Coating with fibronectin and stromal cell–derived factor-1 α of decellularized homografts used for right ventricular outflow tract reconstruction eliminates immune response–related degeneration

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Objective: This study assesses the performance and cellular features of decellularized ovine aortic homografts coated with stromal cell–derived factor-1 α (SDF-1 α) and its natural linker, fibronectin (FN), after implantation in the right ventricular outflow tract of adolescent sheep.

Methods: Right ventricular outflow tract reconstructions using cryopreserved (n = 7), decellularized (n = 8), and decellularized FN/SDF-1 α –coated aortic ovine homografts (n = 6) were performed. Echocardiographic, morphologic, radiographic, histologic, and immunohistochemical examinations were performed 5 months after implantation.

Results: There were no hemodynamic differences between groups, except for the decellularized homografts' tendency to develop more valve regurgitation (3 of 8 grafts had regurgitation >2/4). All decellularized, but coated, grafts had normal hemodynamics. Decellularized valve conduits were less calcified than cryopreserved conduits ($P < .05$), but coated valve conduits were free of calcification ($P < .05$). The same was found for pannus in the outflow parts. Immune response (CD45⁺, CD45R⁺, or CD11b⁺ cells) was decreased in decellularized valves compared with cryopreserved grafts, but was virtually absent ($P < .05$) in coated grafts. Collagen organization and density in the leaflets and walls were decreased in cryopreserved and decellularized valves, but not in coated valves ($P < .05$). Coating improved re-endothelialization ($P < .05$).

Conclusions: Coating of decellularized allografts with FN/SDF-1 α prevents cryopreserved heart valve–mediated immune response, conduit calcification, and pannus formation and stimulates re-endothelialization. (J Thorac Cardiovasc Surg 2014;147:1398–404)

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Clinical problems with cryopreserved homografts after the Ross procedure are not related to the valve leaflets, but rather to supra-avalvular conduit structures (ie, root calcification and intimal hyperplasia of the conduit).¹ Because root calcification is related to calcium deposition on cell remnants in the graft, several investigators hypothesized that acellularization of the graft could prevent these calcifications.² However, immune response is not completely eliminated by decellularization alone, and inflammatory

cell infiltration and intimal hyperplasia in the conduits, pannus formation, conduit degeneration, and calcification remain a hazard. Therefore, additional coating with fibronectin (FN)/stromal cell–derived factor 1 α (SDF-1 α) might be effective.

It is speculated,³ but not confirmed,^{2,4} that in decellularized homografts spontaneous recellularization will occur after implantation, revitalizing the graft with autologous cells. However, spontaneous recellularization of acellular homografts will be no more than a foreign body reaction induced by innate or natural immunity. CD11b⁺ macrophages accumulate at the interface between the native tissue and the graft and participate in the formation of granulation tissue and fibroblasts. Finally, a fibrotic capsula or “pannus,” detrimental for the function of the implant, surrounds the biomaterial implant. Also, stem cells, which participate in inflammation and are recruited to the injury sites, play a role in wound healing and tissue regeneration because of their unique regenerative properties. Recent studies have shown that the implantation of biomaterials may prompt the recruitment and local engraftment of autologous stem cells, having the potential to recellularize the matrices.^{5,6} However, the inflammation-related intimal hyperplasia

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Abbreviations and Acronyms

ASMA	= alpha smooth muscle actin
FN	= fibronectin
IC	= interstitial cell
RVOT	= right ventricular outflow tract
SDF	= stromal cell–derived factor
SMMS	= smooth muscle myosin heavy chain

due to macrophage differentiation is much more pronounced than the true tissue regeneration related to stem cell attraction and adequate differentiation.⁶ Therefore, we were specifically interested in SDF-1 α because this factor was shown, in addition to enhancement of local recruitment of autologous stem cells, to modify and bypass the detrimental inflammatory cell responses at the implant interface.⁶⁻⁸

In this context, we studied the outcome of implanted cryopreserved, acellularized, and FN/SDF-1 α –enriched acellular aortic homografts in a sheep model. We have chosen the aortic, instead of the pulmonary, homograft because it is shown clinically⁹ and experimentally¹⁰ that the aortic homograft has the most from outflow tract obstruction and, thereby, became the second choice. Improvement of the durability of the aortic homograft would expand the available pool of suitable homografts significantly.

METHODS

All animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th edition, 2011). Eleven Lovenaar sheep were used for harvesting/cryopreservation of ovine aortic homografts. Furthermore, 14 aortic ovine valve conduits were purchased from the local slaughterhouse and processed for decellularization and eventual coating, as described later. Twenty-one other Lovenaar sheep were used as valve recipients undergoing right ventricular outflow tract (RVOT) reconstruction, according to the protocol described later.

The study was approved by the ethics committee for animal experiments of the University of Leuven, Leuven, Belgium.

Cryopreserved Homografts

Hearts were harvested from male Lovenaar sheep (n = 11; age, 371 [260–494] days; weight, 59 [50–82] kg) under sterile conditions, after full heparinization, and immediately transferred to European Homograft Bank¹¹ in 4°C sterile saline. Aortic valve conduits were dissected and prepared for cryopreservation in a vertical laminar flow (class A) clean room. Further details about the cryopreservation procedure were already described.¹¹ Four cryopreserved aortic conduits remained unimplanted and were fixed in 4% paraformaldehyde for histology. The other 7 were implanted as described later.

Decellularization and Impregnation

Fourteen aortic ovine valve conduits, obtained from the local slaughterhouse, were decellularized by detergents (sodium dodecyl sulfate and Triton X-100), according to the protocol already described by Ueda and coworkers.¹²

Six of these were impregnated with human FN (32 $\mu\text{g}/\text{cm}^2$; Biomedical Technologies, Stoughton, Mass) and SDF-1 α (3.2 $\mu\text{g}/\text{cm}^2$; Miltenyi Biotec,

Bergisch Gladbach, Germany) (each for 24 hours at 4°C), as already described.^{7,8}

Valve Implantation

Twenty-one female Lovenaar sheep were selected (age, 246 [151–418] days; weight, 43 [30–57] kg) and randomly allocated to 3 groups, of which each animal received an implant in pulmonary position, as listed: (1) cryopreserved homografts (n = 7), (2) decellularized homografts (n = 8), and (3) coated decellularized homografts (n = 6).

The implantation technique was described before.¹⁰ In short, a left thoracotomy was performed. After heparinization, cardiopulmonary bypass was installed after cannulation of the left carotid artery and jugular vein. Under conditions of normothermia and beating heart, the pulmonary artery was crossclamped proximal from its bifurcation and transected just distal from the commissures. The native pulmonary valve leaflets were resected, and the homograft was implanted as a root replacement.

Echocardiography

On all animals undergoing RVOT reconstruction, transthoracic echocardiography was performed at 1 week and 5 months by an experienced echocardiographer using a Vivid Five echo system and a 2.5-MHz GE Ultrasound probe (GE Medical Systems, Milwaukee, Wis). The mean gradient and regurgitation scores were assessed with continuous-wave or color-flow Doppler echocardiography, respectively.

Explantation

At 5 months after implantation, the animals were heparinized (3 mg/kg) and sacrificed by an overdose of pentobarbital (Nembutal; Ovation Pharmaceuticals Inc, Deerfield, Ill) and KCl intravenously. Native pulmonary valve controls (n = 6) were harvested from nonconflicting experiments. Each leaflet was examined with X-ray films (Faxitron; Faxitron Bioptics, Tucson, Ariz) in a horizontal plane perpendicular to the inflow-outflow axis. Calcium content was spectrophotometrically quantified for conduit wall and leaflets with a calcium kit (Chema Diagnostica, Monsano, Italy), as already described.¹⁰

Histology and Immunohistochemistry

Thickness (6-measurement average) and area of pannus were quantified by planimetry on hematoxylin-eosin–stained sections at the wall and leaflet portions. The amounts of collagen and organized collagen were assessed by picrosirius red staining, as already described.¹³

Cryosections were immunohistochemically stained and quantified for the following markers in both leaflet and wall portions¹⁴: vimentin (Dako, Glostrup, Denmark), alpha smooth muscle actin (ASMA) (1A4; Dako), desmin (D33; Serotec, Oxford, UK), smooth muscle myosin heavy chain (SMMS)–1 (Dako), smoothelin (polyclonal; Santa Cruz Biotechnology, Santa Cruz, Calif), endothelial nitric oxide synthetase (3; BD Pharmingen, Franklin Lakes, NJ), CD45 (1.11.32; Serotec), CD11b (CC126; Serotec), CD45R (20.96; Serotec), CD8 (BAT82A; VMD Inc, Pullman, Wash), and BB-1 (BAS9A; VMD Inc). Cell nuclei were visualized with Vectashield 4',6-diamidino-2-phenylindole–containing mounting medium (Vector Laboratories, Burlingame, Calif).

Pictures were taken using an Axioplan 2 imaging microscope with an Axiocam MRc5 camera (Zeiss, Oberkochen, Germany) and these objective lenses: Plan-Neofluar 1 $\times$ /0.025, Plan-Apochromat 1 $\times$ /0.45, 20 $\times$ /0.75, and 100 $\times$ /1.4. Axiovision Rel. 4.4. software (Zeiss) was used for image analysis and morphometry.

For comparison, native ovine aortic and pulmonary valve conduits were obtained from nonconflicting experiments (n = 5) and assessed for histology, immunohistochemistry, and collagen density and organization.

Calculations and Statistical Analysis

Cell counts were obtained by assessing maximally 500 cells (total count) and are expressed as the percentage of positively staining cells on the total count. Functional endothelialization was quantified by measuring the inner luminal length and the segments staining positive for endothelial nitric oxide synthetase and expressed as the fraction of (endothelium length/inner luminal length) $\times$ 100. Collagen density and organization are calculated as previously described.¹⁵

Data were first compared with a Kruskal-Wallis test. If the obtained *P* value was lower than .05, intergroup comparisons were performed with a Wilcoxon-Mann-Whitney test (SPSS 15.0 for Windows; SPSS, Inc, Chicago, Ill). *P* < .05 was considered statistically significant.

RESULTS

Operative Results and Explantation

All 21 valves were successfully implanted and remained functional for up to 5 months, after which they were explanted. None of the valves showed signs of infection or aneurysm formation.

Echocardiography

Echocardiographic data are summarized in Table E1. The diameter of the pulmonary annulus remained unchanged in all subgroups. Peak velocity across the valves did not change significantly over time (*P* > .05). Regurgitation (score $>1/4$) was never seen at 7 days postoperatively but became apparent in 1 of 7 cryopreserved aortic homografts and in 3 of 8 decellularized aortic homografts at 5 months. None of the coated decellularized grafts showed any regurgitation at 5 months.

Calcification

Calcium content data at 5 month postimplantation are summarized in Table 1.

Calcium content in the leaflets is low and not different between the subgroups (*P* = .732). A calcium content level of 3 $\mu\text{g}/\text{mg}$ dry weight is considered as normal in bioprosthetic leaflets.¹³

Cryopreserved homografts showed extremely high calcium contents in the conduit wall (Table 1). Decellularization reduced conduit calcification significantly compared

with cryopreservation (*P* < .05) (Table 1), but could not eliminate it. Typical examples of Faxitron imaging of aortic wall end leaflet sections are shown in Figure 1. Localization of wall calcifications is shown in histologic sections of cryopreserved and decellularized homografts, but not in coated grafts (Figure E1).

Coating combined with decellularization eliminated calcification in the wall portion of the conduits: calcium content levels were approximately 1 $\mu\text{g}/\text{mg}$ dry weight only (Table 1 and Figures 1 and 2, A).

Endothelialization

In cryopreserved aortic homografts, endothelialization at 5 months after implantation was $39\% \pm 8\%$ and $37\% \pm 5\%$ in the leaflet and the wall, respectively. In decellularized homografts, endothelialization is low: only 10% to 15% endothelialization was found at explantation (Figure 2, C). Coating of these significantly improves endothelialization (*P* < .05) (Figure 2, C), and the observed values are comparable to those of cryopreserved homografts. A typical example of partial endothelialization of the leaflet of a coated decellularized graft is shown in Figure E2.

Pannus Formation

Pannus formation (Table E2) is most pronounced in cryopreserved homografts more specifically in the outflow side of the conduit. Decellularization of the homograft significantly reduced pannus formation in this area (*P* < .05). After coating and decellularization, pannus diminished further significantly (*P* < .05) and remains nearly undetectable in the outflow tract (Figure 3).

Immune Response

As markers for the immune response, the incidence of CD45⁺, CD45R⁺, CD11b⁺, CD8⁺, or BB1⁺ cells was calculated. BB1⁺ and CD8⁺ cells were not detected in any of the homograft subgroups. The incidence of CD45⁺, CD45R⁺, and CD11b⁺ cells in the homograft leaflets and conduit portions is listed in Table 2.

Decellularization alone had no effect on the presence of CD45⁺ cells compared with cryopreservation (*P* > .05). However, the incidence of CD45R⁺ and CD11b⁺ cells is systematically lower in the group of decellularized homografts, although only statistically significant in the leaflets.

The coating combined with decellularization decreased the incidence of CD45⁺ cells further, and this decrease was statistically significant in the leaflets and in the wall portion (*P* < .05) (Table 2 and Figure 2, D).

Extracellular Matrix

Collagen density and organization (Table E3) are studied in explanted homografts and compared with those of native

TABLE 1. Calcium content ($\mu\text{g}/\text{mg}$ dry weight) in the cryopreserved, decellularized, and coated decellularized aortic homografts

Variable	Aortic homograft valve		
	Cryopreserved	Decellularized	Decellularized + FN/SDF-1 α
Conduit			
Inflow	28.8 $\pm$ 6.6	6.3 $\pm$ 3.1*	1.2 $\pm$ 0.3*,†
Outflow	157.3 $\pm$ 13.3	10.2 $\pm$ 4.0*	1.0 $\pm$ 0.2*,†
Leaflet	3.8 $\pm$ 0.8	3.1 $\pm$ 0.6	2.5 $\pm$ 1.3

Data are represented as mean $\pm$ SEM. FN, Fibronectin; SDF, stromal cell–derived factor. *Significant difference from cryopreserved homografts (*P* < .05). †Significant difference from decellularized homografts (*P* < .05).

FIGURE 1. Faxitron images of transversal valve segments after 5 months' implantation. The cryopreserved homograft (A) illustrates the wall calcification, a typical complication in these prostheses. The decellularized graft (B) showed limited calcification. The decellularized and coated homograft (C) is devoid of any calcification.

valves and of nonimplanted decellularized homografts. In explanted cryopreserved homografts, collagen organization is significantly decreased in all layers of the leaflets ($P < .05$). Collagen density is also decreased, but mainly in the ventricularis layer of the leaflets ($P < .05$). The decellularization process itself (data on nonimplanted, decellularized homografts) (Table E3) also reduces collagen organization in all layers of the leaflets and in the wall portion ($P < .05$). It typically decreases collagen density in the spongiosa of the leaflets and in the wall.

Five-month implantation of decellularized aortic homografts has little or no effect on the restoration of collagen organization and density.

Coating of decellularized homografts has a profound effect on collagen organization and density after implantation. Recovery of collagen organization and density is complete in practically all layers of the leaflets and in the wall and is superior to that in decellularized or cryopreserved homografts (Table E3 and Figure 2, D).

Interstitial Cells

The density of interstitial cells (ICs) was determined in native pulmonary and aortic valve leaflets of non-implanted cryopreserved and explanted homografts (Table E4). Cell counting was performed within the leaflets (ingrowth). In nonimplanted cryopreserved homografts, the IC density did not differ from that in native leaflets ($P > .05$).

After implantation, cryopreserved homograft leaflets seem to have fewer ICs than before, but the difference is not statistically significant ($P > .05$).

In decellularized homografts, recellularization within the leaflets is scarce after 5 months of implantation: approximately 4% to 5% ($P < .05$ vs cryopreserved). In decellularized homografts coated with FN/SDF1 α , the ingrowth of cells in the leaflets tends to be higher than in noncoated counterparts, but the difference is not statistically significant ($P > .05$) (Table E4).

Immunohistochemical Analysis of IC Phenotypes

Table E5 contains data on the percentage of cells staining positive for vimentin, ASMA, SMMS, smoothelin, or desmin.

In native valves, the difference in IC phenotype between the leaflets and the wall is a higher ASMA⁺ cell fraction in the wall (Table E5).

Cryopreserved valves, and their leaflets as their wall, show an increase in the fraction of vimentin⁺ cells, suggesting an increase in fibrocytes after 5 months of implantation.

Decellularized homografts, coated or not, show high fractions of ASMA⁺ cells, together with low fractions of SMMS⁺, smoothelin⁺, and desmin⁺ cells, in their leaflets. This suggests the presence of mainly myofibroblasts.

The conduit of uncoated, decellularized homografts seems characterized by a relatively low fraction of ASMA⁺ cells and a high fraction of desmin⁺ cells. This is not the

FIGURE 2. The summarized effects of coating of a decellularized homograft on calcification of the homograft wall (A), collagen density in the 3 layers of the leaflet and in the wall (B), endothelialization on the leaflets and the wall (C), and leukocyte population in the leaflets and the wall (D). The data are represented as average and SEM. $P < .05$ versus decellularized grafts (asterisk). FN, Fibronectin; SDF, stromal cell–derived factor.

case in coated decellularized conduit walls: herein, we find a high fraction of ASMA⁺ cells combined with more vimentin⁺ cells.

This suggests that ICs in decellularized conduit walls are characterized by a smooth muscle cell type, whereas coating promotes fibroblast and myofibroblast cell types (Table E5).

DISCUSSION

As could be expected, our results show degeneration of cryopreserved homografts: the leaflets develop an impaired collagen organization; inflammation or accumulation of CD45⁺, CD45R⁺, and CD11b⁺ cells is still present at

5 months. Endothelial coverage and the number of ICs found in the leaflets are reduced, and there is significant pannus accumulation. It remains unknown whether the remaining interstitial cells are still of donor origin or

Data represented as mean $\pm$ SEM. *, # significant difference from their native or cryopreserved counterparts respectively.

FIGURE 3. Summary of decellularization and/or fibronectin (FN)/stromal cell–derived factor (SDF)-1 α -coating effects on pannus deposition on the homograft outflow portion. Decellularization markedly decreases the deposition of pannus, yet FN/SDF-1 α coating further decreases the amount of pannus to levels close to 0. Significant difference from cryopreserved (asterisk) and decellularized (number sign), respectively.

TABLE 2. Immune response in aortic homografts

Marker	Cryopreserved	Decellularized	Decellularized + coated
Leaflet			
CD45	10.7 $\pm$ 1.6	14.3 $\pm$ 2.7	2.9 $\pm$ 1.3*,†
CD45R	7.6 $\pm$ 3.9	0.2 $\pm$ 0.1*	0.4 $\pm$ 0.2
CD11b	1.2 $\pm$ 0.8	0.0 $\pm$ 0.0*	0.0 $\pm$ 0.0
Conduit			
CD45	9.2 $\pm$ 2.2	11.5 $\pm$ 3.4	1.1 $\pm$ 0.4*,†
CD45R	3.8 $\pm$ 1.2	1.3 $\pm$ 0.4	0.1 $\pm$ 0.1
CD11b	1.6 $\pm$ 1.3	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0*,†

Data are represented as mean $\pm$ SEM. *Significantly different from cryopreserved. †Significantly different from decellularized.

autologous. The outflow tract is severely calcified. The phenotype of cryopreserved aortic homograft ICs suggests an accumulation of fibroblasts after 5 months. These results confirm studies by others on cryopreserved homografts.¹⁶ In patients, in case of the Ross operation, not the homograft valve but the conduit may become the problem, mainly because of supralvalvular stenosis in smaller than 14-mm conduit sizes.¹

In our study, homograft decellularization partially reduces wall calcifications and induces a strong tendency of regurgitation: 3 of 8 grafts have a valvular insufficiency of more than 2 of 4. In the leaflets, collagen density and organization are decreased and there is little spontaneous recellularization: we found 10% to 15% endothelial cell coverage, and the total IC count in the leaflets was low. The phenotype of the cells grown in the leaflets shows the presence of myofibroblasts. Cells repopulating the conduit walls, however, have another phenotype: they present a decreased fraction of ASMA⁺ cells in association with a significantly increased fraction of desmin⁺ cells, indicative of differentiation toward smooth muscle cells. Inflammation seems to be reduced after decellularization, but there is still a certain fraction of CD45R⁺ and CD11b⁺ cells detectable. Pannus formation is significantly less pronounced than in cryopreserved homografts. Hopkins and colleagues^{2,3} described that cryopreserved-decellularized pulmonary homografts retain normal function in sheep, but we could show that such aortic homografts are more prone to valvular insufficiency, maybe because of decreases in collagen density and organization. Other researchers³ recognized that decellularized allografts have the potential for autologous recellularization. Yet, none of the valves underwent significant leaflet recellularization beyond the cusp base, potentially limiting the long-term performance. We found a limited degree of recellularization in the leaflets: endothelial coverage was 10% to 15%, and the number of ICs was approximately 3% to 5% of those of native leaflets.

Based on the observation that decellularized allografts have a reduced immunogenicity, human implantations in the right ventricular outflow tract were performed. Cebotari and colleagues¹⁷ found freedom from reoperation of 100% at 5 years, but 3 of 38 patients had moderate regurgitation during follow-up. As shown with our previous studies, the combination of both FN and SDF-1 α yields the best result from the perspective of cell composition and long-term results when applied to a cross-linked biological prosthesis. We showed that impregnation with FN/SDF-1 α of pulmonary valve prostheses, constructed from completely acellular bovine photo-oxidized pericardium, induces complete recellularization of these valves when implanted in the RVOT position.⁷ Although FN and SDF-1 α have had some interchangeable effects on homing of primitive cells, both have distinct actions and functions as well. The large FN molecule bears several moieties, of which the

most important are related to matrix and cell binding. For interaction with graft materials, the collagen binding site is key, yet the cellular binding is mediated mainly by interaction with $\alpha 5 \beta 1$ and $\alpha 4 \beta 1$ integrins and by cell surface glycosylations binding to the heparin-binding domain.¹⁸ Crucial for this application is FN's ability to bind and properly present SDF-1 α to the cells, thereby ascertaining proper function.¹⁹ Although it has not been directly linked to calcification, SDF-1 α plays an important role in regulating the immune response by, among others, preventing leukocyte (mainly T-cell) accumulation,²⁰ of which calcification related to antigen-mediated immune response is the result. We believe that the anti-inflammatory effect of SDF-1 α is the primary mechanism of improved long-term function and not so much the multipotent cell homing capacity. In our experience, the coating clearly influenced the immune response by significantly decreasing the presence of leukocytes (CD45) combined with a complete absence of calcium deposits. Such a decreased content of CD45⁺ cells might be in accordance with the fugetaxis of immune cells observed by others.^{20,21} Another possibility is that SDF-1 α provides an interaction with recipient tissue macrophages to polarize toward an M2 phenotype. In an (unpublished) microarray gene expression study, we found an altered pattern of genes expressed indicative for a transition of the macrophage phenotype toward an alternatively activated macrophage. Intriguingly, we have also observed a clear and positive long-term effect on the matrix. Because there is no increased recellularization, the most probable mechanism relates to the induction of a different phenotype of cells able to regulate extracellular matrix turnover. SDF-1 α has a matrix-stabilizing effect by decreasing the expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1,²¹ but again, this probably relates to a shift of the macrophage phenotype from M1 to M2. A clear association exists between macrophage phenotype and remodeling outcome in extracellular membrane-based scaffolds.²²

Finally, the results relating to pannus formation and endothelialization are less obvious to interpret. There is literature linking SDF-1 α to neointimal hyperplasia in several conditions and experimental setups²³ and to endothelialization by homing of endothelium-related cells or progenitors.²⁴ However, the actions of SDF-1 α are strongly dependent on cellular reactions and interactions with the milieu. For example, the presence of other cytokines induces CD34⁺ cell proliferation and survival; on binding to a substrate, such cells increase the available chemokine (C-X-C-motif)-4 on their surface.²⁵ In this interaction between SDF-1 α and cellular environment, NO, as produced by endothelial nitric oxide synthetase of the present endothelium, has also been shown as an important mediator.²⁶

As such, the long-term results can be explained by homing of the appropriate progenitor cells, but it is more than probable that the milieu provided by the FN/SDF-1 α coating instigates a cellular reaction leading to faster healing and better controlled tissue deposition and inflammatory reaction.

Study Limitations

The repopulating cell phenotypes are assessed at the protein expression level, using immunohistochemistry. These observations are basically qualitative methods converted to a numerical score, and they should be confirmed in a future study by protein biochemical content assays, assessment of collagen remodeling pathways, and procollagen to collagen synthesis pathways.

Clinical Relevance of the Study

Although homografts are still the first choice for RVOT reconstruction, their durability and availability remain severe drawbacks. Mainly, homografts originating from aortic root tissue have outflow tract calcifications and neointimal proliferations. This study suggests that decellularization of homografts, combined with FN/SDF-1 α coating technology, can eliminate these complications. This should allow, in addition to the use of pulmonary homografts, use of the aortic homograft for RVOT reconstruction in congenital heart surgery.

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FIGURE E1. Hematoxylin/eosin-stained sections of cryopreserved (A), decellularized (B), and coated decellularized (C) homografts. The aortic root of the cryopreserved homograft is heavily calcified, and the leaflet is thickened. There is residual calcification in the sinus area of the decellularized graft and complete absence of calcium deposition in the coated graft.

FIGURE E2. Partial endothelial layer (endothelial nitric oxide synthase, green; nuclei, blue) of the leaflet of a coated, decellularized homograft.

TABLE E1. Echocardiographic data 7 days and 5 months after implantation of aortic homografts

Variable	No. of observations	Time	
		7 d	5 mo
Annulus diameter, cm			
Cryopreserved	7	2.03 ± 0.08	2.00 ± 0.09
Decellularized	8	1.90 ± 0.04	1.91 ± 0.06
Coated	6	2.13 ± 0.06	2.22 ± 0.08
Peak velocity, m/s			
Cryopreserved	7	2.11 ± 0.23	1.55 ± 0.16
Decellularized	8	1.96 ± 0.11	2.00 ± 0.23
Coated	6	1.96 ± 0.32	1.54 ± 0.15

Data are represented as mean ± SEM. None of the 5-month data differed from the corresponding 7-day values ($P > .05$).

TABLE E2. Pannus formation on cryopreserved, decellularized, and coated decellularized aortic homografts

Variable	Leaflet	Conduit		
		Inflow	Outflow	Total wall
Area, mm ²				
Cryopreserved	0.34 ± 0.14	1.17 ± 0.50	4.68 ± 0.81	2.92 ± 0.67
Decellularized	0.12 ± 0.05	0.18 ± 0.09	1.59 ± 1.06*	0.88 ± 0.54*
Coated and decellularized	0.25 ± 0.10	0.31 ± 0.25	0.13 ± 0.10*,†	0.22 ± 0.13*

Data are given in mm² and are represented as mean ± SEM. *Significant difference from the cryopreserved counterparts. †Significant difference from the decellularized counterpart.

TABLE E3. Collagen density and organization in the native, cryopreserved, decellularized, and coated decellularized aortic homografts

Variable	Leaflet			Conduit
	Fibrosa	Spongiosa	Ventricularis	
Collagen density				
Native valves	98 ± 1	93 ± 1	99 ± 0	95 ± 1
Unimplanted decellularized homografts	94 ± 3	46 ± 5*,†,‡	96 ± 2†	75 ± 1*,†,‡
Explanted homografts				
Cryopreserved	84 ± 7	79 ± 12	84 ± 5*	80 ± 4*
Decellularized	88 ± 4*	57 ± 8*	86 ± 3*	75 ± 5*
Decellularized and coated	100 ± 0†,§	98 ± 1*,§	100 ± 0*,†,§	98 ± 0*,†,§
Organized collagen				
Native valves	67 ± 4	2 ± 0	28 ± 5	17 ± 3
Unimplanted decellularized homografts	17 ± 7*,†,§	0 ± 0*,†	15 ± 7†,§	0 ± 0*,†,†,§
Explanted homografts				
Cryopreserved	18 ± 8*	0 ± 0*	2 ± 1*	16 ± 5
Decellularized	39 ± 6*	2 ± 1	9 ± 3*,†	25 ± 4
Decellularized and coated	75 ± 5†,§	5 ± 2†	31 ± 7†,§	28 ± 4

Data are represented as mean ± SEM. *Significant difference from the native counterparts. †Significant difference from the cryopreserved counterparts. ‡Significant difference from the decellularized counterparts. §Significant difference from the coated decellularized counterparts.

TABLE E4. Cell count of aortic valve leaflets

Variable	Cell count (ingrowth)
Native valves	7932 ± 1386
Unimplanted homografts	
Cryopreserved	8347 ± 1716
Explanted homografts	
Cryopreserved	5329 ± 1265
Decellularized	334 ± 146*,†
Decellularized and coated	1007 ± 455*,†

Data are represented as mean ± SEM. *Significant difference from the native counterparts. †Significant difference from the implanted cryopreserved counterparts.

TABLE E5. Immunohistochemistry

Variable	Native	Cryopreserved	Decellularized	Decellularized and FN/SDF1α
Leaflet				
Vimentin	33.4 ± 1.7	52.4 ± 9.6*	39.8 ± 7.6†	34.8 ± 6.9‡
ASMA	14.4 ± 0.8	12.3 ± 4.1	27.5 ± 7.5	34.4 ± 8.9*
SMMS	3.7 ± 0.7	0.3 ± 0.2*	0.3 ± 0.1*	1.1 ± 0.8*
Smoothelin	2.0 ± 0.4	0.6 ± 0.3*	0.7 ± 0.3*	0.1 ± 0.1*
Desmin	1.2 ± 0.2	0.7 ± 0.4	0 ± 0*,†	0.9 ± 0.5‡
Wall				
Vimentin	32.5 ± 1.5	81.0 ± 2.2*	35.5 ± 5.6†	61.5 ± 7.9*,‡
ASMA	34.8 ± 5.2	41.6 ± 7.1	19.7 ± 2.2*,†	32.2 ± 10.5
SMMS	6.3 ± 0.8	5.2 ± 1.5	1.5 ± 1.5*	0.9 ± 0.9*,†
Smoothelin	5.1 ± 0.6	2.5 ± 0.8*	0.2 ± 0.1*,†	0 ± 0*,†
Desmin	2.8 ± 0.5	4.8 ± 2.4	11.1 ± 2.1*,†	5.6 ± 3.6

Data are represented as mean ± SEM. FN, Fibronectin; SDF, stromal cell–derived factor; ASMA, alpha smooth muscle actin; SMMS, smooth muscle myosin heavy chain. *Significant difference from the native counterparts. †Significant difference from the cryopreserved counterparts. ‡Significant difference from the decellularized counterparts.