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A novel decellularization protocol with cryopreservation of pulmonary allografts in an ovine model

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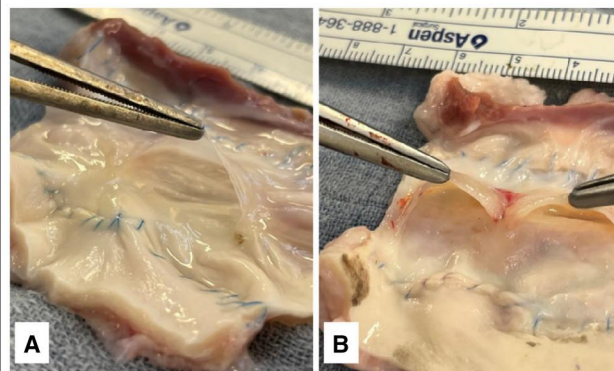
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Summary

We applied a new protocol of pulmonary allograft decellularization in a sheep model and compared decellularized and cryopreserved versus clinical standard cryopreserved allografts at 6 months. Both groups produced equivalent and favorable hemodynamic outcomes. With less inflammation, pannus formation and calcification, histological findings are overall in favor of decellularized grafts.



A. Decellularized valve with supple and thin leaflets. **B.** Cryopreserved valve with thickened leaflets

[†]These authors contributed equally to this work.

Abstract

OBJECTIVES: Pulmonary allografts are commonly used for right ventricular outflow tract reconstruction. Cryopreserved allografts may develop degeneration and stenosis over time due to immune-related issues. Fresh decellularized aortic and pulmonary allografts have shown favourable early outcomes. We used a sheep model to evaluate the safety of a novel decellularization protocol combined with cryopreservation applied on pulmonary allografts, aiming to improve availability and reduce cost.

METHODS: Pulmonary valve replacement was performed in juvenile female sheep using cryopreserved pulmonary allograft ($n = 9$) and decellularized cryopreserved pulmonary allograft ($n = 10$). Haemodynamics were investigated by transthoracic echocardiography and magnetic resonance imaging. Allografts were explanted at 6 months for macroscopic comparison, calcium quantification using X-ray imaging and spectrometry, histological and immunohistochemical characterization.

RESULTS: There was one operative death in each group. There were no significant haemodynamic differences between groups regarding peak gradient ($P = 0.14$) or degree of regurgitation ($P = 0.67$) at 6 months. Calcium content of macroscopically non-calcified tissue was statistically similar ($P = 0.16$). Overall, decellularized tissues were smooth and supple. Cryopreserved allografts developed pannus formation and pronounced subvalvular calcifications. Some grafts in both groups showed luminal fibrin thrombi, calcified in cryopreserved grafts. Histologically, cryopreserved tissues contained more inflammatory cells. Decellularized grafts had lower cell presence, acellular inner media and valve with occasional fibrin covering. No true endothelium was seen in the stained samples except at the anastomoses.

CONCLUSIONS: Haemodynamically, both cryopreserved allografts and allografts decellularized according to the newly-developed protocol produced equivalent and favourable outcomes. Decellularized allografts showed less inflammation, less pannus formation and less calcification on X-ray imaging.

Keywords: decellularized allografts • cryopreservation • pulmonary valve • sheep model

ABBREVIATIONS

Alpha-SMA	Alpha smooth muscle actin
CPA	Cryopreserved pulmonary allograft
DCPA	Decellularized (and subsequently) cryopreserved pulmonary allograft
HR	Heart rate
LVEF	Left ventricular ejection fraction
MPA	Main pulmonary artery
MRI	Magnetic resonance imaging
PVMG	Pulmonary transvalvular mean gradient
PVPG	Pulmonary transvalvular peak gradient

INTRODUCTION

Upon reaching the limits of aortic valvuloplasty and later of the early-generation prosthetic valves, the search for biological valve substitutes was initiated [1]. In 1962, Donald N. Ross conducted the first orthotopic aortic valve replacement using a freeze-dried aortic valve homograft [2]. Throughout the early years, several preservation techniques, including chemical preservation and irradiation, were evaluated with the aim of reducing inflammatory response. Homovital, unprocessed allografts, on the contrary, aimed to deliver a viable allograft [3].

Due to the low rate of immediate complications and superior haemodynamic performance compared to prosthetic valves, cryopreserved allografts used to be the gold standard for the treatment of children [4]. Each year approximately 2.000 allograft valves are implanted in Europe for reconstruction of the right ventricular outflow tract (RVOT) in the setting of congenital heart disease such as tetralogy of Fallot or in the Ross procedure [5].

Cryopreserved allografts show deterioration and failure over time due to immune-related issues from residual antigens, more pronounced in young recipients [6]. Decellularization represents a promising technique to mitigate inflammation, calcification and ultimately conduit/valve stenosis [6, 7]. In animal models

and recent European trials, fresh decellularized aortic and pulmonary allografts have shown favourable outcomes [8–11]. In North America, decellularized and subsequently cryopreserved pulmonary allografts (CPAs) (CryoLife/Artivion, USA) have already shown superior haemodynamic results at mid-term follow-up versus standard cryopreserved allografts [12]. Cost and logistical challenges hinder widespread adoption of currently available fresh decellularized allografts (Corlife, Hannover) in Europe. Therefore, a two-step decellularization protocol using gentle reagents was developed with subsequent cryopreservation, aiming to improve availability and reduce cost [13]. The aim of our pilot study was to evaluate the early *in vivo* outcome and safety of this novel approach.

Sheep are commonly used in preclinical heart valve research to study the biology of prosthetic valve degeneration [14] and have been used to evaluate fresh and decellularized allografts [8, 9]. With pronounced calcification in young sheep [15], the model is ideal for studying decellularization techniques aiming to reduce calcification. Therefore, we used a sheep model comparing the novel protocol of decellularized and subsequently CPAs with clinical standard CPAs, regarding haemodynamics with a focus on transvalvular gradient and regurgitation, macroscopic appearance, qualitative characterization of potential inflammatory infiltrate and presence of micro- and macrocalcifications.

MATERIALS AND METHODS

Study design

This pilot study was approved by the Ethics Committee of the KU Leuven (ECD 163/2020). Animals received humane care and experiments were performed in compliance with the 'Principles of Laboratory Animal Care', formulated by the National Society for Medical Research, and with the European Commission Directive 2010/63/EU [16, 17]. We used female, juvenile Swifter sheep between 4 and 8 months old. Pulmonary valve/artery replacement was performed using clinical standard CPA ($n = 9$)

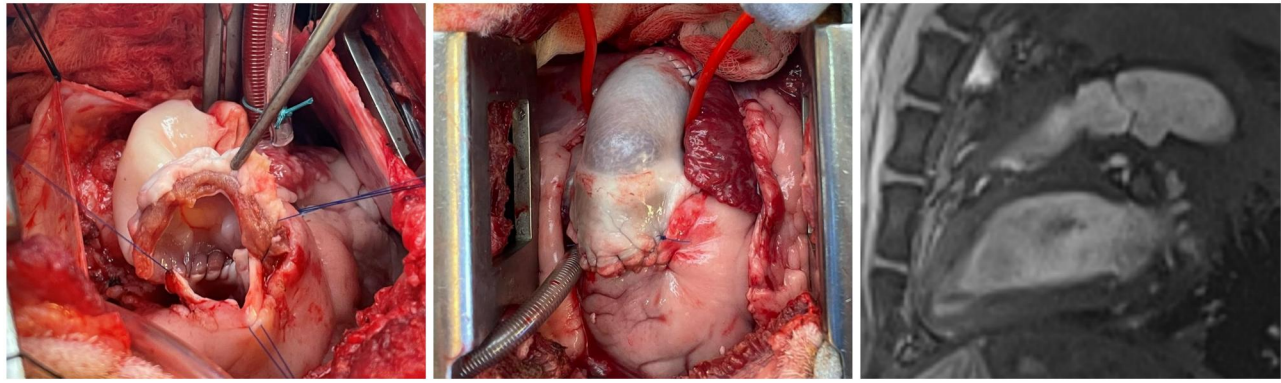


Figure 1: Left: proximal anastomosis, suturing donor infundibular muscle to recipient pulmonary sinus. **Middle:** after implantation. **Right:** MRI with longitudinal image, asterisk indicating partially remaining recipient pulmonary sinuses with leaflets more distally, indicating heterotopic implantation.

and by decellularized CPA (DCPA) ($n = 10$), with all operations performed by the same surgeon. As our primary goal was to determine the early *in vivo* safety of the novel allografts, no sample size calculation was done. Haemodynamics were assessed by echocardiography and magnetic resonance imaging (MRI) at 2 weeks and 6 months, followed by graft explantation. Between the imaging investigations, animals returned to the Zootechnical centre of the KU Leuven.

Preparation of pulmonary allografts

Donor sheep were given heparin (300 IU/kg) and sodium pentobarbital (8000 mg). Hearts were explanted in sterile conditions and stored in 0.9% NaCl at 4°C. The valves were evaluated, measured and competence tests performed. Grafts were immersed in an antibiotic solution containing lincomycin, vancomycin and polymyxin B for 20–48 hours in +4°C [5].

After decontamination, pulmonary allografts for the CPA group were cryopreserved by decreasing temperature progressively to –150°C and stored in liquid nitrogen vapors. Allografts for decellularization were placed in the antibiotic solution at +4°C and shipped to the Barcelona Tissue Bank. The grafts were decellularized performing a two-step protocol with minimal manipulation and using gentle reagents, including hypo- and hypertonic solutions to induce osmotic shock and enzymes to effectively eliminate genetic material. Notably, detergent solutions were deliberately avoided to preserve the integrity of the extracellular matrix, ensuring optimal structural and biochemical properties [13]. This approach is similar in strategy to an established protocol which has been validated experimentally and used clinically [12, 18]. After completion, allografts were returned for cryopreservation. Bacteriology samples were collected from all accepted allografts and tested for presence of aerobic and anaerobic organisms using the BD BACTEC FX Blood Culture System (BD, USA) before and after antibiotic treatment. Pathogen-free allografts were accepted for further processing and implantation [19].

Surgical technique and postoperative care

After sedation (ketamine 4 mg/kg), anaesthesia was induced (isoflurane inhalation 1–5%). A left cervicotomy and thoracotomy in the third intercostal space were performed. After heparinization (300 IU/kg), cardiopulmonary bypass was initiated by

cannulation of the left external jugular vein and common carotid artery. On the beating heart, the main pulmonary artery (MPA) was transected. The pulmonary artery was trimmed to below the sinotubular junction and the leaflets were removed, resulting in a slightly heterotopic implantation (Fig. 1), to minimize bleeding and ventricular fibrillation associated with right ventricular incision on the beating heart. The proximal anastomosis was performed using a 4–0 polypropylene suture and the distal using a running 5–0 polypropylene suture. Animal welfare was checked several times daily with 2-hourly evaluation of discomfort in the first postoperative week. Routine analgesics, postoperative antibiotics and low-dose enoxaparin were continued for 7 days. No long-term anticoagulant or anti-platelet therapy was used.

Echocardiography

Transthoracic echocardiography was performed under sedation at 2 weeks and 6 months postoperatively using a GE Vivid 7 Ultrasound Machine (GE HealthCare, USA). All echocardiography images were reviewed in a blinded fashion. The following parameters were measured using EchoPAC (GE HealthCare, USA): heart rate (HR), pulmonary valve annulus diameter, pulmonary transvalvular peak gradient (PVPg), pulmonary transvalvular mean gradient (PVMg), qualitative description of grafts, pulmonary regurgitation (PR) grade 0–4/4 and left ventricular ejection fraction (LVEF).

Magnetic resonance imaging

A cardiac MRI scan was performed under general anaesthesia at 2 weeks and 6 months postoperatively in a 3.0-Tesla MRI (Magnetom Trio Tim; Siemens Medical Solutions, Germany). For morphological evaluation, cine images were made longitudinally and axially. Phase contrast cine sequences were used to capture velocity vector fields, enabling determination of flow velocities, transvalvular gradient and regurgitant volumes in a blinded fashion after study conclusion.

Graft explantation, X-ray imaging, macroscopic photography and sample division

After final imaging, intravenous heparin (200 IU/kg) was administered, followed by a lethal dose of sodium pentobarbital. The pulmonary allograft was excised and opened longitudinally. To

visualize calcifications, we made X-ray images using a Mammomat Fusion digital mammography (Siemens, Germany). Macroscopic pictures were taken for scoring. Samples were divided with the same region used per analysis for each sheep ([Supplementary Material, Fig. S2](#)).

Scoring system

On X-ray images, calcification was scored for anastomosis, cusp base, sinus/cusp (overlapping on X-ray) and MPA wall. On the photographs, multiple parameters were scored for leaflets: calcification, retraction, neo-intima/pannus, thinning, irregularities and vegetations. For wall, we evaluated: calcification, neo-intima/pannus, thinning, irregularities and vegetations. The following scale was used: 0, 1, 2 and 3 corresponding to none, mild, moderate and severe, respectively. Two independent observers performed scoring; the mean score was calculated and groups were compared using independent-samples Mann-Whitney U test.

Flame atomic absorption spectrometry for calcium content analysis

This assay served to evaluate calcification content in tissue without macroscopically evident calcification, as previously described by our group [15]. One third of the conduit was excised, stored in 4% formaldehyde and later divided: cusp, allograft sinus, allograft MPA and control MPA. After lyophilization, 10–20 mg samples were pulverized and diluted in 20% hydrochloric acid. Calcium content was measured by flame atomic absorption spectrometry.

Histological evaluation

Histological analysis was conducted on 6- μ m-thick sections of tissue embedded in paraffin and stained with hematoxylin and eosin assess morphology, cellular composition and calcifications. Pulmonary roots from unrelated experiments were used as control ($n = 4$). All samples were evaluated by two pathologists, initially blinded. Semi-quantitative scoring (parameters: surface thrombus, neo-intima/pannus, endothelialization, cellular ingrowth, presence of inflammatory cells and degree of calcification) was used to guide descriptive conclusions.

Immunohistochemistry

Formalin-fixed paraffin-embedded slices were stained using a Leica BOND-MAX IHC stainer with the following antibodies: CD3 (ab5690, Abcam) for T-lymphocytes, CD11b (ab133357, Abcam) for myeloid-lineage cells such as monocytes/macrophages/neutrophils, PAX5 (m7307, Dako) for B-lymphocytes, alpha-SMA (M0851, Dako) or smooth muscle cells and myofibroblasts, and finally von Willebrand factor (ab6994, Abcam) for endothelial cells. Baseline allograft samples taken prior to implantation and control samples were stained using alpha-SMA to evaluate residual cells or fragments. Descriptive immunohistochemical evaluation was performed for each sheep and conclusions were reported.

Statistical analysis

Data were reported as mean $\pm$ SD for visual representation. Analyses were exploratory in nature, and there was no prespecified plan to adjust for multiple comparisons. Statistical significance was defined as $P < 0.05$. Normality was evaluated by the Shapiro-Wilk test. Due to sample size and non-parametric distribution of a relevant number of data measures, non-parametric statistics were used with inter-group comparison using the independent-samples Mann-Whitney U test and the related-samples Wilcoxon signed-rank test when comparing paired data between timepoints. SPSS Statistics 26.0 (IBM) and Prism 8 (GraphPad) were used for statistical analysis and visual representation.

RESULTS

Clinical outcome

Nineteen sheep were operated (February 2021–March 2022) with two procedural deaths (refractory ventricular fibrillation upon manipulating the right ventricle and complications related to cannulation), resulting in nine animals for DCPA and eight for CPA. There were no perioperative deaths related to the homografts. All animals recovered well, and there were no deaths during follow-up nor were there episodes of heart failure, fever or clinical deterioration.

Sheep weight and valve size analysis

Mean baseline weight was 43.2 kg (SD 4.7; 36.5–56.1 kg), and animals in the DCPA group had a significantly higher weight (44.8 vs 41.4 kg for CPA, $P = 0.017$). Average implanted valve size, measured during allograft preparation, was statistically similar between both groups. As a result, sheep in the CPA group received a slightly larger valve for their weight ([Supplementary Material, Table S1, Supplementary Material, Fig. S1](#)).

Haemodynamic results

Echocardiography and MRI outcomes are shown in [Supplementary Material, Table S2](#). There were no significant differences between groups regarding transvalvular gradient or PR grade/regurgitation fraction on both early and late echocardiography and MRI. In both groups, there was a limited increase in mean PR grade/regurgitant fraction over time on echocardiography/MRI, only significant for CPA.

Macroscopic examination and calcium quantification

A descriptive summary of macroscopic and X-ray observations, including echocardiography and MRI findings, is shown in [Supplementary Material, Table S3](#), and all photographs and X-ray images are provided in the [Supplementary Material, Appendix S1](#).

There were no pseudo-aneurysms, dehiscence, wall or valve decay in either group. Regarding DCPA, eight (8/9, 88.9%) specimens presented smooth and supple leaflets and wall, resembling pre-implantation tissues ([Fig. 2A–C](#)). Two DCPA (22.2%) had intact leaflets yet small papillomatous irregularities at the

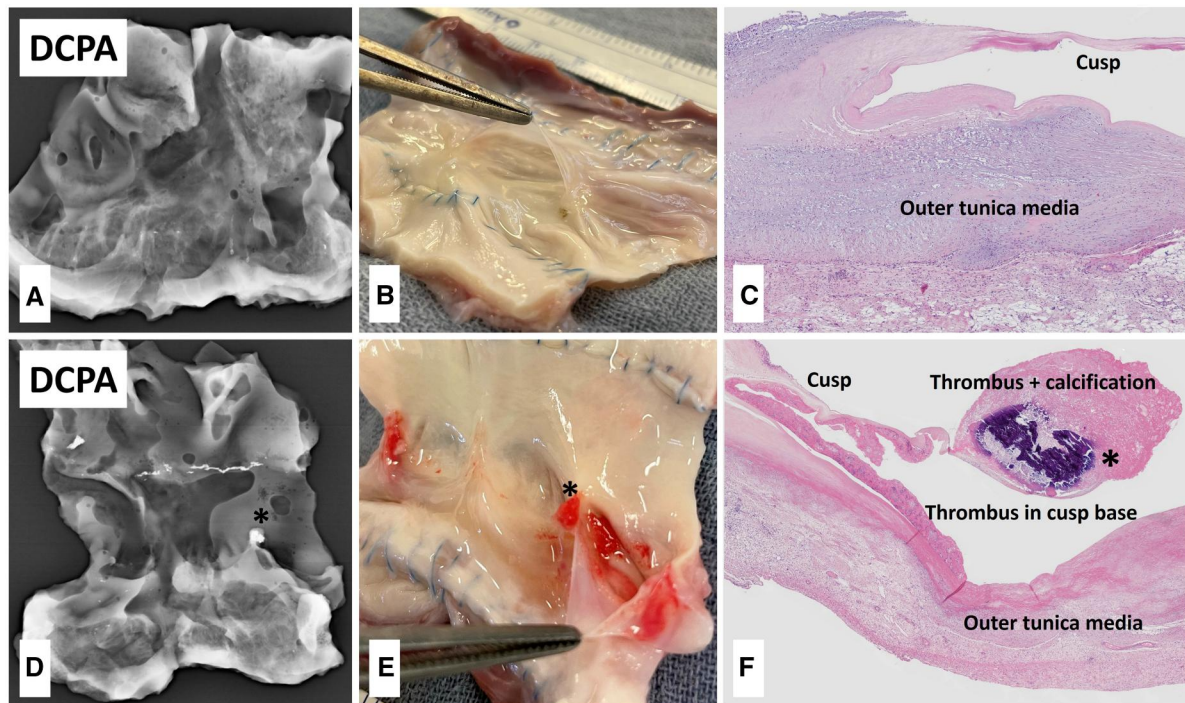


Figure 2: DCPA: X-ray imaging, macroscopy and histology (hematoxylin and eosin, 10× magnification). (A–C) Sheep 10. (D–F) Sheep 11. (A) Absence of calcification. (B) 6/9 DCPA sheep presented with smooth luminal surface, supple and thin leaflets. (C) Acellular cusp, cells present in outer tunica media, shading corresponding to nuclei. (D) Calcification at thrombus on commissure (*). (E) Thin, supple leaflets and smooth wall. Irregularities at 2/3 inter-leaflet commissures. (F) Acellular cusp, cells present in outer tunica media, thrombus in leaflet base extending to larger thrombus at commissure with calcified nodule (*).

inter-leaflet triangle, with a calcified nodule here in one sheep (sheep 11, Fig. 2D–F). One sheep (11.1%) showed extensively calcified nodular lesions, originating from two commissures, with calcification extending onto the leaflets without cusp damage (sheep 12, Fig. 5).

Concerning CPA specimens, a typical finding (7/8 sheep, 87.5%) was the formation of an irregular neo-intima/pannus, predominantly on the leaflets and to a lesser extent the wall (Fig. 3), as confirmed by semi-quantitative scoring (Supplementary Material, Table S4). In four CPA sheep, the papillomatous irregularities noted in DCPA were observed, associated with subvalvular calcification of residual right ventricular muscle of the grafts. In four (50%) sheep, protruding subvalvular calcifications were observed (Fig. 5).

Results of semi-quantitative scoring of X-ray images are shown in Supplementary Material, Table S5. In DCPA, subvalvular calcification was not observed, while it was reported in 6/8 (75%) CPA sheep (mean score 0 vs 1.94/3, $P=0.008$). For other regions, there was no significant difference.

Regarding quantitative calcium content, there were no significant inter-group differences revealed by absorption spectrometry for macroscopically non-calcified samples of leaflet, sinus, MPA or control pulmonary artery (Supplementary Material, Table S6 and Supplementary Material, Fig. S3).

Histological analysis

Histologically, there was no evidence for bacterial endocarditis in either group. DCPA leaflets consisted of connective tissue with scarce cells without inflammatory component (Figs 2 and 4). Intermittently, the cusp surface was covered by a layer of fibrin, with partial organization into loose connective tissue. In

two DCPA sheep (11 and 12), there was formation of a fibrin thrombus with central calcification at the free cusp edge (Figs 2 and 5). CPA specimens comprised thickened, cell-rich cusps with prominent inflammatory infiltrate of lymphocytes, macrophages and plasma-cells, and interstitial cell proliferations (Fig. 4). Apposition of luminal fibrin or organized neo-intima was more extensive than in DCPA with calcification commonly observed at the cusp base (Fig. 3).

Neo-intima formation was most pronounced on CPA wall with inflammatory infiltrates and ongoing cellular organization, as well as mature neo-intima (Fig. 3), corresponding with the macroscopically observed pannus. DCPA wall presented apposition of unorganized fibrin with low cellular content. Cellular populations within the wall are described below. In baseline samples, CPA showed preserved extracellular matrix architecture and cellular content, with nuclei observable at low magnification (Supplementary Material, Fig. S4, Supplementary Material, Appendix S1). DCPA focally contained shades of nuclei, only observable at high magnification.

Immunohistochemical analysis

In DCPA, the alpha-SMA stain showed proliferation of myofibroblasts in the outer tunica media, with virtually acellular inner media and leaflet. The luminal fibrin appositions lacked organization with absence of alpha-SMA-positive cells. CD3, PAX5 and CD11b stain showed a mild inflammatory reaction at the anastomosis and adventitia. Re-endothelialization, assessed by von Willebrand factor stain, was only observed near the anastomoses.

In CPA, the CD3, PAX5 and CD11b stain identified inflammatory cells diffusely scattered throughout the wall and leaflet. In the leaflet base, there were foci of calcification and

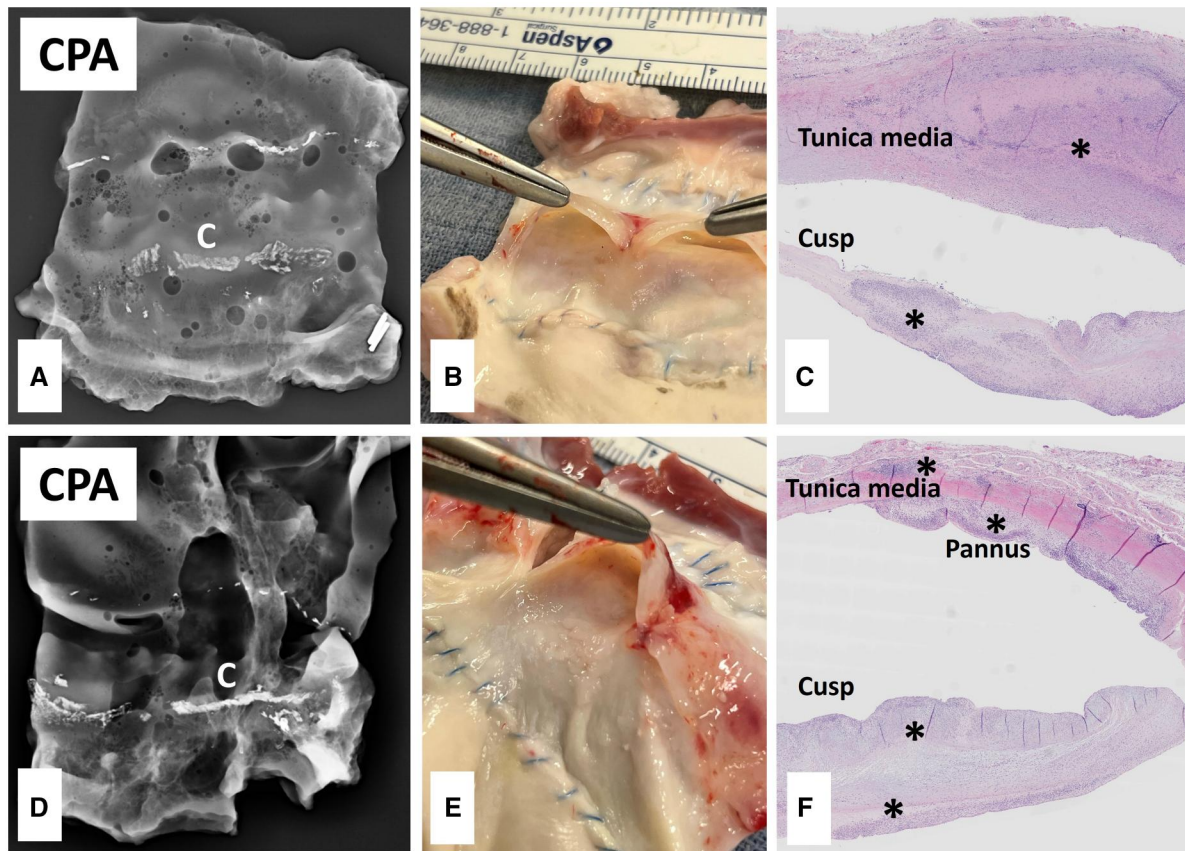


Figure 3: CPA: X-ray imaging, macroscopy and histology (hematoxylin and eosin, 10× magnification). (A–C) Sheep 16. (D–F) Sheep 15. (A) Subvalvular calcifications (C) and at distal anastomosis. (B) Macroscopically thickened leaflets. (C) Cusp thickening with inflammatory infiltrates (shading of nuclei, *) in wall and cusp. (D) Subvalvular calcifications (C). (E) Thickened leaflets, irregularities at commissures and on luminal wall surface. (F) Cusp thickening with inflammatory infiltrates (shading of nuclei, *) in cusp, wall and in pannus on allograft wall.

inflammation. The alpha-SMA stain showed ongoing organization of the neo-intima on wall and leaflet. CD3, PAX5 and CD11b stain identified marked inflammation associated with this neo-intima. In some cases, the neo-intima was highly organized and fibrous. As in DCPA, the anastomoses showed ratios of inflammatory cells typical for remodelling.

In baseline samples, alpha-SMA cellular shading pattern was preserved, indicating a degree of residual presence of this cytoplasmic protein. The hematoxylin counterstain showed remarkably less nuclear shades present in DCPA than in CPA.

DISCUSSION

The primary goal of this pilot study was to validate early *in vivo* outcomes and safety of a novel decellularization protocol, followed by cryopreservation. There were no perioperative or follow-up deaths related to our allografts, and at explantation, we did not observe pseudo-aneurysms, dehiscence or tissue decay. Both graft types were associated with a distinct macroscopic and histological appearance, in line with earlier preclinical studies showing an advantage of decellularized allografts [8]. Overall, DCPA presented smooth leaflets and wall, in most cases closely resembling pre-implantation tissue. With thin and supple leaflets, our findings match with previous ovine models using allografts decellularized via the protocol used in recent clinical trials (Fig. 2) [8, 9]. DCPA wall only contained inflammatory cells at the

anastomoses, and at 6 months, there was virtually no cellular repopulation of DCPA leaflets. In the wall, it was restricted to alpha-SMA-positive cells in the outer media, suggesting entry via the adventitia. Therefore, we conclude that these tissues are immunologically rather inert. In CPA, inflammatory cell infiltrates and alpha-SMA positive cells were found both in the centre of the leaflet, throughout the entire tunica media and on the luminal pannus, suggesting several origins. A previous sheep study with decellularized allografts reported incomplete tissue repopulation even after 20 months implantation [9]. While DCPA had remarkably lower cell content and neo-intimal coverage, we are not able to determine long-term importance of tissue cellularity and potential regenerative ability.

In three DCPA (33%) and four CPA (50%) sheep, papillomatous irregularities originated from the inter-leaflet triangle. Macroscopically and histologically, these lesions were consistent with Libman-Sacks, or ‘non-bacterial thrombotic’, endocarditis, consisting of sterile platelet and fibrin aggregates, typically occurring in patients with oncological or inflammatory disease [20]. In two DCPA sheep (11 and 12), thrombus served as nidus for calcification, and these findings represent the only calcification observed in DCPA. We hypothesize that these lesions were initiated by subclinical thrombosis with turbulent flow at the commissures in a postoperative inflammatory state [15, 21]. Similar proliferations were noted in four CPA sheep, yet here associated with an inflammatory infiltrate. We did not administer anti-aggregants as the incidence of prosthetic valve thrombosis in

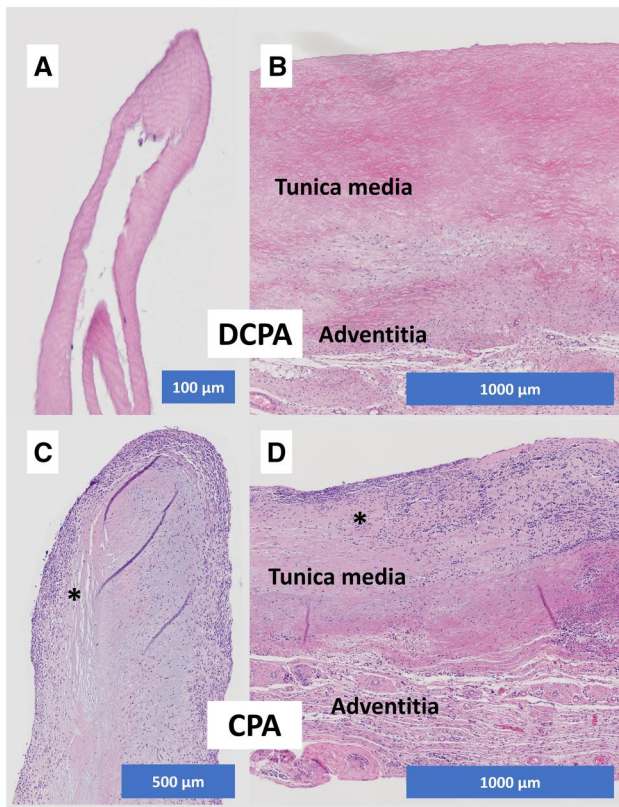


Figure 4: Hematoxylin and eosin, original magnification 20 $\times$. (A, B) DCPA. (C, D) CPA. (A) Sheep 10. Thin and acellular cusp. (B) Sheep 11. Acellular inner media, spindled cells (blue shading) present in outer media and adventitia. (C) Sheep 15. Cusp thickening with extensive infiltration of inflammatory cells (*). (D) Sheep 15. Cells present in entire wall with inflammatory infiltrates (*).

sheep is very low and they are ruminants with unpredictable oral absorption of medication [22]. In patients receiving a pulmonary allograft, aspirin is recommended postoperatively to reduce subclinical valve thrombosis and endocarditis [10].

In CPA, a typical finding was an irregular neo-intima or pannus with inflammatory cells, predominantly on the leaflets. This pannus, together with an inflammatory infiltrate within the cusps, resulted in remarkable leaflet thickening in CPA. Baraki et al. [8] found similar valve degeneration with thickened leaflets, wall calcification and marked leucocyte infiltration when comparing fresh and decellularized allografts in aortic position. This type of reaction was not seen in DCPA which presented with less neo-intimal coverage on histological examination. It seems likely that fibrin apposition and neo-intima formation with heightened inflammation due to residual donor antigens in CPA lead to excessive pannus formation [23]. The calcification we observed after 6 months is in line with the remarkable calcification potential of juvenile sheep [15, 24].

In both groups, there was one outlier sheep with pronounced, exophytic calcifications (Fig. 5 and [Supplementary Material, Appendix S1](#)). DCPA sheep 12 could not undergo early postoperative MRI due to pleuro-pericardial effusion and developed nodular lesions with calcified fibrin thrombus and focal wall damage. At necropsy, there were very dense adhesions, so perhaps a heightened postoperative inflammatory response and subclinical leaflet thrombosis was involved. CPA sheep 17

developed a PVPG of 29 mmHg at 2 weeks and showed thick leaflets with restricted mobility. At necropsy, there were extensive subvalvular calcifications, calcified proliferations on the leaflets as well as typical pannus. In neither case, there was evidence for bacterial endocarditis.

Both groups showed low transvalvular gradients and limited regurgitation, functionally irrelevant at 6 months ([Supplementary Material, Table S2](#)). Calcium content of macroscopically non-calcified wall and valve samples did not reveal inter-group differences at 6 months. Early calcification seems isolated to the leaflet base and sites of thrombus formation. While DCPA appear immunologically inert, we expect that in CPA, both the extensive subvalvular calcification of residual myocardial cells which disintegrate after implantation [25, 26], and inflammation within the wall and leaflets with pannus formation would become clinically relevant in the long-term. Long-term studies would be useful to provide more data on the relation between the observed histological differences and functional performance of grafts.

Limitations

We were not able to perform comparison with decellularized allografts used in recent trials. Our relatively low sample size infers a risk of type II error—not identifying differences which are actually present. On the other hand, histological, macroscopic and X-ray findings were so typical per allograft type, that we believe this mainly applies to potential differences in haemodynamic performance which may develop over time. While we did not administer aspirin, sheep are believed to have lower thrombogenicity than humans, while pigs provide an aggressive model for heart valve thrombosis [27]. Because previous preclinical studies on decellularized allografts were done in sheep, we applied the same model. For future studies, porcine experiments may be considered. As we believe the papillomatous proliferations we observed on several grafts in both groups originated from subclinical thrombosis, perhaps these could have prevented by anti-aggregant therapy. Furthermore, potential, yet unknown, biological differences between sheep and humans must be taken into account regarding calcification, inflammation and thrombosis pathways. As histological findings were consistent, immunohistochemistry served a descriptive purpose. For logistical reasons, operation sequence was not randomized.

CONCLUSIONS

Our findings suggest the early safety of the novel decellularization protocol. Haemodynamically, both clinical standard cryopreserved allografts and allografts decellularized according to a newly-developed protocol, and subsequently cryopreserved, produced equivalent and favourable outcomes. Each type of allograft was associated with a unique macroscopic and histological appearance, in favour of the decellularized technique as these tissues seemed to behave immunologically inert, and there was no pannus formation on the leaflets and less calcification on X-ray imaging.

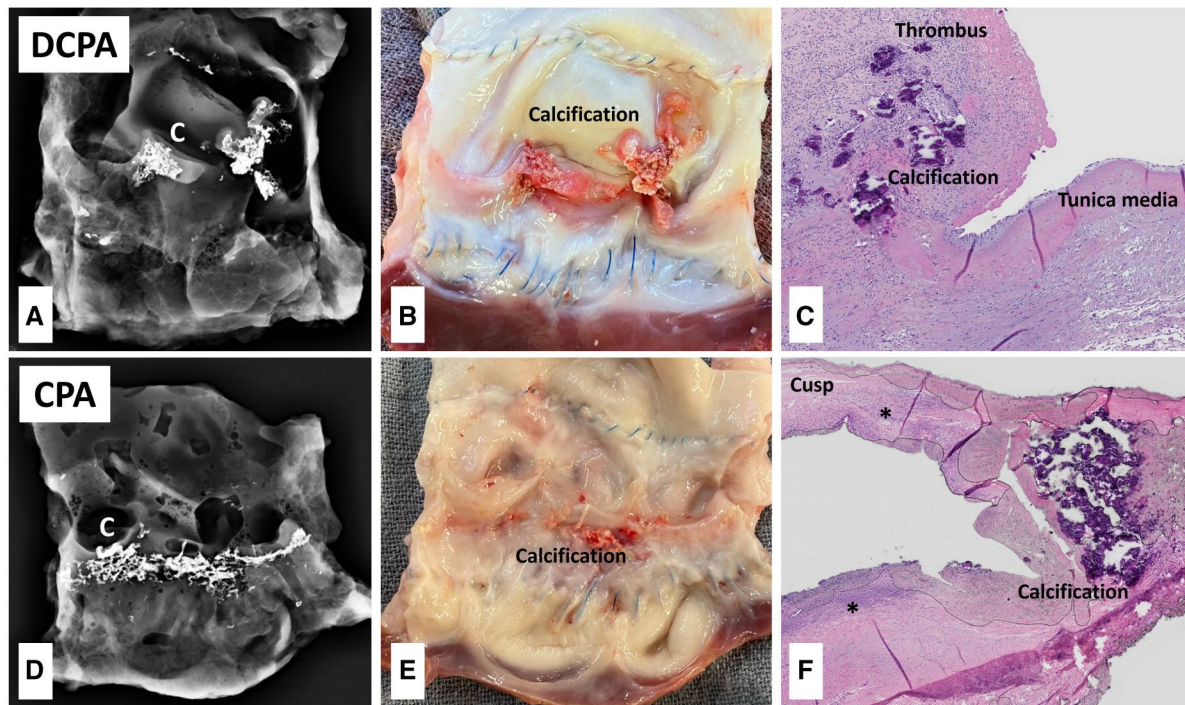


Figure 5: X-ray imaging, macroscopy and histology (hematoxylin and eosin, 10x magnification) for sheep with most pronounced calcifications. **(A–C)** DCPA, sheep 12. **(A, B)** Extensive nodular calcifications **(C)** on wall and on/under cusps. **(C)** Calcified thrombus with local wall ulceration. **(D–F)** CPA, sheep 17. **(D, E)** Extensive subvalvular and protruding calcification **(C)**. **(F)** Leaflet thickening, inflammatory infiltrates (*) and calcification in leaflet base.

SUPPLEMENTARY MATERIAL

[Supplementary material](#) is available at *ICVTS* online.

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CONFLICT OF INTEREST

None declared.

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DATA AVAILABILITY

Underlying data will be made available upon reasonable request.

Author contributions

Lucas Van Hoof, MD: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Visualization; Writing—original draft; Writing—review & editing. **Geoffroy de Beco:** Data curation; Formal analysis; Supervision; Visualization; Writing—original draft. **Manon Van Hecke:** Data curation; Formal analysis; Investigation; Methodology; Writing—review & editing. **Piet Claus:** Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Writing—review & editing. **Alexander Van De Bruaene:** Data curation; Formal analysis; Software; Writing—review & editing. **Tania Roskams:** Data curation; Formal analysis; Methodology; Supervision; Writing—review & editing. **Cristina Castells-Sala:** Investigation; Methodology; Resources; Writing—review & editing. **José Rodríguez Martínez:** Methodology; Project administration; Resources. **Ana Vilarrodona:** Conceptualization; Investigation; Methodology; Project administration; Supervision; Validation; Writing—review & editing. **Stefano Mastrobuoni:** Conceptualization; Methodology; Project administration; Resources; Supervision; Writing—review & editing. **Ramadan Jashari:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing—original draft; Writing—review & editing. **Filip Rega:** Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing—original draft; Writing—review & editing.

Reviewer information

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