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Cadaver donation: structural integrity of pulmonary homografts harvested 48 h post mortem in the juvenile ovine model

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Abstract Cryopreserved pulmonary homograft (CPH) implantation remains the gold standard for reconstruction of the right ventricular outflow tract (RVOT). Harvesting homografts < 24-h post mortem is the international norm, thereby largely excluding cadaveric donors. This study examines the structural integrity and stability of ovine pulmonary homografts harvested after a 48-h post mortem period, cryopreserved and then implanted for up to 180 days. Fifteen ovine pulmonary homografts were harvested 48-h post mortem and cryopreserved. Five CPH served as a control group (group 1; n = 5). CPH were implanted in the RVOT of juvenile sheep and explanted after 14 days (group 2; n = 5) and 180 days (group 3; n = 5). Leaflet integrity was evaluated by strength analysis, using tensile strength (TS), Young's modulus (YM) and thermal denaturation temperature (T_d), and morphology, including haematoxylin and eosin (H&E), Picrosirius red staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and von Kossa stains. Echocardiography confirmed normal function in all implants. In explants, no reduction in TS, YM or T_d could be demonstrated and H&E showed mostly acellular leaflet tissue with no difference on Picrosirius red. TEM demonstrated consistent collagen disruption after cryopreservation in all three groups, with no morphological deterioration during the study period. von Kossa stains showed mild calcification in group 3. No deterioration of structural integrity could be demonstrated using strength or morphological evaluations between the controls and implant groups over the study period. Extending the post mortem harvesting time of homografts beyond 24 h did not appear to negatively affect the long-term performance of such transplanted valves in this study.

Keywords Juvenile ovine model · Homografts · Ischaemic time · Right ventricular outflow tract · Structural integrity

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Introduction

Cryopreserved pulmonary homografts remain the gold standard for reconstruction of the 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, and less than 6 h and definitely less than 24 h 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 24 h 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 24 h. At the Bloemfontein homograft bank, which is largely dependent on cadaver donors, the mean post mortem ischaemic time is 33 h, and it has extended its harvesting times to 48 h 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 90 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, 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, 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 different tissue banks (between 24 and 75 h) (Langley et al. 1996; Barrett-Boyes 1987). Cryopreservation within 3–4 days is accepted for viable cryopreserved homografts (O'Brien et al. 1987a, b). This means that the actual accepted post mortem ischaemic time before cryopreservation is routinely somewhere between 48 and 96 h. Insisting on a post mortem harvesting time of less than 24 h 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 24 and 48-h 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 24 h incubation in an antibiotic solution. The Bloemfontein homograft bank has previously indicated that extending the post mortem harvesting time up to 72 h 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 48 h 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 methods

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 homograft harvesting and preparation

A block removal of all intrathoracic organs as well as the attached liver was performed immediately after slaughtering from fifteen female juvenile Merino sheep [mean age 4.5 months, mean body weight 27.4 ± 1.14 kilograms (kg)]. Each tissue block with remaining blood was lifted from the carcass and sealed in a sterile container without any additives. The tissue blocks were left for 6–8 h at room temperature representing "warm ischaemic time", and then stored at 4 °C for an additional 40 h, representing "cold ischaemic time". At 48 h post mortem the containers were opened and the pulmonary homografts (PH) were dissected from the tissue blocks while being washed in copious amounts of Ringers lactate. The PHs were incubated overnight (mean 20 h) at 4 °C in an antibiotic solution containing 100 ml of Medium199 with Earle's Base (Whitehead Scientific (Pty) Ltd., Brackenfell, 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 sterilization. The homografts were then cryopreserved as previously described (Smit et al. 2015) after tissue samples had been obtained for microscopy, microbiological cultures and sensitivity (MC&S).

Five of these homografts served as controls (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) respectively in order to evaluate their structural integrity and stability (Fig. 1). Leaflet integrity was evaluated by morphology and strength analysis. Strength analyses included TS, YM and T_d, while morphology included H&E, Picrosirius red staining, SEM, 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.

Cryopreservation and thawing

Cryopreservation was performed using an automated programmable freezer (Cryoson BV-9 Biological Freezer, Consarctic, Schöllkrippen, Germany). Before cryopreservation 11 ml of dimethyl sulphoxide (DMSO) (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 (LN_2) between -120 and -160 °C until they were implanted. It was confirmed that all HPs cryopreserved were culture negative on pre-cryopreservation MC&S. 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 35–40 °C for 5–7 min 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 10-min 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, and thereafter the homograft was delivered to the surgical team for final trimming and implantation. Another tissue sample was obtained for MC&S at implantation to confirm sterility.
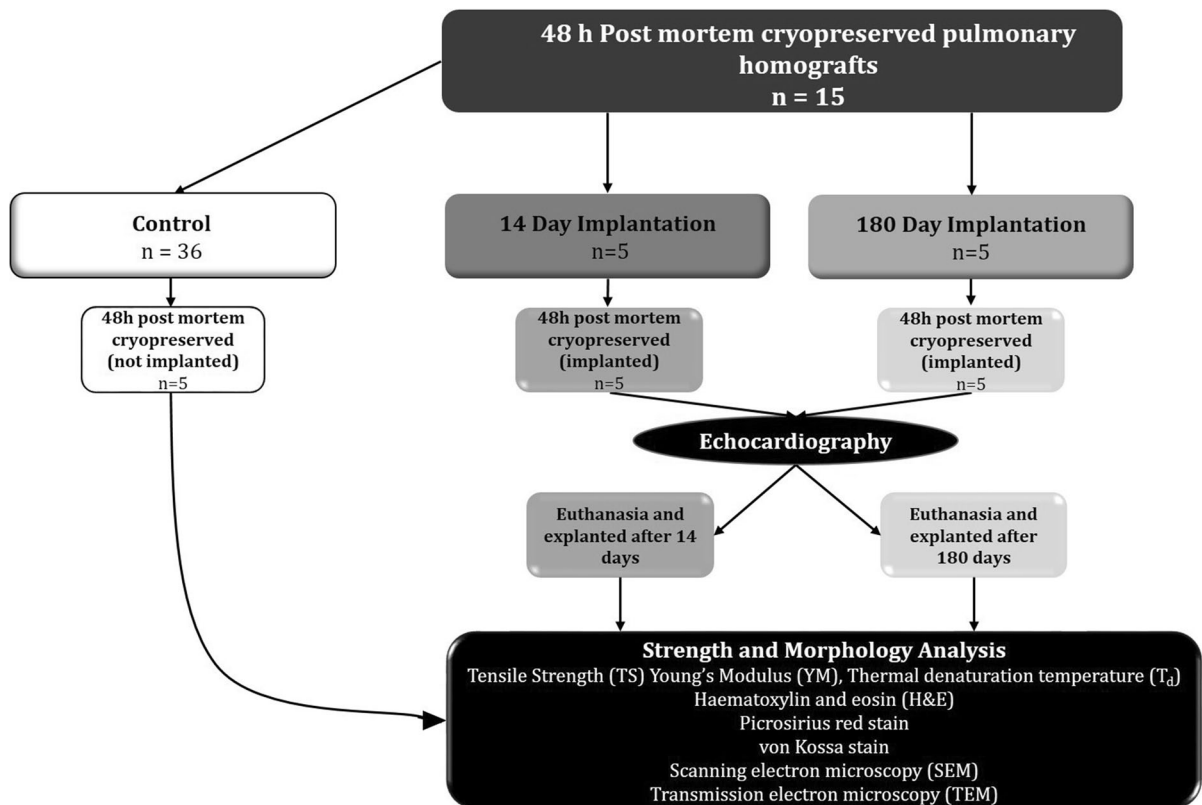
Fig. 1 Study layout

Pulmonary homograft implantation

Recipient sheep (mean age 6.8 months; body weight 40.2 ± 6.38 kg) 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 5 days post-operatively. Animals were extubated between 2 and 4 h 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–strain 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 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 a differential scanning calorimeter (DSC) (Mettler Toledo, T_d 822e, Microsep, Johannesburg, South Africa). Small samples of the pulmonary leaflets (2–5 mg) were placed in the DSC's hermetically sealed pans and subjected to temperature increasing at a rate of 10 °C/min from 25 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 mm × 6 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 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 semi-quantitatively 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% CI for the mean difference (indicating the limits within which the true difference is likely to occur).

Results

Microbiology

All homograft tissue samples were culture negative before cryopreservation as well as before implantation.

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 (Fig. 2).

Gross examination

Blinded visual inspection did not discern differences in the macroscopic appearance of homografts explanted after 14 days and 180 days (Fig. 3).

Biomechanical testing

The strength analysis results are summarised in Table 1. There were no significant differences in TS, YM and T_d between the control and 14-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% CI 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.

Histology

Figure 4 represents the H&E, Picrosirius red and von Kossa staining of the 48 h control, 14-day and 180-day explants.

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
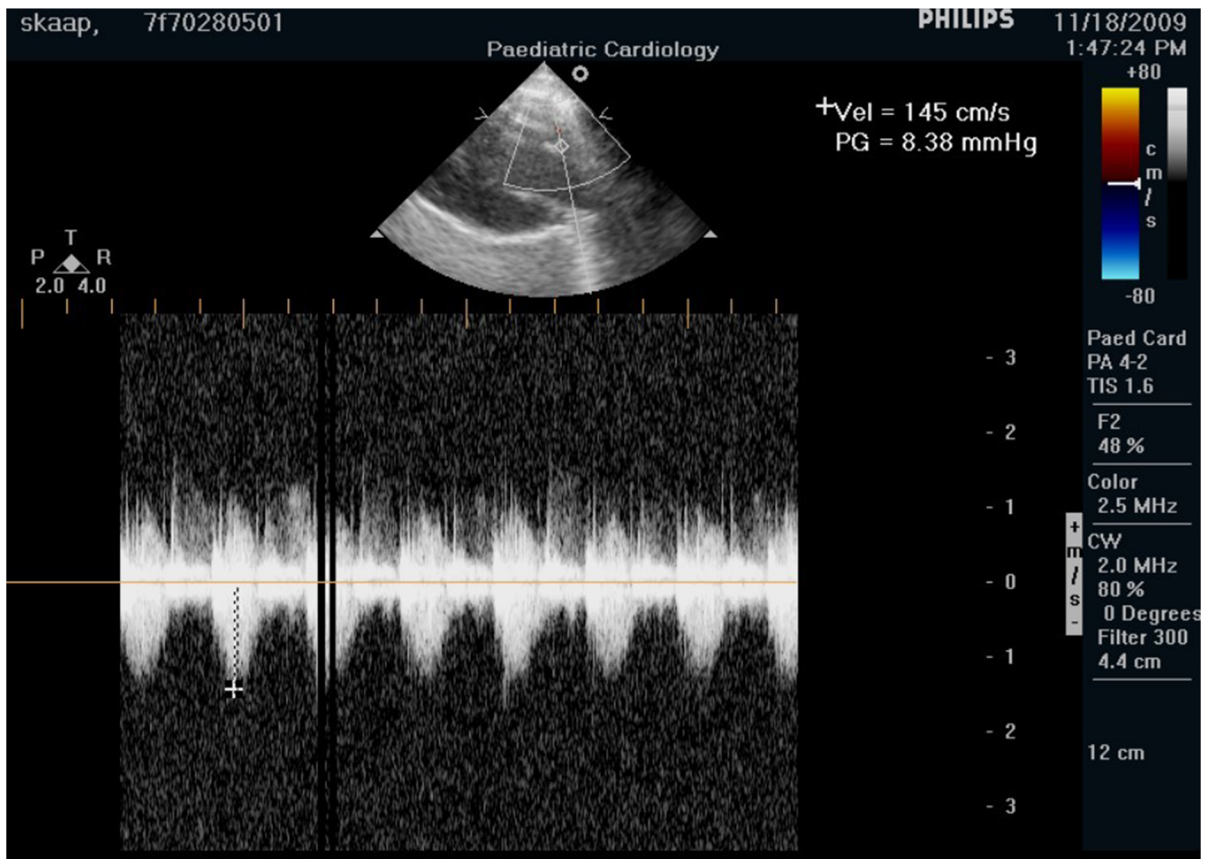
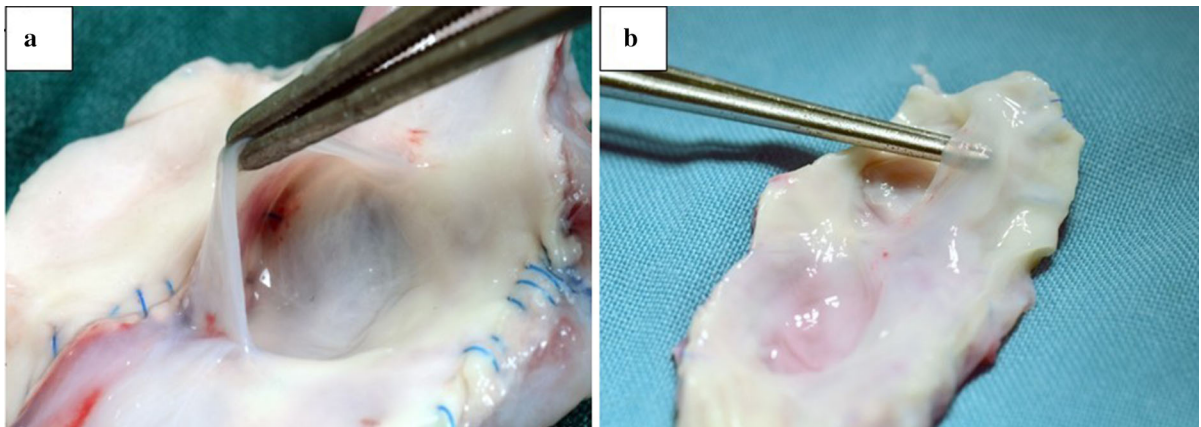Fig. 2 Transvalvular gradient of a 180-day homograft (Smit 2011)

Fig. 3 a Macroscopic appearance of 14-day explanted homograft leaflet. b Macroscopic appearance of 180-day explanted homograft leaflet

14 days explanted leaflets and only mild calcification of the 180 days explanted leaflets (Fig. 4).

Electron microscopy

SEM confirmed an endothelial layer that was visible in the control, the 14-day and the 180-day explants. SEM

Table 1 TS, YM and T_d of 48 h 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% CI control versus 14 day explant	Mean 180-day explant	95% CI control versus 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% CI 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
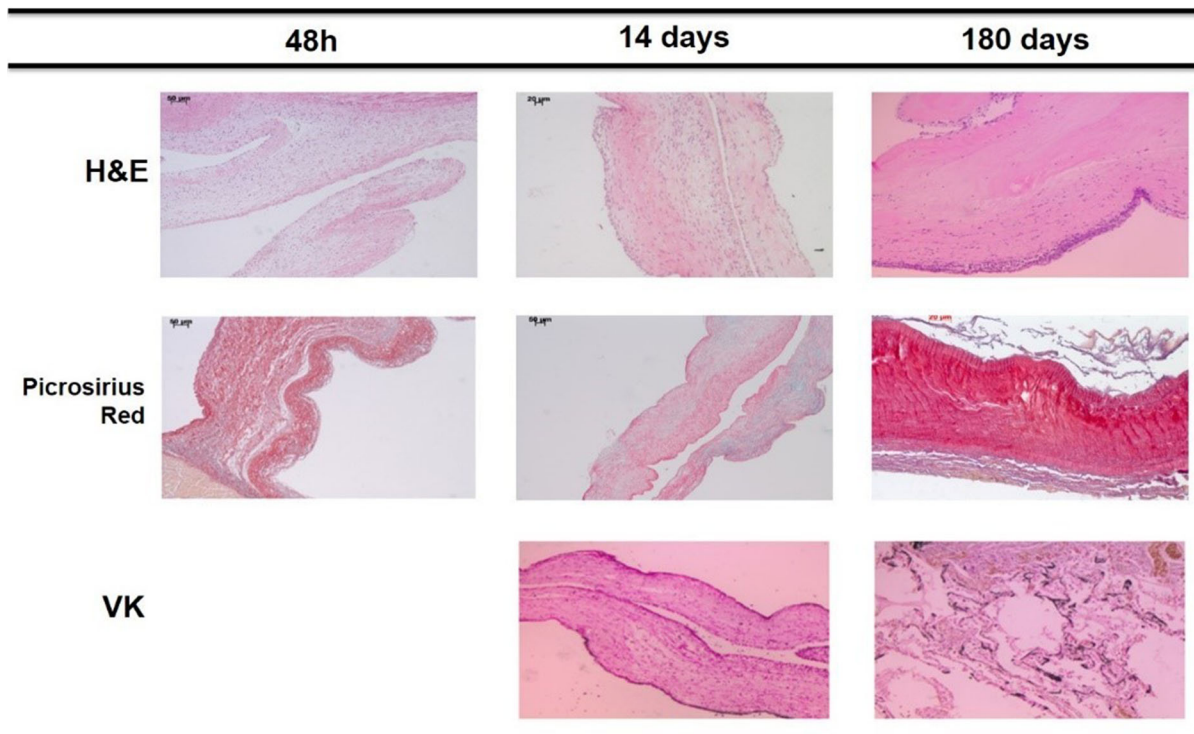
Fig. 4 H&E, Picrosirius red and von Kossa staining of 48 h cryopreserved ovine leaflets before implantation (control, n = 5), and explanted leaflets after 14 days (n = 5) and 180 days (n = 5). h Hours, H&E Haematoxylin and eosin, VK von Kossa

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-day samples show a confluent layer of healthy endothelium (Fig. 5). TEM demonstrates consistent collagen disruption after cryopreservation in the control group as well as the 14 days and 180-day explants with no morphological deterioration or changes during the study period (Fig. 5).

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 homografts sterilized and cryopreserved after 48 h post mortem ischaemic time, a time specifically chosen to simulate a reasonable window of opportunity in order to obtain donor
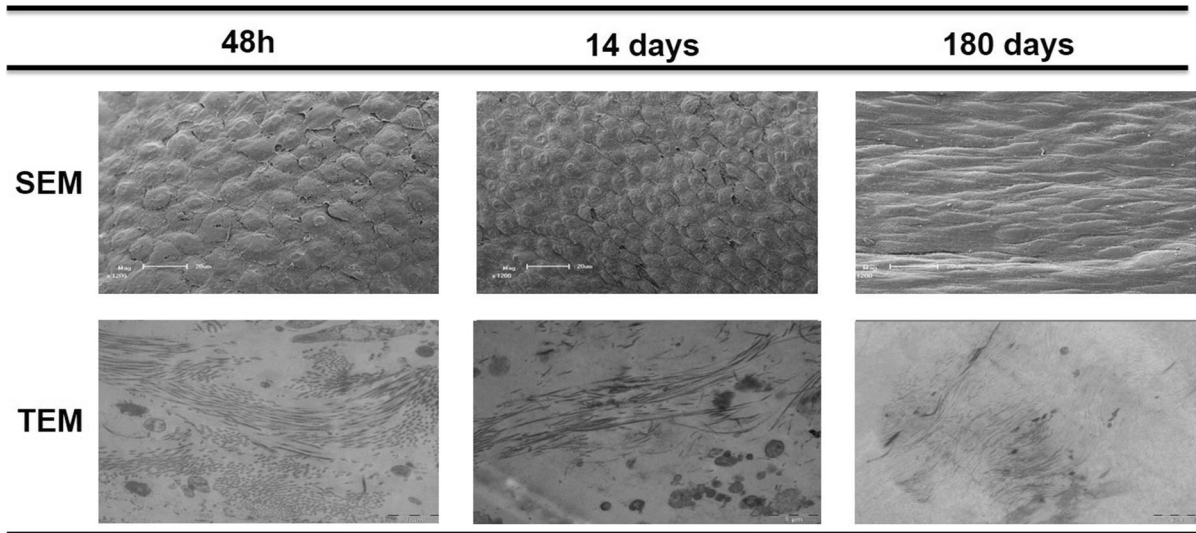
Fig. 5 SEM and TEM of 48 h cryopreserved ovine leaflets before implantation (control, n = 5) and explanted leaflets after 14 days (n = 5) and 180 days (n = 5). *SEM* Scanning electron microscopy, *TEM* transmission electron microscopy, *h* hours

consent in human cadaveric donor programmes in South Africa (mean = 33 h). The ovine model is a widely accepted model for homograft degeneration studies and is well known for its aggressive calcification potential. The ideal is to use primate models for implantation, but this model is very expensive and severely regulated. Although the study revealed excellent results about tissue integrity among the groups, final conclusions and translation to human subjects can only be made once a similar study has been repeated in a primate model.

A limitation of the ovine model is that the fermenting rumen in the large stomach of sheep generates heat post mortem in intact carcasses. In a previous study, the stomach was removed from carcasses otherwise left intact. In that study cultures were obtained from homograft tissue harvested up to 72 h post mortem. The homografts were incubated in Medium199 and antibiotics as described. The homografts were consistently culture negative before cryopreservation and no differences in the outcome at 180 days implantation could be demonstrated between the groups extending harvesting time up to 72 h (Smit et al. 2015). While the organ blocks harvested in this study accurately reflects post mortem ischaemic time as well as confirmed sterility pre-cryopreservation and at implantation (as with human homografts), it does not accurately reflect the effects of temporary bacterial exposure associated with decomposition in human

cadavers that might occur before organ explantation and antibiotic incubation.

The 48 h post mortem harvested cryopreserved pulmonary homografts performed well over the study period, maintained its tissue integrity and stability and exhibited no graft failures. This correlates well with findings by researchers in Sweden that transplanted valves in the pulmonary position from non-beating donors with > 24 h ischaemic time and longer times from retrieval to cryopreservation had a lower reintervention rate compared to < 24 h. They concluded that longer ischaemic times of donor valves might even be preferable and does not affect the long-term outcome of transplanted homografts negatively (Axelsson and Malm 2018).

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-day 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-day explant valves.

We speculate that the increase in TS and YM seen in the 180-day explant group compared to both the control and the 14-day explant can 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 48 h post mortem cryopreserved homografts during the study period. T_d values remained constant between the groups and no deterioration could be demonstrated in the 48 h post mortem cryopreserved homografts over the 180-day 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 14 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 Picrosirius red staining confirmed the presence of collagen in all groups and the von Kossa staining showed mild calcification in the 180-day 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-day study period. In a study that evaluated cryopreservation, ice-free cryopreservation (vitrification) and freeze-drying as preservation methods for decellularized bovine pericardial scaffolds, it was found that cryopreservation resulted in a significant increase in stiffness and tensile strength of the scaffold, which was not observed with the other two preservation methods. Although cryopreservation is the preferred method of preservation for heart valves and other cardiovascular tissues worldwide, it does alter the biomechanical behavior of collagenous tissues (Zouhair et al. 2017).

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?

Study limitations and recommendations

- Direct translation comparison to human subjects cannot be made based on the use of the ovine model.
- To relate the results directly to humans a primate model must be used instead of an ovine model.

Conclusion

Although conditions of human cadaveric tissue donations were not exactly replicated in this study and findings in animal studies can not simply be extrapolated to the human setting, important findings can still be made from the results.

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 48 h post mortem in this study was not associated with early graft failure or loss of tissue integrity. Prolonging harvesting time to 48 h post mortem can attenuate homograft donor shortages.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Angell WW, Oury JH, Lamberti JJ, Koziol J (1989) Durability of the viable aortic homograft. J Thorac Cardiovasc Surg 98:48–56

Axelsson I, Malm T (2018) Long-term outcome of homograft implants related to donor and tissue characteristics. Ann Thorac Surg 106(1):165–171

Barrett-Boyes B (1987) 25 years clinical experience of homograft surgery—a time for reflection 1962–1987. In: Yankah AC, Hetzer R, Miller DC, Ross DN, Somerville J, Yacoub MH (eds) Cardiac valve allografts. Springer, New York, pp 347–358

Botes L, van den Heever JJ, Smit FE, Neethling WML (2012) Cardiac homografts: a 24-year South African experience. Cell Tissue Bank 13:139–146

Brubaker S, Lotherington K, Zhao J, Hamilton B, Rockl G, Duong A, Garibaldi A, Simunovic N, Alsop D, Dao D, Bessemer R, Ayeni OR (2016) Tissue recovery practices and bioburden: a systematic review. Cell Tissue Bank 17:561–571

Dagum P, Green GR, Timek TA, Daughters GT, Foppiano LE, Tye TL, Bolger AF, Ingels NB, Miller DG (1999) Functional evaluation of the Medtronic stentless porcine xenograft mitral valve in sheep. Circulation 100(19 Suppl. 2):70–77

Dawson PE, Brockbank KGM (1997) Human heart cold ischemia and its effects on post cryopreservation viability of heart valves. In: Yankah AC, Yacoub MH, Hetzer R (eds) Cardiac valve allografts, science and practice. Springer, New York

Green MK, Walsh MD, Dare A, Hogan PG, Zhao XM, Frazer IH, Bansai AS, O'Brien MF (1998) Histologic and immunohistochemical responses after aortic valve allografts in the rat. Ann Thorac Surg 66:S216–S220

Hechadi J, Gerber BL, Coche E, Melchior J, Jashari R, Glineur D, Noirhomme P, Rubay J, El Khoury G, De Kerchove L (2013) Stentless xenografts as an alternative to pulmonary homografts in the Ross operation. Eur J Cardiothorac Surg 44:e32–e39

Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council. Guide for the Care and Use of Laboratory Animals [online]. http://www.nap.edu/catalog/5140.html. Accessed 20 Jan 2016

Langley SM, Livesey SA, Tsang VT, Barron DJ, Lamb RK, Ross JK, Monro JL (1996) Long-term results of valve replacement using antibiotic-sterilised homografts in the aortic position. Eur J Cardiothorac Surg 10(12):1097–1106

Methe H, Hess S, Edelmann ER (2007) Endothelial immunogenicity—a matter of matrix microarchitecture. Thromb Haemost 98:278–282

Mitchell RN, Jonas RA, Schoen FJ (1998) Pathology of explanted cryopreserved allograft heart valves: comparison with aortic valves from orthotopic heart transplants. J Thorac Cardiovasc Surg 115:118–127

Mroz TE, Joyce MJ, Steinmetz MP, Lieberman IH, Wang JC (2008) Musculoskeletal allograft risks and recalls in the United States. J Am Acad Orthop Surg 16(10):559–565

O'Brien MF, Stafford G, Gardner M, Pohlner P, McGiffin D, Johnston N, Brosnan A, Duffy P (1987a) The viable cryopreserved allograft aortic valve. J Cardiac Surg 2(Suppl 1):153–167

O'Brien MF, Stafford EG, Gardner MAH, Pohlner P, McGiffin D (1987b) A comparison of aortic valve replacement with viable cryopreserved and fresh allograft valves, with a note on chromosomal studies. J Thorac Cardiovasc Surg 94:812–823

O'Brien MF, Stafford GE, Gardner MAH (1995) Allograft aortic valve replacement: long-term follow-up. Ann Thorac Surg 60:S65–S70

O'Brien MF, Harrocks S, Stafford EG, Gardner MAH, Pohlner PG, Tesar PJ, Stephens F (2001) The homograft aortic valve: a 29-year, 99.3% follow up of 1022 valve replacements. J Heart Valve Dis 10(3):334–344

Rüegg M, Moor U, Blanc B (1975) Hydration and thermal denaturation of beta-lactoglobulin. A calorimetric study. Biochim Biophys Acta 400:334–342

Schenke-Layland K, Madershahian N, Riemann I, Starcher B, Halbhuber KJ, König K, Stock UA (2006) Impact of Cryopreservation on extracellular matrix structures of heart valve leaflets. Ann Thorac Surg 81:918–926

Smit F (2011) The effect of pre-harvest ischemic time on valvular homografty performance - an experimental study. PhD. University of the Free State

Smit FE, Bester D, van den Heever JJ, Schlegel F, Botes L, Dohmen PM (2015) Does prolonged post-mortem cold ischemic harvesting time influence cryopreserved pulmonary homograft tissue integrity? Cell Tissue Bank 16(4):531–544

Smith SH, Judge MD (1991) Relationship between pyridinoline concentration and thermal stability of bovine intramuscular collagen. J Anim Sci 69:1989–1993

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43

Thubrikar MJ, Deck JD, Aouad J, Nolan SP (1983) Role of mechanical stress in calcification of aortic bio prosthetic valves. J Thorac Cardiovasc Surg 86:115–125

Yacoub M, Kittle CF (1970) Sterilization of valve homografts by antibiotic solutions. Circulation 41:SII29–SII31

Yacoub M, Rasmi NRH, Sundt TM, Lund O, Boyland E, Radley-Smith R, Khaghani A, Mitchell A (1995) Fourteen-year experience with homovital homografts for aortic valve replacement. J Thorac Cardiovasc Surg 110:186–194

Yankah AC, Hetzer R (1987) Procurement and viability of cardiac homografts. In: Yankah AC, Hetzer R, Miller C, Ross DN, Somerville J, Yacoub MH (eds) Cardiac valve allografts 1962–1987. Springer, New York, pp 23–26

Yoshikawa Y, Kitamura S, Taniguchi S, Kameda Y, Niwaya K, Sakaguchi H (2000) Pulmonary ventricular outflow reconstruction with a size-reduced cryopreserved pulmonary valve allograft: mid-term follow-up. Jpn Circ J 64:23–26

Zouhair S, Aguiari P, Iop L, Korossis S, Wolkers WF, Gerosa G (2017) Advanced preservation methodologies for decellularized cardiovascular scaffolds. In: 26th Congress of the European association of tissue banks, Treviso, Italy. https://www.eatb.org/images/2017/Final-Program_EATB2017_web.pdf. Accessed Sept 2018