Mechanical Compliance and Immunological Compatibility of Fixative-Free Decellularized/Cryopreserved Human Pericardium

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Abstract

Background: The pericardial tissue is commonly used to produce bio-prosthetic cardiac valves and patches in cardiac surgery. The procedures adopted to prepare this tissue consist in treatment with aldehydes, which do not prevent post-graft tissue calcification due to incomplete xeno-antigens removal. The adoption of fixative-free decellularization protocols has been therefore suggested to overcome this limitation. Although promising, the decellularized pericardium has not yet been used in clinics, due to the absence of proofs indicating that the decellularization and cryopreservation procedures can effectively preserve the mechanical properties and the immunologic compatibility of the tissue.

Principal Findings: The aim of the present work was to validate a procedure to prepare decellularized/cryopreserved human pericardium which may be implemented into cardiovascular homograft tissue Banks. The method employed to decellularize the tissue completely removed the cells without affecting ECM structure; furthermore, uniaxial tensile loading tests revealed an equivalent resistance of the decellularized tissue to strain, before and after the cryopreservation, in comparison with the fresh tissue. Finally, immunological compatibility, showed a minimized host immune cells invasion and low levels of systemic inflammation, as assessed by tissue transplantation into immune-competent mice.

Conclusions: Our results indicate, for the first time, that fixative-free decellularized pericardium from cadaveric tissue donors can be banked according to Tissue Repository-approved procedures without compromising its mechanical properties and immunological tolerance. This tissue can be therefore treated as a safe homograft for cardiac surgery.

Introduction

Pericardium is employed in cardiac surgery to repair congenital heart defects or to perform valve reconstruction. In addition, pericardium of animal origin (pig, cow) is the elective material to fabricate bio-prosthetic valves to be employed in surgical replacement of insufficient and/or stenotic valves. Indeed, thanks to its abundant collagen bundles and elastic fibers composition, pericardium has a relatively high resistance to mechanical stress, comparable to that of the native heart valves leaflets.

The technology commonly used to produce heart valves leaflets from animal-derived pericardium consists in treating the tissue with low concentration aldehydes (e.g. glutaraldehyde, GA), necessary to create chemical bonds among the extracellular matrix (ECM) components, thereby further increasing the tissue mechanical resistance, and to prevent acute host immune rejection [1]. On the other hand, aldehyde treatment has also drawbacks regarding the tissue long-term durability. In fact, clinical data from long term follow up of patients receiving pericardium-made bio-prosthetic valves, has clearly indicated a severe impact of structural valve deterioration (SVD) and calcification, due to permanence of fixative remnants having potent cytotoxic effects. In addition, GA fails in removing animal-specific antigens such as the (α1, 3)-Gal epitope [2-6], which elicits chronic rejection of the implanted prostheses. Importantly, the impact of SVD is highly correlated to the age of recipients, with younger patients being un-favored - and needing redo strategies - as early as 10–15 years following the first surgery in a significant number of cases [7,8].

In order to overcome these limitations, basic research has actively pursued fixation-free protocols (i.e., avoiding the use of aldehydes) aimed at enhancing pericardial tissue durability. In particular, novel decellularization methods have been proposed,
based on treatment with hypotonic “decellularization” buffers, containing detergents and DNA/RNA digesting enzymes to eliminate cell debris and the remaining nucleic material from the tissue. These strategies have been employed in the production of both animal [9] and human-derived [10,11] pericardium with relevant implications for clinical use. Moreover, they have been proposed for the generation of tissue engineered heart valves (TEHVs) [12].

Despite a very active research in this field, animal-derived and human-derived decellularized pericardium have not been yet introduced in the routine clinical practice. This also depends on the absence of convincing evidences that this tissue may be prepared and cryopreserved according to Tissue Repository-compliant procedures, without compromising its mechanical properties and immunological tolerance. In the present study, we aimed at filling this technological gap by setting up a fixative-free decellularization process followed by tissue cryopreservation procedure of human pericardial tissue obtained by cadaveric tissue donors. We validated our protocol with specific histological and biomechanical analysis of the tissue, which revealed, i) a preserved structure of the collagen bundles and elastin fibers, and ii) the maintenance of suitable mechanical properties. Remarkably, the immunological compatibility of decellularized/cryopreserved pericardium was proven by subcutaneous transplantation for up to 60 days into immune-competent mice. From these results, we conclude the feasibility of a Tissue Repository approved procedure for the decellularization and cryopreservation of pericardial samples from tissue donors that may be released as a novel homograft tissue.

Results

Maintenance of tissue integrity and complete genetic material removal by fixative-free decellularization procedure

Histological analysis (Fig. 1A, 1B and 1C) was used as a first line of inspection to qualitatively assess, i) the efficiency of cells removal after decellularization, ii) the degree of the tissue histo-architecture preservation, and iii) the content and distribution of the collagen bundles and elastic fibers in the extracellular matrix (ECM). In detail, Masson’s staining of decellularized (DE) or decellularized/cryopreserved (DE/CR) pericardium transversal histological sections showed a complete removal of cells. The treatment was not found to cause major deterioration of the tissue, as witnessed by preservation of collagen bundles and elastic fibers. In addition, DE or DE/CR samples did not appear swollen compared with fresh tissue. To verify the removal of cells, staining with Hoechst 33258 of the histological sections was also performed [10], followed by fluorescence microscopy analysis. As shown in Figure 2A, this showed an efficient removal of cells, with no evidence of cellular or nuclear residues observed in the sections. Absence of remaining genetic material from DE/CR samples was confirmed by Real Time-PCR on DNA extracted from three pericardium samples, using primer couples specific for the hMYOD1, hGAPDH and hNFkB genes promoter/coding sequences (Figure 2B, Table 1).

Mechanical integrity of human pericardium after cryopreservation procedure

Uniaxial tensile loading (UTL) tests were carried out on fresh, DE and N₂ vapors-cryopreserved DE pericardium (DE/CR) samples to quantitatively characterize the biomechanical properties of the tissue. In particular, we used UTL tests to reveal any potential alteration of the biomechanical characteristics of the
tissue induced by the cryopreservation procedure. Figure 3A–D shows the preparation steps of pericardial specimens for UTL tests, while 3E represents three typical stress/strain curves obtained from fresh, DE and DE/CR specimens strained to rupture. The stress-strain behavior for each specimen of the three groups was analyzed by means of six parameters [13], including: the elastic modulus (i.e., the stress-strain curve slope) at low \(E_{\text{low}}\) and high strain \(E_{\text{high}}\), representative of the tissue resistance due to the contribution of the elastin and collagen fibers composing the ECM, respectively; the tensile stress \(s_{\text{trans}}\) and strain \(e_{\text{trans}}\) values at the transition between the elastin and collagen stress-strain curve slope; the maximum tensile stress \(s_{\text{max}}\) and strain \(e_{\text{max}}\) characterizing the failure phase of the tissue sample. The comparison of the six parameters in fresh vs. DE and DE/CR groups did not show statistically significant differences (Fig. 3F), suggesting that the procedure adopted to obtain the decellularized pericardium not modify the tissue resistance to mechanical strain. This result suggests that the integrity and the arrangement of ECM components were maintained in DE and DE/CR pericardium samples, ensuring a biomechanical performance similar to that of native tissue.

Immunological compatibility of decellularized pericardium, before and after cryopreservation

The immunological compatibility of human- and animal-derived pericardium has been previously tested by transplantation in animals, where the reaction of the host against the graft, and the graft calcification were assessed mainly by ex-post histological analysis of inflammatory/immune cells invasion [11,14–16]. To assess whether the decellularization and the decellularization/cryopreservation procedure of the human pericardium adopted in the present study led to changes in the tissue immune-tolerance, we adopted a similar strategy. Fresh, DE and DE/CR pericardium fragments were transplanted in subcutaneous position into immune-competent mice for 30 and 60 days followed by histology, immunohistochemistry and analysis of inflammatory-competent cells in peripheral circulation. As shown in Figure 4A, the histological appearance of DE and DE/CR samples was strikingly different from that of the fresh tissue. In fact, high levels of round cells with macrophage/lymphocyte morphology, forming deep layers of granulation tissue were observed in fresh pericardium samples; by contrast an overall lower number of cells with a predominant spindle-shape morphology and elongated nuclei, was observed in DE and DE/CR samples. Of note, foci of donor-derived inflammatory cells infiltration and tissue matrix reabsorption were never observed in any of the DE and DE/CR samples explanted from mice at both time points, indicating a high - but not different - degree of compatibility of DE and DE/CR vs. fresh samples.
pericardium. To confirm the overall lower infiltration by host cells, an automatic counting of cellular nuclei was performed in fixed area images (0.15 mm²) of hematoxylin/eosin stained sections of fresh, DE and DE/CR samples. Results of this survey confirmed an overall lower presence of cells in DE and DE/CR compared with fresh pericardial samples (Fig. 4B).

We and others have found that patients receiving allograft valve transplantation show increased serum levels of anti HLA-1 antibodies [17] and of circulating cytotoxic and helper T-lymphocytes (CTLs, HTLs) [18,19]. Since an elevation of inflammatory markers and immune cell circulation has been indicated as one of the main causes of SVD, a time course analysis of circulating T-lymphocytes was performed in blood samples obtained from mice receiving fresh, DE and DE/CR pericardium at 15, 30, 45 and 60 days after implantation. As a reference for the dynamics of the T-cell mediated rejection of pericardial tissue, the relative ratio of the CD4⁺/CD8⁺ lymphocytes was calculated after appropriate recognition of total circulating T-lymphocytes stained with the pan T-cell marker CD3. As shown in Figure 5, a statistically significant decrease of this ratio, caused by a relative increase of circulating CD8⁺ cells, was observed at all times in mice receiving fresh pericardial samples. By contrast, a constant CD4⁺/CD8⁺ cell ratio around or above 3 was observed in mice implanted with DE or DE/CR, suggesting absence of a strong T-cell-mediated immune response. Finally, no statistically significant difference between DE and DE/CR receiving mice was observed, suggesting that cryopreservation did not alter the immunological compatibility of human decellularized pericardium.

The host immune response has a striking impact on in vivo durability of GA-fixed and fixative-free bio-prosthetic valve grafts, due to chronic inflammatory process [20] and calcification [21], which leads to progressive SVD and decay of mechanical performance. To reveal the inflammatory cellular species invading the implanted pericardium specimens, an immunohistochemistry staining of fresh, DE and DE/CR samples was performed using anti mouse CD3 and CD11b antibodies, which recognized host-derived lymphocytes and macrophages, respectively (Fig. 6A). The results showed the presence of an elevated number of CD3⁺ and CD11b⁺ cells in fresh pericardial samples explanted at 30 and 60 days; these numbers were significantly higher than in DE or DE/CR explanted pericardial tissue (Fig. 6B). Interestingly, a lower T cells number in fresh pericardium was observed at 60 compared with 30 days, suggesting an attenuation of the host T-cell-mediated immune rejection at longer times post-implantation. A similar decrease in macrophages (CD11b⁺ cells) content was not observed, showing a sustained innate immunity response.

Finally, to assess whether calcification secondary to inflammatory/immune response occurred in the transplanted pericardial specimens, Von Kossa staining was performed on histologic...
sections of tissue explants at 60 days post-surgery. As shown in
Figure S1, no staining was observed in DE or DE/CR explanted
samples; small calcium deposits were occasionally found in fresh
pericardium specimens recovered after 60 days, showing that
inflammatory response was not associated to major pericardium
calcification in any of the conditions considered in the present
study.

Discussion

Devising novel procedures to produce replacement allograft or
bio-prosthetic valve conduits offers significant expectations to
ameliorate the clinical outcome of the current heart valves disease
treatments. In fact, the durability of these devices is still
unacceptably limited, with an elevated burden for the patients’
quality of life. As an example, studies reporting the clinical
outcome of patients receiving bio-prosthetic valve implants clearly
indicated a progressive SVD due to host inflammatory response
and immune rejection. Strikingly, the clinical data indicated a
higher propensity of younger subjects susceptible to develop
implant failure more rapidly than older patients and thus requiring
re-operation [7,8]. Depending on the type of the employed
prostheses, different causes are at the basis of the insufficient
durability. For example, the employment of non-decellularized/
cryopreserved valve homografts obtained from cadaveric tissue
donors does not resolve the problem of the allogenic (man to man)
immune response [20], while the employment of the so called
“biological” valves - valve conduits of animal origin (porcine
valves) or bio-prosthetic valves fabricated with leaflet mimicking
biological tissues (typically pig and cow pericardium) - has its
major limitation in the use of GA-based fixation protocols, which
leave residual free aldehyde groups in collagen abundant tissues
[22], form chemical bonds between ECM components, and fail in
removing xeno-antigens such as the (a1, 3)-Gal epitope [5,6],
recognized as “non-self” by the human species. Although
advanced protocols to reduce the overall cytotoxicity of GA-fixed
tissues have been devised [14,16,23–25], novel procedures to
prepare leaflet and pericardial tissue patches preventing the use of
aldehydes have been also explored. These procedures are based on
decellularization of the tissue through osmotic lysis followed by
exposure to ionic (e.g. SDS) and/or non-ionic (e.g. Triton-X 100)
detergents to efficiently remove cell remnants. The apparent
advantage of these procedures over the GA-based fixation is the
lack of aldehyde residues, the absence of chemical bonds between
ECM components, and the removal of xeno-antigens. On the
other hand, whether decellularized tissues have or not an
improved immunologic compatibility and an optimal mechanical
performance is still matter of debate requiring further investigation
[4,9,26].
To our knowledge, human-derived pericardium decellularized with an osmotic/detergent-based procedure has not yet clinically employed. In fact, preservation of mechanical properties and in vivo compatibility of this tissue were previously assessed [10,11]; however, the absence of data related to the maintenance of the tissue material properties after a cryopreservation storage period does not allow concluding whether this tissue may be eventually routinely prepared, banked and distributed by officially recognized homograft Repositories. This particular issue is not trivial. In fact, similar to the production process of cells for cell-based therapy according to the Good Manufacturing Practice [27], the homografts approval for clinical employment requires crucial validation steps to demonstrate the biological and the functional integrity of the tissue. In this regard, the UTL tests performed in the present study (Figure 3) showed that none of the parameters describing the mechanical properties of the human pericardium were modified by the decellularization or the decellularization/cryopreservation procedure. Thus, our data extend the observations by Mirsadraee et al. [10] and underline the relevance of fixation-free decellularization procedures to prepare human pericardium with intact mechanical properties for future clinical employments.

The result of subcutaneous implantation into immune-competent mice was performed taking as a reference the human fresh pericardium. This was done to assess the efficiency of the decellularization protocol at removing cellular material representing a source of allogenic tissue rejection, or promoting secondary calcification. As shown in Figure 4, the overall invasion by host cells was significantly lower in mice receiving DE and DE/CR tissue samples; moreover, in both cases, the number of infiltrating cells was significantly lower than in mice implanted with fresh pericardium. In addition, DE and DE/CR samples showed no signs of reabsorption and formation of granulomas. This finding is in line with the lower numbers of CD3$^+$ cells and CD11b$^+$ cells in DE and DE/CR compared with the fresh pericardium (Fig. 6) [11]. To further explore the role of systemic inflammation in mice implanted with human pericardial samples, the ratio of circulating CD4$^+$/CD8$^+$ lymphocytes, a parameter that is commonly taken as a reference to monitor rejection in solid organ transplantation [28] and mouse models [29], was also determined. These tests (Fig. 5) revealed a striking increase in the relative amount of circulating CD8$^+$ cells as early as at 15 days post-implantation in mice receiving the fresh pericardium, suggesting a rapid initiation of the rejection process. By contrast, DE and DE/CR pericardial implants were never found to cause an imbalance in lymphocytes ratio for the whole duration of the experiment. Although we did not specifically investigate the subclasses of CD8$^+$ circulating cells, this observation appears particularly relevant in the view of homograft transplantation of the DE/CR tissue. In fact, GA-fixed pericardial implants are known to cause an increase of circulating cytotoxic [18] or helper [19] T cells and production of anti HLA-I antibodies, which correlate with graft failure [17]. Thus, the fixation-free decellularization/cryopreservation method used in the present study may be helpful to reduce the inflammatory and cell-mediated immune response in recipients, improving the long-term durability of the implants.

In summary, thanks to unaltered mechanical properties, the potentially reduced cytotoxicity and the lowered immunogenicity,
we propose the decellularized human pericardium produced with the protocol described here as a novel and safe homograft tissue to be clinically employed. In addition, since the adoption of fixative-free decellularization strategies is recommendable for production of biological-derived material for recellularization with living cells and derivation of TEHVs [16,25,30–33], the procedure described here may also be useful for devising novel living implants, with higher degree of immunological compatibility and lower potential for SVD, with significant benefits for the patients.

Materials and Methods

Ethics statement

Human pericardial samples were obtained as discharge material during heart dissection in the routine processing of valve homografts preparation at the Lombardy Cardiovascular Tissue Repository, the official Lombardy’s Facility for collection of cadaveric cardiovascular tissues and cardiovascular homograft preparation and storage (www.cardiologicomonzino.it/Clinica/ChirurgiaCardiovascolare; authorization number: DGR VII/12848 (April 28, 2003 – Official Lombardy Bulletin). Samples from a total of six cadaveric tissue donors were used in the present study. Collection of hearts from these donors was performed after signature of an approved informed consent by donor relatives.

The procedures concerning animal care, surgery, and euthanasia were carried out in accordance to the “Guide for the Care and Use of Laboratory Animals” and the Helsinki declaration.

They were further compliant with European directives and guidelines (Legislative Decree September 19, 1994, n. 626; 89/391/CEE, 89/654/CEE, 89/655/CEE, 89/656/CEE, 90/269/CEE, 90/270/CEE, 90/394/CEE, 90/679/CEE). The procedures used in the present study, were further approved and authorized by the Italian Ministry of Health and the University of Milan Ethical Committee (approval number:1242003-A 13/10/2003).

Decellularization procedure and histology

In the present study, a decellularization procedure similar to that developed by Mirsadree et al. [10,11] was adopted. After an accurate surgical elimination of fat, pericardial tissue was first washed for 90 min in phosphate-buffered saline (PBS) containing protease inhibitors (aprotinin, 10 KIU/ml, Trasylol, Bayer, Germany; 0.1% w/v EDTA, BDH, Lutterworth, United Kingdom) under continuous agitation, and with buffer changing every 30 min. To achieve cells removal from the tissue, the tissue was treated with hypotonic buffer (10 mM Tris-HCl; pH 8.0) for 16 h under continuous agitation at 4°C in the presence of protease inhibitors, followed by incubation for 24 h in 0.1% (w/v) sodium dodecylsulphate (SDS) in hypotonic buffer at room temperature under continuous agitation. To remove nucleic acid material, the samples were washed in sterile PBS three times for 30 min under agitation and incubated for 3 h at 57°C in a reaction buffer containing 50 U/mL deoxyribonuclease I from bovine pancreas [DNase, Sigma–Aldrich, Germany] and 1 U/mL ribonuclease A.
from bovine pancreas [RNase, USB] in 10 mM Tris-HCl (pH 7.5; Sigma-Aldrich) under gentle agitation by a gyrrosraker. Finally, samples were washed in PBS for three periods of 30 min under agitation. To check the completeness of cell removal, pericardial tissue was fixed immediately after the end of the decellularization procedure and embedded into paraffin. Tissue sections (4–6 µm) were cut and stained with haematoxylin/ eosin, Masson’s trichrome

The load (F) and displacement (l) data were acquired from the load cell and the stroke of the cross-head of the testing machine and the engineering stress (σ) and strain (ε) were calculated for each data point, according to equation 1 and 2:

\[ \sigma = F/A \]  

\[ \varepsilon = (l - l_0)/l_0 \]

where A is the cross-sectional area of the specimen at zero strain, expressed as (eq. 3):

\[ A = wxt \]

with, w, t, l, and l₀ representing the specimen width, average thickness, and length at zero strain, respectively.

The stress strain behavior was described by means of 6 parameters according to Korossis et al. [13]: elastic modulus at low (E low) and high (E high) strain values, transition stress (σtrans) and strain (εtrans), maximum tensile stress (σmax) and strain (εmax). The biomechanical parameters calculated for verifying pericardium mechanical properties are based on engineering stress and strain values assuming that the cross-sectional area reduction is negligible during specimen deformation. Even if the real calculated stress, considering the cross-sectional reduction of the specimens may be more accurate, its measurement requires the use of optical-based systems, equipment not commonly integrated in the standard UTL apparatuses. On the other hand, most of the published UTL data consider the calculation of engineering stress and strain [10,33,36,37].

**Surgical procedures, peripheral blood collection, pericardium recovery and image analysis**

Subcutaneous implantation of pericardial fragments (10×10 mm) was performed in male CD1 mice (n = 54; 9 mice/group) (Charles River, Calco Italy) of about 30gr weight. Before implantation, mice were anesthetized with a ketamine (100 mg/ Kg) - xylazine (20 mg/kg) solution injected intraperitoneally; at the same time, blood sampling was performed. Peripheral blood was also collected in 5% EDTA containing tubes at 15, 30, 45 and 60 days after implantation following mild anesthesia with isoflurane (1% in 100% oxygen, v/v). Pericardial tissue specimens recovered at 30 and 60 days after implantation were placed into 4% buffered formalin immediately after recovery from the animals and processed for conventional histological analysis (see below) and immunohistochemistry. Tissue sections were observed under light/fluorescence microscopes (Axiovert/Akioskop) equipped with image analysis software (Zeiss, Cell Quest). Images were acquired at a fixed magnification and analyzed using Image-J software (http://rsbweb.nih.gov/ij), after appropriate grayscale filtering, for automatic/manual nuclei/cells counting.

**Immunohistochemistry**

For immunohistochemistry, dewaxed and re-hydrated sections (5 µm thick) were incubated with 5% bovine serum albumin (BSA) in PBS to block nonspecific binding. The sections were then immunostained with rabbit mAbs recognizing mouse CD3 (Abcam, Cambridge, UK, ab5690) or CD11b (Abcam, Cambridge, UK, ab75476), followed by biotinylated goat anti-rabbit IgG, using an avidin–horseradish peroxidase–biotin complex method (Vector Laboratories, Burlingam, CA) and DAB or NovaRed substrate (Vector Laboratories). A manual counting

DNA extraction and Q-PCR methods

Total DNA was isolated with TriPure Isolation Reagent (Roche Applied Science) from 100 mg of fresh and DE pericardium samples. Gycogen was added to samples before precipitation to see pellets. Quantitative real-time PCR (qRT-PCR) amplifications were carried out for MYOD, GAPDH and NFKB gene promoters using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7900 Fast Real-Time PCR System (Applied Biosystems). Primer sequences were as follows:

**hMYOD promoter**: sense-5′-CCTCTTTTCGTCCCTTTTCCT-CTTTC-3′, antisense-5’-ATGGTATAGGGCTGTAGA-3′;

**hGAPDH**: sense-5′-TACTACGTTTTACGACGGCC-3′, antisense-5′-TCGAACAGGAGGACAGAGGCGA-3′;

**hNFKB promoter**: sense-5′-CAGCCGATGAGAGCCGGCAG-3′, antisense-5′-CCGGACCTCCACGCTGACA-3′.

Cryopreservation and thawing

Pericardium samples were frozen and thawed according to the procedures used by Lombardia Cardiovascular Tissue Bank. Briefly, samples were frozen in a double sterile bag containing freezing solution (RPMI 1640 plus 10% of DMSO) by the use of liquid nitrogen ultra-low-temperature cooler (Planer - SOL S.p.A., Milan, Italy) and stored in N₂ vapors in temperature range between −140°C and −185°C. The samples were thawed inside the sterile bag and washed three times in fresh RPMI 1640 [34,35] immediately before use.

Mechanical testing

Uniaxial tensile loading (UTL) was performed on native (fresh), decellularized (DE) and decellularized/cryo-preserved (DE/CR) pericardial specimens. Before the test, the samples were maintained hydrated with PBS for the whole test duration. Tissue specimens were preloaded up to 0.01 N and subjected to a constant velocity of 10 mm/min. The samples were maintained immersed in PBS at 4°C until the UTL tests begun. All the tests were performed at room temperature and the specimens were maintained hydrated with PBS for the whole test duration.

The biomechanical parameters calculated for verifying pericardi um mechanical properties are based on engineering stress and strain values assuming that the cross-sectional area reduction is negligible during specimen deformation. Even if the real calculated stress, considering the cross-sectional reduction of the specimens may be more accurate, its measurement requires the use of optical-based systems, equipment not commonly integrated in the standard UTL apparatuses. On the other hand, most of the published UTL data consider the calculation of engineering stress and strain [10,33,36,37].

**Chondrogenic differentiation of mesenchymal stem cells (MSCs)**

For chondrogenic differentiation, MSCs were cultured in chondrogenic differentiation medium [27] for 28 days. The cells were then stained with safranin O and counterstained with Fast Green FCF. Images were captured using an optical microscope (Axiovert 200M, Zeiss) equipped with AxioCam MRc5 camera (Zeiss) and analyzed using AxioVision 4.8 (Zeiss) software. The total amount of proteoglycan was quantified by ImageJ software (NIH, Bethesda, MD) as the percentage of total stained area.
Flow cytometric evaluation of circulating monocytes

Before staining with antibodies, blood was treated with ammonium chloride to lyse red blood cells. Lymphocyte population (CD3+ cells) and CD11b+ cells were identified by staining with allophycocyanin-conjugated anti-mouse CD3 (Ab553066), and fluorescein-isothiocyanate-conjugated anti-mouse CD8 (Ab553056), while the percentage of CD4+ population was evaluated by the use of phycoerythrin-conjugated anti-mouse CD4 (Ab553086). All antibodies were from BD Biosciences, Milan, Italy. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Milan, Italy). Data on CD4+/CD8+ cells ratio were derived by acquisition of at least 10⁴ events with appropriate logical gating of the CD3+ cellular population.

Statistical analyses

Statistical comparisons were performed with Graph-Pad 5 (Prism) statistical software. All data were analyzed with Shapiro-Wilk normality test. In case of normal data distribution, statistical comparisons were performed using parametric tests - 1-way/2ways ANOVA with Newman-Keuls/Bonferroni post-hoc analysis. In case of non-normal data distribution, the statistical methods used to compare data were the Kruskall-Wallis test with Dunn’s multiple comparison post-hoc analysis or the Mann-Whitney rank sum test. The methods adopted to perform statistical comparisons are indicated in each figure legend.

Supporting Information

Figure S1 Von Kossa staining of fresh, DE and DE/CR pericardium specimens recovered from mice at 60 days following implantation. Except for few small calcium deposits (arrows) in fresh samples, calcification was not observed. (TIF)

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Author Contributions

Conceived and designed the experiments: MCV MS GP MP LS. Performed the experiments: MCV GT LG FP MS RS F. Colazzo F. Consolo BM. Analyzed the data: MCV MS MP. Contributed reagents/materials/analysis tools: BM F. Colazzo MS MC VP RS GT. Wrote the paper: MP.

References

1. Carpenter A, Lemaigue G, Robert L, Carpenter S, Dubost C (1990) Biological factors affecting long-term results of vascular heterografts. J Thorac Cardiovasc Surg 99: 467–475.
2. Grabenwoger M, Sier J, Fiztal F, Zelenka G, Windberger U, et al. (1996) Impact of glutaraldehyde on calcification of pericardial bio-prosthetic heart valve material. Ann Thorac Surg 62: 772–777.
3. Vincentelli A, Latremouille C, Zegdi R, Shen M, Lajos PS, et al. (1996) Does glutaraldehyde induce calcification of bio-prosthetic tissues? Ann Thorac Surg 66: 8253–258.
4. Bloch O, Golde P, Dolmen PM, Posner S, Konertz W, et al. (2011) Immune response in patients receiving a bio-prosthetic heart valve: lack of response with decellularized valves. Tissue Eng Part A 17: 2399–2405.
5. Galili U (2005) The [alpha]-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. Immunol Cell Biol 83: 674–686.
6. Konnacki KZ, Bohle B, Blumer R, Hoetzenrecker W, Roth G, et al. (2005) Alpha-Gal on bioprostheses: xenograft immune response in cardiac surgery. Eur J Clin Invest 35: 17–23.
7. McClure RS, Narayanasamy N, Wiegerink R, Lipsitz S, Maloney A, et al. (2004) Late Outcomes for Aortic Valve Replacement With the Carpentier-Edwards Pericardial Bioprosthesis: Up to 17-Year Follow-Up in 1,000 Patients. The Annals of Thoracic Surgery 89: 1410–1416.
8. Puvimanasinghe JPA, Takkenberg JMJ, Eijkemans MJC, Seyberg EW, van Herwerden L, et al. (2005) Prognosis After Aortic Valve Replacement With the Carpenter-Edwards Pericardial Bioprosthesis: Use of Microinflation: The Annals of Thoracic Surgery 80: 825–831.
9. Mendoza-Novelo B, Avila EE, Cauich-Rodriguez JV, Jorge-Herrero E, Rojo FJ, et al. (2010) Decellularization of pericardial tissue and its impact on tensile viscoelasticity and glycosaminoglycan content. Acta Biomaterialia 7: 1241–1248.
10. Mirsadraee S, Wilcox HE, Korosoi SA, Kearney JN, Watterson KG, et al. (2006) Development and characterization of an acellular human pericardial matrix for tissue engineering. Tissue Eng 12: 763–773.
11. Mirsadrae S, Wilcox HE, Watterson KG, Kearney JN, Hunt J, et al. (2007) Biocompatibility of acellular human pericardium. J Surg Res 143: 407–414.
12. Dainese L, Guarino A, Burba I, Esposito G, Pompilio G, et al. (2012) Heart valve engineering: decellularized aortic homograft seeded with human cardiac stromal cells. J Heart Valve Dis 21: 125–134.
13. Korosoi SA, Booth C, Wilcox HE, Watterson KG, Kearney JN, et al. (2002) Tissue engineering of cardiac valve prostheses II: biomechanical characterization of decellularized porcine aortic heart valves. J Heart Valve Dis 11: 463–471.
14. Jorge-Herrero E, Fonseca C, Barge AP, Turnay J, Olmos N, et al. (2010) Biocompatibility and calcification of bovine pericardium employed for the construction of cardiac bioprostheses treated with different chemical crosslink methods. Artif Organs 34: E165–176.
15. Sinha P, Zurakowski D, Susheel Kumar TK, He D, Rossi C, et al. (2012) Effects of glutaraldehyde concentration, pretreatment time, and type of tissue (porcine versus bovine) on postimplantation calcification. The Journal of Thoracic and Cardiovascular Surgery 143: 224–227.
16. Manzoco MJS, Pires MD, Cauias M, Higa OZ, Pitombo RNM, et al. (2008) Histological Evaluation of Biocompatibility of Lyophilized Bovine Pericardium Implanted Subcutaneously in Rats. Artificial Organs 32: 268–271.
17. Pompillo G, Polvani G, Piccolo G, Guarino A, Nocco A, et al. (2004) Six-year monitoring of the donor-specific immune response to cryopreserved aortic allograft valves: Implications with valve dysfunction. The Annals of Thoracic Surgery 78: 557–563.
18. Oei FBS, Welters MJP, Knoop CJ, Vaessen LMB, Stegmann APA, et al. (2000) Circulating donor-specific cytotoxic T lymphocytes with high avidity for donor human leukocyte antigens in pediatric and adult cardiac allograft valve conduit recipients. European Journal of Cardio-Thoracic Surgery 18: 466–472.
19. Welters MJP, Oei FBS, Vaessen LMB, Stegmann APA, Bogers AJJC, et al. (2001) Increased numbers of circulating donor-specific T helper lymphocytes after human heart valve transplantation. Clinical & Experimental Immunology 124: 353–358.
20. Koolbergen DR, Hazekamp MG, de Heer E, Bruggemann ES, Hoymann HA, et al. (2002) The pathology of fresh and cryopreserved homograft heart valves: An analysis of forty explanted homograft valves. The Journal of Thoracic and Cardiovascular Surgery 124: 689–697.
21. Manji RA, Zhu LF, Nijjar NK, Rayner DC, Korbutt GS, et al. (2006) Glutaraldehyde-Fixed Bio-prosthetic Heart Valve Conduits Calcify and Fail From Xenograft Rejection. Circulation 114: 318–327.
22. Levy RJ, Schoen FJ, Sherman FS, Nicholls J, Haslsey MA, et al. (1986) Calcification of subcutaneously implanted type I collagen sponges. Effects of formaldehyde and glutaraldehyde pretreatments. Am J Pathol 122: 71–82.
23. Guldner NW, Bastian F, Weigel G, Hazekamp MG, de Heer E, et al. (2012) Nanocoating with titanium reduces (33P and granulocyte-activating immune response against glutaraldehyde-fixed bovine pericardium: A new technique to improve heart valve prosthesis durability? The Journal of Thoracic and Cardiovascular Surgery 143: 1152–1159.
24. Guldner NW, Jasmund I, Zimmermann Hr, Heinlein M, Girndt B, et al. (2010) Detoxification and Endothelialization of Glutaraldehyde-Fixed Bovine Pericardium With Titanium Coating. Circulation 119: 1653–1660.
25. Rodas ACD, Polak R, Haro PH, Lee EF, Pitombo RNM, et al. (2011) Cytotoxicity and Endothelial Cell Adhesion of Lyophilized and Irradiated Bovine Pericardium Modified With Silk Fibroin and Chitosan. Artificial Organs 35: 502–507.
26. Kierle E, Sebacher G, Kasimir M-T, Eichmair E, Winter B, et al. (2005) Tissue Engineering of Heart Valves: decellularized porcine and human valve scaffolds differ importantly in residual potential to attract monocyte cells. Circulation 111: 2792–2797.
27. Gaipa G, Türeml M, Straino S, Barba I, Zacagnini G, et al. (2010) GMP-based CD133+ cells isolation maintains progenitor angiogenic properties and enhances standardization in cardiovascular cell therapy. J Cell Mol Med 14: 1619–1634.
28. Creemers P, Brink J, Wainwright H, Moore K, Shepherd E, et al. (2002) Evaluation of peripheral blood CD4 and CD8 lymphocyte subsets, CD69 expression and histologic rejection grade as diagnostic markers for the presence of cardiac allograft rejection. Transplant Immunology 10: 285-292.

29. Chen CH, Yeh YC, Wu GJ, Huang YH, Lai WF, et al. (2010) Tracking the rejection and survival of mouse ovarian iso- and allografts in vivo with bioluminescent imaging. Reproduction 140: 105-112.

30. Vesely I (2005) Heart Valve Tissue Engineering. Circulation Research 97: 743-755.

31. Wong ML, Leach JK, Athanasiou KA, Griffiths LG (2012) The role of protein solubilization in antigen removal from xenogeneic tissue for heart valve tissue engineering. Biomaterials 32: 8129-8138.

32. Dong X, Wei X, Yi W, Gu C, Kang X, et al. (2009) RGD-modified acellular bovine pericardium as a bio-prosthetic scaffold for tissue engineering. J Mater Sci Mater Med.

33. Tedder ME, Liao J, Weed B, Stabler C, Zhang H, et al. (2009) Stabilized collagen scaffolds for heart valve tissue engineering. Tissue Eng Part A 15: 1257-1268.

34. Barili F, Dainese L, Cheema FH, Dell’Antonio G, Topkara VK, et al. (2007) Rates of cycling cells in cryopreserved valvular homograft: a preliminary study. Artif Organs 31: 152-154.

35. Dainese L, Barili F, Topkara VK, Cheema FH, Formato M, et al. (2006) Effect of cryopreservation techniques on aortic valve glycosaminoglycans. Artif Organs 30: 259-264.

36. Lee JM, Boughner DR (1985) Mechanical properties of human pericardium. Differences in viscoelastic response when compared with canine pericardium. Circulation Research 57: 477-481.

37. Dahms SE, Piechota HJ, Dahiya R, Lue TF, Tanagho EA (1998) Composition and biomechanical properties of the bladder acellular matrix graft: comparative analysis in rat, pig and human. Br J Urol 82: 411-419.