydperk bestendig en stabiel gebly met normale kliniese funksie en minimale verkalking.

Danksy die voorgenoemde skaapmodelstudies wat uitgevoer is ten einde eksperimentele bewyse te lewer dat dit veilig is om koue *post mortem*-oestye te verleng, is daar afgelei dat *in vitro*- en *in vivo*-studies geen nadelige effek getoon het op die weefselintegriteit van homotransplantate wat ten minste agt-en-veertig uur en moontlik selfs tot twee-en-sewentig uur *post mortem* geoes is. Die veiligheid van homotransplantate wat agt-en-veertig uur na afsterwe geoes is en daarna kriobewaarde pulmonale homotransplantate is spesifiek bestudeer deur die menslike kliniese omstandighede na te boots waartydens die stabiliteit van die homotransplantate tydens twee studietydperke wel bevestig is.

Daar word tot die slotsom gekom dat hierdie studies eksperimentele, wetenskaplike bewyse lewer om *post mortem* homotransplantaat-oestye tot na ten minste agt-en-veertig uur te verleng. Hierdie studies voorsien verder kollektief eksperimentele steun om menslike kadawer homotransplantaatskenkerbanke te herevalueer in ‘n poging om internasionale homotransplantaat-tekorte te verminder.

# Chapter 1
Introduction

## 1.1 Historical perspective

The first usage of fresh aortic homografts was reported by Gordon Murray in 1956, who implanted these valves in the descending aorta of patients with aortic valve insufficiency. Although the procedure was only partially successful in controlling aortic valve regurgitation, the valves were remarkably durable. Four patients had no calcification with normal valve function at thirteen years and two other patients had preserved valve function at twenty years (Heimbecker, 1986). This is a remarkable result.

The use of aortic homografts in the treatment of aortic valve disease was described in 1962 by Donald Ross (a native of Kimberley, South Africa, working in England at the time) and also independently by Barrett Boyes in New Zealand (Ross, 1962; Barratt-Boyes, 1964).

**"This kicked off the age of homograft surgery."**

Early homograft valves were freshly harvested, implanted, with minimal treatment, relatively "quickly" after harvesting and with no attempt at ABO blood group matching. These valves delivered excellent haemodynamic results and were remarkably durable. Therefore, demand soon outstripped donor availability.

Attempts to establish homograft banks soon followed. Various storage techniques were explored. This included freeze drying and antibiotic sterilisation with grafts being stored in a fridge at 4°C. The use of aggressive antibiotic regimes to prevent transmission of infection, irradiation, flash freezing and glutaraldehyde sterilisation and fixation were also explored. However, this resulted in such a severe reduction in valve durability, to the extent that the use of homografts became unpopular, especially in an age where newer mechanical and xenograft valves that had been developed during the sixties and seventies were more readily available (Merin and McGoon, 1973; Heimbecker *et al*., 1968). Despite this, valve survival of up to 50% at seven years was described, equivalent to that of the xenografts and mechanical valves of the time (Wain *et al*., 1980; Baratt-Boyes *et al*., 1977).

However, a number of centres continued to use homografts and by the late seventies a number of cadaver programmes were established world-wide, with valves harvested at variable ischaemic times, antibiotic sterilised and stored at 4°C or "fresh-wet stored" (Hopkins, 2005).

Encouraging medium to long-term results were being reported. In 1980 Ross reported on 615 homografts followed for up to fifteen years. It included a group of valves that was freeze dried as well as 179 pulmonary autografts, of which 90% of these patients had not succumbed to valve related issues at ten years (Penta *et al*., 1984).

Various studies analysed and compared the clinical outcomes of fresh antibiotic sterilised, 4°C homografts, cryopreserved homografts and viable homografts. A number of important studies are discussed hereafter to assess the impact on homograft degeneration and freedom from re-operation when the different and bearing in mind, at the time, evolving techniques of homograft processing were being compared.

## 1.2 Classification of homografts

Homograft valves are classified based on the method of preservation. Homovital is untreated valves, harvested under sterile conditions, usually from the recipient at the time of heart transplantation and kept in nutrient media. They are considered viable if implanted within three days (Yacoub *et al.,* 1995). Antibiotic sterilised are valves stored at 4°C in nutrient media and are considered to be nonviable valves (Yacoub and Kittle, 1970). Cryopreserved valves are sterilised in an antibiotic solution and subsequently cryopreserved. These valves are considered viable if cryopreserved within four days of procurement (O'Brien *et al.,* 1987). Cryopreservation remains the most commonly used method for valvular preservation and storage. Homografts are cryopreserved and stored in the vapor phase of liquid nitrogen at minus 140°C.

Since 1968 homograft valves have been sterilised using antibiotic drugs and stored in culture medium at 4°C (Yacoub and Kittle, 1970). The short- and mid-term clinical results with antibiotic sterilised valves were superior to those with chemical sterilisation and similar to those with untreated fresh homograft valves (Barratt-Boyes *et al*., 1977). Antibiotic sterilised valves stored at 4°C are considered to have a storage time of about six to eight weeks before they are regarded as unusable. Valve storage through cryopreservation in liquid nitrogen in the 1970s allowed much longer periods, most likely indefinitely (Mermet *et al*., 1970).

### 1.2.1 Important published studies

Yacoub and his group in Harefield, England reported on 679 patients operated between 1979 and 1980 with actuarial patient survival rates of 87% at five years and 81% at eight years (Thompson *et al*., 1979; Ross and Yacoub, 1969). This was followed by a 1984 series of 140 patients, followed for a mean of eleven years with freshly wet stored homografts using the Ross methods (Penta *et al*., 1984). Freedom from valve failure was 72% at ten years. However, in this series, prolonged warm ischaemic harvest times and older age of the recipient were associated with worse outcomes (Penta *et al*., 1984). This was one of the earliest series that showed warm ischaemic post mortem harvest time was associated with worse outcomes setting in motion what eventually became the viable homograft "movement".

In 1999, Lund *et al*. reported on a twenty-five-year follow-up of primary aorta valve replacement in 618 patients from the Yacoub group. Of these patients, 479 received fresh antibiotic sterilised valves, only 12 received cryopreserved valves and the remaining 127 received a viable valve. In this study the authors concluded that viable valves had a homograft survival benefit. Post mortem harvest times in the fresh antibiotic sterilised group was 40±22 hours with a range of 0–102 h. Viable valves were harvested and placed in sterile medium in a fridge at 4°C. However, implantation of the viable group occurred at a mean of fourty-eight hours post harvesting, 31% of implants occurred between fourty-nine to ninety-six hours and 20% occurred after ninety-six hours. Remarkably, the implantation time ranged between three hours and thirty days. In another series of 275 homovital valves, most implants occurred within three days, but with a range of three hours to sixty-two days (Yacoub *et al*., 1995).

However, the question begs: how viable were these valves when implanted at three days? It is also important to note that Barrett-Boyes (1987) also considered valves stored for eight days to be nonviable.

Mark F. O'Brien described a cryopreservation technique and the use of viable homografts in 1987. In his first series of 124 fresh antibiotic sterilised homografts and 192 cryopreserved valves, he described valve selection to be from a donor less than sixty years old, harvested within twenty-four hours after death, no malignancy or systemic diseases and infection, incubated for twenty-four hours in a low dose antibiotic solution in a nutrient medium and cryopreserved by day three to four post mortem (O'Brien *et al*., 1987a & b). He projected an actuarial survival benefit of viable cryopreserved valves over that of fresh antibiotic treated homografts (essentially nonviable). However, it must be noted that the follow-up of the

cryopreserved group was only five years, compared to thirteen years of the fresh antibiotic sterilise group. Excellent results were obtained in both groups with 84% actuarial freedom of operation in the fresh antibiotic group versus 92% in the cryopreserved group.

Importantly, in this article O 'Brien (1987a) stated that after antibiotic sterilisation in either a balanced salt solution or nutrient medium, valve viability declines and consequently most of these valves would be nonviable, as was stated by Ross before.

In 1995 O'Brien *et al*. reported a modification of donor policy, now only accepting homografts within six hours post mortem and cryopreserved within twenty-four hours. Once again he could show an actuarial freedom of structural deterioration advantage in the viable cryopreserved group.

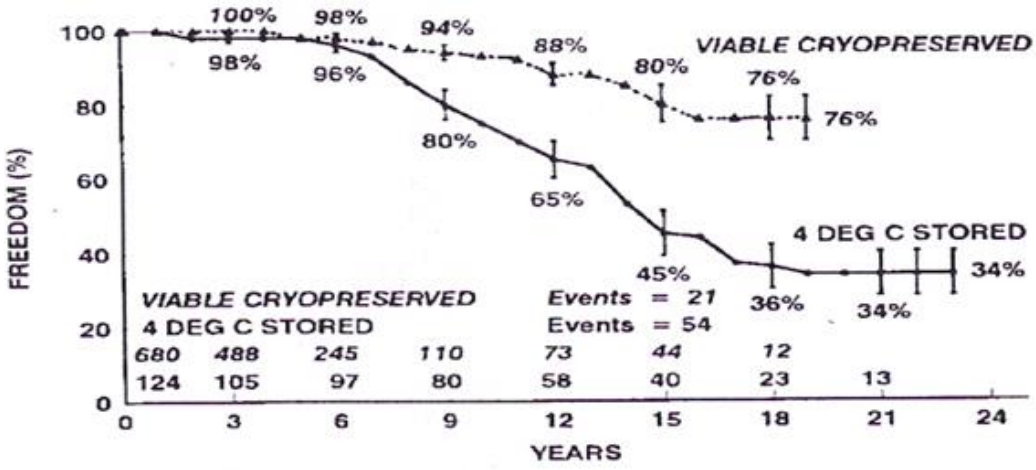


**Figure 1.1** **Actuarial percent freedom from structural deterioration for series I (events = 54) and for series II (events = 21).** P-value for difference is 0 (adapted from O'Brien *et al*., 1995).

However, two important observations were made. All explanted valves in the fresh antibiotic group were acellular and this in the viable cryopreserved group (now harvested at less than six hours post mortem and cryopreserved within twenty-four hours) thickened and retracted leaflets were seen in all homografts showing viability before implantation. This clearly points at some immunological process.

In 2001, O'Brien *et al*. published a twenty-nine-year follow-up series of homografts in three subsets (Table 1.1). He found that the earlier advantage of viable cryopreserved homografts disappeared and that the freedom from structural degeneration curves in all groups meet. The series also demonstrated the reduced homograft survival rate in recipients less than twenty years old.

**Table 1.1** **Patient cohort (n=1022), series, preservation, dates and type of implantation technique for homograft aortic valve replacement** (adapted from O'Brien *et al*. 2001).

| SERIES I | SERIES II | SERIES III |
|---|---|---|
| **4°C antibiotic-stored** | Early cryopreservation | Early cryopreservation |
| **Nonviable at implantation** | Viable at implantation | Viable at implantation |
| **n = 124** | n = 546 | n = 352 |
| **December 1969 to May 1975** | June 1975 to April 1995 | November 1985 to December 1998 |
| **Sub-coronary implantation** | Sub-coronary/cylinder implantation | Root replacement |

In the 1989 series from the Ross group, Bodnar *et al*. (1989) demonstrate a difference between the freedom of re-operation and graft survival between different preservation techniques, including freeze drying, frozen, and antibiotic storage.

The Ross group reported an 89% graft survival after six years in the aorta position in 1978 (Ross *et al*., 1979) in the fresh antibiotic sterilised group stored at 4°C. With regard to fibroblast viability, using titrated thymidine studies, Ross demonstrated that no donor fibroblasts were viable after six hundred days and although the valves appeared histologically normal and showed some metabolic activity, those stored for more than a few days were not viable within months of implantation (Livi *et al*., 1987; Yankah *et al*., 1987). Valves harvested twenty-four to fourty-eight hours post mortem had a 50% twelve-year homograft survival rate. In an autograft series, Ross reported an 82% allograft survival at fourteen years (Bodnar *et al*., 1980; Ross, 1967), showing the excellent survival of homografts in the right ventricular outflow tract (RVOT). In a later study it was demonstrated that autograft survival was better than that of allografts (Albertucci *et al*., 1994), which could be related to immunological factors rather than homograft viability.

In a landmark study of 252 isolated aorta homografts with a nine year to sixteen-and-a-half-year follow-up, Barrett- Boyes *et al*. (1987) recorded a 95% survival at five years, 78% at ten years and 42% at fourteen years' freedom from valve degeneration and re-operation. Of great consequence, in this series he could not find a correlation between increased warm ischaemic post mortem time and valve failure. The salvage time in this series was not recorded in fourty-three patients, 147 were harvested within twenty-four hours, eighty-eight between twenty-four to fourty-eight hours and seven at fourty-nine to seventy-five hours.

In South Hampton at the Wessex Cardiothoracic Center, Langley *et al*. (1996) reported an 87.9% freedom from aorta valve replacement at ten years, 71.7% at fifteen years and 49.7 at twenty years. Valves in this series were harvested within four days after death (mean 1.23±0.9 days).

In congenital cardiac surgery, restoration of the right ventricle to pulmonary artery (RV–PA) continuity frequently requires a conduit repair. In this setting the homograft truly came to its rightful position as a valve conduit since 1966. Conduit failures plague congenital surgeons due to calcification and degeneration of xenograft valves, peel formation in the Dacron tubes and thromboembolic events (Agarwal *et al*., 1981). In a series from Great Ormond Street Hospital for Sick Children (GOSH) in London, the mean patient age was six years with only 27% of xenografts not requiring replacement by year five (Shore *et al*., 1982). In a series of 201 children from Boston Children's Hospital, a Dacron tube or porcine valve conduit failed by 50% at eight years and 100% by year ten (Jonas *et al*., 1985).

In a very important study from the perspective of this paper, Fontan *et al*. (1984) postulated a homograft survival rate of ten to fifteen years with an actuarial homograft survival of 80% at nine years, using fresh antibiotic sterilised homografts. Similarly, GOSH demonstrated a survival rate of 85% at five years and 75% at nine years in sixty-five patients with a mean age of six-and-a-half years at operation. Although the homograft conduit walls calcified over time, leaflet function was well retained (DiCarlo *et al*., 1982).

Mitchell *et al*. (1995) analyzed twenty homograft explants from the RVOT. He could find no deep tissue, minimal inflammation was present and mild cuspal haematomas and calcification of the aorta homograft walls were observed. TEM showed nonviable cells and cell debris with minimal or no viable cells in the deeper layers of the valve. The collagen was largely intact and the tissue essentially acellular and nonviable.

Koolbergen *et al.* (2002) studied fourty explanted homografts. They showed a strong reduction in cellularity of the tissue within the first year. The trilaminer architecture of leaflets disappeared and they did not observe an endothelial layer. Valve tissue ingrowth consisted of host cells and they could not demonstrate a convincing continuing immunological process.

In a 1998 study thirty-three explanted cryopreserved allografts were compared to non-implanted allografts and valves explanted during re-transplantation of orthotropic heart transplants (Mitchell *et al*., 1998). They concluded once again that allografts are morphologically nonviable, that the collagen is flattened, but largely preserved and that these

allografts are unlikely to grow or have any metabolic functions. They also argued that their degeneration is probably non-immunogenic. In contrast, valves from explanted heart transplants demonstrated normal architecture, even in the setting of acute rejection.

## 1.3. The Bloemfontein experience

The Bloemfontein Homograft Bank was established in 1984 (Botes *et al*., 2012). Cadaver donors constitutes the backbone of this bank. By December 2016, 3135 valves were harvested and 1820 valves were processed, of which 1092 were aorta and 728 were pulmonary homografts. One thousand four hundred and seventy-nine valves were supplied to thirty units in South Africa and 591 homografts to the academic hospitals in Bloemfontein. Post-mortem harvest time was at a mean of thirty-three hours in the period 1984 to 2008 and presently is 29.8 hours (1984 to 2016), thus exceeding the twenty-four hour cut–off period.

No beating donor homografts were processed and unsterile harvesting takes place in the State Mortuary. Consent is routinely obtained and the homografts are processed as described in appendix C.

A clinical case series involving 253 children was assessed (Table 1.2 & 1.3), in which no difference between pulmonary homografts could be observed, as far as homograft degeneration was concerned. Homograft survival and freedom of degeneration were compared. A total number of 107 children received homografts harvested after twenty-four hours post–mortem, compared to 107 children who received homografts harvested before twenty-four hours post mortem.

**Table 1.2 Age at first operation, gender, follow-up and freedom from homograft failure**

| VARIABLE | | GROUP 1 <24 h PM | GROUP 2 >24 h PM | TOTAL |
|---|---|---|---|---|
| **Total number of grafts** | | | | 253 |
| **Number of Implants available for analysis** | | 107 in 102 patients | 107 in 99 patients | 214 |
| **Gender** | | | | |
| Female | n (%) | 40 (39.2%) | 35 (35.4%) | 75 (37.3%) |
| Male | n (%) | 62 (60.8%) | 64 (64.6%) | 126 (62.7%) |
| **Age at implantation (years)** | Mean (SD) | 10.9 (11.6) | 13.7 (11.9) | 12.3 (11.8) |
| **Follow-up time (days)** | Mean (SD) | 1291.5 (1534.1) | 1646.2 (1742.1) | 1468.9 (1647.2) |
| **Years** | | 3.5 ± 4.2 | 4.5 ± 4.8 | 4.0 ± 4.5 |
| **Freedom from valve failure** | Mean | 4043.7 | 3550.6 | 3797.2 |
| **Years** | | 11.1 | 9.7 | 10.4 |

(PM = post mortem)

**Table 1.3 Statistical analysis included Log-Rank, Wilcoxon and 2 Log(LR) tests**

| TEST OF EQUILITY OVER STRATA | | | |
|---|---|---|---|
| **Test** | **Chi-Square** | **DF** | **Pr > Chi-Square** |
| Log-Rank | 0.1868 | 1 | 0.6656 |
| Wilcoxon | 0.0059 | 1 | 0.9385 |
| -2 Log(LR) | 0.2249 | 1 | 0.6353 |

In order to fully provide a perspective of the Blomfontein Homograft Bank's *modus operandi*, the reality of homograft practices must be evaluated in an international context. In the Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013), 57% of homografts were obtained from organ donors, 15% from domino hearts and only 28% from non-organ donors.

In reality, the number of hours from death to excision ranged from two hours to fifty hours in the same report and in an analysis of the practices of twenty-three banks, twelve accepted cardiectomy or receipt by the bank up to twenty-four hours post mortem, while nine accepted tissue harvested or receipt by the bank longer than twenty-four hours post mortem, mostly up

to fourty-eight hours, being at six out of nine, according to the Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013).

The impact of short term pre-processing bacterial contamination and the impact of that on long term homograft performance is unclear (Brubaker *et al*., 2016).

## 1.4 The impact of cryopreservation on the cell biology of cardiac valves

Once homografts had been established in the field of cardiac surgery, emphasis shifted to the improvement and development of more advanced preservation techniques. Different techniques of sterilisation, preservation and storage were modified and improved with time (Salles *et al*., 1998; Parker *et al*., 1978). Currently, mainly two storage techniques are applied, namely cryopreservation in the vapor phase of liquid nitrogen and fresh-wet storage at 4°C after antibiotic sterilisation (Delmo Walter *et al*., 2012).

Despite advantages like long-term storage, superior haemodynamic properties, resistance to infections, and the low incidence of thromboembolic complications, the long-term durability of cryopreserved valves remains limited. Cryopreserved graft dysfunction, and eventual re-operations are, in the majority of cases, the result of tissue deterioration, which manifests as structural and calcific degeneration of the valves (O'Brien *et al*., 2001; Baskett *et al*., 1996). For years, various authors engaged in identifying and possibly understanding multifactorial mechanisms involved in cryopreserved homograft failure. The majority of these investigations were aimed at the relevance of cellular viability (Armitage *et al*., 2005; Kitagawa *et al*., 2001), the role of immune responses, biochemical aspects of the extracellular matrix such as elastin, proteoglycans or collagen (Schenke-Layland *et al*., 2006) as well as the impact of damage caused by ice formation during cryopreservation (Brockbank *et al*., 2000).

Although excellent early aortic valve replacement results were reported for cryopreserved allograft valves, eventual failure of these tissues is common (LeBlac *et al*., 1998; Mitchell *et al*., 1998; Salim *et al*., 1995). Despite the long-standing and widespread use of cryopreserved allograft valves, the influence of cryopreservation on the basic cellular biology still remains controversial.

### 1.4.1 Cellular viability

Several studies initially suggested that the preservation of cell viability (intact endothelial and fibroblast cells) after cryopreservation was one of the most recognised influencing factors of long-term valve durability, for it would result in grafts with some degree of regenerative capacity (Angell *et al*., 1989; O'Brien *et al*., 1987). However, it is still disputable whether viable donor cells like intrinsic cuspal interstitial tissue cells, mainly consisting of fibroblasts and myocytes, are present at the time of cryopreserved valve implantation and whether they persist over the long term. Another factor that remains controversial is whether long term haemodynamic performance of the implanted cryopreserved allograft is linked to donor cell viability and the regeneration of the intrinsic extracellular matrix, which could not be demonstrated in the 2001 study by O' Brien himself (O'Brien *et al*., 2001).

Hillbert *et al*. (1999) reported apoptosis in the endothelial cells and in the cuspal interstitial tissue cells of implanted cryopreserved allograft valves, which might contribute to the loss of valvular cellularity. The apoptosis can be the result of various factors, including immunological and chemical injury, hypoxia during valve processing and reperfusion injury at the time of valve implantation. Whether or not the cryopreservation technique is responsible for the apoptosis and acellularity or patchy cellularity commonly seen in clinical explanted valves, still needs clarification (Mitchell *et al*., 1995) (Figure 1.2).

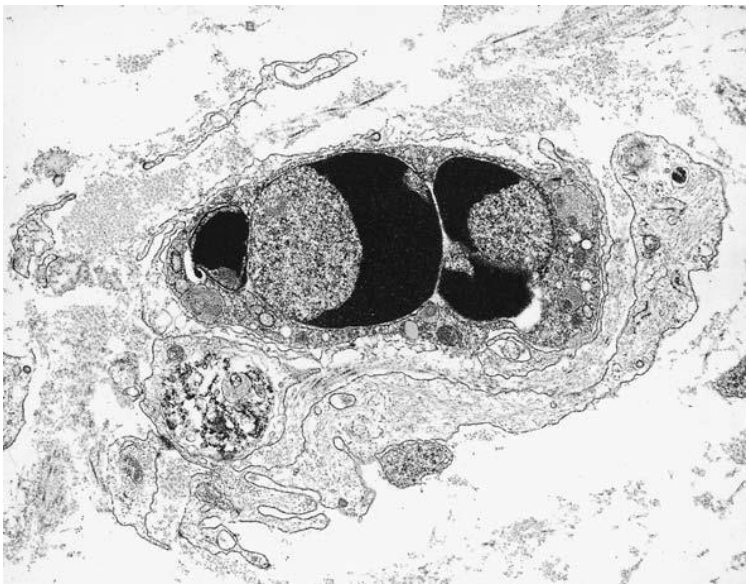

**Figure 1.2** **Transmission electron micrograph depicting the ultrastructural appearance of an apoptotic body.** Note the presence of discrete nuclear fragments and crescent-shaped condensed nuclear chromatin. Cryopreserved aortic valve allograft implanted for 30 days. Uranyl acetate/lead citrate stain. X 6,000 magnification (adapted from Hilbert *et al*., 2005).

### 1.4.2 Fibroblast viability

Fibroblasts are crucial in determining the long-term fate of heart valves and are responsible for protein synthesis and structural integrity. The best way to maximise fibroblast viability is by freezing at a constant of -1°C per minute (Van der Kamp *et al*., 1981; Mochtar *et al*., 1984). Therefore, an ideal harvested homograft needs the presence of a high percentage of fibroblasts capable of resynthesizing the collagenous matrix to maintain structural integrity (Brockbank *et al*., 1992; Hu *et al*., 1989).

The viability of any tissue after cryopreservation is influenced by many variables, such as handling methods during harvesting, ischaemic times, sterilisation (antibiotics, including antifungal media for twenty-four hours), freezing (fluid shifts and ice crystal formation), storage and thawing (Gall *et al*., 1995; Wassenaar *et al*., 1995).

Mark F. O'Brien described a cryopreservation technique and the use of viable homografts in 1987. Niwaya *et al*. (1995) studied twelve human pulmonary valves, using flow cytometry, and demonstrated cell viability after processing and thirty-day cryopreservation with a warm ischaemic time of less than 8.7 hours. However, some clinical studies did not favour donor viability after implantation (Mitchell *et al*., 1995). They reported that after implantation, cryopreserved homografts showed acellularity or rare cellularity or patch cellularity of the leaflets. Therefore, the ability of fibroblasts (homogenised freshly cryopreserved aortic valve tissue) to incorporate tritiated glycine into collagen after short-term implantation (Al-Janabi *et al*., 1972; Kano *et al*., 2001) does not necessarily mean the ability to repair and regenerate leaflet structure over the long-term as implied by the term "viable".

### 1.4.3 Endothelial cell viability

Besides functions like resistance to thrombosis, maintenance of haemostasis, modulation of vascular smooth muscle, vascular endothelium also plays an important role in the mediation of immunologic and inflammatory responses (Rocca *et al*., 2000). Whether endothelial cells are important to the long-term survival of homograft valves remains unknown. Endothelium is considered the most immunostimulatory component of whole organ allografts but whether this is applicable to the endothelium of dynamic valves, which can be markedly altered by cryopreservation, remains to be investigated.

Yankah *et al*. (1987) showed a 70% to 80% endothelial cell viability in cryopreserved valves, compared to 0% to 8% viability in grafts stored at 4°C. These findings were supported by

several other authors (Tominga *et al*., 2000; Killinger *et al*., 1992; Lupinetti *et al*., 1993). However, it must be noted that the majority of published data examining cellular viability were done on allografts immediately after harvest, disinfection, or thawing, but not after subsequent implantation, thus excluding the influence of immunological responses.

Christy *et al*. (1991) examined rat aortic grafts stored at 4°C and found that 95% of endothelial cells were viable immediately after harvest. With storage at 4°C the percentage viability declined in linear fashion to 92% at three days, 86% at seven and ten days, 83% at fourteen days and 64% at twenty-one days. The study demonstrated that storage at 4°C not only has the ability the preserve endothelial cell viability but also demonstrated that preservation is probably limited to a relatively short time.

Lupinetti *et al*. (1993) reported that viable endothelial cells were present in only 16% of cryopreserved allografts. By contrast, examination of native valve leaflets and arterial walls removed at operation found endothelial cells in 78% of allografts. These results demonstrated that routine cryopreservation methods carried out in routine clinical practice result in the complete loss of endothelium in the overwhelming majority of cases. Pompilio *et al*. (1997) studied the impact of ischaemic time (from nought to thirty-six hours) on valve endothelium on twenty-five nine-month old swine. The endothelium was resistant to ischaemic damage for up to six hours, but after twelve hours exhibited progressive irreversible damage by twenty-four and thirty-six hours. Smit *et al*. (2015) and the results presented in this dissertation supported Pompilio's findings (Pompilio *et al*., 1997).

According to Tominaga *et al*. (2000), cryopreservation causes serious damage to cytosolic and mitochondrial functions of endothelial cells. Furthermore, Lu *et al*. (1997) also demonstrated diminished mitochondrial dehydrogenase activity in porcine valves after cryopreservation. Cell membranes can be easily damaged soon after harvesting due to handling, processing, sterilisation, freezing and thawing. Mitochondria serves as the centre for the intracellular energy source and the more the mitochondrial function is damaged by the cryopreservation method the more the cell membrane deteriorates due to energy depletion.

### 1.4.4 Immunogenicity

Cryopreserved and fresh valve allografts have been regarded as tissues with low antigenicity and showed good long-term clinical results after implantation, especially in adults (Hoekstra *et*

*al*., 2005). The loss of endothelial cells may increase the longevity of allografts by minimising the host immune response, which may contribute to valve degeneration.

Donor specific antibodies were demonstrated in all homograft recipients in a study performed by a Munich group using cyto-immunological techniques (cold ischaemic time), although this response was transient and reversed spontaneously without immunosuppressants (Shutz *et al.,* 1994). The same group reported that cryopreservation could attenuate immune responses (Fischlein *et al*., 1995). Motomura *et al*. (1995) also demonstrated the attenuation of histological appearance of chronic rejection after cryopreservation in a rat study.

To clarify the mechanism and impact of immune responses in allografts, investigators turned to explanted valves after heart or heart-lung transplantations and compared these valves with explanted allografts. Simone *et al*. (1998) found that cardiac valve endothelial cells express molecules capable of initiating an immune response and might play a role in valve degradation. Valves from transplant patients that were receiving immunosuppressive therapy as well as having been HLA matched, were virtually free from degeneration. They speculated that the long-term durability of allografts might be improved by HLA matching and by administering immunosuppressant treatment.

However, the same cannot be said for cryopreserved valves (functioning up to nine years) explanted from human recipients for various reasons. These valves demonstrated progressive severe loss of normal layered structures and were predominantly acellular.

As mentioned before, Koolbergen *et al*. (2002) studied fourty explanted homografts and demonstrated largely acellular valve leaflets as well as leaflets that suffered from a loss of the trilaminar structures within one year. The valves implanted were mostly harvested from transplant patients and could be considered viable at the time of implantation. All grafts were examined using *in situ* hybridisation procedures and revealed that host cells were present but donor cells were either completely absent or nearly so (Figure 1.3). They also suggested that host cell ingrowth makes no significant contribution to valve tissue structure integrity and concluded that homografts maintain their function through the relatively good preservation of its collagen structure. Furthermore, they found no evidence of immune-mediated injury and thought that the reduction of cell numbers represents apoptosis, rather than immune based necrosis.

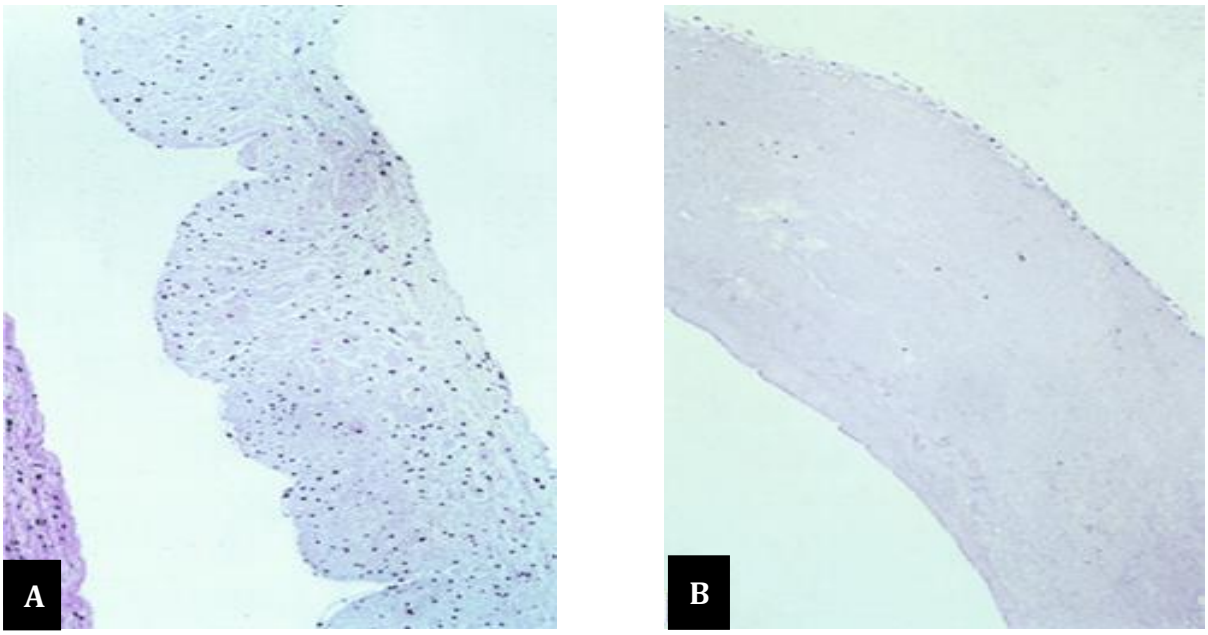


**Figure 1.3** **Photomicrograph of radial sections of an unimplanted cryopreserved aortic valve and midterm explant. (Routine H&E staining; original magnification 250X).** (A) Un-implanted cryopreserved aortic homograft valve. Note the carefully arranged collagen bundles and rippled outflow surface as an expression of leaflet elasticity. (B) Cryopreserved aortic homograft from a beating-heart donor implanted in a twenty-year old woman in the RVOT and explanted after 2.5 years because of stenosis caused by thickening of the vessel wall. The leaflet shows the typical loss of tissue architecture and cellular elements, stretching of collagen (loss of elasticity) and increase in ground-substance volume (adapted from Koolbergen *et al*., 2002).

Morphologically cryopreserved allografts are considered nonviable and are unlikely to grow, remodel, or exhibit active metabolic functions. By comparing explanted cryopreserved allograft heart valves with aortic valves explanted from orthotropic heart transplants, transmission electron microscopy of cryopreserved explants showed nonviable cells but the collagenous skeleton remained largely intact (Mitchell *et al*., 1995). They concluded that cryopreserved allograft heart valves have minimal if any viable cells and maintained function by retaining an intact original collagen network, although flattened, and suggested that immune responsiveness has little or no impact on late allograft degeneration (Mitchell *et al.,* 1995).

Investigators also started to pay attention to young recipients known for allograft failure, suggesting that immunological mechanisms might be imported in this sub-group of patients. Yankah *et al*. (1995) identified ABO incompatibility, the type of allograft and young age as independent factors contributing to early graft failure in implants during the first two years of life.

Baskett *et al*. (2003) studied ninety-six homografts implanted in the RVOT of eighty-three children after recognising that many homografts in children become rapidly insufficient post implantation. The use of an aorta homograft and short antibiotic preservation time were associated with re-operation. Younger age, ABO mismatch and diagnosis were associated with echocardiographic failure and in a sub-analysis with human leukocyte typing, age, aortic homograft and human leukocyte antigen-DR mismatch were associated with echocardiographic failure. The authors concluded that matching for human leukocyte antigen-DR, blood group and avoiding short preservation times, offer the potential to improve long-term results of these valves.

### 1.4.5 Collagen synthesis and collagenolysis

The long-term performance of cryopreserved allograft valves might be addressed by preserving the intactness of the original collagen network that provides the basis to the valvular matrix (Hilbert *et al*., 1999)(Figure 1.4).

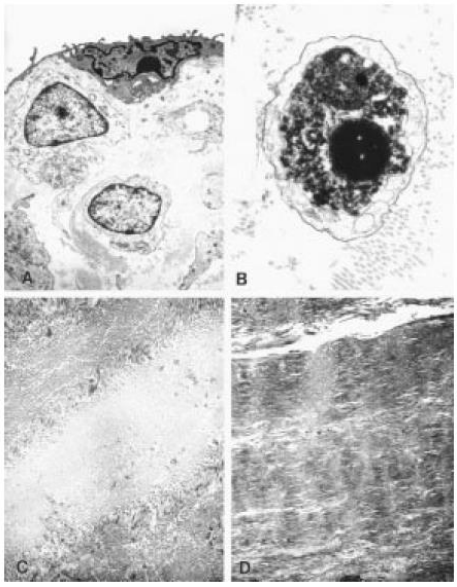


**Figure 1.4** **Cryopreserved allograft valve implanted for thirty days.** Ultrastructural appearance of pyknotic endothelial cell lining the fibrosa. Note marked condensation of the nuclear chromatin and contraction of the cytoplasm. Compare with the normal chromatin of adjacent fibroblasts. (Uranyl acetate/lead citrate stain; original magnification, X4800). **B**, Cryopreserved allograft valve implanted for thirty days. Transmission electron micrograph of a macrophage contains a phagocytosed apoptotic body composed of fragmented nuclear chromatin. (Uranylacetate/lead citrate stain; original magnification, X10,000). Transmission electron micrographs depict changes in the crimping of collagen in the cusps of **(C)** cryopreserved allograft valves after thirty days of implantation and **(D)** fresh allograft valves after twenty weeks of implantation. Collagen crimp is preserved at thirty days, although prominent regions of collagen straightening is noted at twenty weeks. (Uranyl acetate/lead citrate stain; original magnification, [C] x3500 and [D] x2900)(adapted from Hilbert *et al*., 1999).

Mitchell *et al*. (1998) suggested that controlled collagenolysis ability plays an important role in the first step of the valve regeneration process and consequently collagen synthesis is the second step. However, the balance of collagen synthesis and collagenolysis ability in cryopreserved valves have not yet been clearly understood (Lester *et al*., 1992; Mcgregor *et al*., 1976). The expression of α1-(I) in allograft aortic valves and the presence of procollagen after implantation for three days suggest donor fibroblast viability (Lupenetti *et al*., 1997; Song *et al*., 1997). Fibroblasts found in the valve matrix are known to participate in valve remodelling through collagen metabolism (Broom, 1978).

In a study conducted by Kano *et al*. (2001) collagen content in cryopreserved cusps was kept at the same degree as that of fresh cusps and the collagen synthesis ability in the cryopreserved cusps was relatively well preserved. However, a significant decrease was noted in the protein synthesis ability, including collagen synthesis of the cryopreserved leaflet cusps. Lupinetti *et al*. (1997) and Song *et al*. (1997) supported these findings but the significant reduction in protein synthesis may have had a detrimental impact on long-term valvular cellular viability.

Matrix matalloproteinases (MMPs) are able to digest a wide range of extracellular matrix proteins e.g. MMP-1, and interstitial collagenase, mainly produced from fibroblast, degrade structural type I collagen in the matrix. MMP-2 and MMP-9 mainly produced from endothelial cells are involved in the degradation of type IV collagen, which constitutes a major part of the sub-endothelial basement membrane.

Tominaga *et al*. (2000) stated that cryopreservation and thawing damage both the cytosolic and mitochondrial function of endothelial cells and may cause a latent cytosolic and mitochondrial injury even in fibroblasts. Therefore, the activity of MMP-1 and MMP-2 in the supernatant of the cryopreserved valves was quantitatively the same as that of the fresh valves, despite reduced cellular viability (Kano *et al*., 2001). These findings confirm that cryopreservation and thawing cause the release of MMP-1 and MMP-2 from the endothelial and fibroblast cells of the allograft cusps and activate them before implantation. Consequently, the possibility exists that the activated MMP-2 will degrade the basement membrane consisting of type IV collagen and the activated MMP-1 will destruct the cusp matrix. The majority of cryopreserved valves have the potential to degrade and autolyse the matrix extensively during processing, intraoperative preparation, and possibly after implantation.

## 1.5 Study layout and aims

Retaining the cellular viability of allografts probably has the disadvantage of preserving endothelial cells and dendrite cells of the donor valve with the ability to evoke an immunological response. This could produce a rejection reaction similar to HLA mismatching in cardiac transplants. Endothelial cells express HLA class 1 and class 2 antigens and immune stimulatory and adhesion molecules, playing a role in inflammatory and rejection processes. Dendritic cells, the antigen presenting cells in the sub-endothelial matrix of heart valves, also express HLA class 2 antigens.

It is clear that allografts can trigger both cellular and humoral responses that are donor specific (Hoekstra *et al*., 1994, Hoekstra *et al*., 1996) and that HLA-DR matching reduced reaction and T lymphocyte showed cytotoxicity directed against donor specific class 1 and class 2 HLA antigens.

*In vivo* studies discussed, showed humoral immune responses to donor specific class 1 and 2 antigens.

It is certainly possible that immune responses might play a role in the long-term degeneration of homografts. Dignan and colleagues (2003) were able to show that class 2 HLA mismatch had a significant impact on valve deterioration in a subset of patients followed for more than five years.

The role of ABO mismatching is unclear as cardiac valve endothelium does not express blood group antigens, or endothelium might just be lost during processing. Long-term series have not shown any benefit in ABO matching.

This differs from other workers, who did show a more intense reaction in ABO mismatched grafts, but the reaction was reversible without immunosuppression (Fischlein *et al*., 1995).

The work on decellularised valves has to be noted and despite the view and conclusions from reputable investigators, speculating that immune mediated degeneration is unlikely in the long term, certainly in young patients, immune mediated degeneration seems to be a reality.

Another important fact is that in most series where immune mediated degeneration has been found, it has to be noted that valves were viable and a large percentage were obtained from transplant recipients and beating heart donors.

Longer preservation times and prolonged WIT seem to attenuate the initial immune responses, especially in younger patients. The loss of endothelium and viable cells theoretically presents a lesser immunological target, which might be beneficial to long–term performance as long as this does not come at the price of a damaged collagen scaffold.

On the other hand, Mitchell *et al.* (1995), questions the role of immune responses in the degeneration of homografts. They argue that homografts are essentially nonviable and even if some metabolic and cellular activity is present initially, it is lost quite soon after implantation. Leaflets are thus essentially acellular structures with a relatively intact collagen network.

In order to justify a re-evaluation of cadaver donor programmes and also to educate the Department of Cardiothoracic Surgery at the University of Bloemfontein's local tissue engineering programme about decellularization of homografts, it is important to understand the exact impact of post mortem harvest times as well as cryopreservation on tissue integrity.

**This was addressed step-by-step through the following in *vitro* and in *vivo* studies:**

## Article 1

### Does prolonged post-mortem cold ischaemic harvesting time influence cryopreserved pulmonary homograft tissue integrity?

*In this article where the author of this paper is the second author, this author conducted all in vitro testing and bench tests. This author also ran the CPB in the animal lab. Data processing of all bench test data was conducted by this author and was also involved in all aspects of both the research project as well as the preparation of the manuscript.*

## Article 2

### Morphology of unprocessed and cryopreserved pulmonary homograft leaflets with post mortem harvest times up to seventy-two hours

*During analyses of the results of the first study, it became obvious that detailed studies of leaflet structures were warranted in order to evaluate the effect of extended post mortem times on the*

*quality of the structure and substructures of homografts harvested at different post mortem times. The impact of cryopreservation was assessed independently in comparison to control tissue as well as on tissue harvested at extended post mortem times. The impact of extended homograft harvest times on morphology was compared to cryopresrved tissue at similar donor times.*

## Article 3

### Is the impact of cryopreservation on the tissue strength of pulmonary artery homograft leaflets more important than extending ischaemic harvest times?

*The impact of extended harvest times is studied in relation to tissue strength, using TS, YM and $T_d$. Strength is evaluated and compared to fresh unprocessed leaflets and the impact of prolonged harvest time is assessed on unprocessed and cryopreserved tissue.*

## Article 4

### Cadaver Donation: Structural integrity of pulmonary homografts harvested fourty-eight hours post mortem in the juvenile ovine model

*In the final study, the short and medium term stability as well as the tissue integrity of cryopreserved tissue harvested at fourty-eight hours, which is the Department of Cardiothoracic Surgery at the University of Bloemfontein's limit of tissue acceptance in its homograft bank, was assessed over a period of implantation. Analyses of morphology and tissue strenght were conducted after fourteen days' implantation and after 180 days' implantation. This study addresses the impact of in vivo processes on homograft tissue integrity over a short term (fourteen days implantation) as well as a longterm (180 days implantation) in vivo model.*

# Chapter 2
Article 1

*Cell Tissue Bank* (2015) 16: 531–544

# Does prolonged post-mortem cold ischaemic harvesting time influence cryopreserved pulmonary homograft tissue integrity?

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## Abstract

**Introduction:** This study investigated cryopreserved pulmonary homograft (CPA) structural integrity after prolonged cold ischaemic harvesting times in a juvenile sheep model.

**Methods:** Three groups with different post-mortem cold ischaemic harvesting times were studied, i.e. Group 1 (24h, n=10); group 2 (48h, n=10); group 3 (72 h, n = 10). In each group, 5 CPAs were studied *in vitro* after cryopreservation and thawing. The other 5 CPAs were implanted in juvenile sheep for a minimum of 180 days. Serology samples were obtained and echocardiography was performed before euthanasia. Hematoxylin and eosin (H&E), scanning electron microscopy (SEM), von Kossa, Picrosirius red, a-actin, immunohistochemistry [von Willebrand factor (vWF), CD4, CD31 and CD34] and calcium content analyses were performed on explanted CPAs.

**Results:** The *in vitro* and *in vivo* studies failed to demonstrate any change in tensile strength, Young's Modulus and thermal denaturation ($T_d$) results between the groups. SEM demonstrated a reduction in endothelial cells (50 % at 24 h, 60.9 % at48 h and 40.9 % at 72 h), but H&E could not demonstrate autolysis in any CPA in vitro. All cultures were negative. In the explanted

groups, IgE, IgM and IgG results were inconclusive. Echocardiography demonstrated normal valve function in all groups. H&E and Picrosirius red staining confirmed tissue integrity. vWF, CD31 and CD34 staining confirmed a monolayer of endothelial cells in all explanted valves. Calcium content of explanted CPA leaflets was similar.

**Conclusion:** This experimental study supports the concept of prolonging the cold ischaemic harvesting time of cryopreserved homografts to reduce homograft shortage.

**Keywords:** Cryopreserved homografts–Warm ischaemic time–Right ventricular outflow tract reconstruction–Juvenile sheep model

## Introduction

The gold standard for reconstruction of the right ventricular outflow tract (RVOT) is cryopreserved pulmonary homografts (CPAs). Although Chambers *et al*. (1997) showed that freedom from reoperation following homograft reconstruction of the RVOT using the Ross procedure to be as high as 80 % at 25 years of follow-up, it is generally agreed that homografts degenerate over time. This degeneration is mostly due to immune responses (Welters *et al*. 2002; Neumann *et al*. 2014). Alternatives to homografts are xenogenic heart valves, which are available off-the- shelf. However, these valves are treated with glutaraldehyde and therefore present serious limitations for young patients due to early tissue degeneration and calcification (Holmes *et al*. 2012; Homann *et al*. 2000). A further alternative is tissue engineered pulmonary heart valves, which show promising results for up to 10 years if the scaffold is a homograft (Dohmen *et al*. 2011; Cebotari *et al*. 2011). Since CPA use is limited by donor availability, especially with respect to small valve sizes, investigations are needed to increase the pool of potentially available human heart valves without compromising quality. The international norm for harvesting valves either from heart transplant recipients, beating heart donors or non-beating heart donors, is limited to 24 h post-mortem. The reason for this restraint is that increased homograft durability was demonstrated with retained cellular viability in cryopreserved homografts (Angell *et al*. 1989; O'Brien *et al*. 1995). However, Mitchell *et al*. (1998) argue that most homografts are acellular and that valvular performance is linked to retention of structural integrity of the valve scaffold and not cellular viability.

This study investigated CPA structural integrity after prolonged cold ischaemic harvesting times in a juvenile sheep model.

## Materials and methods

All animal experiments and surgical procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). Approval of the study protocol was obtained from the Animal Ethics Committee of the University of the Free State (ETOVS nr. 12/06). A schematic presentation of the study layout is provided in Figure 1.1.

Three groups with different post-mortem cold ischaemic harvesting times were studied in vitro as well as in vivo, while a fourth group with valves (n = 5) harvested within 6 h post-mortem and non- cryopreserved, were used as control in the in vitro studies: group 1 (24 h, n = 10); group 2 (48 h, n=10); group 3 (72 h, n=10). In each of the three groups, 5 CPAs were studied in vitro after processing and thawing to evaluate tissue integrity and impact of processing. The other 5 CPAs from each group were studied in vivo through implantation in juvenile sheep, for a minimum of 180 days (group 1: ñ = 210 days; group 2: ñ = 206 days; group 3: ñ = 188 days), to evaluate valve performance, biological interaction and modes of degeneration. The population median values between the different days of implantation in the three groups proved not to differ significantly ($p > 0.05$) from each other. Wethers of the Dorper strain were used and each carcass and recipient animal received an ear tag with a unique identification number.

### Pulmonary valve harvesting and preparation

For the control group, hearts were obtained from a local abattoir directly after slaughtering and transported on ice to the laboratory. The valves were dissected and washed in cold (4 °C) Ringers-lactate (Bodene Pty, Ltd trading as Intramed, Port Elizabeth, SA) and all required samples taken for evaluation. For the other three groups, thirty juvenile sheep were sacrificed by intravenous injection of an overdose of potassium chloride (Adcock Ingram Critical Care, Johannesburg) and pulmonary valve conduits for the preparation of cryopreserved ovine homografts were harvested at 24, 48 and 72 h respectively. Ischaemic time was defined as the time between death and harvesting of the heart valve. Ischaemic time was composed of a 6 h period of warm ischaemia (from death to refrigeration of the cadaver in the mortuary) followed by a longer period of cold ischaemia until the heart was removed from the cadaver at the designated times. The carcasses were skinned after euthanasia and the large stomach was removed to limit the effect of the ongoing intestinal fermentation process, which is typical in ruminants and would increase the body temperature and subsequently the rate of autolysis. In group 1 (n = 5) the pulmonary heart valves were removed as a valve conduit and processed after 24 h, in group 2 (n = 5) after 48 h and in group 3 (n=5) after 72 h. The valves were rinsed, inspected for fenestrations or other abnormalities and the thickness of the myocardium was

trimmed to approximately 5 mm. A competency test was performed to evaluate regurgitation control. Each valve was immersed in 100 ml of M199 with Earle's Base (Highveld Biological (Pty) Ltd., Johannesburg, South Africa (SA) containing 2.5 mg Fungizone (Bristol-Meyers Squibb, Johannesburg, SA), 25 mg Amikacin (Fresenius, Bodene (Pty) Limited trading as Intramed, Johannesburg, SA), 50 mg Cefoxitin (Sabax, Johannesburg, SA) and 50 mg Piperacillin (Sabax, Johannesburg, SA) to sterilize for 24 h. Then the pulmonary valves were cryopreserved under sterile conditions using a Cryoson BV-9 Biological Freezer (Consarctic, Schöllkrippen, Germany). Valves were placed in a cryobag containing a solution of 100 ml M199 with 11 ml dimethylsulfoxide (DMSO) and cryopreserved at a rate of approximately -1 °C/min until -140 °C was reached. Quality control for the cryopreservation of the heart valves procured for this study was performed according to ISO 9000 standards.

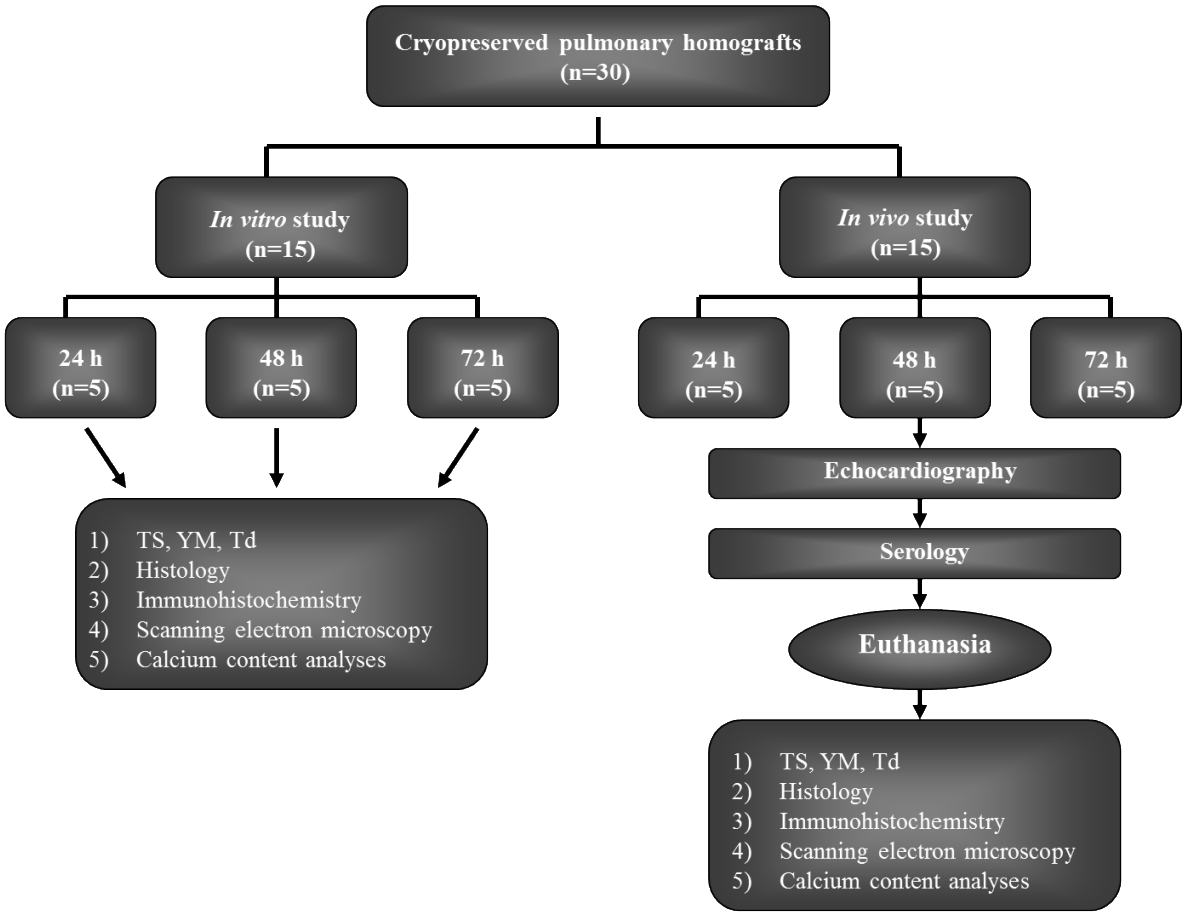


**Figure 1.1** **Outline of the study methodology** (h = hour; TS = tensile strength; YM = Young's modulus; Td = Thermal denaturation temperature)

Cryopreserved valves were stored in the vapor phase of liquid nitrogen ($LN_2$) for a minimum period of 2 weeks till implantation. At thawing, the cryobags were placed on a shelf at room temperature for ±5 min to eliminate the excessive cold temperature, followed by immersion in a

waterbath at 30 °C for another 5–7 min until most of the liquid inside the bag have melted. The bag was then opened sterile and the contents placed gently in two changes of 500 ml of M199 at 4 °C for 10 min each, before implantation.

## *In vitro* study

### Biomechanical testing

Biomechanical properties of the tissue samples were examined using a tensile testing apparatus (Lloyds LS100 Plus tensile strength (TS) tester, IMP, Johannesburg, SA). TS and YM were performed on tissue samples fixated with clamps at both ends and gradually stretched (0.1 mm/s) by applying constant tension on the ends (Thubrikar *et al*. 1983). Both pulmonary valve leaflets and wall samples underwent biomechanical testing.

Determination of Thermal Denaturation Temperature (Td) is a technique for thermal analysis, performed using differential scanning calorimetry (DSC) (Mettler Toledo, DSC 822e, Microsep, Johannesburg, SA). In DSC, the rate of heat flow to the sample is compared to the rate of heat flow to an inert material, while the materials are heated or cooled concurrently. 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 (Smith and Judge 1991). This was recorded as Td for each group (Rüegg et al. 1975).

### Microbiological examinations

Intra cardiac blood samples were taken from each intact donor heart before harvesting the heart for further dissection of the valves (groups 1–3). These samples were analyzed using standard microbiological techniques for detecting aerobic and anaerobic microbes, as well as for fungi. At cryopreservation, tissue and fluid samples from each valve were also taken for microbiological testing, and compared to the organ- isms cultured from blood samples taken intra-cardially at harvesting.

### Histological evaluation

Specimens, for light microscopy examination, were taken from the middle of the pulmonary leaflet of each CPA as well as the wall of each CPA. These specimens were embedded in paraffin wax and two micrometer thick longitudinal sections were then prepared and routinely processed with H&E, von Kossa and Picrosirius red staining's. Specimen numbers were randomly allocated to each tissue sample, allowing completely blinded evaluation by all the

evaluators for histology, immunohistochemistry and scanning electron microscopy (SEM) samples.

**Immunohistochemistry**

Immunohistochemical staining was performed with anti-von Willebrand factor (vWF) rabbit polyclonal antibody [Abcam, Cambridge, United Kingdom (UK)], anti-CD4 rabbit polyclonal antibody [Bioss, Atlanta, United States of America (USA)], anti-CD31 mouse monoclonal antibody (Abcam, Cambridge, UK), anti- CD34 goat polyclonal antibody (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) and anti-a-smooth muscle actin rabbit polyclonal antibody (Abcam, Cambridge, UK). Different valvular regions were investigated, due to differing shear stresses at the leaflets compared with the rest of the valves. From each region representative samples were obtained including the inflow and outflow aspects of the valve.

**Scanning electron microscopy**

All valves were fixed in 2.5 % Glutaraldehyde [Merck, Johannesburg, South Africa (SA)]. Valve leaflets were divided into two specimens of approximately 3 x 6 mm. Similar specimen samples were taken from the pulmonary wall, sinus area and pulmonary trunk. Tissue specimens were dried using the critical point method and were metalized using gold.

Evaluations were performed with a Shimadzu SSX 550 scanning electron microscope (Kyoto, Japan). The surface area of each specimen was examined and photographed at either four or five different positions, and all images were then evaluated by three independent evaluators and a score allocated. An average score for each specimen was then calculated. A three category scoring system, adapted from the six categories de- scribed by Krs *et al*. (2006) was used to define endothelial integrity and to evaluate the quality of the extracellular matrix (ECM) surface area. The classification of tissue was based on two key markers, endothelium and basal membrane. Category I; there is a virtual absence of both endothelium and basal membrane and the bare scaffold of the tissue is exposed. Category II; the basal membrane predominantly covers the scaffold and the endothelium is largely missing. Category III; the endothelium is virtually intact.

## *In vivo* study

**Echocardiography**

Hemodynamic evaluation was performed by one experienced investigator using trans-thoracic echocardiography prior to sacrifice. A Philips Envisor Ultra Sound system (Philips, Johannesburg, SA) was used with a 3.5-MHz probe and all data were recorded. Two-dimensional trans-thoracic echocardiography was per- formed to evaluate morphological conditions of the

valve conduit and leaflets. Additionally, the diameters of the valve annulus, sinotubular junction (ST-junction) and pulmonary artery wall were measured. Pulmonary insufficiency was evaluated semi-quantitatively using pulsed wave, continuous wave and color Doppler flow on the parasternal short axis view. The regurgitation jet across the valve was graded by identification length and width into the right ventricular outflow tract and mapped as: none/trivial, mild, moderate or severe using standard echocardiography criteria. The mean flow velocities across the implanted valves were obtained by the use of continuous wave Doppler. Each measurement was repeated six times and the mean value over the measurements was calculated. Again animals were only identified by their individual ear tag numbers, allowing blinded evaluation by the sonographer.

**Serology samples**

Full blood counts were analyzed on a Sysmex XE 2100 (Roche, Johannesburg, SA) according to the TF/ DC detection method, hydrodynamic focusing (DC detection), flow cytometry method and a SLS-hemoglobin method. Immunoglobulins (IgG, IgA and IgM) in serum were determined quantitatively by means of immunonephelometry on a Siemens BN ProSpec Nephelometer (Siemens, Johannesburg, SA).

**Gross examination**

The explanted CPAs were inspected and color photographs were taken before fixation. The leaflets were inspected for fenestrations, retraction, thrombotic material, atheroma and calcification.

**Calcium content analysis**

Quantitative calcium analyses were performed on samples dried in a temperature controlled Scientific series 100 incubator (Lasec, Johannesburg, SA) at 45 °C for 48 h. Samples were weighed and hydrolyzed in 1 ml 50 % nitric acid and 50 µl hydrogen peroxide. Extractable calcium content was determined by inductively coupled plasma mass spectrometry Agilent ICP-MS 7500c (Chemetrix, Midrand, SA) and expressed as µg calcium per mg dry weight tissue. Only calcium content of the pulmonary leaflet and wall of the control group (<6 h) were determined pre- implantation and used as baseline values, and com- pared to the explanted tissues of the three ischaemic groups.

**Statistical analysis**

For statistical analysis purposes, the cold ischaemic time was described by the time effect and the source of a sample, i.e., whether the sample was sourced from the aortic leaflet or the aortic wall, was described by the source effect. Various measurements ($T_d$, Calcium, etc.) were analyzed separately using linear models in order to determine whether there were significant time or source effects and whether there was an interaction between time and source. Time and source were treated as simple fixed effects and an interaction effect was also considered. Standard ANOVAs were also done over time and over source to obtain additional clues as to whether either effect was significant. The study was not statistically powered to detect differences due to the cost restraints involved.

Where the interaction effect in the linear model was significant, further investigation was done using ANOVA methods by separating the sources. In a few significant cases (where the time effect was significant for a specific source) the results were investigated even more deeply using Welch Two Sample t tests. This involved comparing the three timestamps in pairs. This top-down approach (starting global and drilling down) was followed throughout in order to avoid spurious results and false positives/negatives.

Where the above method was not appropriate, other methods were applied. For example, the sonar data was analyzed using Hotelling's test and the SEM data was analyzed with Pearson's Chi Square test. For Hotelling's test we have to assume multivariate normality as the sample size is too small to conduct tests for this.

**Surgical implantation**

The juvenile sheep were male with a mean age of 4–6 months and a mean body weight of 34–40 kg. Premedication was administered with 0.175 mg/kg Neurotranq [VirbacRSA (Pty) Ltd, Halfway House, SA] and 0.2 mg/kg Atropine [Bayer (Pty) Ltd, Animal Health Division, Isando, SA] intramuscularly, and anesthesia was induced with 12 mg/kg Bomathal [Merial SA (Pty) Ltd, Halfway House, SA] intra-venously. The sheep were intubated, ventilated and positioned in a lateral decubitus position. Arterial cannulation for cardiopulmonary bypass was obtained via the left carotid artery. The stump pressure of the tied-off distal carotid artery was used for invasive arterial pressure measurement. A left mini-thoracotomy was performed and the fourth rib removed. The pulmonary artery was transected and the native pulmonary valve leaflets were excised. The CPAs, with a diameter of ±16 mm, were implanted as an interposition, with two continuous 4/0 polypropylene suture anastomoses. The sheep was weaned from cardiopulmonary bypass, the mini-thoracotomy was closed in layers and a chest drain was

inserted. Systemic pain medication, in the form of 2 mg Morphine sulphate [Bodene (Pty) Ltd, trading as Intramed, Port Elizabeth, SA] intramuscularly twice a day and 5 mg Depomycin [INTERVET SA (Pty) Ltd, Johannesburg, SA] daily as antibiotic, was administered for 5 days post-operatively. Animals were extubated 2–4 h post-operatively. Underwater drains and pressure lines were removed before animals were transferred to an overnight facility.

## Results

### *In vitro* study

#### Biomechanical testing

The following baseline mechanical results were obtained in pulmonary homografts harvested within 6 h after death. These samples were obtained from valves of juvenile sheep from a local abattoir. The mean TS were 1.04 ± 0.36 MPa for the pulmonary wall and 1.24 ± 0.79 MPa for the pulmonary leaflet. The mean TS for the pulmonary wall in the three ischaemic groups were as follows: (group 1: 1.03 ± 0.36 MPa; group 2: 0.95 ± 0.22 MPa; group 3: 1.16 ± 0.34 MPa). The mean TS for the pulmonary leaflets in the three ischaemic groups were as follows: (group 1: 2.57 ± 1.07 MPa; group 2: 2.93 ± 0.50 MPa; group 3: 3.03 ± 1.02 MPa).

The mean YM baseline value was 2.06 ± 0.77 MPa for the pulmonary wall and 5.68 ± 4.57 MPa for the pulmonary leaflet. The mean YM for the pulmonary wall in the three ischaemic groups were as follows: (group 1: 3.21 ± 0.90 MPa; group 2: 3.26 ± 0.93 MPa; group 3: 3.81 ± 1.31 MPa). The mean YM for the pulmonary leaflets in the three ischaemic groups were as follows: (group 1: 9.93 ± 2.63 MPa; group 2: 9.25 ± 5.02 MPa; group 3: 10.73 ± 4.33 MPa).

The mean $T_d$ baseline values were 70.57 ± 1.03 °C for the pulmonary wall and 67.16 ± 1.31 °C for the pulmonary leaflets. The mean $T_d$ for the pulmonary wall in the three ischaemic groups were as follows: (group 1: 70.57 ± 1.12 °C; group 2: 71.19 ± 1.13 °C; group 3: 72.44 ± 2.85 °C). The mean $T_d$ for the pulmonary leaflets in the three ischaemic groups were as follows: (group 1: 70.25 ± 0.76 °C; group 2: 71.25 ± 1.17 °C; group 3: 71.01 ± 0.36 °C).

In vitro evaluation of the TS, YM and $T_d$ temperatures did not reveal any statistically significant differences ($p > 0.05$) between the 24, 48 and 72 h groups.

#### Microbiological examinations

Microbiological examination detected that the initial microbes present on the CPAs in the different groups were as follows: group 1 (n = 5): no aerobic organisms and 3 positive cultures

for anaerobic microorganisms; group 2 (n = 5): 2 positive samples for aerobic organisms and 1 positive sample for anaerobic organ- ism; group 3 (n = 5): 3 samples were positive for aerobic microorganisms and 3 samples were positive for anaerobic microorganisms. The most common microorganisms were gram + bacilli and coagulase negative staphylococci. After 24 h of sterilization in the nutrient antibiotic solution no organisms could be cultured in the pre-cryopreserved specimens. No fungal cultures were positive at any point in time.

**Histological evaluation**

The histological findings in the control group (harvested<6 h post-mortem) are described as reference. H&E staining showed endothelial-like cells on the surface of the control valves; however, a confluent layer was not present in all of the specimens. The ECM exhibited a normal configuration with an absence of abnormalities in the collagen and elastin structures, and this was confirmed with Picrosirius red staining. The spindleform-shaped fibroblast-like cells were arranged in a parallel layout; however, the number of cells in some of the samples was limited. Endothelial-like cells were confirmed to be endothelial cells by positive expression of vWF and CD31. Interstitial cells were confirmed to be myofibroblasts since these cells were $\alpha$-actin positive. No differences between the groups were observed post processing and thawing, compared to the control group.

**Scanning electron microscopy**

According to the modified Krs *et al*. (2006) and Smit, PhD, unpublished data, 2011 classification, the baseline samples of the pulmonary leaflets were all in category III (100 %) and, for the pulmonary wall samples, 86.7 % were in category II and 13.3 % were in category I. In group 1 (24 h), 12.5 % of the pulmonary leaflets fell in category I, 37.5 % in category II and 50 % in category III, while 96 % of the pulmonary wall samples fell in category I and 4 % in category II. In group 2 (48 h), 21.7 % of the pulmonary leaflets fell in category I, 60.9 % in category II and 17.4 % in category III, while 100 % of the pulmonary wall samples fell in category I. In group 3 (72 h), 18.2 % of the pulmonary leaflets fell in category I, 40.9 % in category II and 40.9 % in category III, while 100 % of the pulmonary wall samples fell in category I.

No statistically significant differences ($p > 0.05$) in SEM was found between any of the three ischaemic groups.

**Calcium content analysis**

The mean baseline quantitative calcium content was 1.8 ± 0.09 μg/mg of dry weight for the pulmonary leaflet samples and 0.80 ± 0.06 μg/mg of dry weight for the pulmonary wall samples.

Only the baseline values of the control group (<6 h) were determined and compared to the explanted tissues in the three ischaemic groups.

## *In vivo* study

### Echocardiography

Echocardiographic examinations showed limited morphological calcification. The calcification was in the range of none to mild for the valve leaflets in all the groups. Mild to moderate calcification was observed in the valve annulus and in the pulmonary wall. There were however no differences between the groups.

The mean pressure gradients at the CPAs within the different groups were 12.0 ± 5.6 mmHg in group 1, 10.7 ± 3.6 mmHg in group 2 and 10.5 ± 2.6 mmHg in group 3 respectively. There were no statistically significant differences between the groups (group 1 vs 2, p = 0.70; group 1 vs 3, p = 0.61; group 2 vs 3, p = 0.94). The mean annulus diameter in group 1 was 20.2 ± 2.2 mm, in group 2 it was 20.4 ± 2.6 mm, and in group 3 it was 20.5 ± 1.5 mm. The mean diameter at the ST-junction was 18.4 ± 1.4 mm in group 1, 19.3±1.5mm in group 2 and 20.1±2.1mm in group 3. The mean diameter of the pulmonary artery was 20.2±1.5mm in group 1, 20.5±1.3mm in group 2 and 20.5 ± 1.4 mm in group 3.

### Serology samples

The mean total IgG for group 1 was 7.5 ± 8.9 kU/l, for group 2, 7.3 ± 10.9 kU/l and for group 3, 37.0 ± 31.1 kU/l. The mean IgA and IgM values were below 0.25 g/l for all three groups. There was no obvious trend that would identify a marked immuno- logical process in any specific group.

### Gross examination

There was an absence of aneurysm formation, deformations and thrombi in all of the explanted CPAs from the three groups (Fig. 1.2). The leaflets were pliable without fenestrations in all the samples from the three groups (Fig. 1.3).

### Biomechanical testing

The TS, YM and $T_d$ of the leaflets and wall specimens are summarized in Table 1. No statistically significant differences were found between groups, nor did they differ from the baseline results. Certainly no loss of strength could be demonstrated in any of the groups compared to the baseline values.

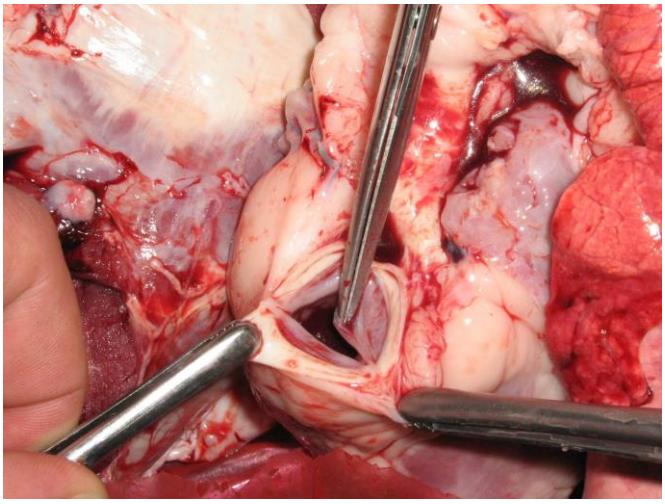

**Figure 1.2 Explanted cryopreserved pulmonary homograft of group 1 (24 h).** Note the pliable leaflet after 150days of implantation in the juvenile sheep.

**Histological evaluation**

The histological examinations are presented in Figure 1.4a–c. In the H&E staining results there was evidence that all samples from group 1 were covered by a monolayer of endothelial-like cells. Acellularity of the ECM in group 1 was between 30 and 90 %. Eighty percent of the CPAs in group 2 were covered by endothelial-like cells. The ECM was completely acellular in 2 samples. The other CPAs in group 2 were between 0 and 90 % acellular. In group 3, all of the CPAs were covered by a monolayer of endothelial- like cells. Acellularity was found in one valve, whereas the other valves were between 20 and 80 % acellular. von Kossa staining detected severe calcification of a leaflet in one of the explanted CPAs from group 3.

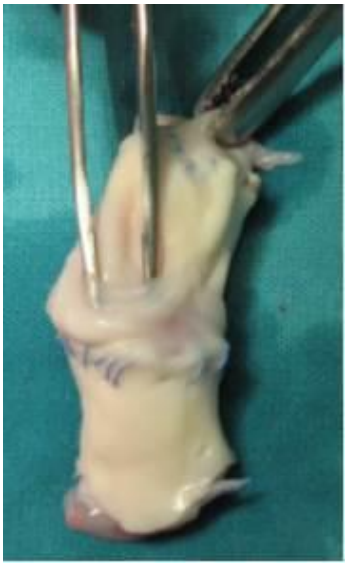

**Figure 1.3 Inverted cryopreserved pulmonary homograft (24 h) after 150 days implantation.**

None of the other explanted CPAs showed calcification of the leaflets. The pulmonary wall of only one of the CPAs from group 1 showed mild calcification. In group 2, there was severe wall calcification in two of the CPAs, moderate calcification in two CPAs and no calcification in one of the CPAs. Moderate calcification was observed in the wall of one of the explanted CPAs from group 3. None of the other explanted CPAs in group 3 exhibited calcification. Picrosirius red staining showed a well preserved ECM in all samples from the three groups. Endothelial–like cells covering the valve surfaces were identified as such since they were vWF, CD31 and CD34 positive. Interstitial cells were α-actin positive, however, since no CD4 positive cells were found, none of the samples showed inflammatory cells within the ECM.

**Scanning electron microscopy**

After explantation, SEM was able to demonstrate that 88.0 % of the pulmonary leaflets from group 1 fell in category III and 12.0 % fell in category II. In group 2, 62.7 % of the leaflets were classified as category III, 20.0 % as category II and 17.3 % as category I. In group 3, 64.0 % of the leaflets fulfilled the criteria for category III, 12.0 % for category II and 24.0 % for category I.

In group 1, 37.3 % of the CPA pulmonary wall specimens were classified as category III, 37.3 % as category II, and 25.4 % as category I. In group 2, 26.7 % of the pulmonary wall specimens fulfilled the criteria for category III, 37.3 % for category II and 36.0 % for category I. Finally, group 3 had 12.0 % of pulmonary wall specimens in category III, 61.3 % in category II and 26.7 % in category I.

Analysis of the SEM data for both the pulmonary leaflet and wall showed a time-related change over the categories ($p<0.005$ respectively). Although a gradual deterioration in the endothelial covering of both the pulmonary leaflets and walls in relation to the ischaemic time was observed, no differences in structural integrity could be demonstrated.

**Calcium content analysis**

Mean quantitative calcium content in the pulmonary leaflets was 0.18 ± 0.13 μg/mg dry weight in group 1, 0.30 ± 0.57 μg/mg dry weight in group 2 and 0.05 ± 0.05 μg/mg dry weight in group 3. There is no difference between calcification in the explanted pulmonary leaflets between the three groups ($p = 0.518$). For the pulmonary wall the mean quantitative calcium content was 16.12 ± 24.40 μg/mg dry weight in group 1, 84.95 ± 16.07 μg/mg dry weight in group 2 and 68.11 ± 41.64 μg/mg dry weight in group 3. The pulmonary wall calcification does not differ between the groups ($p = 0.218$). Note that only one pulmonary wall had significantly elevated calcium content.

**Table 1.1 Cryopreserved pulmonary homograft structural integrity testing**

| Variable | ≤ 6 h Baseline (n = 20) | 24 h Group (n = 5) | 48 h Group (n = 5) | 72 h Group (n = 5) | *p* values |
|---|---|---|---|---|---|
| **Tensile strength** | | | | | |
| Mean | 1.24 | 1.58 | 1.54 | 2.18 | 0.8882 ∗ |
| SD | 0.79 | 0.93 | 0.19 | 0.67 | |
| Median | 1.01 | 1.75 | 1.61 | 2.40 | |
| Minimum | 0.25 | .62 | 1.29 | 1.40 | |
| Maximum | 3.73 | 2.72 | 1.72 | 3.06 | |
| **Young's modulus** | | | | | |
| Mean | 5.68 | 6.55 | 5.94 | 7.76 | 0.5820 ∗ |
| SD | 4.57 | 4.71 | 1.65 | 2.60 | |
| Median | 4.78 | 7.75 | 6.14 | 8.55 | |
| Minimum | 0.84 | 1.42 | 3.98 | 4.01 | |
| Maximum | 18.42 | 11.36 | 8.21 | 10.57 | |
| **Thermal denaturation temperature ($T_d$)** | | | | | |
| Mean | 67.16 | 70.96 | 68.53 | 71.99 | 0.1697 ∗ |
| SD | 1.31 | 1.40 | 0.36 | 1.98 | |
| Median | 66.68 | 71.22 | 68.52 | 72.36 | |
| Minimum | 66.1 | 68.63 | 68.03 | 69.71 | |
| Maximum | 69.35 | 72.11 | 68.94 | 74.26 | |

*SD* standard deviation, *h* hour
∗ *p* value for the time effect in a mixed model

## Discussion

The pulmonary homograft remains the gold standard for right ventricle outflow tract (RVOT) reconstruction; its use is however limited by supply. The worldwide practice of limiting homograft harvesting to beating heart donors, or within 24 h post-mortem, may contribute to this shortage. If post mortem harvesting times can be extended safely, thereby allowing more time to obtain consent or fulfill other legal requirements, cadaver-based donor program can be expanded or in some cases be re-opened. This depends on whether viability of homografts contributes significantly to attenuate homograft failure as claimed by O'Brien *et al*. (1991). On the other hand Mitchell *et al*. (1998) considers all homografts to be eventually acellular and thus nonviable. It is thus possible to view homografts as biological scaffolds.

If all homografts eventually become acellular and function as a biological scaffold, for the purpose of this experimental animal study, it was important to firstly evaluate the impact of extending ischaemic harvesting times on the morphology, structural and strength characteristics of homografts, and taking the historical perspectives on homograft biology and degeneration into consideration.

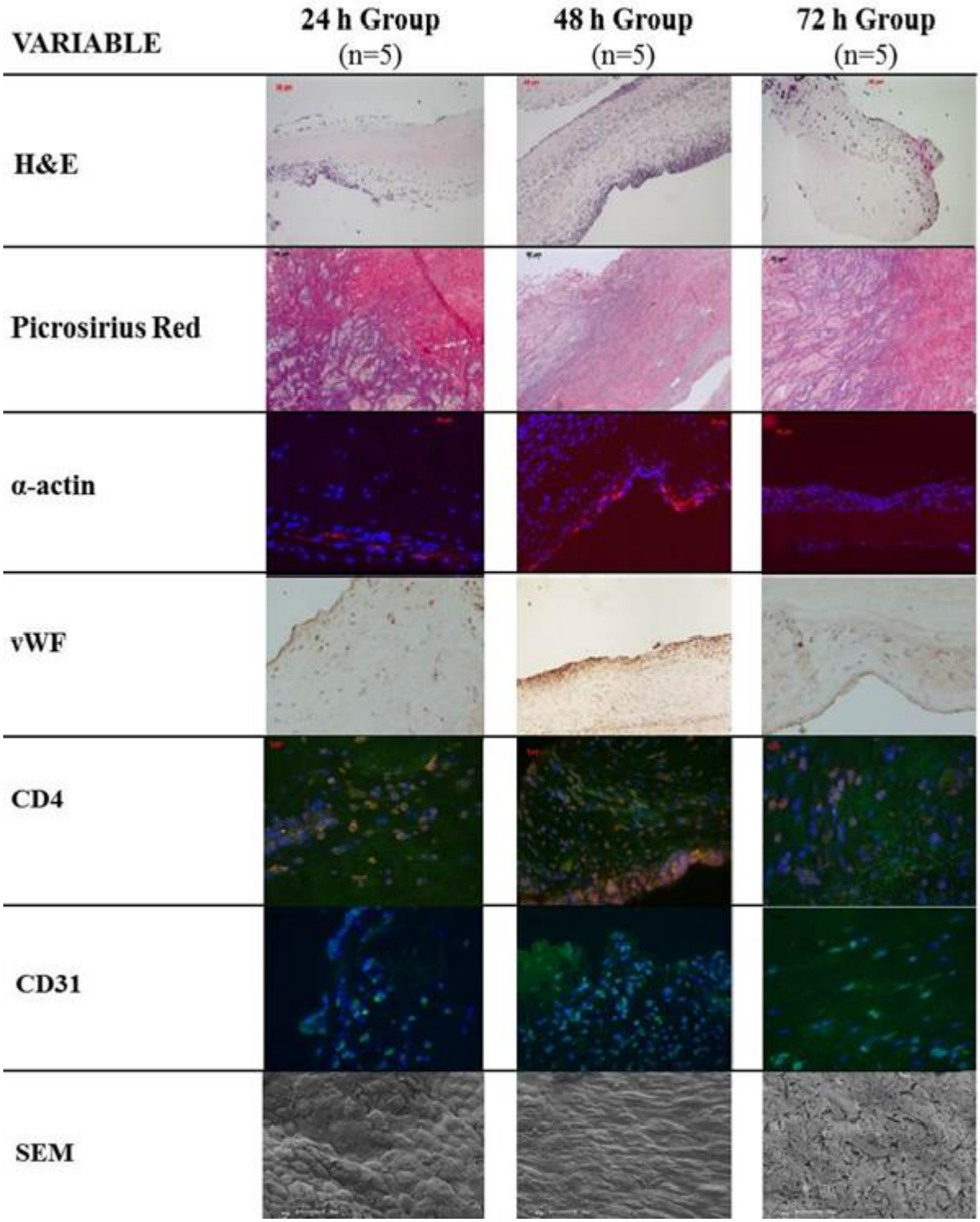


**Figure 1.4** **Histological findings of explanted CPAs**. **a** group 1 (24 h), **b** group 2 (48 h), and **c** group 3 (72 h). For each group; 1 H&E staining, 2 Picrosirius red staining, 3 α-actin staining, 4 vWF staining, 5 CD4 staining, 6 CD31 staining and 7 SEM. [*h* hours, *H&E* hematoxylin and eosin, *vWF* von Willebrand factor, *SEM* scanning electron microscopy]

Secondly, valve function and outcomes of the biological interactions post implantation were evaluated in vivo in a standard juvenile ovine model. Long-term durability of CPAs has

previously been correlated with warm- and cold ischaemic times. Cell metabolism is unchanged if the cold ischaemic time is <24 h and the warm ischaemic time is <12 h (Lang *et al*. 1994; Hu *et al*. 1989). O'Brien *et al*. (1991) stated that long-term durability is dependent on the viability of a homograft and therefore the CPA needed to be prepared within 24 h post-mortem. There is, however, no definition of preferred warm and cold ischaemic times. In the literature, different homograft banks have customized protocols for processing and cryopreservation of homografts. There are also variations in the ischaemic time between harvesting and cryopreservation.

Several studies have addressed homograft viability. Yankah and Hetzer (1987) reported that only 24 % of the endothelial cells survive after 2 h of exposure to room temperature. Crescenzo *et al*. (1992) noted the relationship between ischaemic time and progression of fibroblast cell damage, which is reversible with a warm ischaemic time of up to 12 h. However, a warm ischaemic time longer than 12 h will lead to apoptosis. Fibroblast response to warm ischaemic time is correlated with morphometric measurements (St Louis *et al*. 1991; Brockbank and Bank 1987).

Significant decreases in freedom of reoperation in the cryopreserved homografts compared to the 4°C stored homografts were observed in medium term studies. However, in a recent single-center study including 1,022 patients, O'Brien *et al*. (2001) compared 4°C stored homografts with viable cryopreserved homografts. In this 29-year follow-up study the viability of homografts progressively reduced, result- ing in non-viability after a few days, regardless of whether the homografts were cryopreserved or stored in the refrigerator, and whether they were stored in a balanced salt solution or a nutrient medium. Thus the viable homograft theory did not survive the test of time.

Barrat-Boyes *et al*. (1987) conducted a long-term follow-up study including 248 patients in which valve incompetence was assessed. Significant freedom of incompetence of antibiotic-sterilized aortic homografts was found in 95 % after 5 years, 78 % after 10 years and 42 % at 14 years. Harvesting time was <24 h in 147 donors, between 24 and 48 h in 88 donors and between 49 and 75 h in 7 donors. Harvesting time was not recorded for 43 of the donors. A multivariate analysis showed that advanced donor valve age ≥55 years (CI 1.12 ± 0.30; p = 0.0002), aortic root size >30 mm (CI 1.39 ± 0.41; p = 0.0007), and recipient age <15 years (CI 2.03 ± 0.61; p = 0.0008) are independent risk factors for homograft incompetence.

Langley *et al*. (1996) reported, based on a series of 249 patients, a freedom of reoperation of sub-coronary implanted antibiotic sterilized valves with a warm ischaemic time of 24 h and cold

ischaemic time of a maximum of 3 months. The freedom of reoperation of the aortic valve was excellent with 49.7 ± 5.6 % at 20 years follow-up.

The mean warm ischaemic time of homografts prepared at the European Homograft Bank is <6 h. However, due to post-mortem delay, the average cold ischaemic harvesting time is 24 h, up to a maximum of 36 h (Goffin *et al*. 1996). The clinical data of these homografts, presented by Meyns *et al*. (2005) shows that the ischaemic time has no statistical influence on the long-term durability of these homografts when implanted into pediatric patients. Only non-anatomic position ($p = 0.001$), smaller graft size ($p < 0.0001$), younger age (on square root scale, $p < 0.0001$) and clamp time ($p = 0.01$) remain as independent risk factors.

On the other hand, Tweddell *et al*. (2000) investigated the longevity of homografts used to reconstruct the RVOT in 205 patients with congenital heart disease and a mean age of 6.9 ± 7.6 years (range 3 days to 48 years). Freedom from homograft failure was 54 ± 7 % at 10 years follow-up. They found that independent risk factors for homograft failure in a multivariate analysis were younger age ($p < 0.001$), longer warm ischaemic time ($p < 0.001$), Z-value < 2 ($p = 0.03$), and aortic homograft ($p = 0.04$).

Importantly, Kadoba *et al*. (1991) performed a study of cryopreserved aortic homografts in a lamb model in which the cold ischaemic time was extended up to 48 h. The 48 h group performed as well as the fresh and 24 h groups. The authors concluded that expanding the pool for homografts is feasible by increasing the cold ischaemic time. However, no specific tests or histological examinations were performed to systematically evaluate the allograft scaffolds or the quality of the valvular tissue as in the present study.

In the present in vitro study, we could demonstrate the presence of endothelial cells in all groups. However, no pattern was observed between the presence of endothelial cells and cold ischaemic harvesting times. These findings suggest that the number of endothelial and interstitial cells present in the 72 h group were comparable with the number of cells in the 24 and 48 h groups. Thus, if the presence of endothelial cells was used to assess suitability, there should be no limitation on increasing the cold ischaemic harvesting time. No testing was performed to compare the viability of the different cell types in this study as all samples were harvested after 24 h ischaemic time. This 24 h period was conclusively demonstrated in previous studies to be associated with non-viability (Arminger 1995).

The *in vivo* part of this study showed that implanted CPAs, from the different cold ischaemic harvesting time groups, exhibited intra-luminal coverage with endothelial cells. These endothelial cells are most probably of host origin since, at implantation, most of the endothelial cells were either no longer present or nonviable after at least 24 h post- mortem. However, additional investigation will be required to confirm the origin of these endothelial cells. Gradual loss of endothelial cells with prolonged ischaemic times might not be detrimental to the clinical durability of the homograft, as the immune response of the recipient to the implant is severely diminished.

Previous studies have revealed that re-endothelialization is commonly seen in the juvenile sheep model (Dohmen *et al*. 2006a, b). This can however not be generalized to humans, since this process seems to be more restricted and takes longer in humans (Dohmen *et al*. 2007; Konertz *et al*. 2011). Further investigation is needed to better understand the recellularization of decellularized heart valves implanted into patients. The endothelial cells also need to be investigated for their functionality, as they have a major determinant function. Endothelium normally inhibits thrombus formation and leukocyte adhesion, regulates vasomotor function, and inhibits smooth muscle cell proliferation. If there is damage to the endothelium interaction with inflammatory cells and interstitial cells, by expression of vascular adhesion, molecules such as VCAM-1, ICAM-1 and ELAM-1 will change (Ardehali *et al*. 1995). The intercellular network via macrophages, T-lymphocytes, endothelial cells and smooth muscle cells is generated by a variety of stimulatory cytokines (IL-1, IL-2, IL-6, and tumor necrosis factor-a) and growth factors (PDGF, IGF-1, FGF, HB-EGF, EGF, GM-CSF, and TGF-b) (Duquesnoy and Demetris 1995). Therefore, it is of great interest that autologous endothelial cells overgrow the CPAs to restore the function of the interstitial cells and decrease the activation of the inflammatory cells. On the contrary, it is important to avoid a pseudo-intima formation on the leaflet surface since this will lead to leaflet retraction, resulting in central valve regurgitation (Affonso da Costa *et al*. 2004). Pseudo-intima formation was not observed in any of the implanted valves in this study.

A well-known disadvantage of viable CPAs in transplant surgery is that donor endothelial and interstitial cells evoke an immune response from the host (Jane-wit *et al*. 2013). Decellularization of a CPA could attenuate the humoral immune response to donor HLA after implantation of a CPA as was shown by Kneib *et al*. (2012). Homografts with viable endothelium and interstitial cells have a significantly higher number of immunogenic epitopes, for HLA classes I and II, than the decellularized groups exhibit, and should therefore initiate a more dramatic immune response from the recipient. In this study, with harvesting times in excess of 24 h, endothelial viability is highly unlikely.

On a cellular level there were no differences between the groups with varying cold ischaemic times. The extracellular matrix was tested to evaluate if the strength of the scaffold would change by extending the cold ischaemic harvesting time. No differences in strength of the valve tissue were detected. Further- more, there was no increase in tissue denaturation, evaluated by measuring the $T_d$, in any of the groups. The extracellular matrix was also confirmed to be intact by histological examination. From the in vitro data we are able to conclude that tissue strength is unaltered and therefore extending the cold ischaemic harvesting time is possible.

From the *in vivo* investigations, with a minimum follow-up of 150 days in a juvenile sheep model, hemodynamic data shows no differences between the groups when the cold ischaemic time was increased. Furthermore, the histological examinations showed a normal intact extracellular matrix. The immunohistochemistry results in this study did not show any differences in inflammatory reactions when cold ischaemic time was increased. An overgrowth of endothelial cells was observed in all groups. There- fore, in this study, there were no contra-indications to extending the cold ischaemic harvesting time in order to increase the number of viable homografts.

### Limitations

The limitations of this study are that data provided by the juvenile sheep model cannot be unconditionally applied to human patients, since re-endothelialization is more extensive in this model than in humans. Also, the functionality and viability of the endothelial and interstitial cells were not investigated in this study.

## Conclusions

This experimental study supports the concept that, with a limited warm ischaemic time, the cold ischaemic harvesting time of cryopreserved homografts can be prolonged. This could be a way to reduce homograft shortage since by increasing time limits for harvesting, the opportunities for obtaining consent and facilitating cadaver donor programs are increased. However, long-term clinical hemodynamic evaluation is needed to confirm this approach.

We certify that all authors named in this manuscript deserve authorship, and that all authors have agreed to be listed and have read and approved the manuscript and its submission to Cell and Tissue Banking. The authors have no conflicts of interest to declare.

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# Chapter 3
Article 2

## Morphology of unprocessed and cryopreserved pulmonary homograft leaflets with post mortem harvest times up to seventy-two hours

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### Abstract

**Introduction:** Homograft reconstruction of the right ventricle outflow tract (RVOT) remains the ideal conduit for lesions requiring RVOT reconstruction. Most homograft banks now restrict post mortem harvesting times, regarded as warm ischaemic time, to less than twenty-four hours, which resulted in the disappearance of cadaver donor programmes. Morphological changes in homografts in the first twenty-four hours post mortem have been well documented but morphological changes after extending harvest times beyond fourty-eight hours are less clear. The aim of this study was to investigate the possibility of extending harvest times safely up to seventy-two hours post mortem.

**Method:** The homograft valve leaflets of fourty sheep were harvested at six, twenty-four, fourty-eight and seventy-two hours post mortem. Morphological and ultra-structural changes over increased post mortem times as well as the impact of the cryopreservation process on valves with extended post mortem harvest times were investigated.

**Results:** The unprocessed pulmonary homografts showed no morphological changes for Haematoxylin and Eosin (H&E), Picrosirius red, scanning electron microscopy (SEM), transmission electron microscopy (TEM) up to fourty-eight hours post mortem. At seventy-two

hours post mortem TEM revealed cellular swelling and oedema with retention of the collagen structure and organisation of the leaflets. The cryopreserved pulmonary homografts did not show any morphological differences between the groups harvested at six hours, twenty-four hours, fourty-eight hours and seventy-two hours post mortem. The cryopreserved homografts showed changes in the collagen as well as appearing more compact and flattened as per the Picrosirius red stain and TEM demonstrated cellular swelling and oedema as well as signs of disrupted and damaged collagen structures. Despite the establishment of homograft banks through the development of cryopreservation, the demand for homografts exceeds supply. In this study, the ischaemic post mortem period up to seventy-two hours had very little demonstrable affect on tissue morphology in the unprocessed groups, except for a more oedematous appearance on TEM in the seventy-two hour group. Thus, as a whole, leaflet tissue integrity was well maintained during the ischaemic period. Cell death and disruption of the collagen scaffold could be observed in all cryopreserved groups (after thawing), irrespective of post mortem harvest times. The affects of cryopreservation were thus immediate, disruptive and lethal. Yet cryopreservation remains standard procedure in virtually all tissue banks. **Conclusion:** The study showed that post mortem harvest times can safely be extended to at least fourty-eight hours, alluding to the obvious and necessary re-evaluation of the current format of cadaver programmes. Strategies, including decellularisation, should be further explored to reduce or to avoid the impact of cryopreservation on tissue scaffolds during processing in tissue banking.

**Keywords:** Cryopreservation–Homografts–Ischaemic time–Morphology

## Introduction

Homograft reconstruction of the right ventricle outflow tract (RVOT) remains the ideal conduit for lesions requiring RVOT reconstruction. The availability of homografts remains the most important limiting factor for its surgical use. O'Brien *et al*., 1987(a & b), popularised the concepts of viable homografts and cryopreservation. This resulted in the development of the present cryopreserved homograft banks.

Most homograft banks now restrict post mortem harvesting times, regarded as warm ischaemic time, to less than twenty-four hours Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013). This resulted in the disappearance of cadaver donor programmes, further limiting homograft supplies. Despite the fact that O'Brien published a very long-term follow-up where no outcome difference could be shown between fresh antibiotic

preserved valves (kept at 4˚C) and viable homografts harvested less than six hours post mortem and cryopreserved within three to four days post mortem (O'Brien *et al*., 1988).

At the Bloemfontein Homograft bank, the mean post mortem harvest time was thirty-three hours post mortem (Botes *et al*., 2012). As this was in excess of the generally accepted twenty-four hour limited post mortem donor times, it became essential for an experimental study that was subsequently conducted in the ovine model. Pulmonary homografts were implanted in the RVOT of juvenile sheep, which were monitored for six months. No difference could be observed in the function, strength or morphology of the explants between homografts harvested twenty-four, fourty-eight and seventy-two hours post mortem (Smit *et al*., 2015).

Post mortem cellular and structural changes associated with warm ischaemia have been extensively studied (Crescenzo *et al*., 1992; Mitchell *et al*., 1998; Schenke–Layland *et al*., 2006; Smit *et al*., 2015) and ultra-structural changes associated with irreversible cell injury occurs in 40% of leaflet cells within sixteen to twenty hours post mortem (Crescenzo *et al*., 1993).

Despite all efforts to retain cells and have viable homografts through early post mortem or beating heart harvesting as well as immediate processing and cryopreservation, Mitchell *et al*. (1998) still concluded that most homografts actually are nonviable and consists mostly of acellular scaffolds.

During cryopreservation, crucial homograft leaflet matrix elements are affected negatively and unfavourably. Significant modification and degradation of collagen and elastic fibres and disfigurement of the leaflet histo-architecture during cryopreservation can also be caused by extracellular ice formation (Schenke-Layland *et al*. 2006). Mitchell *et al*. (1998) found that in cryopreserved allografts the collagen is mostly preserved albeit flattened.

Crescenzo and co-workers (1992) found that shortly after donor death, cellular injury begins and escalates during the period up to thirty-six hours. They also found a connection between permanent cellular injury and increasing warm ischaemia up to thirty-six hours. Their findings suggested that escalating leaflet cell injury was associated with increased ischaemic time after donor death.

As most cryopreservation programmes still require three to four days of harvesting, dissection, incubation and sterilisation before cryopreservation, insisting on a harvest time of less than twenty-four hours (< 6 h in several tissue banks), becomes a contradiction in terms. Although

biological loads might increase, which could influence the long-term survival of homografts (Gall *et al*., 1995; Brubaker *et al*., 2016), a definite limit to extension of the post mortem harvest time has not been defined (St Louis *et al*., 1991). Viability studies confirmed that by twenty-four to thirty-six hours after death, most if not all, cells will have sustained irreversible cellular injury (Hopkins *et al*., 2005).

In addition, viable endothelium can evoke an immune reaction to the detriment of graft survival (Wheatley & McGregor, 1977; Yankah & Hetzer 1987); therefore unprocessed valves of less than six hours should best be avoided.

Overall, morphological changes in homografts in the first twenty-four hours post mortem have been well documented, but morphological changes after extending harvest times beyond fourty-eight hours are less clear.

In order to investigate the possibility of extending harvest times safely, this experimental study explored the comparative morphological and ultra-structural changes of valve leaflets harvested less than six hours, and up to seventy-two hours post mortem. It also studied valve leaflets harvested up to seventy-two hours post mortem, which were cryopreserved and thawed after seven days in order to evaluate the impact of the cryopreservation process on valves with extended post mortem harvest times.

## Method and materials

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). Approval of the study protocol was obtained from the Animal Ethics Committee of the University of the Free State (ETOVS Number: UFS–AED2016/0101).

### Pulmonary homograft harvesting

The heart, lungs and major blood vessels of fourty sheep, weighing between twenty-five and thirty kilograms were harvested as a block after they were slaughtered at the Bloemfontein abattoir. Heart-lung blocks were kept at room temperature for eight hours and thereafter stored at 4°C in a refrigerator until the pulmonary homografts were excised.

Four groups of ten homografts each were harvested at less than six hours post mortem: (group A) at twenty-four hours, (group B) at fourty-eight hours, (group C) and (group D) at seventy-two hours post mortem. In each group, five homografts were left unprocessed (group 1) five

were cryopreserved (group 2). The cryopreserved homografts were thawed after seven days and leaflets resected for analyses (Figure 1.1).

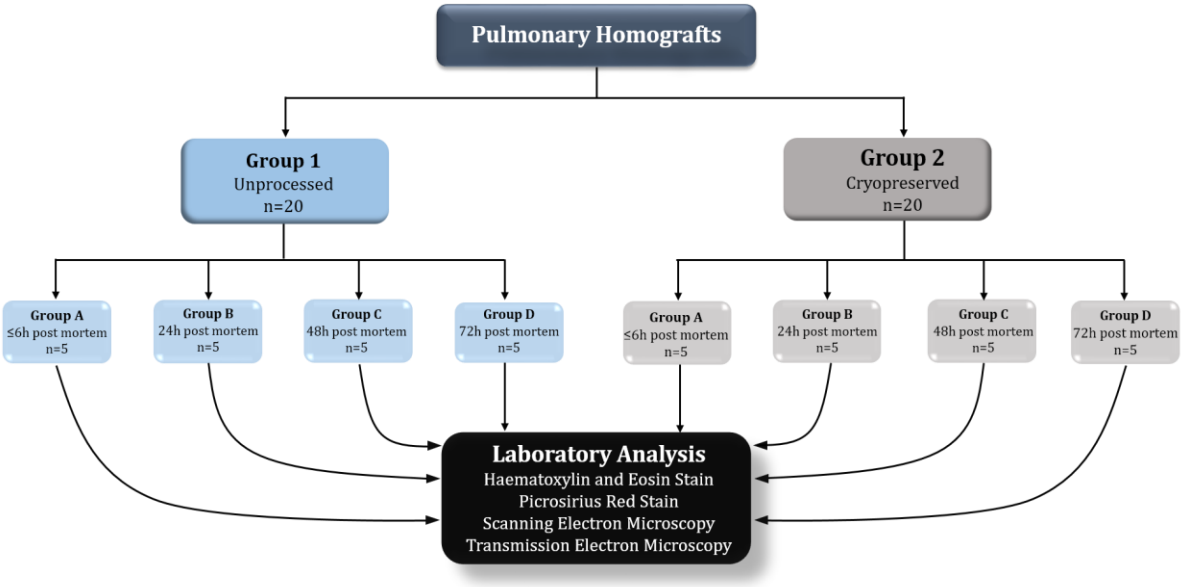


**Figure 1.1 Study layout** (h = hours; n = sample size).

## Tissue preparation and cryopreservation

Cultures were obtained before cryopreservation was performed using an automated biological freezer (Cryoson BV-9 biological freezer, Cryoson, Krefeld, Germany). An amount of 11 ml of dimethyl sulphoxide [DMSO, Sigma-Aldrich, Midrand, South Africa (SA)] was added to the 100 ml of M199 and the homograft was cryopreserved. Homografts were stored in a double package tri–laminated aluminium cryobag (Kapak Corporation, Minneapolis, USA) in a storage tank in the vapor phase of liquid nitrogen between -120°C to -160°C until they were used.

Before laboratory analysis, homografts were thawed by placing it on a shelf at room temperature for ±5 min. to eliminate excessive cold temperature followed by immersion in a water bath at 30°C for five to seven minutes until they were about 80% thawed. The outer package was opened and the homograft was transferred into a bowl with 500.ml cold (4°C) M199 medium (GibcoBRL, United States of America), allowing a ten-minute rinse period with gentle shaking to extract most of the DMSO from the homograft tissue. This procedure was repeated in a second bowl of cold M199 medium, and thereafter the homograft was dissected and samples taken for analysis.

## Histological and morphological analyses

### Light microscopy

Samples from the centre of each pulmonary homograft leaflet were processed by the department of Anatomical Pathology of the National Health Laboratory Services (NHLS) in Bloemfontein, using standard operative procedures. The specimens were embedded in paraffin wax and two micrometre thick longitudinal sections were prepared and routinely processed for haematoxylin and eosin (H&E) and Picrosirius red staining. The impact of ischaemic time and cryopreservation on the valvular leaflet tissue was evaluated by performing H&E stains to display cytoplasmic, nuclear, and extracellular matrix features as well as Picrosirius red stains to study the collagen networks.

### Electron microscopy

The electron microscopy (scanning electron microscopy and transmission electron microscopy) of each pulmonary homograft was processed and analysed by the Centre for Microscopy, at the University of the Free State, as per standard protocol.

### Scanning electron microscopy (SEM)

Each pulmonary homograft tissue sample for SEM was prepared by the Centre for Microscopy at the University of the Free State. All samples were fixed in 2.5% glutaraldehyde (Merck, Johannesburg, South Africa). Homograft leaflets were divided into two specimens of approximately 3x6 mm. Tissue specimens were dried using the critical point method (Tousimis critical point dryer, Rockville, Maryland, USA, ethanol dehydration, carbon dioxide drying gas) and were metallised using gold (BIO-RAD, Microscience Division Coating System, London, UK; Au/Ar sputter coating @ 50-60 nm). Evaluations were performed with a Shimadzu SSX 550 scanning electron microscope (Kyoto, Japan, with integral imaging (SDF, TIF and JPG format)). The surface area of each specimen was examined and photographed in either four or five different positions, and all images were then evaluated by three independent assessors and a score allocated. SEM micrographs were used to assess endothelial integrity and the effect of cryopreservation on endothelium integrity, and to evaluate the quality of the extracellular basal membrane.

### Transmission electron microscopy (TEM)

Pulmonary leaflet samples were fixed in 3.0% glutaraldehyde overnight, post fixated in Palade's osmium tetroxide, and dehydrated in a graded acetone series. Dehydrated samples were impregnated/embedded in an epoxy (Spurr, 1969) to facilitate the creation of ultra-thin sections for the TEM evaluation. Ultra-thin sections were cut from the sample imbedded in the

epoxy using a Leica ultra-microtome (Leica Ultracut UC7, Vienna, Austria). After sectioning, the samples were stained with uranyl acetate and lead citrate. Sections of the leaflet samples were evaluated by using a Philips (FEI, Netherlands) CM100 transmission electron microscope and photographed using an Olympus Soft Imaging System Megaview III digital camera, with Soft Imaging System digital image analysis and documentation software (Olympus, Tokyo, Japan).

## Results

All cultures obtained from homografts were negative. No difference could be demonstrated on H&E between the less than six hour, the twenty-four hour, fourty-eight hour and seventy-two hour groups in both the unprocessed (group 1A-D) and cryopreserved groups (group 2A-D). Also, no difference could be demonstrated between the unprocessed (group 1A-D) and the cryopreserved group (group 2A-D). Endothelial layers were present in both the unprocessed and cryopreserved leaflets up to seventy-two hours (Figure 1.2).

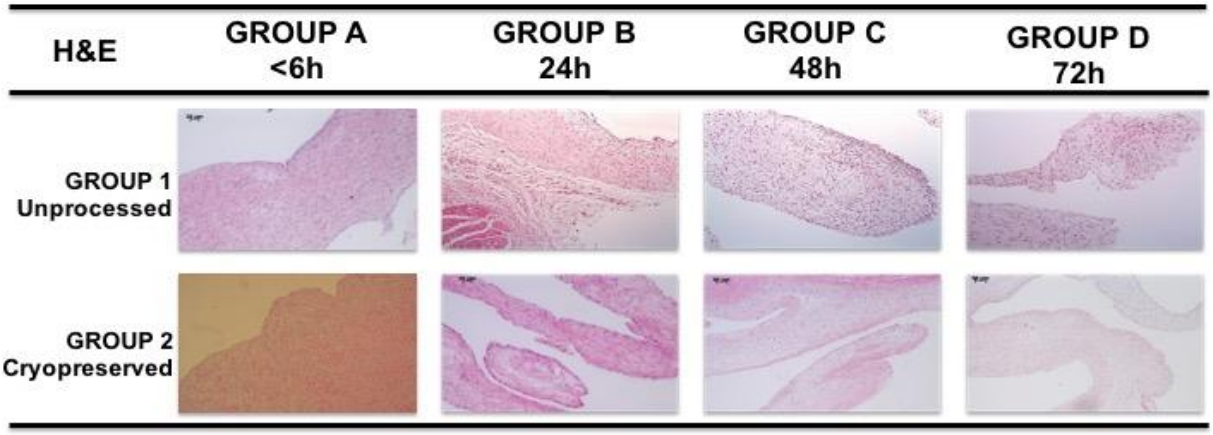


**Figure 1.2 H&E stain of unprocessed (group 1A-D) and cryopreserved groups (group 2A-D).** Unprocessed group (group 1); unprocessed <6 h group (group 1A); unprocessed 24 h group (group 1B); unprocessed 48 h group (group 1C); unprocessed 72 h group (group 1D); Cryopreserved group (group 2); cryopreserved <6 h group (group 2A); cryopreserved 24 h group (group 2B); cryopreserved 48 h group (group 2C); cryopreserved 72 h group (group 2D); H&E=haematoxylin and eosin; h=hours.

Picrosirius red also demonstrated no difference between the unprocessed groups (group 1A-D). There were no differences between the cryopreserved groups 2B, 2C and 2D either, but clearly the collagen structure and organisation were modified by cryopreservation. In the cryopreserved groups, collagen appeared to be more compact or flattened (Figure 1.3).

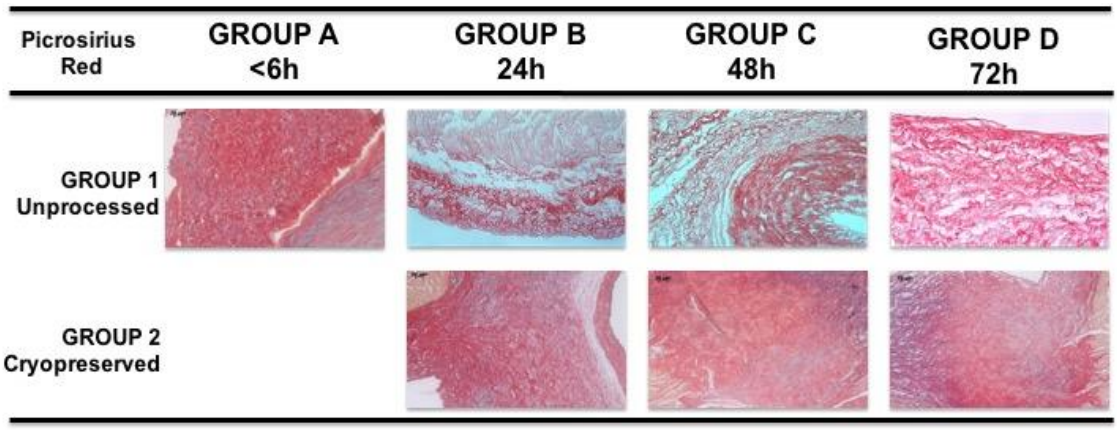


**Figure 1.3 Picrosirius red stain of unprocessed (group 1A-D) and cryopreserved groups (group 2A-D)** Unprocessed group (group 1); unprocessed <6 h group (group 1A); unprocessed 24 h group (group 1B);unprocessed 48 h group (group 1C); unprocessed 72 h group (group 1D); Cryopreserved group (group 2); cryopreserved <6 h group (group 2A); cryopreserved 24 h group (group 2B); cryopreserved 48 h group (group 2C); cryopreserved 72 h group (group 2D); H&E=haematoxylin and eosin; h=hours

SEM of the endothelial surface of the pulmonary leaflets demonstrated intact endothelial layers in all groups (group 1A-D and group 2A-D), although the prominence of nuclei may indicate abnormal cells and probably nonviable cells (Figure 1.4).

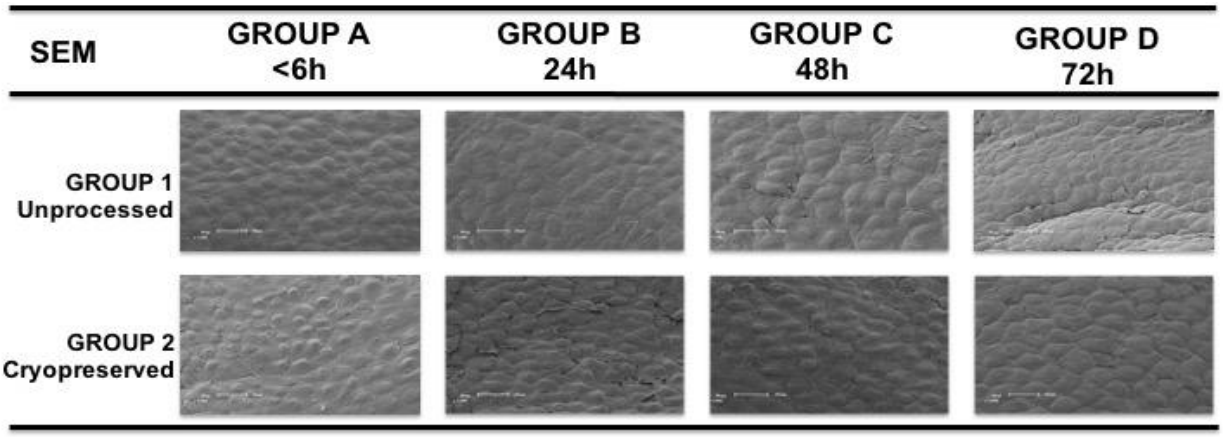


**Figure 1.4 SEM of unprocessed (group 1A-D) and cryopreserved groups (group 2A-D)** Unprocessed group (group 1); unprocessed <6 h group (group 1A); unprocessed 24 h group (group 1B); unprocessed 48 h group (group 1C); unprocessed 72 h group (group 1D); Cryopreserved group (group 2); cryopreserved <6 h group (group 2A); cryopreserved 24 h group (group 2B); cryopreserved 48 h group (group 2C); cryopreserved 72 h group (group 2D); H&E=haematoxylin and eosin; h=hours

TEM demonstrated some oedema and cellular swelling in the seventy-two hour unprocessed pulmonary leaflets (group 1D) with retention of collagen structure and organisation. In all cryopreserved groups (group 2A-D) collagen was disrupted and damaged with cell death (Figure 1.5).

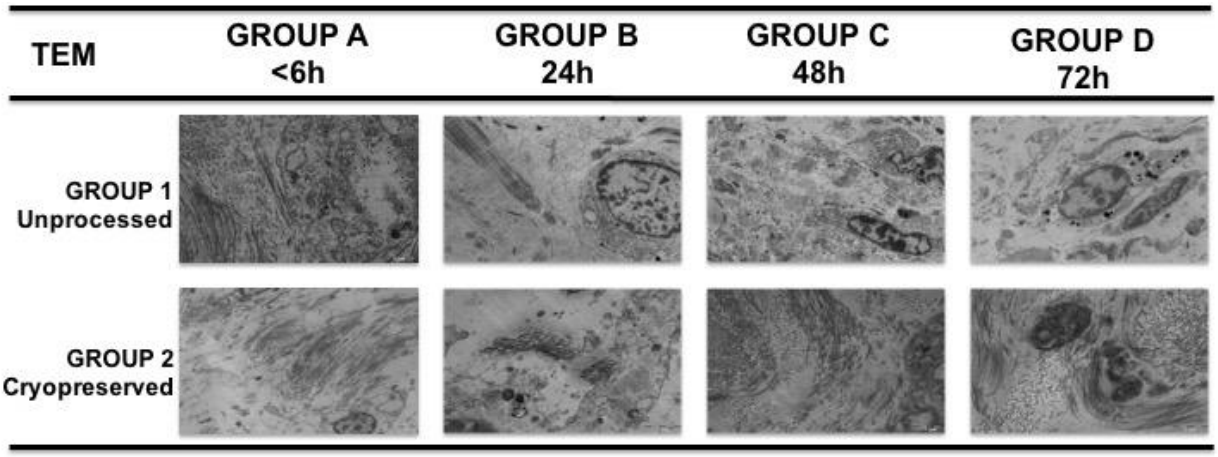


**Figure 1.5 TEM of unprocessed (group 1A-D) and cryopreserved groups (group 2A-D)**
Unprocessed group (group 1); unprocessed <6 h group (group 1A); unprocessed 24 h group (group 1B); unprocessed 48 h group (group 1C); unprocessed 72 h group (group 1D); Cryopreserved group (group 2); cryopreserved <6 h group (group 2A); cryopreserved 24 h group (group 2B); cryopreserved 48 h group (group 2C); cryopreserved 72 h group (group 2D); H&E=haematoxylin and eosin; h=hours

## Discussion

Despite the establishment of homograft banks through the development of cryopreservation, demand for homografts exceeds 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 harvesting time of less than twenty-four hours (Livi *et al.,* 1987; O'Brien *et al*., 1988) in order to ensure cellular viability, particularly of endothelial cells and fibroblasts, which in turn was thought to influence long-term valve stability and durability (O'Brien *et al*., 1995; Angell *et al*., 1989). Therefore, this study was designed to evaluate whether morphological changes appear in pulmonary homografts harvested at six, twenty-four, fourty-eight and seventy-two hours post mortem. By extending the post mortem harvesting time the shortage of homografts required for surgical procedures can be addressed.

No morphological differences could be observed between the unprocessed and cryopreserved groups with regard to H&E and SEM after seventy-two hours post mortem. The unprocessed pulmonary homografts showed no morphological changes (H&E, Picrosirius red, SEM, TEM) up to fourty-eight hours post mortem. At seventy-two hours post mortem TEM revealed cellular swelling and oedema with retention of the collagen structure and organisation. The cryopreserved pulmonary homografts did not show any morphological differences between the groups harvested at six, twenty-four, fourty-eight and seventy-two hours post mortem. However, when compared to the unprocessed homograft group, the collagen in the cryopreserved homograft group appeared to be more compact or flattened (Picrosirius red stain). Furthermore, TEM demonstrated cellular swelling and oedema (necrosis) as well as disrupted and damaged collagen structures occurring in all cryopreserved groups, irrespective of

post mortem harvest times (six to seventy-two hours). Fischlein *et al*. (1994) described that cryopreserved homografts from non-beating heart donors exhibit altered tissue structure with oedema and vacuolisation within the spongiosa of the leaflets as well as irreversible cell damage when examined under transmission and electron microscopy.

Cryopreservation is therefore associated with structural damage with significant changes in collagen and elastic fibres as well as loss of glycosaminoglycans (GAGs) of the extracellular matrix (ECM)(Wollmann *et al*., 2011).

For a considerable period of time, it was believed that the preservation of cell viability was important, for it would result in grafts with some degree of regenerative capacity and consequently, greater durability. Wheatley and McGregor (1977) have raised important questions about the presence of excessive viable endothelium cells during implantation. In their study, large numbers of viable cells (fresh homografts) led to immune reactions, which accelerated the calcification of these valves (Wheatley and McGregor, 1977). Mitchell *et al*. (1995) reported that after implantation, cryopreserved homografts rapidly become acellular, and are subjective to progressive tissue degeneration. This finding was confirmed in an earlier study by this research group (Smit *et al*., 2015).

In this study the TEM results of the cryopreserved leaflets demonstrated cell death and disruption of the collagen scaffold in all cryopreserved groups. In addition, this research group found that the ischaemic post mortem period up to seventy-two hours had very little demonstrable effect on tissue morphology in the unprocessed groups, except for a more oedematous appearance on TEM in the seventy-two-hour group. Thus, as a whole, leaflet tissue integrity was well maintained during the ischaemic period.

On the other hand, cell death and disruption of the collagen scaffold could be demonstrated in all cryopreserved groups (after thawing), irrespective of post mortem harvest times. The effects of cryopreservation were therefore immediate, disruptive and lethal. Yet cryopreservation remains standard procedure in virtually all tissue banks and despite the demonstrable detrimental effects of cryopreservation, cryopreserved homografts have excellent medium to long-term results in human studies (O'Brien *et al*., 2001).

## Conclusion

The detrimental effect of post mortem ischaemia on the morphology of ovine pulmonary homografts up to fourty-eight hours could not be demonstrated. The only difference was

demonstrated by TEM revealing cellular swelling in the seventy-two-hour post mortem group. Cryopreservation had an immediate and disruptive impact on ovine pulmonary homograft leaflets. This study demonstrated that harvest times can safely be extended to at least fourty-eight hours, prompting a re-evaluation of the use of cadaver programmes. Strategies, including decellularisation, should be further explored to reduce or to avoid the impact of cryopreservation on tissue scaffolds during processing in tissue banking.

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# Chapter 4
Article 3

## Is the impact of cryopreservation on the tissue strength of ovine pulmonary artery homograft leaflets more important than extending ischaemic harvest times?

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### Abstract

**Introduction:** The availability of homografts, the gold standard of surgical correction of congenital defects of RVOT, remains a challenge. The fact that leading homograft banks limit post mortem harvest times to less than twenty-four hours is the main cause. The aim of this study was to address the availability by assessing the effect of ischaemic times of up to seventy-two hours before harvest on the strength of ovine pulmonary artery homograft leaflets. The research group also assessed the effect of cryopreservation at the longer ischaemic times on these leaflets.

**Method:** Pulmonary homograft leaflets were harvested from the pulmonary arteries of eighty-four sheep. The arteries were stored at 4°C for six hours, twenty-four hours, fourty-eight hours and seventy-two hours before harvesting. Twelve of the leaflets harvested at the time-points were unprocessed. Twelve of those harvested at twenty-four, fourty-eight and seventy-two hours were cryopreserved. The strength of the leaflets was assessed by determining tensile strength (TS), Young's modulus (YM) and thermal denaturation ($T_d$).

**Results**: TS did not differ significantly between the twenty-four, fourty-eight and seventy-two hour valves for both the unprocessed and cryopreserved leaflets. Cryopreservation tended to decrease, but not significantly, the TS in the cryopreserved leaflets when compared to the six hours unprocessed leaflet. A reduction in strength of the cryopreserved leaflets was demonstrated in the twenty-four and seventy-two hour groups compared to unprocessed leaflets, which did not reach significance in the fourty-eight hour groups.
**Conclusion:** The results of this study strongly indicate that the strength of valve leaflets was not negatively affected by increased post mortem harvest times. There is a slight possibility that cryopreservation may cause some decrease in the strength. Nevertheless, the results strongly suggest that post mortem harvest times may be extended to fourty-eight hours and perhaps beyond.

**Keywords:** Pulmonary homografts–Tissue strength–Prolonged ischaemic harvest time–Cryopreservation

## Introduction

Homografts remain the gold standard for surgical correction of congenital defects of the RVOT. However, availability remains a challenge. The post mortem harvesting time of homografts has been restricted to a maximum of twenty-four hours by most tissue banks internationally (Dawson and Brockbank, 1997). This practice has led to the demise of most cadaver based donor programmes. According to The Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013), 57% of homografts were obtained from organ donors, 15% from domino hearts and only 28% from non-organ donors.

In reality, the hours from death to excision ranged from between two hours to fifty hours in the same report and in an analysis of twenty-three banks' practices, twelve accepted cardiectomy or receipt by the bank up to twenty-four hours post mortem, while nine accepted tissue harvested or receipt at the bank longer than twenty-four hours post mortem, and six out of nine banks mostly up to fourty-eight hours (Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide, 2013).

Extending the post mortem harvest time to fourty-eight hours for example, might allow the resurgence of cadaver banks, attenuating the international shortage of homograft availability.

The deleterious affect of prolonged ischaemia on homografts has been well documented (Crescenzo *et al*., 1992; St Louis *et al*., 1991) as has been the impact of cryopreservation in various experimental studies (Koolbergen *et al*., 2002; Mitchell *et al*., 1998; Schenke–Layland *et al*., 2006). However, prolonging the ischaemic harvest time up to seventy-two hours in a clinical setting could not be shown to have a deleterious effect on cryopreserved pulmonary homograft functioning, calcification, strength or structural integrity in an experimental six-month implantation study in the ovine model (Smit *et al*., 2015).

As cryopreservation occurs as a common endpoint after a variety of incubation and sterilisation processes in all cryopreservation tissue banks, the comparative effect on tissue strength between prolonged ischaemic times and the impact of processing and cryopreservation itself, needs to be clarified. It makes very little sense to reject homograft donors after twenty-four hours ischaemic time, if the processing and cryopreservation itself has a greater impact on tissue strength than prolonged ischaemic harvest times.

In assessing the structural integrity of pulmonary homografts, morphology as well as the tissue strength of tissue need to be evaluated and the impact of post mortem ischaemic harvest times as well as cryopreservation needs to be carefully assessed.

The aim if this study was to compare the impact of extended ischaemic cold harvest times on the tissue strength of pulmonary homografts to that of cryopreserved homografts in an *in vitro* ovine model.

## Materials and method

### Pulmonary homograft harvesting

The heart, lungs and major blood vessels were harvested as a block from eighty-four sheep, slaughtered at the Bloemfontein abattoir. Sheep selected weighed between twenty-five and thirty kilograms. The blocks were stored at 4°C until the pulmonary homografts were harvested as previously described (Smit *et al*., 2015). The schematic representation of the experimental procedure is illustrated in Figure 1.1. Cryopreservation was done as described by Smit *et al.* (2015). All homografts were culture negative before cryopreservation.

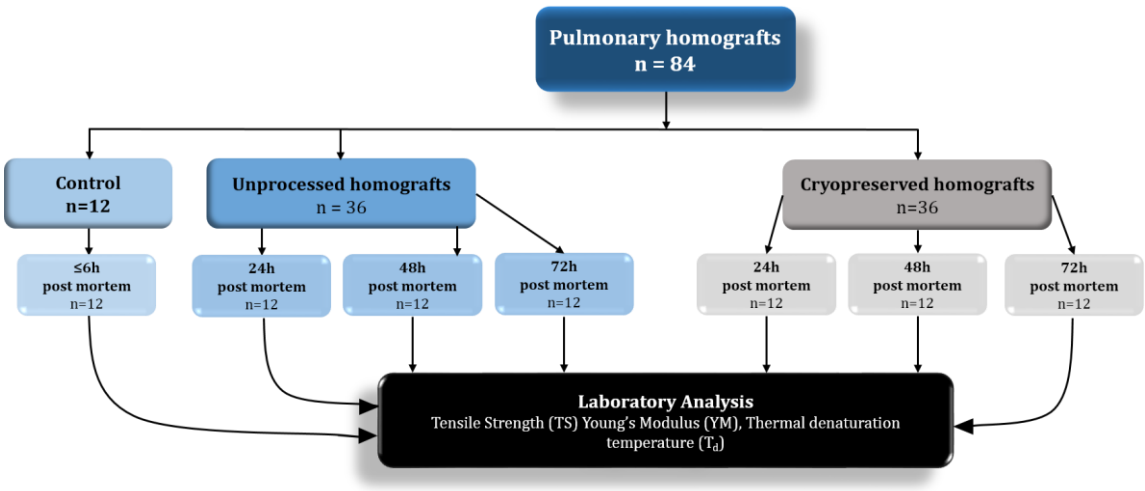


**Figure 1.1** **Schematic representation of the experimental approach that was used to assess the impact of longer ischaemic harvest times and cryopreservation on ovine pulmonary leaflet strength.**

## Strength analysis

The tensile strength of leaflets was assessed using the automated and computerised tensile strength testing apparatus (Lloyds LS100 Plus, IMP, Johannesburg, South Africa). Leaflet samples were prepared by dissecting 5 mm wide strips and fixed by clamps at both ends and gradually stretched. A random leaflet sample was obtained from all pulmonary homografts in every group. Tensile strength curves of the leaflets stretched at 0.1 mm/s were automatically recorded. The derivatives calculated from the tensile strength curve were the tensile stress and strain. The tensile strength (TS, MPa) is represented by the stress. The Young's modulus (YM, MPa) were calculated by dividing the stress by the strain, i.e. the ratio of stress to strain.

Thermal denaturation ($T_d$) was determined on a small sample of leaflets as described (Lovekamp and Vyavahare, 2001). Leaflet samples were randomly selected from ten sheep in each group, of which five were from the cryopreserved set and five from the unprocessed pulmonary homografts set. These samples (3.0 mm X 3.0 mm) were patted dry, weighed and then placed in the hermetically sealed aluminium crucibles/pans of a differential scanning calorimeter (Mettler Toledo, $T_d$ 822e, Microsep, Johannesburg, South Africa). $T_d$ was determined by increasing the temperature at a rate of 10°C/min from 25°C to 95°C. The temperature of thermal denaturation was recorded electronically and $T_d$ regarded as the peak maximum of denaturation.

## Statistical analysis and ethics approval

The means were calculated. Confidence intervals of 95% of the differences were calculated to test for the significance between measurements. The $Chi^2$ test was used to test for differences in

frequency between slipped and non-slipped results. The project was approved by the Interfaculty Animal Ethics Committee of the University of the Free State (UFS-AED2016/0101).

## Results

The results of strength testing and $T_d$ analyses are summarised in Tables 1.1A and B.

### Tensile strength (TS)

The six hour TS values in the unprocessed leaflets were lower than the twenty-four and seventy-two hour values and although the six hour values were not significantly different from the fourty-eight hour values, it tended to be lower since the lower limit of the 95% CI was close to zero. For the TS there were no significant differences between the twenty-four, fourty-eight and seventy-two hour values of the unprocessed leaflets (Table 1.1A).

The six hour TS values in the cryopreserved leaflets were significantly higher than the twenty-four, fourty-eight and seventy-two hour values, i.e. –twenty-four hours vs. six hours: -4.1; 0.3, fourty-eight hours vs. six hours: -3.5; -0.2, seventy-two hours vs. six hours -4.0; -1.0. There were no significant differences between the twenty-four, fourty-eight and seventy-two hour values of TS in the cryopreserved leaflets (Table 1.1B).

A reduction in strength of the cryopreserved leaflets was demonstrated in the twenty-four and seventy-two hour groups compared to unprocessed leaflets, which did not reach significance in the fourty-eight hour groups (Table 1.2).

The effect of cryopreservation on tensile strength compared to the six hours unprocessed leaflets showed a reduction in strength, which did not reach significance in any of the groups (Table 1.3). However, the lower values are closer to zero than the upper values, suggesting that TS decreased slightly as a result of cryopreservation.

### Young's modulus (YM)

The YM values in the unprocessed leaflets did not differ significantly for all comparisons (Table 1.1A). Also, no difference could be demonstrated in the YM values when the twenty-four, fourty-eight and seventy-two hour cryopreserved leaflets were compared (Table 1.1B).

When comparing unprocessed leaflets to cryopreserved leaflets, a significant reduction of YM values were observed in the seventy-two hour cryopreserved group.

In Table 1.3 the cryopreserved values of YM at twenty-four, fourty-eight and seventy-two hours were similar.

### Thermal denaturation temperature ($T_d$)

Although there were few instances where the $T_d$ values differed significantly at the different time points (Tables 1.1A and B), the physiological and functional value of this result is unclear. As implanted homografts will function at temperatures in the order of 37°C, it can only be inferred that structural integrity will be maintained in all groups at this temperature.

**Table 1.1 The mean and 95% confidence intervals of the differences between six, twenty-four, fourty-eight and seventy-two hours.** The difference in the 95% confidence intervals (CI) that include zero, is statistically not significant ($\alpha > 0.05$). (TS = tensile strength; n = 12 per time point, YM = Young's modulus; n=12 per time point, $T_d$ = thermal denaturation; n=5 per time point).

**Table 1.1A Unprocessed homografts**

| Variable | Time (h) | Mean | 95 % CI | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 24 vs. 6 | 48 vs. 6 | 72 vs. 6 | 48 vs. 24 | 72 vs. 24 | 72 vs. 48 |
| **TS (MPa)** | 6 | 2.63 | 0.9;2.6* | -0.7;2.2 | 0.6;3.0* | -2.4;0.3 | -1.0;1.0 | -1.7;1.2 |
| | 24 | 4.39 | | | | | | |
| | 48 | 3.37 | | | | | | |
| | 72 | 4.45 | | | | | | |
| **YM (MPa)** | 6 | 9.8 | -1.9;8.3 | -7.2;8.3 | -1.0;11.4 | -10.4;5.0 | -4.1;8.1 | -15.2;8.1 |
| | 24 | 13.0 | | | | | | |
| | 48 | 10.3 | | | | | | |
| | 72 | 15.0 | | | | | | |
| **Td (°C)** | 6 | 69 | -2.0;0.4 | -2.8;-0.8* | -2.8;-0.9* | -2.8;0.8 | -2.8;0.7 | -1.4;3.3 |
| | 24 | 69 | | | | | | |
| | 48 | 67 | | | | | | |
| | 72 | 68 | | | | | | |

**Table 1.1B Cryopreserved homografts**

| Variable | Time (h) | Mean | 95 % CI | | |
|---|---|---|---|---|---|
| | | | 48 vs. 24 | 72 vs. 24 | 72 vs. 24 |
| **TS (MPa)** | 6 | | -1.1;1.9 | -1.6;1.1 | -1.3;2.8 |
| | 24 | 2.1 | | | |
| | 48 | 2.5 | | | |
| | 72 | 1.8 | | | |
| **YM (MPa)** | 6 | | -10.5;7.6 | -12.1;4.5 | -9.3;19.7 |
| | 24 | 11.5 | | | |
| | 48 | 10.0 | | | |
| | 72 | 7.7 | | | |
| **Td (°C)** | 6 | 68 | -1.8;0.4 | -1.5;1.7 | -0.7;2.2 |
| | 24 | 70 | | | |
| | 48 | 70 | | | |
| | 72 | 71 | | | |

**Table 1.2 Comparison of unprocessed and cryopreserved leaflet values at different time-points.** Those 95% confidence intervals that do not include zero are statistically significant ($\alpha < 0.05$). (TS = tensile strength; n=12 per time point, YM = Young's modulus; n=12 per time point, $T_d$ = thermal denaturation; n=5 per time point, unp = unprocessed, cryo = cryopreserved, h = hours).

| Variable | Time (h) | UP Mean | Cryo Mean | 95% CI |
|---|---|---|---|---|
| TS (MPa) | 6 | 2.63 | | |
| | 24 | 4.39 | 2.10 | -3.64 ; -0.94* |
| | 48 | 3.37 | 2.46 | -2.41 ; 0.59 |
| | 72 | 4.45 | 1.82 | -3.65 ; -1.60* |
| YM (MPa) | 6 | 9.75 | | |
| | 24 | 12.98 | 11.46 | -10.33 ; 7.28 |
| | 48 | 10.28 | 10.00 | -8.23 ; 7.67 |
| | 72 | 14.97 | 7.65 | -12.59 ; -2.05 |
| $T_d$ (°C) | 6 | 69 | 69 | -1.87 ; 0.65 |
| | 24 | 69 | 70 | 0.22 ; 3.59* |
| | 48 | 68 | 70 | 1.09 ; 3.37* |
| | 72 | 68 | 71 | 1.39 ; 4.65* |

**Table 1.3 Comparison of unprocessed six-hour control values and cryopreserved leaflet values at different time points.** Those 95% confidence intervals that do not include zero are statistically significant ($\alpha < 0.05$). In order to compare the effect of cryopreservation, the six hour unprocessed values were used as the control TS and YM. (TS = tensile strength; n=12 per time point, YM = Young's modulus; n=12 per time point, unp = unprocessed, cryo = cryopreserved, h = hours, ** for comparison with the cryopreserved TS and YM the six hour unprocessed TS an YM were used).

| Variable | Time (h) | Cryopreserved Mean | 95% CI |
|---|---|---|---|
| TS (MPa) | 6 | 2.63** | |
| | 24 | 2.10 | -0.60 ; 1.82 |
| | 48 | 2.46 | -0.76 ; 2.53 |
| | 72 | 1.82 | -0.33 ; 3.24 |
| YM (MPa) | 6 | 9.75** | |
| | 24 | 11.46 | -4.30 ; 13.65 |
| | 48 | 10.00 | -5.82 ;15.19 |
| | 72 | 7.65 | -4.13 ; 22.24 |
| TS (MPa) | 6 | 2.63** | |
| | 24 | 2.10 | -0.60 ; 1.82 |
| | 48 | 2.46 | -0.76 ; 2.53 |
| | 72 | 1.82 | -0.33 ; 3.24 |

## Data Validation

While conducting the strength analyses, it was observed that, in some of the tests, there was slippage during stretching of the leaflets. As a result, it was decided to assess the effect of slippage on TS and YM. Slippage was defined as is illustrated in Figure 1.2. Slippage was observed in sixteen of thirty-six of the cryopreserved leaflets and in twenty of thirty-six of the unprocessed leaflets. The $Chi^2$-test found no significant differences in the frequency of slippage in the cryopreserved ($p = 0.89$) and the unprocessed ($p = 1.00$) leaflets.

The results of the effect of slippage are summarised in Table 1.4. Both TS and YM were significantly higher in the cryopreserved slipped leaflets, suggesting that it was overestimated as a result of slippage. Taken as a whole, it strongly suggests that slippages overestimate the strength of leaflets.

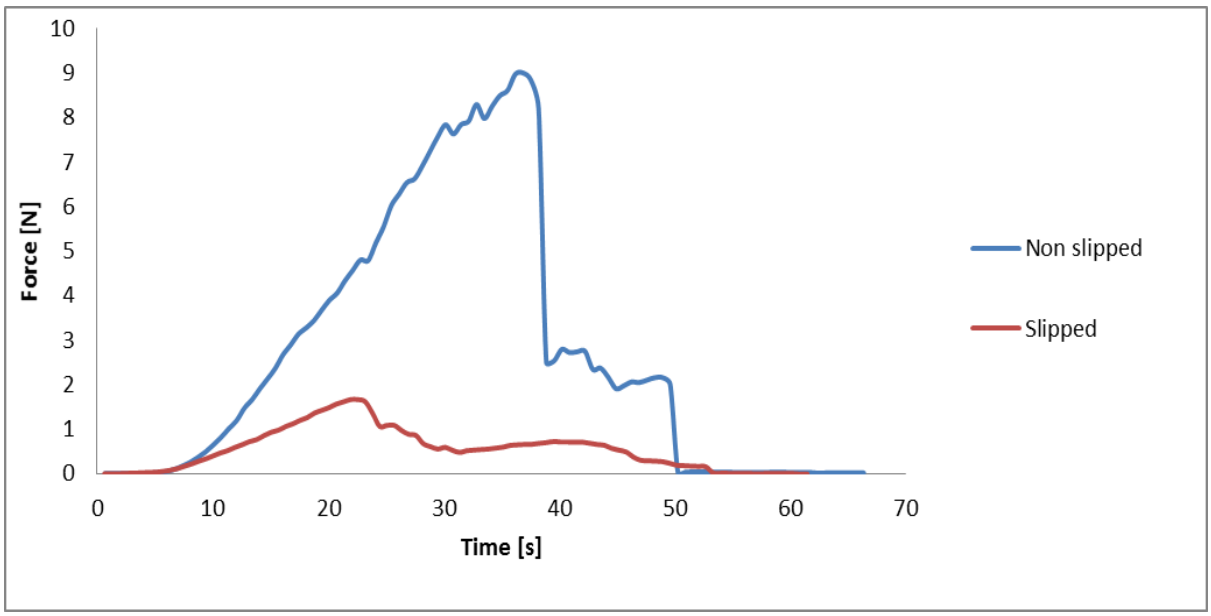


**Figure 1.2** **Slipped versus non slipped material assessing TS.** Slippage was regarded as the force of stretch reaching a maximum but then gradually decreases. Non-slippage was regarded as the force reaching a maximum and then decreasing rapidly.

**Table 1.4 The effect of slippage on the values of tensile strength (TS) and Young's modulus (YM).** Values are given as a mean and the 95% confidence interval (CI) of the difference between non-slip and slip. (*Significant at the 5% level).

| Material | Variable | Slip | Non-Slip | 95% CI |
|---|---|---|---|---|
| **Cryo (n = 48)** | TS (MPa) | 5.44 | 3.46 | 0.82 ; 3.16* |
| | YM (MPa) | 31.00 | 16.33 | 6.13 ; 23.20* |
| **Unprocessed (n = 48)** | TS (MPa) | 4.46 | 4.15 | -0.61 ; 1.23 |
| | YM (MPa) | 20.98 | 13.39 | 1.45 ; 13.72* |

When comparing the TS data of non-slipped cryopreserved leaflets to that of unprocessed leaflets over the different harvest periods, there was a significant reduction in TS in the twenty-four and seventy-two hour groups. No difference could be demonstrated in YM in any of the groups (Table 1.5).

**Table 1.5 Comparison of the effect on cryopreservation on tensile strength (TS) and Young's modulus (YM) on the non-slipped measurements at the different time points.** Values are given as a mean and the 95% confidence interval (CI) of the difference between non-slip and slip. (*Significant at the 5% level, n for YM was the same as for TS).

| Variable | Time (h) | Cryopreserved | Unprocessed | 95% CI |
|---|---|---|---|---|
| **TS (MPa)** | 6 | | 3.32 (n = 4) | |
| | 24 | 1.83 (n = 8) | 3.87 (n = 7) | -3.71 ; -0.28* |
| | 48 | 3.37 (n = 2) | 4.43 (n = 9) | -3.49 ; 1.38 |
| | 72 | 2.45 (n = 6) | 4.84 (n = 8) | -4.02 ; -0.76* |
| **YM (MPa)** | 6 | | 17.84 | |
| | 24 | 7.50 | 11.61 | -10.03 ; 1.82 |
| | 48 | 15.08 | 10.91 | -6.62 ; 14.99 |
| | 72 | 8.93 | 15.54 | -14.26 ; 1.05 |

## Discussion

An increase in the post mortem harvest time that is allowed for homograft donors, can have an attenuating effect on the availability of homografts, still the gold standard for use in right ventricle reconstruction. Although in practice, harvest times are extended in a significant number of units, generally accepted practise dictates harvest times of less than twenty-four hours (Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide, 2013). It must be noted that there is an interchangeable use of post mortem harvest time and receipt times, which can lead to some confusion.

This study focused on the impact of the extension of post mortem harvest times on the strength of pulmonary homograft leaflets in an ovine model. The impact of prolonged harvest time was assessed compared to a fresh less than six hours control sample. The impact of cryopreservation was also compared to the less than six hours control sample. The effect of cryopreservation was assessed at different harvest times and the unprocessed values were compared to the cryopreserved groups. In addition, the study attempted to validate TS and YM data by excluding data deemed to reflect slippages.

Smit *et al.* (2015), in an *in vitro* and *in vivo* ovine study, found that cryopreserved ovine homografts that were harvested after fourty-eight and seventy-two hours, were not different from those harvested after twenty-four hours cold ischaemic times. Cellular reaction, the extracellular matrix, the strength of the tissue and histological characteristics were not negatively affected by cold harvest time of up to seventy-two hours. In that study though, the findings on cryopreserved homografts were not compared to that of control homografts that were not cryopreserved. Neither was the impact of cryopreservation on leaflet strength compared to control samples for each group harvested at twenty-four, fourty-eight and seventy-two hours. However, this study compared the effect of extended cold harvest times on the strength, elasticity and thermal denaturation of cryopreserved tissue with non-cryopreserved homografts, i.e. unprocessed homografts harvested at the same time points.

As far as extending post mortem harvest times, this study demonstrated that TS and YM were not affected by the longer ischaemic harvest times, since both TS and YM after fourty-eight and seventy-two hours were not significantly different from the twenty-four hour values (Table 1.1A). Leaflets therefore did not deteriorate when stored as part of a heart lung block in a refrigerator at 4°C for up to seventy-two hours. Similarly, no deterioration in TS and YM values could be demonstrated between the twenty-four, fourty-eight and seventy-two hours cryopreserved leaflets (Table 1.1B). However, when data were compared between unprocessed and cryopreserved leaflets in the different harvest time groups, there was a reduction in TS in the twenty-four and seventy-two hour groups (Table 1.2), but no difference in the YM values.

In comparing the unprocessed control group with the cryopreserved groups, TS values were reduced, but did not reach significance (Table 1.3). After reanalyses, albeit with smaller groups, a reduction in TS was once again observed in the twenty-four and seventy-two hour cryopreserved groups compared to unprocessed leaflets. YM values were not different between all the groups and the control group.

Thus, the TS and YM data once again supported the concept that prolonging post mortem harvest times up to seventy-two hours was not associated with a significant reduction in tissue strength. On the other hand, cryopreservation has a deleterious effect on TS (Tables 1.1 to 1.3). This was confirmed in the data validation process, albeit with reduced numbers (Tables 1.4 and 1.5). The YM data are not different between any of the groups, which points at the retention of the elasticity of homograft leaflets in all groups.

It should be noted that determining TS and YM on small tissue samples is not unproblematic since, based on previous experience, slippages occur regularly. This may affect the accuracy of calculating TS and YM. However, based on the minimum and maximum values of slipped and non-slipped tests (Table 1.4), it is believed that the results can be combined to draw conclusions, albeit with the necessary caution.

$T_d$ was also not affected by longer ischaemic times. It is not known what the relevance of determining $T_d$ is. The results indicate that thermal denaturation occurs at a temperature of approximately 70°C (Table 1.1) and that none of the groups have values below the control values. The value of this test with regard to tissue integrity is not known. One must also bear in mind that the temperature is much higher than the known temperature at which tissue damage occurs *in vivo* (Yu *et al*., 2010). The results nevertheless show that the leaflets were not easily damaged when subjected to high temperatures *in vitro*. As yet, there is no ready explanation for this.

It is known that cryopreservation damages tissue on the cellular level as is evidenced by electron microscopy, histological analyses and elasticity testing (Mitchell *et al*., 1998; Schenke–Layland *et al*., 2006; Pukacki *et al*., 2000). The results of this study suggest that it may result in some reduction of TS, but not YM. On the other hand, cold storage of unprocessed leaflets does not appear to damage tissue, at least not when compared to the leaflets that were harvested after twenty-four hours. $T_d$ increased significantly as a result of cryopreservation (Table 1.2). It is uncertain if this is of clinical relevance.

It is important to note that slippage resulted in values of TS and YM that were markedly overestimated. Therefore, results of studies in this field must be carefully scrutinised so that these results can be reanalysed or methodological alternatives be explored. Small tissue samples remain a challenge. However, it is important to note that the values for TS and YM were not significantly different when compared between the cryopreserved and unprocessed

leaflets if the calculations included only the non-slipped results. This in effect confirms the conclusion that cryopreservation might affect the strength of the leaflets (Table 1.5).

## Conclusion

In conclusion, the results of this study strongly suggest that the strength of valvular leaflets were not negatively affected by increased post mortem harvest times. However, cryopreservation might result in some reduction in TS. It supports prolonging post mortem harvest times and raise a note of caution about the impact of processing and cryopreservation, in line with previously described morphological effects of cryopreservation.

It also supports the continuing research efforts in reducing cryopreserved damage to tissue and the development of tissue engineering techniques, which would result in the development of non–immunogenic three dimensional tissue scaffolds with extended lifespans.

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# Chapter 5
Article 4

## Cadaver donation: Structural integrity in the juvenile ovine model of pulmonary homografts harvested fourty-eight hours post mortem

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### Abstract
**Introduction:** Cryopreserved pulmonary homograft implantation remains the gold standard for reconstruction of the right ventricular outflow tract, but availability remains a challenge worldwide. Harvesting homografts less than twenty-four hours post mortem has become the norm, thereby reducing the availability of cadaveric donors. However, excellent performance of pulmonary homografts harvested up to seventy-two hours post mortem have been described in the juvenile ovine model. This study examines the structural integrity and stability of pulmonary homografts harvested after a fourty-eight-hour post mortem period, cryopreserved and then implanted.

**Methods:** Fifteen ovine pulmonary heart valve homografts were harvested fourty-eight hours post mortem and cryopreserved. Five pulmonary homografts were cryopreserved but not implanted and served as a control group (group 1; n=5). The right ventricular outflow tracts of ten juvenile sheep were reconstructed with cryopreserved homografts and explanted after fourteen days (group 2; n=5) and 180 days (group 3; n=5). Leaflet integrity was evaluated by morphology and strength analysis. Strength analyses included tests for tensile strength, Young's modulus and termal denaturation temperature ($T_d$), and morphology included haematoxylin

and eosin, Picrosirius red staining, scanning electron microscopy, transmission electron microscopy and Von Kossa stains.

**Results:** All animals had uncomplicated postoperative courses and all homografts functioned well clinically and on echocardiography. Tensile strength and Young's modulus of group 3 were significantly increased ($p<0.05$) compared to that of group 1 and group 2. Thermal denaturation temperature was not significantly different between groups. Haematoxylin and eosin (H&E) demonstrated mostly acellular leaflet tissue in both groups 2 and 3, with an endothelial layer in all explanted homografts, confirmed with scanning electron microscopy. No difference could be demonstrated by Picrosirius red staining between the groups. Transmission electron microscopy demonstrated consistent collagen disruption after cryopreservation in all three groups, with no morphological deterioration or changes during the study period. Von Kossa stains showed mild calcification in group 3.

**Conclusion:** No deterioration of structural integrity could be demonstrated using strength (tensile strength, Young's modulus or thermal denaturation temperature) or morphological evaluations between the controls and implant groups over the study period. Mild calcification was observed in group 3. Expanding homograft harvesting time to fourty-eight hours post mortem is not associated with early graft failure.

**Keywords:** Juvenile ovine model–Homografts–Ischaemic time–Right ventricular outflow tract–Structural integrity

## Introduction

Cryopreserved pulmonary homografts remain the gold standard for reconstruction of the right ventricular outflow tract (RVOT) (Hechadi *et al*., 2013). However, availability has been the Achilles heel of homograft-based surgery, because of donor shortages (Yoshikawa *et al*., 2000). In 1987, O'Brien *et al*. (1987a) described the use of viable cryopreserved homografts, which introduced the era of homograft banking. The possibility of delaying homograft degeneration by using viable cryopreserved homografts or homovital homografts (Yacoub *et al*., 1995) became a well-established concept and homograft banks across the world started using cryopreserved homografts or homovital homografts. Viable cryopreserved homografts are harvested from beating heart donors less than six hours and definitely less than twenty-four hours post mortem in the case of cadaveric donors (O'Brien *et al*., 1987a). Homovital homografts are untreated homografts harvested under sterile conditions, usually from the recipient at the time of the heart transplantation, and kept in nutrient medium. These homografts are considered viable if implanted within a few days (Yacoub *et al*., 1995). The post mortem harvesting time of homografts is restricted to a maximum of twenty-four hours by most tissue banks internationally (Dawson and Brockbank, 1997). Strict adherence to this criterion reduces the

potential homograft pool, as the so-called donor consent window of opportunity is restricted, thereby reducing the potential cadaver donor pool to mainly in-hospital deaths. Potential cadaveric donors who never reach the hospital, for example suicide or traffic deaths, are not recruited either, due to the absence of specific cadaver donor programmes. In South Africa, nearly 70 000 medico-legal autopsies are performed annually. Reactivating cadaver donor programmes has the potential to address general shortages; however, this might require extending the post mortem harvesting time to beyond twenty-four hours. At the Bloemfontein homograft bank, which is largely dependent on cadaver donors, the mean post mortem ischaemic time is thirty-three hours, and it has extended its harvesting times to fourty-eight hours with sound results (Botes *et al*., 2012). Despite detailed studies of homograft viability, endothelial changes (Angell *et al*., 1989; O'Brien *et al*., 1995; Yankah and Hetzer, 1987) and post mortem cellular changes, the importance of preserved structure and function is still uncertain.

Historically, homografts were harvested from cadavers, and harvesting times varied widely (Botes *et al*., 2012; O'Brien *et al*., 1987a). Homografts were stored at 4°C in an antibiotic solution for up to ninety days (Yacoub and Kittle, 1970). Despite O'Brien's claims to the contrary in a medium-term follow-up series (O'Brien *et al*., 1995; O'Brien *et al*., 2001), no difference could be demonstrated between fresh antibiotic homografts, group 1, and viable cryopreserved homografts, groups 2 and 3, in a long-term follow-up series (O'Brien *et al*., 1987b; O'Brien *et al*., 2001). Several other centres have also reported good medium-term results with fresh antibiotic-preserved homografts (Yacoub *et al*., 1995; Langley *et al*., 1996).

It is known that humoral and cellular immunological responses to viable endothelium are pronounced (Methe *et al*., 2007) and that homovital homografts might be rejected (Green *et al*., 1998). Mitchell *et al*. (1998) believe that most, if not all, homografts, are eventually essentially dead and acellular and only survive as a scaffold.

The period between harvesting and cryopreservation comprises a culture and a sterilisation phase, which range in length between tissue banks (between twenty-four and seventy-five hours) (Langley *et al*., 1986; Barrett-Boyes, 1987). Cryopreservation within three to four days is accepted for viable cryopreserved homografts (O'Brien *et al*., 1987a; O'Brien *et al*., 1987b). This means that the actual accepted post mortem ischaemic time before cryopreservation is routinely somewhere between fourty-eight and ninety-six hours. Insisting on a post mortem harvesting time of less than twenty-four hours is therefore questionable from a structural point of view. However, extending homograft harvesting times can increase bacterial exposure, escalating the bio-burden and therefore contributing to graft failure and calcification (Brubaker

*et al*., 2016; Mroz *et al*., 2008). However, microbiological examinations yielded similar results in the twenty-four and fourty-eight hour post mortem groups harvested from intact sheep carcasses as described by Smit *et al*. (2015). Furthermore, no fungi were cultured and all samples were free of organisms after twenty-four hours incubation in an antibiotic solution. The Bloemfontein homograft bank has previously indicated that extending the post mortem harvesting time up to seventy-two hours had no deleterious effect on homograft structure and functioning in the juvenile ovine model (Smit *et al*., 2015).

In this study, cryopreserved homografts that had been harvested fourty-eight hours post mortem were implanted in juvenile ovine models for either 14 or 180 days and, after explantation, compared to non-implanted cryopreserved homografts. The aim of this experimental study was to evaluate the structural stability and integrity of the homografts and to assess modes of failure, if applicable.

## Materials and method

All experiments were performed in accordance with the *Principles of Laboratory Animal Care* prepared by the National Society of Medical Research, and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the US National Institute of Health 1996 (http://www.nap.edu/catalog/5140.html.). Approval of the study protocol was obtained from the Animal Ethics Committee of the University of the Free State (ETOVS Number: UFS–AED2016/0101).

Pulmonary homografts were harvested fourty-eight hours post mortem from fifteen male juvenile sheep (mean age four to six months, mean body weight thirty- four to fourty kilograms) and cryopreserved (Figure 1.1). Five of these homografts acted as the control (n=5) and were not implanted into recipient sheep. The ten remaining pulmonary homografts were implanted into recipient sheep and explanted after 14 days (n=5) and 180 days (n=5) in order to evaluate the structural integrity and stability. Leaflet integrity was evaluated by morphology and strength analysis. Strength analyses included tensile strength (TS), Young's modulus (YM) and thermal denaturation temperature ($T_d$), while morphology included H&E, Picrosirius red staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and Von Kossa stains. Wethers of the Dorper strain were used as recipient animals, and each recipient received an ear tag with a unique identification number.

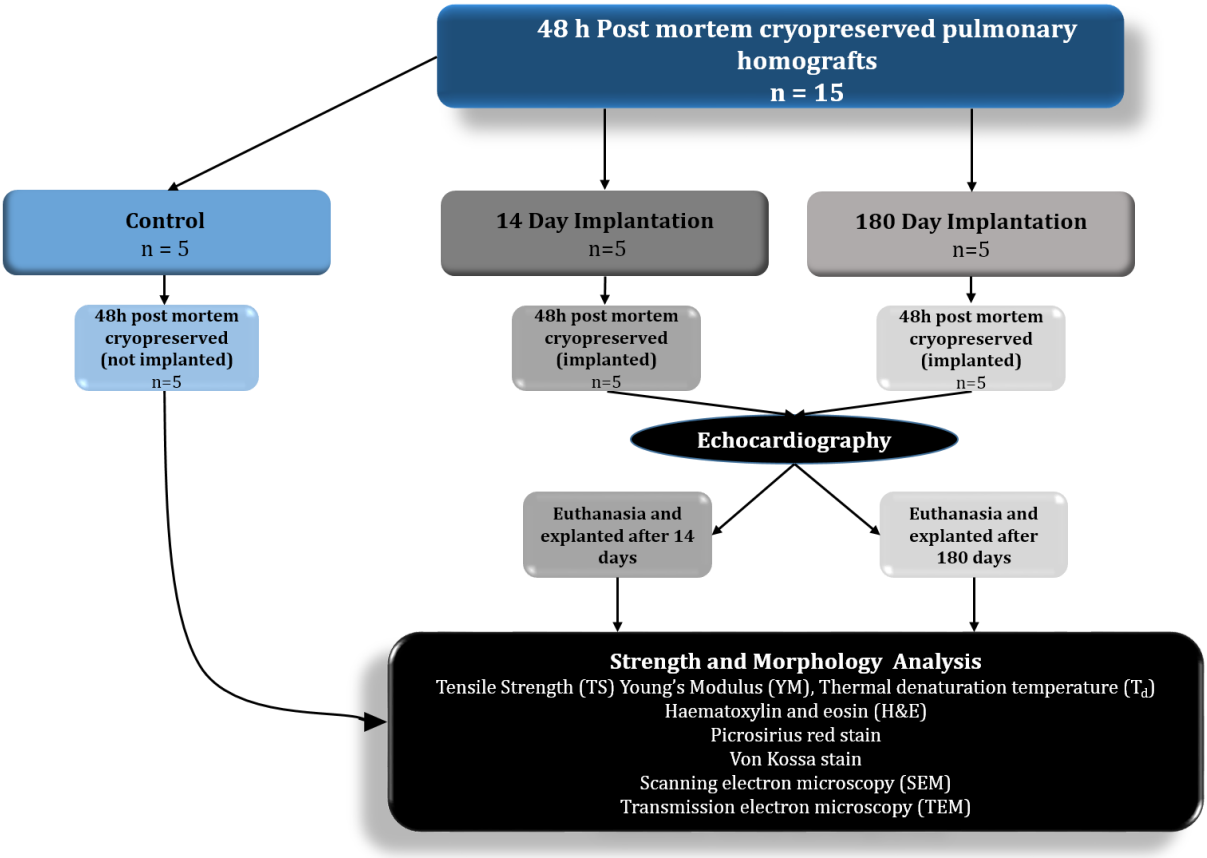


**Figure 1.1 Study layout.**

## Pulmonary homograft harvesting and preparation

Homografts were dissected from fifteen Merino ewe heart blocks obtained from the abattoir, after storage in a sealed container for fourty-eight hours. For the first eight hours post mortem containers were kept at room temperature and thereafter at 4°C. The pulmonary homografts were immersed in an antibiotic solution containing 100 ml of M199 with Earle's Base (Highveld Biological (Pty) Ltd., Johannesburg, South Africa), 2.5 mg Fungizone (Bristol-Meyers Squibb, Johannesburg, South Africa), 25 mg Amikacin (Fresenius, Bodene (Pty) Limited trading as Intramed, Johannesburg, South Africa), 100 mg Vancomycin (Gulf Drug Company (Pty) Ltd, Mount Edgecombe, South Africa) and 50 mg Piperacillin (Sabax, Johannesburg, South Africa) for sterilisation. The homografts were incubated overnight at 4°C and cryopreserved (Smit *et al.*, 2015) after tissue samples had been obtained for microscopy, culture and sensitivity.

Cryopreservation was performed using an automated programmable freezer (Cryoson BV-9 Biological Freezer, Consarctic, Schollkrippen, Germany). Before cryopreservation 11 ml of dimethyl sulphoxide (Sigma-Aldrich, Midrand, South Africa) was added to 100 ml of M199. Homografts were stored in a double package sachet in a storage vessel in the vapor phase of

liquid nitrogen between -120°C and -160°C until they were used. Five of these pulmonary homografts were used as controls and the remaining ten homografts were implanted into male juvenile sheep.

Before implantation, homografts were thawed in a water bath at 35°C -40°C for five to seven minutes until they were about 80% thawed. Both packages were opened aseptically and the homograft was transferred into a bowl with 500 ml cold (4°C) M199, allowing a ten minute rinse period with gentle shaking to extract most of the dimethyl sulphoxide from the homograft tissue. This procedure was repeated in a second bowl of cold M199, and thereafter the homograft was delivered to the surgical team for final trimming and implantation.

### Pulmonary homograft implantation and explantation

Following, ten recipient sheep were premedicated with 0.175 mg/kg Neurotranq (VirbacRSA (Pty) Ltd, Halfway House, South Africa) and 0.2 mg/kg Atropine (Bayer (Pty) Ltd, Animal Health Division, Isando, South Africa) intramuscularly, and anaesthesia was induced intravenously with Bomathal (12 mg/kg IV, Merial SA (Pty) Ltd, Halfway House, Johannesburg, South Africa). After positioning the sheep in a lateral decubitus position they were intubated and ventilated. Cardiopulmonary bypass was obtained by arterial cannulation via the left carotid artery and venous cannulation via the right atrium (Dagum *et al*., 1999). The stump pressure of the tied-off distal carotid artery was used as an index of invasive arterial pressure. A central line was inserted into the left jugular vein and the surgical procedure was performed on a beating heart.

A mini-thoracotomy was performed on the left side of the sheep and the fourth rib was removed. The pulmonary artery was transected, the native pulmonary valve leaflets resected and the pulmonary homograft was implanted as an RVOT conduit with two continuous 4/0 polypropylene suture anastomoses.

After implantation, the recipient was weaned off cardiopulmonary bypass, the mini-thoracotomy closed in layers and a chest drain was inserted. Systemic pain medication (2 mg Morphine sulphate, Bodene (Pty) Ltd, trading as Intramed, Port Elizabeth, South Africa) was administered intramuscularly twice a day and 5 mg Depomycin (Intervet SA (Pty) Ltd, Johannesburg, South Africa) as antibiotic was administered daily for five days post-operatively. Animals were extubated between two and four hours post-operatively. Chest drains were removed before the animals were transferred to an overnight facility with a companion sheep to alleviate stress on the recipient sheep.

## Biomechanical testing

The biomechanical properties of the control homograft leaflets and the ten explanted valvular leaflets were examined using a TS testing apparatus (Lloyds LS100 Plus, IMP, Johannesburg, South Africa). Briefly, the pulmonary leaflets were fixed into clamps at both ends and gradually stretched (0.1 mm/s) by applying tension on both ends (Thubrikar *et al*., 1983). TS and YM were calculated from the stress-stain curves and automatically recorded on the computerised apparatus. The YM was calculated by taking the derivative of the stress-strain curve, where the largest value was chosen before breakage. The TS, measured in Pa, is calculated as the force divided by the cross-sectional area of the leaflet.

In thermal denaturation temperature ($T_d$), the rate of heat flow to the sample is compared to the rate of heat flow to an inert material, while the materials are heated or cooled concurrently. For proteins, the thermally induced process detectable by $T_d$ is the structural malting 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 $T_d$ (Smith and Judge, 1991). This was recorded as $T_d$ for each group (Rüegg *et al*., 1975). $T_d$ analysis was performed using $T_d$ (Mettler Toledo, $T_d$ 822e, Microsep, Johannesburg, South Africa). Small samples of the pulmonary leaflets (2-5 mg) were placed in the $T_d$'s hermetically sealed pans and subjected to temperature increasing at a rate of 10°C/min from 25°C to 95°C. The maximum temperature of protein $T_d$ was electronically recorded for each sample.

## Histology

Samples from the middle of each pulmonary homograft leaflet was processed by the Department of Anatomical Pathology of the National Health Laboratory Services in Bloemfontein using standard operative procedures. The specimens were embedded in paraffin wax (Siemens, Johannesburg, South Africa) and two micrometer-thick longitudinal sections were prepared and routinely processed for H&E, Picrosirius red and von Kossa staining. The impact of ischaemic time on the structural integrity of pulmonary valvular leaflet tissue was evaluated by performing H&E staining to display cytoplasmic, nuclear, and extracellular matrix features, Picrosirius red staining to study the collagen networks and von Kossa staining to observe the degree of leaflet calcification.

## Electron microscopy

### Scanning electron microscopy

Each pulmonary homograft tissue sample for SEM was prepared by the Centre for Microscopy at the University of the Free State. All samples were fixed in 2.5% glutaraldehyde (Merck, Johannesburg, South Africa). Homograft leaflets were divided into two specimens of approximately 3 mmx6 mm. Tissue specimens were dried using the critical point method (Tousimis critical point dryer, Rockville, Maryland, USA, ethanol dehydration, carbon dioxide drying gas) and were metallised using gold (BIO-RAD, Microscience Division Coating System, London, UK; Au/Ar sputter coating @ 50-60 nm). Evaluations were performed with a Shimadzu SSX 550 scanning electron microscope (Kyoto, Japan, with integral imaging (SDF, TIF and JPG format)). The surface area of each specimen was examined and photographed in either four or five different positions, and all images were then evaluated by three independent assessors and a score allocated. SEM micrographs were used to assess endothelial integrity and the effect of cryopreservation on endothelium integrity, and to evaluate the quality of the extracellular basal membrane.

### Transmission electron microscopy

Pulmonary leaflet samples were fixed in 3.0% glutaraldehyde overnight, post fixated in Palade's osmium tetroxide, and dehydrated in a graded acetone series. Dehydrated samples were impregnated/embedded in an epoxy (Spurr, 1969) to facilitate the making of ultra-thin sections for the TEM evaluation. Ultra-thin sections were cut from the sample embedded in the epoxy using an ultra-microtome (Leica Ultracut UC7, Vienna, Austria). After sectioning the samples they were stained with uranyl acetate and lead citrate. Sections of the leaflet samples were evaluated by using a transmission electron microscope (CM100, FEI, Netherlands) and photographed using an Olympus Soft Imaging System Megaview III digital camera with Soft Imaging System digital image analysis and documentation software (Olympus, Tokyo, Japan).

## Echocardiography

The ten implanted pulmonary homografts were explanted after either 14 days (n=5) or 180 days (n=5). Before the animal was sacrificed a transthoracic echocardiograph was done using a Philips Envisor Ultra Sound system (Philips, Johannesburg, South Africa) with a 3.5 MHz probe to ensure patency of the implanted homografts. Pulmonary insufficiency was evaluated semiquantitatively with pulsed wave, continuous wave and colour Doppler flow on the parasternal short-axis view. Each measurement was repeated six times and mean values were calculated. The regurgitation jet across the homograft was graded by identification length and width into the RVOT and mapped as: none/trivial, mild, moderate or severe, using standard

echocardiography criteria. The mean flow velocities across the implanted homografts were obtained by the use of continuous wave Doppler. Each measurement was repeated six times and the mean value over the measurements was calculated. Animals were only identified by their individual ear tag numbers, allowing blinded evaluation by the sonographer.

### Gross examination

One surgeon and five researchers inspected the explanted homografts visually in a blinded manner. The general appearance of the homograft leaflets was evaluated for fenestrations, retraction, thrombotic material and atheroma or calcification.

### Statistical analysis

The unpaired t-test was used to compare the TS, YM and $T_d$ explant results after 14 days and 180 days to that of the control. The null hypothesis assumes that the population means of the two independent samples are equal. The mean difference (positive or negative) was calculated, as well as a 95% confidence interval for the mean difference (indicating the limits within which the true difference is likely to occur).

## Results

All homografts were proved to be culture negative before cryopreservation.

### Echocardiography

All the homografts functioned normally and no more than mild regurgitation was recorded in any of the homografts. None of the homografts had more than minimal regurgitation or demonstrated a maximum instantaneous gradient of more than 20 mmHg. No leaflet calcification could be demonstrated (Figure 1.2).

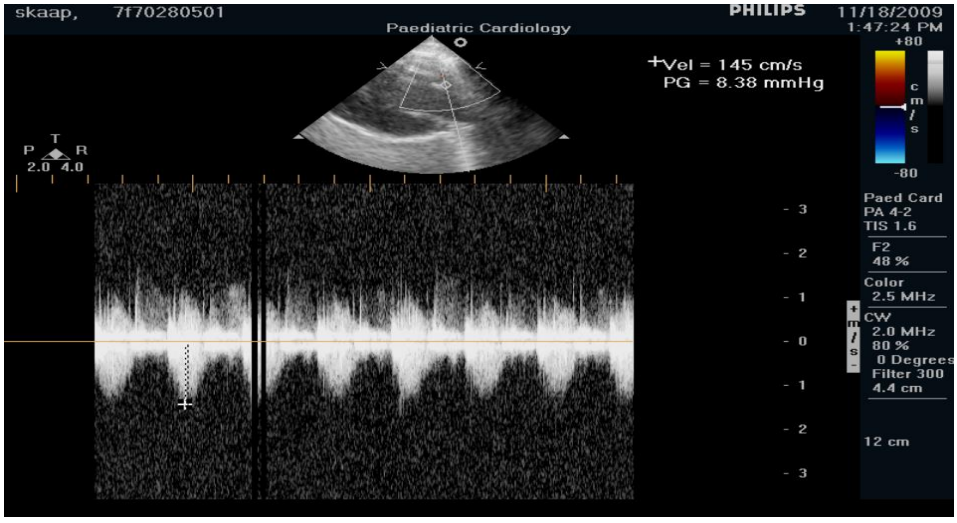


**Figure 1.2** **Transvalvular gradient of a 180 day homograft** (adapted from Smit, Ph.D., unpublished data, 2011).

## Gross examination

Blinded visual inspection did not discern differences in the macroscopic appearance of homografts explanted after 14 days and 180 days (Figure 1.3).

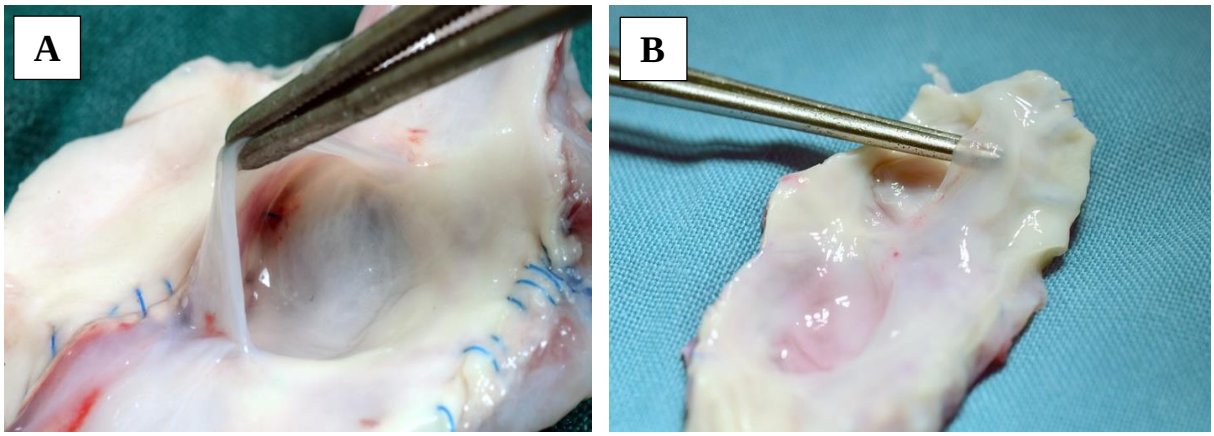


**Figure 1.3** **A) Macroscopic appearance of 14 day explanted homograft leaflet B) Macroscopic appearance of 180 day explanted homograft leaflet.**

## Biomechanical testing

The strength analysis results are summarised in Table 1.1. There were no significant differences in TS, YM and $T_d$ between the control and fourteen-day explanted leaflets. Although TS of the control and the 180 day explanted leaflets did not differ significantly, the trend was towards stronger explanted leaflets, because the value of the lower limit of the 95% confidence interval was much closer to zero than the upper limit. YM of the 180 day explanted leaflets was significantly higher than that of the control leaflets. $T_d$ of control and 180 day explants did not differ significantly. No deterioration of strength could be demonstrated over the study period.

**Table 1.1** **TS, YM and $T_d$ of 48 hour cryopreserved ovine leaflets before implantation (control, n=5) and explanted leaflets after 14 days (n=5) and 180 days (n=5)**

| Variable | Mean control | Mean 14 day explant | 95% confidence interval Control vs. 14 day explant | Mean 180 day explant | 95% confidence interval Control vs. 180 day explant |
|---|---|---|---|---|---|
| **TS** | 2.461 | 2.788 | -1.273 ; 1.927 | 4.801 | -0.303 ; 4.983 |
| **YM** | 10.001 | 11.057 | -7.695 ; 9.770 | 25.388 | 1.819 ; 28.954* |
| **$T_d$** | 70 | 72 | -0.765 ; 4.265 | 70 | -3.785 ; 4.005 |

Values are given as a mean and the 95% confidence interval of the difference between the control and 14 and 180 days after implantation (*p<0.05). [TS=tensile strength; YM=Young's modulus; $T_d$=thermal denaturation temperature].

## Histology

Figure 1.4 represents the H&E, Picrosirius red and Von Kossa staining of the 48 h control, 14 days and 180 days explants.

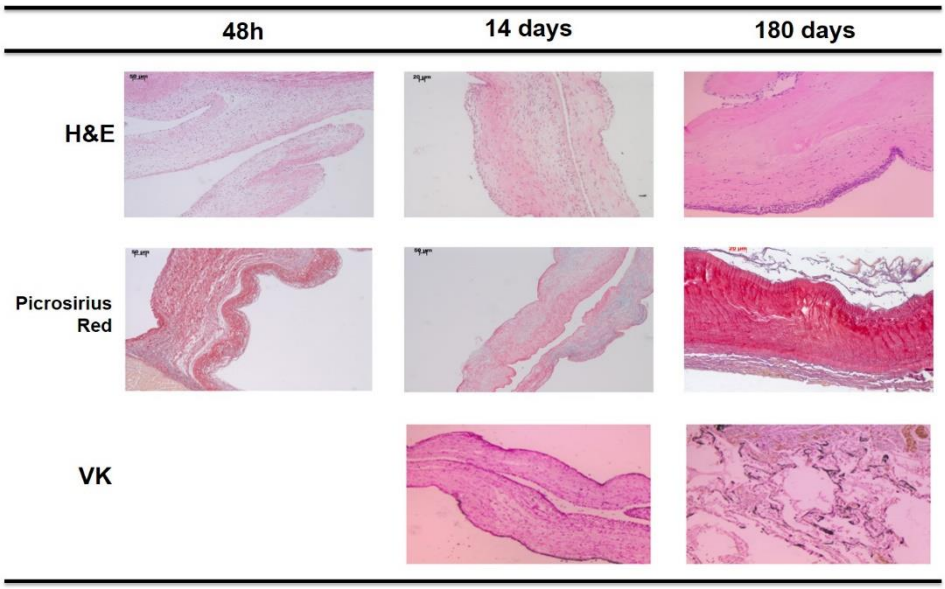


**Figure 1.4** **H&E, Picrosirius red and von Kossa staining of 48 hours cryopreserved ovine leaflets before implantation (control, n=5), and explanted leaflets after 14 days (n=5) and 180 days (n=5)** [h=hours; H&E=haematoxylin and eosin; VK=von Kossa].

The H&E stains show that endothelium covered the leaflets in a monolayer in the control, the 14 days and the 180 days explanted leaflets. Picrosirius red stains for collagen did not differ between the three groups and the Von Kossa stains showed no calcification of the 14 days explanted leaflets and only mild calcification of the 180 days explanted leaflets (Figure 1.4).

## Electron microscopy

SEM confirmed an endothelial layer that was visible in the control, the 14 days and the 180 days explants. SEM after cryopreservation and at 14 days shows endothelial cells with prominent nuclei, collapsed extra-nuclear areas and areas of dehiscence from the basal membrane. In contrast, the 180 days samples show a confluent layer of healthy endothelium (Figure 1.5). TEM demonstrates consistent collagen disruption after cryopreservation in the control group as well as the 14 days and 180 days explants with no morphological deterioration or changes during the study period (Figure 1.5).

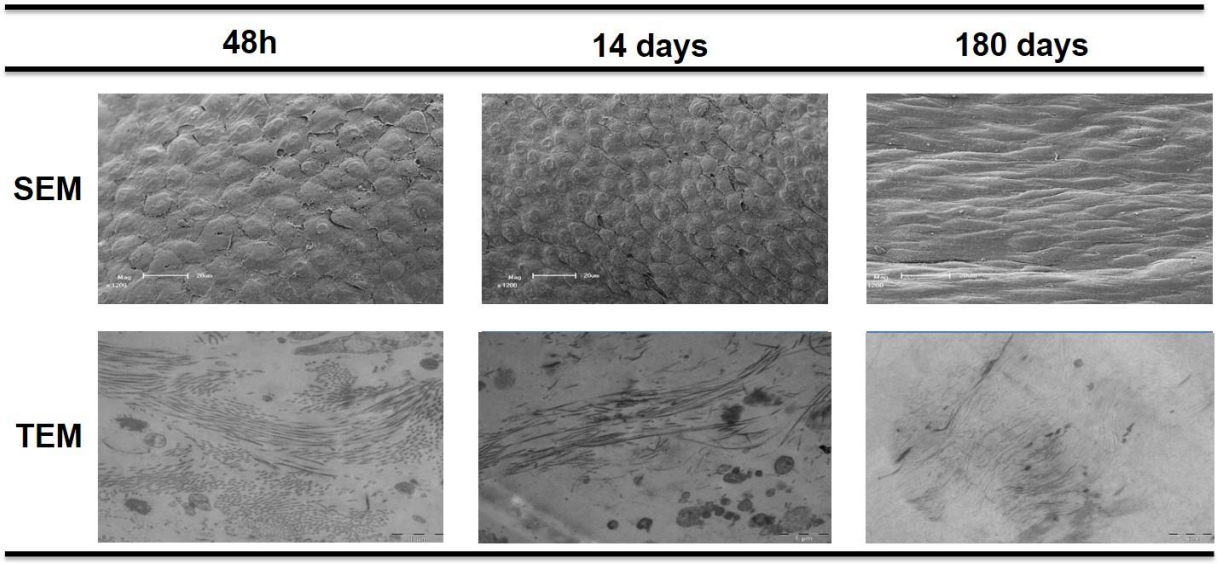


**Figure 1.5** **SEM and TEM of 48 hours cryopreserved ovine leaflets before implantation (control, n=5) and explanted leaflets after 14 days (n=5) and 180 days (n=5)** [SEM=scanning electron microscopy; TEM=transmission electron microscopy; h=hours].

## Discussion

In an attempt to broaden the scientific basis for extending cadaveric homograft harvesting times, which could attenuate the worldwide shortage of homografts, this study was conducted in the juvenile sheep model. The study focuses on the structural integrity and stability of fourty-eight hours post mortem cryopreserved homografts, a time specifically chosen to mimic a reasonable window of opportunity in order to obtain donor consent in human cadaveric donor programmes. The ovine model is a widely accepted model for homograft degeneration studies and is well known for its aggressive calcification potential.

The fourty-eight hour post mortem harvested cryopreserved pulmonary homografts performed well over the study period, maintained its tissue integrity and stability and exhibited no graft failures.

All the homografts functioned well, as assessed by echocardiography before euthanasia, after a minimum post-operative follow-up period of 180 days. None of the homografts showed gradients in excess of 20 mmHg or more than mild pulmonary regurgitation (>2/4). No calcification could be demonstrated in the leaflets in either the 14 day or 180 day groups, and only mild calcification occurred in the homograft wall, but only in the 180 days group.

Gross macroscopic inspection after euthanasia confirmed the absence of homograft degeneration in both groups. Mild, spotty calcification in the homograft walls occurred in two of the 180 days explant valves.

The increase in TS and YM seen in the 180 days explant group compared to both the control and the 14 days explant, we speculate to be related to pannus or neo–collagen. It is important to note that no deterioration of strength occurred during the study period. It was therefore concluded that strength is maintained in the fourty-eight hours post mortem cryopreserved homografts during the study period. $T_d$ values remained constant between the groups and no deterioration could be demonstrated in the fourty-eight hours post mortem cryopreserved homografts over the 180 days implantation period.

Histology showed progressive loss of cellularity over the study period, with 180 days explanted homograft leaflets being essentially acellular, as previously described by Mitchell *et al*. (1998). The fourteen days explanted homografts showed mononuclear infiltrates, composed primarily of T-lymphocytes, that were diffuse but not prominent in most valves and which were generally comparable with those seen in the control group. The endothelial cells appeared focally and variably demonstrable in the control group, tattered in the 14 days explant group but "healthy" in the 180 days explants (group 3), because these cells are probably of recipient origin. The Picosirius red staining confirmed the presence of collagen in all groups and the von Kossa staining showed mild calcification in the 180 days explant group.

SEM studies highlighted the abnormal endothelium after cryopreservation maintained in the 14 day group; however, a pristine endothelial layer was present in the 180 day explants. TEM clearly demonstrated damage to the collagen scaffold caused by cryopreservation, as previously described by Schenke-Layland *et al*. (2006). This disruption of the collagen matrix was sustained throughout the study period and was similar between all groups.

This study demonstrated severe disruption caused by cryopreservation and this damage remained constant during the study period. The fact that no reorganisation of the collagen scaffold occurred correlates with the progressive acellularity observed in the implanted homograft leaflets over the 180 days study period.

It is certainly interesting to note that the leaflet strength did not deteriorate over this period, despite confirmed cryopreservation damage to the collagen scaffold as well as progressive

acellularity of the leaflets. This acellularity leaves very little hope that damage will be repaired by cells in the leaflet.

The pristine endothelial lining of the 180 day explants is most likely of recipient origin and may perform an important antithrombotic function as well as other anti-inflammatory endothelial functions, which can attenuate thrombotic and inflammatory processes initiated by deterioration and calcification. It is unlikely to contribute to valvular maintenance functions in the absence of valvular interstitial cells.

The damaged, albeit stable, state of the post cryopreservation collagen scaffold, combined with the lack of leaflet cellularity and absence of indications of collagen scaffold repair or new valvular interstitial cells by 180 days, begs the question: How do these cryopreserved homografts retain their strength, as they clearly do in this study, as well as in thousands of homograft recipients over many years?

## Conclusion

Post mortem harvesting time might be less important in homograft survival than attenuating damage to the collagen scaffold and providing a scaffold where recipient cells can infiltrate, proliferate and function, thereby maintaining a normal leaflet structure. This remains the goal of tissue-engineered heart valves and valvular conduits.

As cryopreserved homografts still form the backbone of clinical homograft application, prolonging homograft harvesting times to fourty-eight hours post mortem by this study was not associated with early graft failure or loss of tissue integrity. Prolonging harvesting time to fourty-eight hours post mortem can attenuate homograft donor shortages.

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# Chapter 6
## General discussion and conclusion

### General discussion

The initial homograft bank experience in Bloemfontein commenced with the cadaver harvesting of aorta homografts in 1984. Initially, these homografts were harvested from cadavers, antibiotic sterilised and stored at 4°C. In 1992 cryopreservation was introduced and pulmonary homografts were harvested from each donated heart as well. Today, the Bloemfontein homograft bank is the only homograft bank in South Africa and, to its knowledge, the only homograft bank in sub-Saharan Africa. The bank is the sole supplier of homografts to South African cardiac surgery units.

The world-wide practice of ever fresher post mortem and beating heart harvest policies, the homo-vital concepts favoured by Yacoub *et al.* (1995) and the published series discussed (*vide supra*) demonstrating enhanced homograft survival and increased freedom of re-operation by reducing post mortem harvest times to a maximum of twenty-four hours, mandated the scientific evaluation of the cadaver donor based Bloemfontein Homograft programme with harvest times exceeding a mean of twenty-four hours.

The less than six hours and less than twenty-four hours policy adopted by most of the leading homograft banks world-wide lead to the demise of most cadaver banks. However, in Bloemfontein's opinion, this also directly contributed to the already international donor shortage. As homograft valved conduits remain the gold standard for the management of congenital lesions requiring reconstruction of the RVOT, this specific entity was chosen for clinical and experimental evaluation.

The Bloemfontein homograft experience was summarised by Botes *et al.* (2012) and discuss aspects of harvesting, processing, evaluation, storage and distribution. In an unpublished clinical evaluation of pulmonary homografts from the Bloemfontein homograft bank implanted in the RVOT of children younger than fourteen years old (n=253), in which the medium term outcomes of pulmonary homografts harvested less than twenty-four hours post mortem

(n=107) were compared to those harvested after twenty-four hours (n=107), no difference could be observed in homograft degeneration and required re-operations.

The complexity of homograft harvesting and the impact of post mortem harvest times, homograft viability, cryopreservation and processing on homograft survical and degeneration remain an area worthy of ongoing research and was highlighted in chapter one of this manuscript. It is also important to note the harvest time to processing times of homografts reflecting actual homograft banks as listed in the Directory of European Cardiovascular Tissue Banks and Tissue Bank Addresses World Wide (2013). In this report, 57% of homografts were obtained from organ donors, 15% from domino hearts and only 28% from non-organ donors.

In reality, hours from death to excision ranged between two hours and fifty hours in the same report and in an analysis of twenty-three banks' practices, twelve accepted cardiectomy or receipt by the bank for up to twenty-four hours post mortem, while nine accepted tissue harvested or receipt at the bank longer than twenty-four hours post mortem, mostly up to fourty-eight hours (six out of nine banks).

The deleterious effects of cryopreservation and the lack of continuing survival of viable and metabolically active cells in implanted homografts were noted. In the essentially acellular explanted homograft leaflet studies, the impact of immunological processes are questionable and degeneration might be linked to other factors linked to non-viability and lack of leaflet maintenance and regeneration. The implication for the development of decellularisation processes based on the concept of reduced immunological degeneration is important if no significant re-celluarisation takes place. The impact of processing on the biological scaffold is therefore highlighted.

Thus, instead of concentrating on homograft viability, it is necessary to evaluate two of the most important unavoidable factors in the preservation of homografts in homograft banks, being the impact of post mortem harvesting times and processing as well as cryopreservation itself on the tissue integrity of homografts. This should be examined in both the *in vitro* and *in vivo* settings.

A series of experimental studies were thus embarked upon to firstly validate the post mortem harvest time policy and practice of the Bloemfontein Homograft Bank, but also by implication all cadaver donor based programmes. Secondly, the impact of cryopreservation on tissue integrity was studied at various harvest times.

In this thesis, the outcomes of some of these studies on pulmonary homografts in the ovine model were combined in four articles (one published and three ready for submission for publication).

**Findings of the studies are briefly summarised below:**

**1.)** In studying the impact of extended harvest times on homograft performance in the ovine model, homografts were harvested at different post mortem times and implanted for a minimum of 150 days.

The *in vitro* and *in vivo* studies failed to demonstrate any change in tensile strength, Young's Modulus and thermal denaturation ($T_d$) results between the groups. SEM demonstrated a reduction in endothelial cells (50% at 24 hours, 60.9% at 48 hours and 40.9% at 72 hours), but H&E could not demonstrate autolysis in any CPA *in vitro*. It is important to note that all cultures were negative before implantation. In an attempt to show a difference in the evoked immunological response to the different groups, based on theoretical differences in endothelial cell viability and therefore immune reactivity, IgE, IgM and IgG results were analysed but were inconclusive. All valves at gross examination showed no abnormalities and echocardiography demonstrated normal valve function in all groups. The histological examination by H&E and Picrosirius red staining, confirmed preserved tissue integrity. In all explanted homografts, vWF, CD31 and CD34 staining confirmed a monolayer of endothelial cells. Minimal calcification occurred and the calcium content of explanted CPA leaflets was similar. It was concluded that this experimental study supports the concept of prolonging the cold ischaemic harvesting time of cryopreserved homografts to reduce homograft shortage.

**2.)** In order to evaluate the morphological impact of prolonged ischaemic harvest times and cryopreservation on pulmonary homografts, an *in vitro* was conducted. In the unprocessed pulmonary homografts no morphological changes could be demonstrated when using Haematoxylin and Eosin (H&E), Picrosirius red staining and scanning electron microscopy (SEM) up to seventy-two hours post mortem harvest times. However, at seventy-two hours post mortem TEM revealed cellular swelling and oedema with retention of the collagen structure and organisation.

The cryopreserved pulmonary homografts did not show any morphological differences between the groups harvested at six hours, twenty-four hours, fourty-eight hours and seventy-two hours post mortem. The cryopreserved homografts showed changes in the

collagen, which apeared compacted and flattened on the Picrosirius red stain. TEM demonstrated cellular swelling, oedema, disrupted and damaged collagen structures in all samples. Thus, the ischaemic post mortem period up to seventy-two hours had very little demonstrable effect on tissue morphology in the unprocessed groups, except for a more oedematous appearance on TEM in the seventy-two hour group and as a whole, leaflet tissue integrity was well maintained during the ischaemic period. Cell death and disruption of the collagen scaffold could be observed in all cryopreserved groups (after thawing), irrespective of post mortem harvest times. The effects of cryopreservation were thus immediate, disruptive and lethal. It was concluded that post mortem harvest times can safely be extended to at least fourty-eight hours, prompting a re-evaluation of the implementation of cadaver programmes. Strategies, including decellularisation, should be further explored to reduce or to avoid the impact of cryopreservation on tissue scaffolds during processing in tissue banking.

**3.)** To assess the impact of extended post mortem harvest times on tissue strenght, the impact of cryopreservation *per se*, as well as the impact of extended harvest times versus cryopreservation was studied *in vitro*. The TS did not differ significantly between the twenty-four, fourty-eight and seventy-two hour values when compared in both the unprocessed and cryopreserved leaflets groups. Cryopreservation tended to decrease, though not significantly, the TS in the cryopreserved leaflets when compared to the six hours unprocessed leaflet. A reduction in strength of the cryopreserved leaflets was demonstrated in the twenty-four and seventy-two hour groups, compared to unprocessed leaflets, which did not reach significance in the fourty-eight hour groups. Most significantly, the results strongly indicate that the strength of valve leaflets was not negatively affected by increased post mortem harvest times. There is a slight possibility that cryopreservation may cause some decrease in the strength. Nevertheless, the results strongly suggest that post mortem harvest times may be extended to fourty-eight hours and perhaps beyond.

**4.)** An assessment of the tissue stability of implanted cryopreserved pulmonary homografts, harvested at fourty-eight hours post mortem, was conducted. This study allows short term and long–term immunological process impact and bio-modification over a period of time, in which homograft explants were assessed after periods of 14 days and 180 days *in vivo***.** All animals had an uncomplicated postoperative course and all homografts functioned well clinically and on echocardiography. TS and YM of the 180–day period were significantly increased ($p<0.05$) when compared to the control and 14 day explants. $T_d$ was not significantly different between the groups. H&E demonstrated

mostly acellular leaflet tissue in both the 14 day and 180 day explants. An endothelial cell monolayer was observed in all explanted homografts and confirmed by SEM. No difference could be demonstrated between the groups on Picrosirius red staining. TEM demonstrated consistent collagen disruption after cryopreservation in all valves with no morphological deterioration or changes during the study period. von Kossa stains showed very mild calcification in the 180 day group. It was concluded that no deterioration of structural integrity could be demonstrated using strength (TS, YM and $T_d$), or morphological evaluations between the control and implant groups over the study period. Only mild calcification was observed in the 180–day explants. Thus, expanding homograft harvesting times to fourty-eight hours post mortem is not associated with early graft deterioration in cryopreserved pulmonary homografts in the ovine model.

## Conclusion

The afore-mentioned studies that were conducted in the ovine model, were undertaken to provide experimental evidence for the safe extension of cold post mortem harvest times. In both *in vitro* and *in vivo* studies no detrimental effect could be observed in tissue integrity up to at least fourty-eight hours and possibly to seventy-two hours. The safety of fourty-eight hour post mortem harvested and then cryopreserved pulmonary homografts was specifically studied to mimic the human clinical scenario and the stability of the homografts was confirmed at two study periods.

It is concluded that this study provides experimental scientific evidence to safely increase post mortem homograft harvest times to at least fourty-eight hours.

These studies provide experimental support for the re-evaluation of human cadaver homograft donor banks in order to attenuate international homograft shortages.

### Limitations

All implants were confirmed to be sterile after processing and antibiotic sterilisation before cryopreservation. Pre-processing and antibiotic sterilisation bacterial loads were not recorded and did not form part of this series of studies. This could be a limitation of the studies. However, as outcomes did not differ between groups studied, we can only speculate that if there were a significant difference in the pre-sterilisation bacterial loads between groups, it did not impact on experimental results.

# CHAPTER 7
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# Appendix A

Ethical Clearance (Article 1)

UNIVERSITEIT VAN DIE VRYSTAAT
UNIVERSITY OF THE FREE STATE
YUNIVESITHI YA FREISTATA

**Direkteur: Fakulteitsadministrasie / Director: Faculty Administration**
**Fakulteit Gesondheidswetenskappe / Faculty of Health Sciences**

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Me / Ms H Strauss 2007-01-30

PROF FE SMIT
DEPT OF CARDIOTHORACIC SURGERY
FACULTY OF HEALTH SCIENCES
UFS

Dear Prof Smit

**ANIMAL EXPERIMENT NR 12/06**
**PROJECT TITLE: "EFFECTS OF PROLONGED PRE-CRYOPRESERVED ISCHEMIC TIMES ON HOMOGRAFT PERFORMANCE"**

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 (Dorper)* | 30 | 31 JANUARY 2008 |

A report regarding this project has to be submitted after completion.

***REMARKS:*** *None*

Regards

H Strauss

For CHAIR:
CONTROL COMMITTEE FOR ANIMAL EXPERIMENTATION

Cc Dr F Potgieter, Animal Experimentation Unit

339, Bloemfontein 9300,RSA ☎ (051) 405 2812
Republiek van Suid-Afrika / Republic of South Africa

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# Appendix B

## Ethical Clearance (Article 2–4)

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

**Animal Research Ethics**

02-Aug-2016

Dear Mnr Dreyer Bester

**Student Project Number: UFS-AED2016/0101**

**Project Title:** The Impact of Extended Harvesting Times on Tissue Integrity of Cryopreserved Ovine Pulmonary Homografts

**Department:** Cardiothoracic Surgery (Bloemfontein Campus)

You are hereby kindly informed that, your project has been approved by the chair of the committee and will be submitted for final condonation to the Interfaculty Animal Ethics Committee on 04 August 2016.

**Kindly take note of the following:**

1.
A signed progress report with regard to the above study has to be submitted electronically to EthicsFHS@ufs.ac.za while a hard copy has to be submitted to the Ethics Office, Room D104, Francois Retief building, Faculty of Health Sciences. A report has to be submitted when animals are physically involved and after completion of the study. Guidelines with regard to progress reports are available from the Ethics Office and on the Faculty Intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht. A copy of the quotation has to be submitted with the application before the application will be considered for approval.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Digitally signed by Derek Litthauer
Date: 2016.08.02 10:40:02 +02'00'

Prof. Derek Litthauer Chair: Animal Research Ethics Committee

# APPENDIX C

Protocol for the dissection and sterilisation of heart valves

## Dissection and sterilisation of heart valves

### 1. PURPOSE

The purpose of this document is to describe the procedure for the complete dissection of the aortic and pulmonary valves and other cardiac tissue from the donor heart.

### 2. MATERIALS

2.1. **Media & antibiotics**

| | | | |
|---|---|---|---|
| 2.1.1. | Tazobax (Tazobactam) | 4mg / 20ml | -0.25 ml |
| 2.1.2. | Vancomycin | 500mg / 10 ml | -1.0 ml |
| 2.1.3. | Amikacin | 100mg / 2 ml | -0.5 ml |
| 2.1.4. | Fungizone (Amphoteracin B) | 50mg / 10ml | -0.4 ml |
| 2.1.5. | Earles Medium 199 | | -100 ml |
| | (Antibiotic solutions are freshly made just prior to sterilization) | | |
| 2.1.6. | Ringers lactate | | -1000 ml |
| | For rinsing the valve before sterilization in the antibiotic solution. | | |

2.2 **Consumables**

2.2.1 2cc sterile syringe

2.2.2 18G sterile needle

2.3 **Dissection set: A ready pre-packaged dissection set consisting of**

2.3.1 2 x 500 ml sterile stainless steel bowls

2.3.2 Set of Hegar dilators consisting of sizes 15 mm to 26mm double dilators or 21mm to 30 mm single dilators (dilators of smaller sizes are kept separately).

2.3.3 1 x Debakey vascular forceps

2.3.4 1 x Russian forceps

2.3.5 1 x Metzembaum dissection scissors

2.3.6 1 x stainless steel ruler

2.3.7 2 x Schwartz vessel clips

2.3.8 1 x 250 ml Schott bottle

2.3.9 ± 8-10 Sterile wipes

2.3.10 Dissection tray is wrapped in 2 x reliance blue coversheets.

2.4 **Clothing**

2.4.1 A clean sterile doctor's theatre gown

2.4.2 Sterile gloves

2.4.3 Mask

2.4.4 Cap

## 3. PROCEDURE

3.1 **Dissection**

3.2.1 Dissection of the valves will take place under clean conditions in the allocated dissection area.

3.2.2 Doctor's scrubs, shoes, cap, mask and unsterile gloves will be the suitable attire when entering the dissection area.

3.2.3 Spray the work surface in the dissection area with Surfacide, before placing the sterilised dissection set, wound packs, etc. on it.

3.2.4 Open the dissection set and lay the sterile reliance blue worksheets on the work surface, using aseptic technique.

3.2.5 Using aseptic technique open a 1L bag of Ringers lactate onto the work surface.

3.2.6 Place a pack containing a sterile gown at the end of the dissection table and open it. Also place and open sterile gloves next to it.

3.2.7 Take the bag containing the donor heart from the fridge, spray the outer surface with 70% IPA as well as the scissors used to cut open the bag. The heart should be opened just before scrubbing after preparation for dissection.

3.2.8 Do the same with the inner bag.

3.2.9 You should be able to remove the heart without touching the sides of the plastic bag.

3.2.10 The person performing the dissection scrubs as for surgery.

3.2.11 He/she puts on the sterile theatre gown and sterile gloves for the dissection.

3.2.12 Pour equal volumes of the cold Ringer's lactate into the two 500 ml medium bowls.

3.2.13 With a Russian forceps grasp the auricle of the heart, lift it out of the bag and move it to the sterile dissection surface.

3.2.14 Shake lightly for a couple of seconds to get rid of excess blood and fluid before beginning dissection.

3.2.15 The proximal ends of both right and left coronary arteries are dissected free of surrounding tissue and left intact to the aorta.

3.2.16 The aortic and pulmonary valves are now carefully separated using scissors, trimmed to the required dimensions and appearance, and size measured using Hegar dilators.

3.2.17 Appropriate quality checks are done, inspecting for any injuries, fenestrations, etc.

3.2.18 Glass tube of appropriate size is connected to the distal end of the artery, the tube and valve filled with Ringers lactate and competency / leakage of the valve evaluated. Coronary arteries of the aortic valve are temporarily closed with Schwartz vessel clips to perform the test.

3.2.19 During dissection the valves should be rinsed regularly in the Ringers Lactate to keep the tissue moistened.

3.2.20 The dissected valves and forceps are now placed in a bowl with Ringers lactate and are ready to be moved to the sterilisation area.

3.2.21 Document (hard copy and electronically) all measurements, findings and abnormalities of the valve immediately after dissection is completed. Donor data, valve identification numbers, dates and times should also be recorded.

## 3.3 **Sterilisation**

3.3.1 Spray the benchtop next to the laminar flow cabinet with Surfacide and place bowl with dissected valves on it. Also spray working area of laminar flow cabinet with Surfacide.

3.3.2 Light Bunsen gas burner inside laminar flow cabinet.

3.3.3 Open sterilised 125 ml / 250 ml Schott glass bottle using aseptic technique and place it in laminar flow cabinet.

3.3.4 Spray 500 ml bottle of Medium 199 as well as 4 vials with different antibiotics down with Surfacide and place on left side in laminar flow cabinet.

3.3.5 Open 2 cc sterile syringe and 18 G sterile needle in laminar flow cabinet using aseptic technique.

3.3.6 Open pack of sterile gloves on the benchtop next to the laminar flow cabinet and put it on.

3.3.7 Move the bowl with valve(s) and forceps also inside the laminar flow cabinet.

3.3.8 Pour 100 ml/200 ml of Medium 199 into the required Schott glass bottle and add the appropriate volumes of antibiotics for the cocktail.

3.3.9 Carefully place the valve(s) with the forceps into the Schott glass bottle and secure cap tightly.

3.3.10 Mark the bottle properly with valve identification number(s), size(s) and date, and place in designated fridge overnight at 4°C till cryopreservation the next day.

3.3.11 Clean all used areas/surfaces properly and dispose of all garbage, towels, packaging, etc as prescribed.