Vitrification of Heart Valve Tissues

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Abstract

Application of the original vitrification protocol used for pieces of heart valves to intact heart valves has evolved over time. Ice-free cryopreservation by Protocol 1 using VS55 is limited to small samples where relatively rapid cooling and warming rates are possible. VS55 cryopreservation typically provides extracellular matrix preservation with approximately 80 % cell viability and tissue function compared with fresh untreated tissues. In contrast, ice-free cryopreservation using VS83, Protocols 2 and 3, has several advantages over conventional cryopreservation methods and VS55 preservation, including long-term preservation capability at −80 °C; better matrix preservation than freezing with retention of material properties; very low cell viability, reducing the risks of an immune reaction in vivo; reduced risks of microbial contamination associated with use of liquid nitrogen; improved in vivo functions; no significant recipient allogeneic immune response; simplified manufacturing process; increased operator safety because liquid nitrogen is not used; and reduced manufacturing costs.

Keywords

1. Introduction

1.1 Overview of Heart Valve Tissue Banking

Three types of heart valves are employed for replacement of defective valves in patients: mechanical, xenogeneic tissue, and allogeneic human valves derived from donors' post-mortem. Most patients receive either xenogeneic tissue or mechanical valves; however, the use of cryopreserved human valve allografts became established during the 1970s and 1980s for certain patient subsets. Two trileaflet valves, the aortic and pulmonary, with associated arterial conduit and cardiac muscle band, are dissected from cadaver donor hearts for patient use. Following cryopreservation, aortic and pulmonary valve allografts have typically been used to reconstruct the left or right ventricular outflow tract for repair of diverse congenital cardiac anomalies. Cryopreserved allografts have especially benefited children with congenital heart disease since the use of alternative mechanical and xenogeneic tissue valves has historically been limited in this patient population. They are also used in women of

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[4]Notes

childbearing age and older patients with memory problems who may not be relied upon to keep up with the medications required for mechanical valves.

In the last century, cryopreserved, control rate frozen Me_2SO-protected, human heart valves were used in approximately 20 % of the tissue heart valve procedures performed annually [1–4]. They are currently less utilized because of new xenogeneic valves, which are impacting aortic valve utilization, and because of failures in young patients [5], particularly infants [6,7]. The pathophysiology of allograft heart valve failure is not fully understood [8–10]. Immunology definitely played a significant role in failure in young patients; this led to the development of decellularization methods to minimize the recipient immune response upon implantation. We, however, hypothesized that the rapid deterioration seen in some allograft heart valve recipients might be due, at least in part, to disruptive interstitial ice damage that occurred during cryopreservation leading subsequently to accelerated valve degeneration upon implantation. About 75 % of the area of conventional Me_2SO-cryopreserved heart valve leaflets, the most important functional component of a heart valve, was occupied by ice [11] (*see* Fig. 1). It was hard to imagine that such extensive ice formation was not damaging the tissue. Both light and electron microscopy demonstrated that the ice was extracellular. Upon thawing and rehydration, the leaflets looked normal histologically because the ice domains within the extracellular matrix closed up as the ice melted.

Development of ice-free cryopreservation by vitrification methods for heart valves was initially stimulated by our demonstration of large ice crystal domains within heart valve tissues [11] (*see* Fig. 1). Vitrification as we have practiced it for heart valve tissues is a non-equilibrium approach (Protocol 1) in contrast to the equilibrium approach developed by Pegg and his colleagues (*reviewed in* 12). This method for the avoidance of ice was based on the early work of Fahy and Rall [13–15]. The basic principles of this approach have been discussed and reviewed in great detail many times [12, 13, 15–20]. In addition to heart valve tissues, we have used Protocol 1 for many other types of tissue including blood vessels [21–24], cartilage [25–28], pancreatic islets [29], and engineered tissues [30–32]. The functional survival of these vitrified tissues was approximately 80 % or higher, whereas the frozen counterparts yielded less than 20 % survival (see the "The emerging 80/20 rule" [12]). This marked contrast (~80 % vs. 20–30 %) was consistent irrespective of the nature of the tissue except in the case of cardiovascular tissues where equivalent post-cryopreservation cell viability can be obtained by either freezing or vitrification methods. In the case of tissues that were particularly susceptible to cryoprotectant cytotoxicity, the final steps in addition and first steps in removal of the cryoprotectants could be performed at subzero temperatures.

Our first heart valve vitrification studies utilized pieces of porcine aortic valves and intact rat aortic valves in small volumes of a vitrification solution (VS) called VS55 to reflect its 55 % (w/v) of cryoprotectant solutes that was originally designated VS41A by its originators [14]. VS55 consists of an 8.4 M mixture of 1,2- propanediol, formamide, and dimethyl sulfoxide in Euro-Collins (EC) solution. EC is an extracellular-type preservation solution that was developed in the 1970s by Collins and subsequently modified in Europe, where it was the gold standard for hypothermic storage of human kidneys for several years. The protocol is described in US Patent #6,740,484 [33, 34] and in Protocol 1 below. In the porcine studies,

we demonstrated that in contrast to conventionally frozen heart valves, vitrified leaflets had no visible ice by light microscopy. There was some discussion of whether or not very small ice crystals could be observed by electron microscopy. In vitro studies with animal tissues have repeatedly demonstrated that Protocol 1 maintained ~80 % heart valve leaflet cell viability immediately after rewarming, returning to control values over a few days in tissue culture (example, Table 1). The emphasis in our rat studies was assessment of transplant outcomes. Two implant models were employed (1) a subcutaneous implantation calcification model and (2) descending thoracic aorta implants [35]. The calcification rate of frozen valves was significantly greater ($p < 0.01$) than that of vitrified valves in both syngeneic and allogeneic recipients, supporting prior observations that ice-free cryopreservation reduces allogeneic heart valve calcification. However, cryopreservation by freezing and vitrification resulted in only mild morphological changes in 2- and 4-week syngeneic explants, a slight decrease in leaflet cellularity, and a more rapid onset of intimal hyperplasia than in fresh valve explants. The allograft explant groups exhibited changes, fresh allograft leaflets were relatively acellular, and frozen leaflets were hypercellular due to acute inflammatory reactions compared with the vitrified allografts (*see* Fig. 2). At that time, we decided that "these observations provided only weak support for the hypothesis that the rapid deterioration seen in some allograft heart valve recipients was due to disruptive interstitial ice damage that occurs during cryopreservation by freezing" [35]. In hindsight it is likely that the decreased inflammation observed in ice-free vitrified heart valve explants was actually a sign of the decreased immune response subsequently observed in sheep.

1.2   Development of a Vitrification Protocol

When our rat studies were published in 2004 [35], our vitrified heart valve research had encountered a significant hurdle; a stronger demonstration of superiority to conventional cryopreservation was needed to warrant further development. In 2006 the publication of a paper using advanced morphological methods that demonstrated significant differences in extracellular matrices of fresh and Me_2SO-cryopreserved frozen heart valves [36, 37] provided the tools and experience to compare vitrified and frozen heart valves. Autofluorescence and particularly second harmonic generated microscopy images revealed that conventional frozen cryopreservation of cardiac valves, when compared with fresh or vitrified tissues, led to the loss of normal ECM structures in valve leaflets [38]. Similar results were found in associated cardiac muscle and artery suggesting that structural deterioration of the ECM is a common consequence of frozen cryopreservation. These observations led to a renewed enthusiasm for further development and optimization of the vitrification protocol for heart valves. Our first protocol was excellent for small-volume tissue samples where relatively rapid cooling and warming rates that avoided ice formation could be achieved. However, upon scale up to full-sized heart valves, 80–100 mL volume, we encountered two problems: (1) cracking at vapor-phase nitrogen temperatures, particularly in dry nitrogen shippers, and (2) the VS55 solution demonstrated ice formation during rewarming [39]. The solution to both problems was to increase the concentrations of the three cryoprotectants in VS55 from 55 to 83 % to make VS83. Furthermore, modulated differential scanning calorimetry studies indicated that this new formulation was potentially stable at −80 °C, free from ice at −80 °C [39], which would make it easier and cheaper to store and ship the tissue samples.

Three in vivo juvenile sheep implant studies were performed to test the VS83 protocol (Protocol 2). The first two studies are unpublished. Protocol 2 was similar to Protocol 1 except that VS83 was employed and after cooling the tissues were stored at −80 °C and shipped on dry ice to the implant site. There were differences in the post-warming wash in each study. Another issue that should be made clear is that in contrast to early work with VS55, there was no effort to keep heart valve tissue cells alive, viable, with VS83. The reason for this is that at this time there is no convincing evidence in the literature suggesting that maintenance of graft cell viability after cryopreservation is beneficial in allogeneic heart valve recipients. In the early years, 1980s and 1990s, of allograft heart valve processing, cell viability was thought to be very important for long-term maintenance of the heart valve post-transplant in patients. Since then analysis of explanted heart valves has supported the assumption that living cells are not necessary in a functioning transplant. Explant analyses indicated that most of the original cells either vanished, especially in the leaflet cusps, or were replaced by donor fibroblasts [40, 41]. Only a few living cells from the original donor could be detected after long-term transplantation and normal graft leaflet cellularity was not observed in a single explant [42]. Furthermore, many groups are now decellularizing heart valves in order to reduce their concerns regarding immunogenicity and its consequences in patients [43, 44]. Therefore, we did not attempt to preserve cell viability during further development of the VS83 method for heart valves. We had previously demonstrated that the VS83 formulation cryoprotectant components are cytotoxic for cultured porcine myofibroblasts at high concentrations [45].

In the first study, we compared conventional frozen aortic sheep heart valves with VS83 vitrified valves stored at −80 °C or in vapor-phase nitrogen over a period of 6 months in vivo. These valves went through a multistep wash protocol similar to that described in Protocol 1. Explant pathology studies indicated that frozen heart valves had most deterioration at explant and 2/4 explants had changes that may have been due to ice damage (Fig. 3 and Table 2). Even though all heart valves exhibited changes, the VS83 formulation combined with −80 °C storage demonstrated the least structural deterioration. Explant gross pathology and histology revealed little difference, except that two cryopreserved by freezing controls had single holes present at explant that could have been due to ice damage (example, Fig. 3d). There were no significant differences in calcium content in explanted leaflet or artery samples (Fig. 4) and least development of aortic insufficiency (AI) (Table 2).

These test results suggested that preservation methods based upon modifications of VS83 combined with −80 °C storage in a mechanical freezer may provide an alternative for liquid nitrogen storage of allogeneic heart valves, reducing the costs of both long- term storage and distribution. Due to the small size of each experimental group ($n = 4$), it was not possible to state that the benefits of the VS83 protocol combined with −80 °C storage were significantly different from cryopreservation by freezing. However, this test group demonstrated less structural deterioration and less aortic insufficiency than traditionally cryopreserved by freezing allograft heart valves after 6 months in vivo in sheep.

The second study was an unpublished Current Good Laboratory Practice (cGLP) study in which decellularized arterial tissue, sheep pulmonary artery, was vitrified ($n = 5$ sheep) and compared with cryopreserved tissue ($n = 2$ sheep) by implantation as patches in the

pulmonary artery of juvenile female sheep (results on file pending future regulatory submissions). The cryopreserved/decellularized/vitrified samples had an average 99.8 ± 0.05 % reduction in extractable DNA. Two patches were implanted in each sheep, and they were explanted after 140 days. One explant from each sheep was used for calcium analysis (non-GLP inductively coupled plasma optical emission spectroscopy) and the other for histopathology. Histopathology included review of H&E, α-actin smooth muscle, TUNEL for apoptosis, Hsp47 (marker for the production of type I and III collagens) (Fig. 5), and factor VIII endothelial cell marker-stained sections. Panel reactive antibody analysis was performed using serum obtained pre-implant and 10 and 20 weeks post implant. None of the animals demonstrated MHC Class I antibodies. One of the two controls demonstrated a 54.65 % shift to the right over baseline for MHC Class II. One of five experimental sheep experienced an 11.16 % shift over baseline for MHC Class II. There were no differences in blood work, echocardiography, or necropsy results between the experimental and control groups. The cryopreserved patches decreased in cellularity and calcified. The decellularized vitrified patches recellularized and resisted calcification, and the infiltrating cells were producing α-smooth muscle actin, factor VIII, and Hsp47 after 20 weeks in vivo. An increase in TUNEL-positive cells was observed in vitrified tissue compared with cryopreserved controls, 45 vs. 34 % positive. The cellularity was the result of fibroblast and myofibroblast infiltration, and the amount of inflammation seen was little to none. The results were similar to published work on the decellularization method employed using ovine patch grafts in the same animal model [46–48]. These results suggest that VS83-preserved tissues should be safe in humans. However, the increased apoptosis noted in the TUNEL staining led to in vitro studies to determine whether rewarmed, washed vitrified tissues were cytotoxic (discussed later).

The third study was a research study in which the VS83 protocol was employed with simplified washing steps to process ovine pulmonary heart valves (Protocol 2 [49, 50]). Vitrified and conventionally frozen valves were then stored at −80 °C and in vapor-phase nitrogen, respectively, for approximately 1 year. Vitrified pulmonary valves were shipped from Charleston, South Carolina, to the implantation site in Berlin, Germany, on dry ice and stored at −80 °C until implantation. The control frozen pulmonary valves were shipped in a nitrogen dry shipper and stored at −135 °C in Berlin. Aortic valve tissues were kept in Charleston to evaluate the impact of preservation without implantation. The pulmonary valves were implanted in the pulmonary position. We had concerns that our earlier unpublished sheep studies, presented above, utilized sheep that may have been highly inbred because crossbred Whiteface heart donors and recipients were used. Therefore, two different strains of sheep were chosen, crossbred Whiteface heart valve donors from Minnesota and Heidschnucke, a Nordic short-tailed breed, recipients from Germany to increase the possibility of using a true allogeneic model.

Multiphoton microscopy revealed reduced, but not significantly, damaged extracellular matrix before implantation in frozen valves compared with ice-free tissues. Viability assessment revealed significantly less metabolic activity in the ice-free valve leaflets and artery samples compared with frozen tissues ($p < 0.05$). After 3 and 6 months, in vivo valve function was determined by two-dimensional echo-Doppler, and at 7 months the valves were explanted. Severe valvular stenosis with right heart failure was observed in recipients

of frozen valves; the echo data revealed increased velocity and pressure gradients compared to ice-free valve recipients ($p = 0.0403$, $p = 0.0591$). Histopathology showed significantly thickened leaflets in the frozen valves ($p < 0.05$) and infiltrating CD3+ T-cells ($p < 0.05$) compared with ice-free vitrified valve leaflets. Multiphoton microscopy at explant revealed reduced inducible autofluorescence and extracellular matrix damage in the frozen explants and well-preserved structures in the ice-free explant leaflets. We concluded that ice-free cryopreservation of heart valve transplants at −80 °C avoids ice formation and tissue-glass cracking and preserves extracellular matrix integrity resulting in minimal inflammation and improved hemodynamics in allogeneic juvenile sheep [49–51].

Biomaterial properties of ice-free preserved heart valves have also been assessed [39]. We observed occasional tissue cracking and fracturing of the vitrified solution in experiments involving storage of heart valves in VS55 below the solution glass transition temperature in vapor-phase nitrogen. Substitution of VS83 for VS55 corrected this for samples stored at −135 °C. Biomechanical testing was performed on fresh untreated control and VS83 cryopreserved leaflets and stored at −80 and −135 °C. There were no significant differences in Young's modulus and ultimate stress (strain) observed between the −80 and −135 °C stored leaflets for any of the mechanical test comparisons; similarly neither group differed significantly when compared with the fresh untreated control leaflet samples (Fig. 6). Thakrar [52] reported that vitreous cryopreservation, employing 40 % w/v 1,2-propanediol in DMEM culture medium maintains the viscoelastic property of human vascular grafts in contrast to conventional cryopreservation by freezing with Me_2SO. We also assessed the impact of VS83 preservation upon ECM integrity at the two storage temperatures. Multiphoton imaging of VS83-preserved heart valves stored at each temperature demonstrated branched elastic fibers and wavy bundles of collagen in almost all vitrified heart valve leaflets, aortic trunk, and cardiac muscle specimens. There were no significant differences in SHG signal intensities (Fig. 7) or in qualitative mean rating scores on tissue quality determined by the operator during imaging. These studies provided further support for the storage of VS83-preserved tissues at −80 °C provided that cell viability was not required. However, these tissues are technically not vitrified since we are storing them above the glass transition temperature. Therefore, in most of our more recent publications, we refer to tissues preserved at −80 °C as being ice-free cryopreserved.

Further process simplification has since been performed (Protocol 3) with in vitro evaluation studies to assess the impact of VS83 vitrification on cell viability and tissue hemocompatibility. During the development of Protocol 3, we examined the impact of varying the number of addition or removal steps in combination with cooling and found that it made little if any difference for cell survival. Room temperature incubation in VS83 without subsequent cooling and storage had similar effects on cell viability. The vitrified artery and leaflet components were significantly less viable than either fresh or Me_2SO-cryopreserved frozen tissues. The muscle component, while less viable, was not significantly different. The changes in tissue viability were a function of cryoprotectant exposure and cytotoxicity, not temperature reduction during storage. Therefore, the simple one-step addition at room temperature was selected for use in further experiments. Multiple changes of washing solution were employed to minimize the residual cryoprotectant concentrations after rewarming. Subsequent studies confirmed the impact of VS83 on heart valve tissue cell

viability using Protocol 3 [52] and demonstrated that heart valve tissues were hemocompatible using multiple assays [53, 54]. Protocol 3 will be tested in vivo with either ice-cold or room temperature wash solutions in future studies.

1.3 Current State

In the evolution of our vitrification protocols, we started with Protocol 1 based upon VS55 (reviewed in 12, 55). This protocol resulted in excellent avoidance of ice formation with retention of cell viability and matrix integrity provided that the sample size and geometry permitted rapid cooling and warming. Ice formed during slow rewarming of VS55 but not during rewarming of a more concentrated cryoprotectant solution, VS83 [39]. The use of Protocol 2 with VS83 permitted the use of large sample volumes and heart valve tissue storage at −80 °C at the expense of cell viability.

A variety of reasons for allograft heart valve failure were discussed in the past, and most investigators have emphasized immunological issues [41, 56]. Standard quantitative and qualitative cellular and matrix evaluations such as biochemical, immunohisto-chemical screening, and routine histology did not help to solve the controversial discussion whether remaining allogeneic cells or potentially altered extracellular matrix contributed to the observed degeneration. Preliminary data on patients treated with decellularized allografts has recently demonstrated that decellularization did not significantly improve outcome in terms of pressure gradients and structural deterioration compared to nondecellularized allografts [57]. These early clinical results question the validity of theories suggesting that an immune reaction to the remaining donor cells in allogeneic heart valves is the sole cause of structural deterioration. The impact of vitrification on the immunoreactivity of tissue grafts was first clearly observed in the in vivo sheep model study employing different sheep strains discussed above [49, 50]. Explant leaflets demonstrated well-preserved extracellular matrix structures, with few CD3+ T-cells and significantly improved hemodynamics. In marked contrast, leaflets in the frozen control group demonstrated significant matrix damage and T-cell-mediated inflammation. The observation that explanted vitrified leaflets were nearly devoid of active immune cells suggested that a state equivalent to decellularization was accomplished by the ice-free cryopreservation step. Further support for this observation was obtained in studies combining human cells with rewarmed, washed tissues in vitro [58]. Concerns that the apparent benefits of vitrification were due to cytotoxic cryoprotectant residuals were dismissed by demonstration that cells cultured with these tissues survived [58] (Figs. 8 and 9). Our current working hypothesis to explain these changes in vitrified tissue immunoreactivity is that this vitrification method is modifying or masking tissue signals perceived by responder cells. These tissue signals are most likely damage-associated molecular pattern molecules that can initiate and perpetuate immune responses.

The potential advantages of VS83 heart valve storage at −80 °C employing Protocol 3 includes reduced infrastructural needs for preservation, storage, and shipping in comparison with traditional freezing methods while maintaining extracellular matrix integrity and material properties. The loss of cell viability is probably a benefit contributing to the reduction of immunogenicity observed in vivo and in vitro. The typical methods employed for conventional cryopreserved frozen heart valves require a control rate freezer, storage in

nitrogen-cooled tanks, and a continuous supply of liquid nitrogen. Furthermore, conventionally cryopreserved valves also require shipment in nitrogen dry shippers to the implantation site where the valves need to be kept in nitrogen-cooled freezers until implantation. Dry shippers are expensive, and the cost of return shipment adds to the cost of the heart valve to the end user. Liquid nitrogen is also a safety hazard for employees, and precautions must be taken during operation of nitrogen-cooled equipment. In contrast, no expensive equipment and liquid nitrogen are needed for the Protocol 3 VS83 storage method that has evolved. There may also be a reduced risk of microbial contamination associated without use of liquid nitrogen. The only equipment required is a mechanical storage freezer, and long-term preservation can be performed at −80 °C. Shipping requires only an insulated box with dry ice. The removal of liquid nitrogen from the process also reduces the training required and safety hazards for employees. In the future, we anticipate removal of the cooling below −100 °C step in the initial cooling procedure in order to further reduce the complexity and cost of this method. In vivo large animal studies of VS83 preservation combined with −80 °C storage employing pulmonary valves in juvenile sheep have already demonstrated better function and explant pathology than traditionally frozen cryopreserved heart valves [49, 50]. VS83 preservation protocols combined with −80 °C storage method should be particularly beneficial in developing countries with limited financial and logistic resources that have a high incidence of aortic heart valve disease.

2 Materials

Prepare all solutions using deionized water (prepared with an activated carbon filter, a mixed bed working deionizer, and a mixed bed polishing deionizer to attain a sensitivity of 10 MΩ cm at room temperature). The solutions are prepared with raw materials that meet or exceed requirements of the American Chemical Society. If no such specifications exist, chemicals of the highest purity available should be used. The solutions are made at room temperature and stored at 4 °C for up to 4 weeks. The solution formulations described are for 1 L batches.

2.1 Euro-Collins Solutions

1. Put 0.5 L water in a container (graduated cylinder or glass beaker), then add a stir bar followed by addition of each component in the following order, 174.76 g Dextrose, 10.2 g KH_2PO_4, 36.5 g K_2HPO_4, 5.6 g KCl, 4.2 g $NaHCO_3$ make up to 1 L with water to make a 5× stock solution. Stir continuously during formulation; when the solution is clear of particles, it should be filtered using 0.2 μm filters. We do not test the osmolality or pH of 5× Euro-Collins. Dilute 1:5 with water to make 1× Euro- Collins and check the pH (~7.4) and osmolality (365 ± 5). Store at 4 °C.

2.2 Vitrification Solutions

1. VSs are formulated by placing a stir bar and then each component, in the order that they appear in the list below (Table 3), in a graduated container. Stir continuously; when finished and it is clear of particles, it should be filtered. We use bottle top 0.2 μm filters, and it takes a long time (hours) to filter due to the

high viscosity of the solutions. At the conclusion of preparation, check the final pH and make a 1:20 dilution for assessment of osmolality. Store at 4 °C.

2.3 Vitrification Solutions

1. Sequential vitrification solutions, both addition and removal solutions, are used in certain protocols (*see* Note 1). The VS chemical component concentration can be increased from 1/8, ¼, ½, and ¾ to 100 % of the final full-strength VS formulation being employed. The diluted VS solutions are prepared by the addition of appropriate volumes of 1× EC. The solutions employed for removal are similar to those employed for addition except that 200–400 mM mannitol is added to the formulation as an osmotic buffer. For example, using seven steps, the VS CPAs are decreased from full strength to 0 M plus mannitol to 0 M, plain EC, and finally Dulbecco's Modified Eagle Medium (DMEM).

3 Methods

3.1 Protocol 1: Suitable for Small Heart Valve Tissue Samples (3 mL Total Volume, Sample Plus Solution) with Maximum Retention of Cell Viability and Matrix Integrity

1. Tissues should be gradually infiltrated with VS55 at 4 °C (*see* Note 1). Precooled diluted VS solutions are employed in six 15 min steps at 4 °C of increasing CPA concentration as previously described [12] (*see* Note 2).

2. The top of the vitrification solution is then covered with 2-methylbutane (isopentane; *see* Note 3).

3. The samples should be cooled rapidly to −100 °C followed by slow cooling to −135 °C (*see* Note 4).

4. Finally the samples should be stored at −135 °C in a mechanical storage freezer or at   −135 °C near the top in a vapor-phase nitrogen freezer.

5. Rewarming of vitrified tissues is a two-stage process including slow warming to −100 °C and then rapid warming to ~4 °C (*see* Note 5). Wipe the container with

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[1]·Stepwise VS addition and removal procedures are employed in Protocol 1 in order to minimize damage to cells as they respond to osmotic changes in their environment as a consequence of being placed in VS solutions with high concentrations of CPAs (*reviewed in* 12). This is not an issue in Protocols 2 and 3 because we are not trying to maximize cell viability. Loss of cell viability is considered advantageous because it appears to reduce immunogenicity in vivo [49, 50].

[2]·In the last step, the tissues are placed in vials; we usually use glass scintillation vials (25×60 mm), containing 1–2 mL of precooled full-strength VS solution. In circumstances where unexpected cytotoxicity is observed, the last one or two steps can be performed at subzero temperatures in a cold bath to minimize cytotoxicity.

[3]·We use 0.7–1.0 mL of 2-methylbutane for the glass vials described in Note 2. The 2-methylbutane has a freezing point of −160 °C and density of 0.62 g/cm^{3}. The 2-methylbutane layer prevents direct air contact minimizing the risk of ice nucleation. Alternatively, the vial can be purged with a dry gas, such as nitrogen, or a container without excess air space can be used.

[4]·Rapid cooling to < −100 °C is done by placing the vials in a precooled (−135 °C) 2-methylbutane bath; slow cooling is performed by placing the vials in air in the top of storage freezer, either a −135 °C mechanical freezer or near the top of a vapor- phase nitrogen-cooled freezer. More rapid cooling can be obtained, if needed, using liquid ethane at ~ −183 °C. However, this strategy may result in the development of thermal stresses within the sample because the edges will cool rapidly and the inside more slowly. Such stresses may lead to cracking during storage or upon rewarming.

[5]·A dummy sample prepared under the same conditions as the preserved samples with a thermocouple is used to monitor cooling and warming. Make sure that the thermocouple has been calibrated using a NIST standard. The dummy sample is placed in close proximity to the samples to be rewarmed in the storage freezer. Plug the dummy sample thermocouple into a reader. Place the samples and dummy sample at the top of the storage freezer and hold just inside the lid. When the temperature reaches −100 °C (~1 min),

70 % ethanol to minimize the risk of microbial contamination and use sterile
pickups to transfer the tissue to the first VS removal solution.

6. The vitrification solution is then removed in a stepwise manner at 4 °C in six or
    seven sequential, 15 min steps of increasingly dilute VS solutions containing
    mannitol as previously described [12] (*see* Note 1). Finally the tissues are placed
    in DMEM culture medium.

3.2 Protocol 2: Suitable for Large Heart Valve Tissue Samples (Up to 100 mL Total Volume) with Preservation of Matrix Integrity and Minimal Residual Cell Viability

1. Tissues are gradually infiltrated with VS83 in six 15 min steps of increasing
    concentration at 4 °C.

2. After the final step, the tissues are placed individually in sterile polyester bags
    containing 70–80 mL of the vitrification solution. Each bag is then evacuated of
    air by squeezing (*see* Note 3) and heat-sealed.

3. The bags containing tissues need to be cooled rapidly to −100 °C in a precooled
    2-methylbutane bath and then transferred to storage (*see* Note 4).

4. Store at −80 °C in a mechanical storage freezer (*see* Note 6).

5. Rewarming needs to be performed by placing each bag in a 37 °C water bath
    until the solution moves freely.

6. The bag is then transferred onto ice and the bag externally cleaned with 70 %
    ethanol to reduce the risk of microbial contamination. Cut the bag open with
    sterile scissors and use sterile pickups to transfer the tissue to the first VS
    removal solution.

7. A multistep VS removal protocol was employed in our first unpublished sheep
    study, similar to that used in Protocol 1, above. Simpler, washing methods have
    been employed in a subsequent sheep study [49, 50] and an unpublished study
    with arterial patches (*see* Note 7).

3.3 Protocol 3: Simplified Versions of Protocol 2 in Development for Heart Valves

1. Place the tissues in sterile polyethylene bags containing 80 mL of VS83 at room
    temperature (*see* Note 1).

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initiate the rapid warming step. Place both vials in a room temperature 30 % Me$_2$SO bath and swirl the vials manually until the glass
begins to melt (the VS starts to move). Open the vial and remove the VS with a transfer pipette and add the first dilution solution. Start
timer for 15 min.

[6.]The samples can be stored in vapor-phase nitrogen but VS solution fractures may be observed. Although the visual impact of the
fracturing is disturbing, we have rarely seen tissue fractures, and on the rare occasions when tissue cracking has occurred, it has been
during the use of nitrogen dry shippers for transport. If samples are stored in vapor-phase nitrogen, the two-step rewarming should be
performed as described in Protocol 1.

[7.]Ice-free artery patches were rewarmed by placing the bag containing the tissue in a 37 °C water bath for 1 min and then washed by
transferring the contents of the bag to an AlloFlow® continuous gradient washout chamber with 1 L of room temperature isotonic
saline over 20 min. Ice-free cryopreserved valves were rinsed briefly in ice-cold EC solution containing 200 mM mannitol. Then three
sequential rinses were performed for 15 min each with continuous careful agitation in ice-cold EC solution with 200 mM mannitol,
followed by EC solution alone and finally DMEM. The valves were stored briefly on ice in DMEM culture medium until implantation
or evaluation.

2. Evacuate the air and heat-seal the bags (*see* Note 3).

3. Incubate the tissues with continuous shaking for at least 1 h at room temperature prior to cooling.

4. The cooling process can then be achieved by placing the bags for 10 min in a precooled bath of 2-methylbutane (< −100 °C, *see* Note 4).

5. The tissues can then be placed at −80 °C for storage.

6. Rewarming of the tissue is then performed by submersion of the tissue in its bag in a 37 °C water bath.

7. The bag is then dried and externally cleaned with 70 % ethanol to reduce the risk of microbial contamination. Cut the bag open with sterile scissors and use sterile pickups to transfer the tissue to the first wash step.

8. Washing to remove CPAs has been performed using five wash steps of 5 min duration each with ice Euro-Collins solution or with several washes with Lactated Ringers solution containing 5 % dextrose (*see* Note 8).

Acknowledgments

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[8.]We are presently evaluating the use of Lactated Ringers solution containing 5 % dextrose (LRD5) for washing because it is commonly available in surgery suites where ice-free cryopreserved tissues may be used. Human vessels generated in the laboratory by culturing human smooth muscle cells on a polyglycolic acid scaffold [31] were employed. The tissues were subjected to a total of 2, 4, 8, or 15 min room temperature wash steps in Lactated Ringers solution containing 5 % dextrose to remove residual cryoprotectants. In control decellularized tissues, the cellular material was removed using enzymes and detergents leaving an extracellular matrix tube as previously described [31], which was refrigerated in phosphate-buffered saline until tested. Trypan blue excluding human umbilical vein endothelial cells was added to each tissue sample under physiological cell culture conditions in non-tissue culture tubes (to minimize cell migration from the tissues). There were no statistically significant viability differences comparing endothelial cell seeded decellularized vessels and ice-free preserved tissue-engineered blood vessels that were not decellularized after 2, 4, or 8 washes in cell culture (Fig. 8). The endothelial cells formed confluent monolayers on all surfaces of the washed tissues, regardless of the number of post-rewarming washes (Fig. 9), demonstrating that any residual cryoprotectants present were not cytotoxic. In parallel xenograft tissue studies, we have also demonstrated that viability of human peripheral blood mononuclear cells is not impacted by culture with rewarmed tissues washed 5× with EC.

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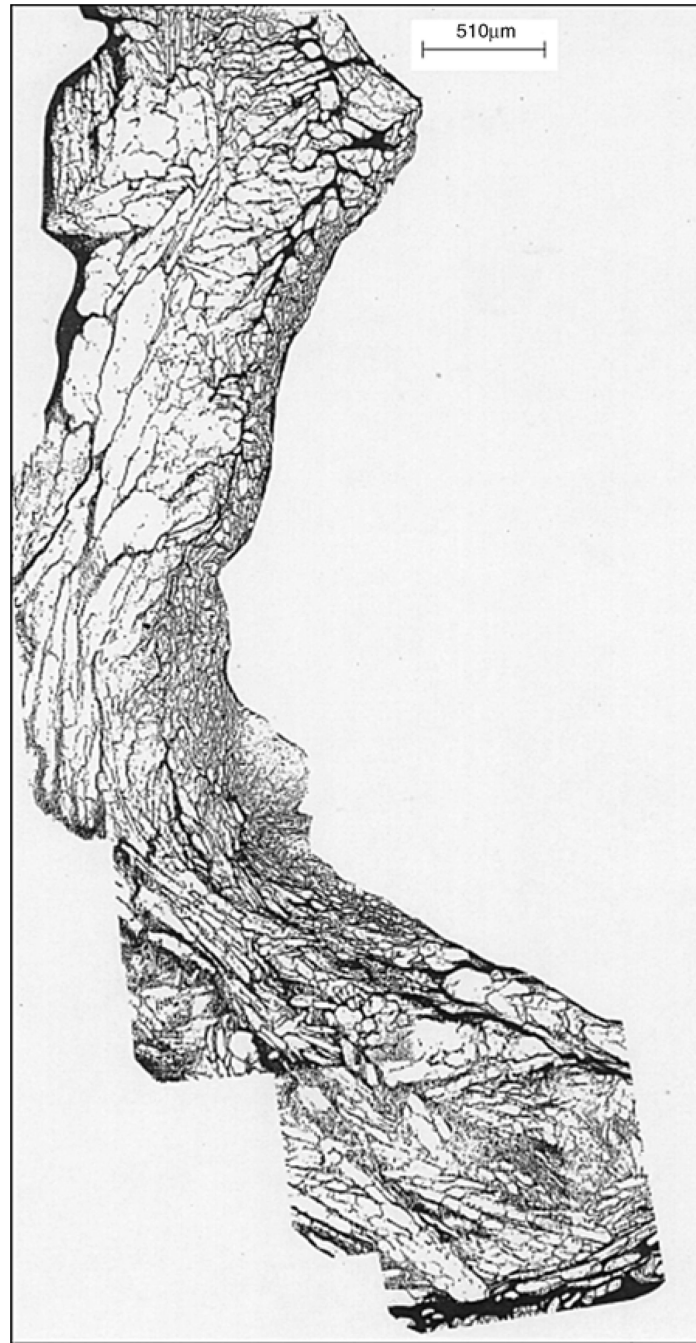
Fig. 1.
Montage of micrographs from a cryo-substituted porcine heart valve leaflet demonstrating
the presence of ice (*white spaces*) throughout the tissue. This tissue sample was slow frozen
in culture medium containing 1 M Me_2SO. Cryo-substitution was performed with methanol
at −90 °C as previously reported [21]

Syngeneic Valves

Allogeneic Valves

Fresh   **Conventionally Frozen**   **VS55**
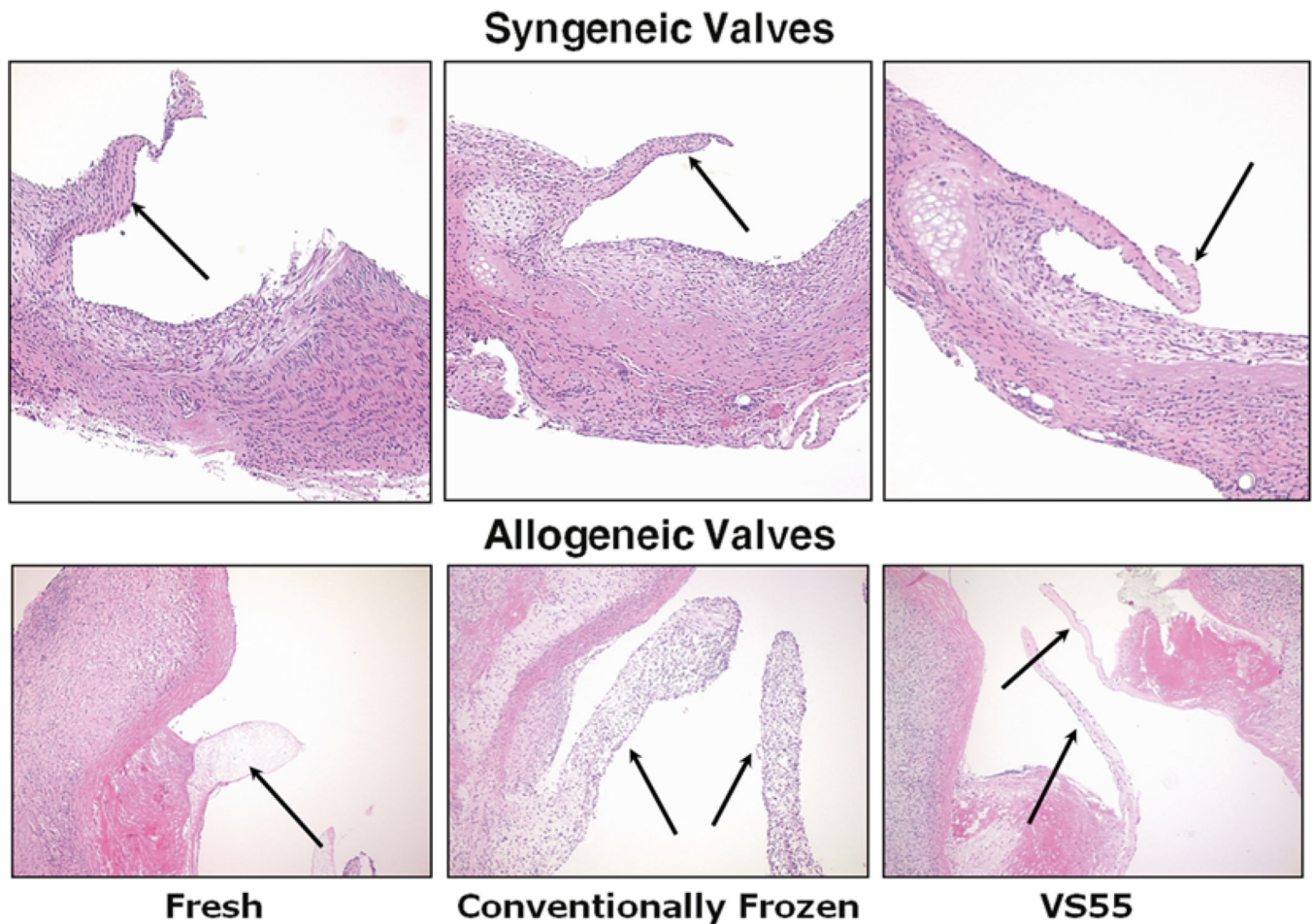
Fig. 2.
Representative heterotopic rat heart valve explant histology. The valves were explanted at 2 weeks (4 week explants were similar in appearance). The syngeneic valves all appear similar; however, both the fresh allograft and frozen valve leaflets appear thickened relative to vitrified valves. Fresh allograft leaflets were relatively acellular, and frozen leaflets were hypercellular due to acute inflammatory reactions. Leaflets indicated by *arrows*, hematoxylin and eosin stain, 10× magnification
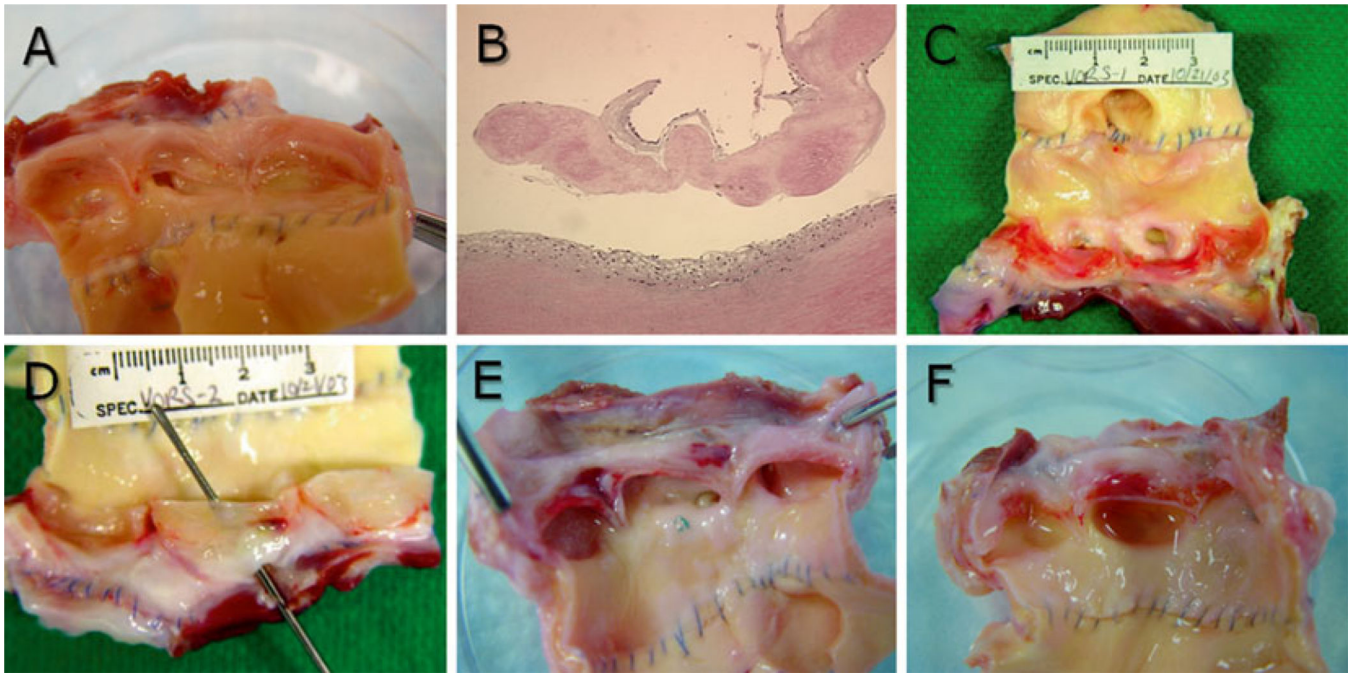
Fig. 3.
Sheep heart valve explant gross and histopathology. Heart valves were explanted at 6 months and photographed before further processing. (**a**) VS83 formulation stored at −80 °C in a mechanical storage freezer, (**b**) H&E-stained section of VS83 −80 °C explant after 6 months in vivo demonstrating cell-free leaflet with recipient cell-derived pannus overgrowth on the artery (*bottom* and leaflet *top*), (**c**) and (**d**) cryopreserved by freezing controls, (**d**) also demonstrating a hole in the base of leaflet, (**e**) and (**f**) VS83 formulation stored in vapor-phase nitrogen
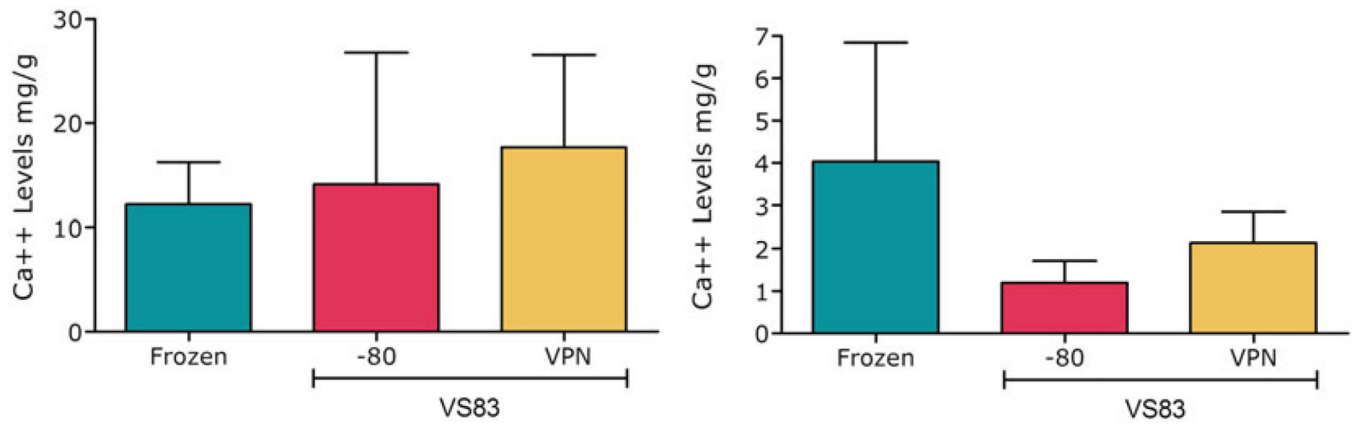
Fig 4.
Calcium contents in sheep heart valve explants. Leaflet results are on the *left* and conduit results on the *right*. VPN = vapor-phase nitrogen. Calcium-specific mineralization occurring in aortic allografts was quantified using atomic absorption spectroscopy [59]. There were no significant differences
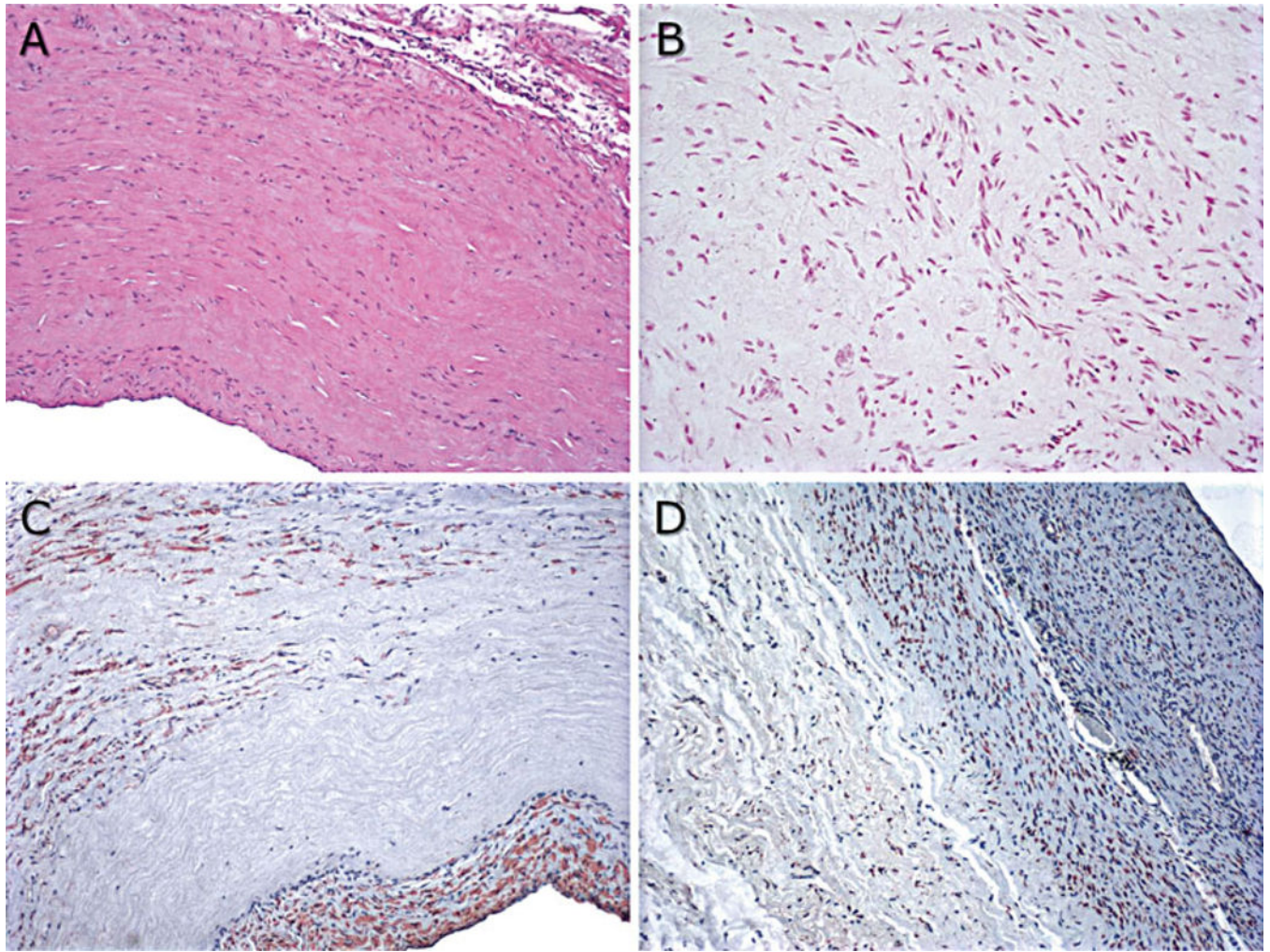
Fig. 5.
Representative explant histology from 20-week decellularized VS83-preserved artery patch explants: (**a**) hematoxylin and eosin stain, 100×; (**b**) TUNEL stain to identify apoptotic cells, 200×; (**c**) actin stain to identify smooth muscle cells, 100×; and (**d**) HSP47-stained cells to identify cells actively producing collagen, 100×. Factor VIII-positive endothelial cells were also observed (not shown)
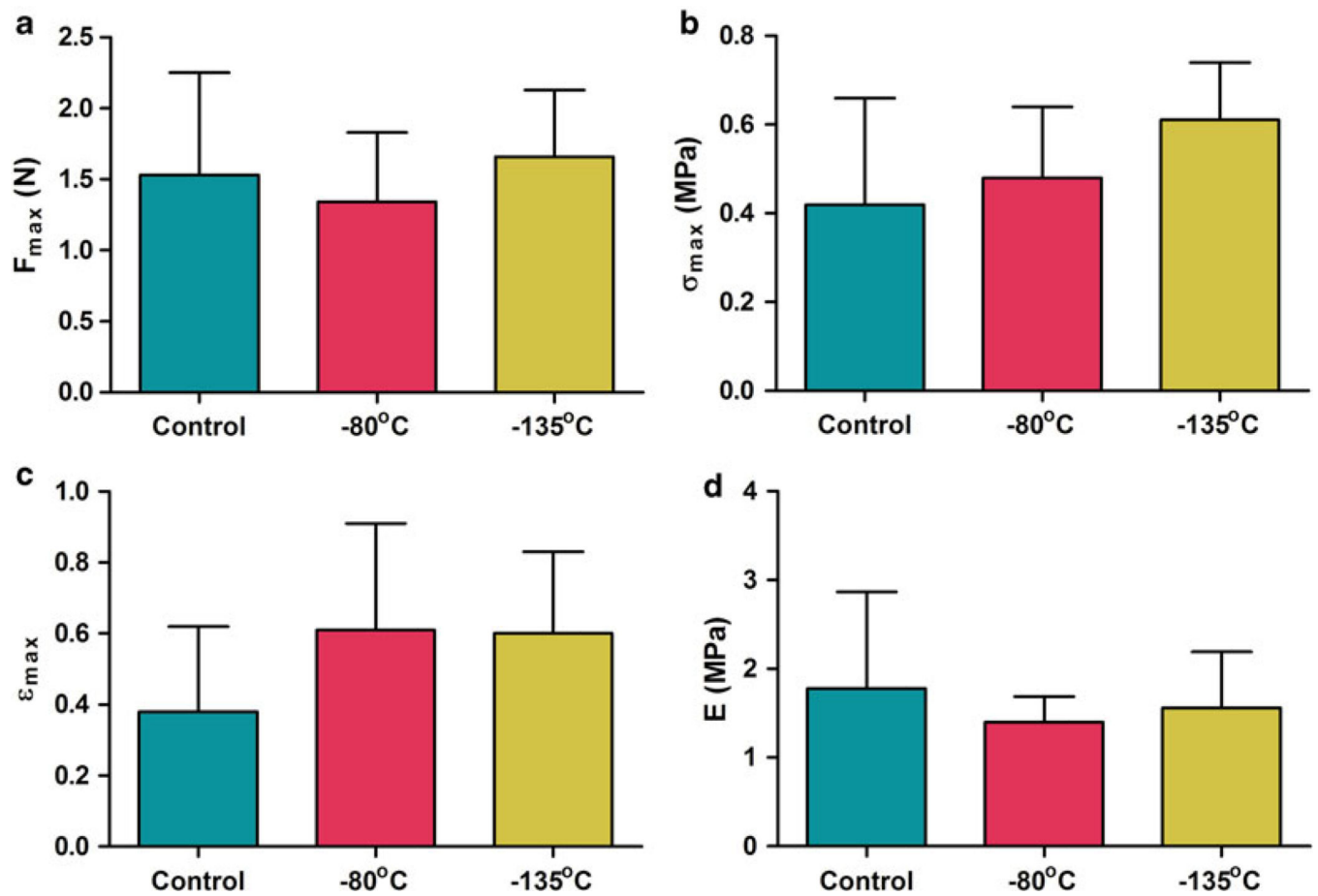
Fig. 6.
Summary of VS83 biomechanical testing. Results comparing VS83-preserved leaflets, stored at –80 or –135 °C, and fresh untreated control leaflets are presented as (**a**) ultimate force, (**b**) ultimate stress, (**c**) ultimate strain, and (**d**) Young's modulus. Data presented as the mean ± 1 standard deviation, $n = 5$–6, for each individual sample mean. There were no significant differences [39]
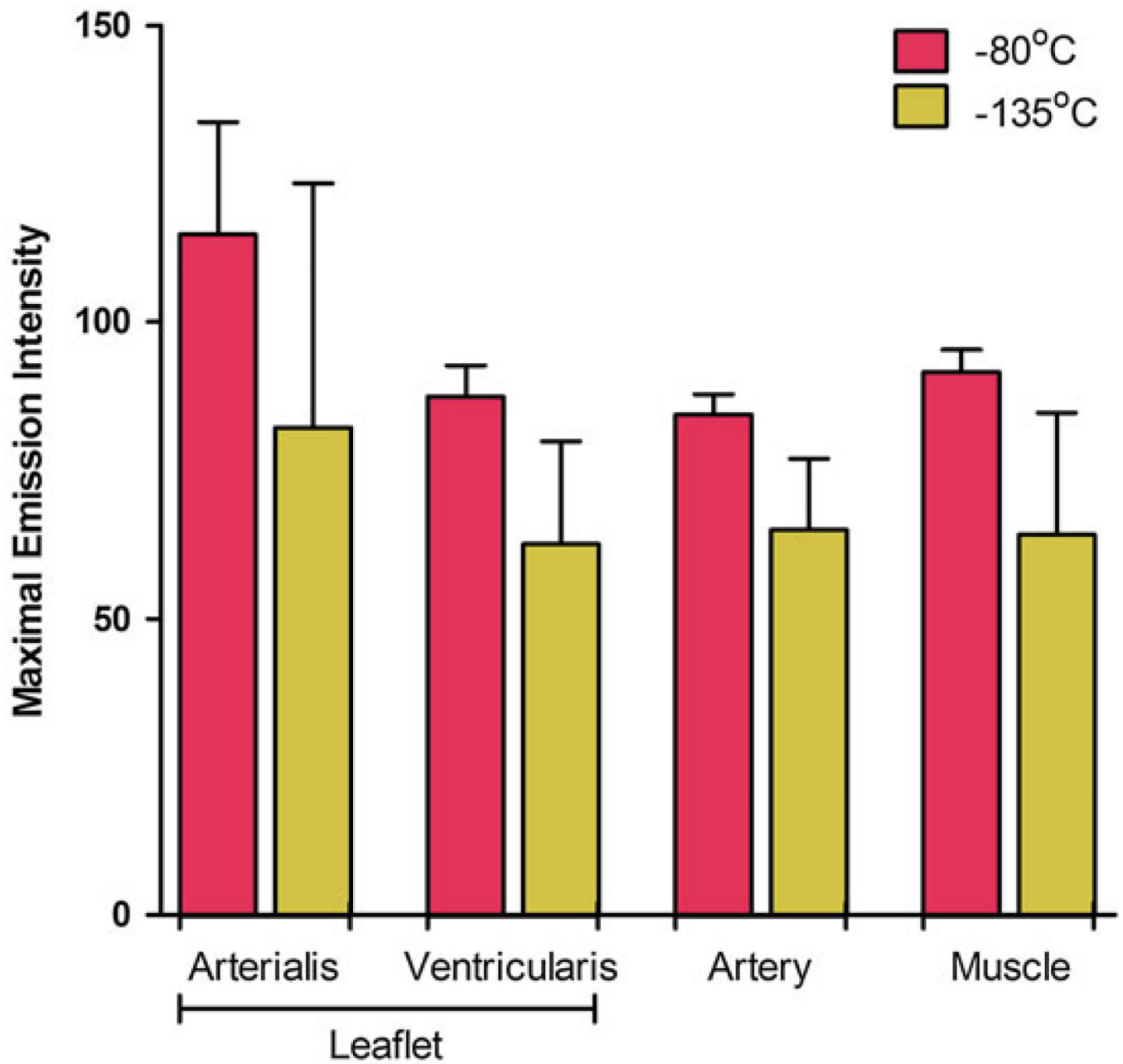
Fig. 7.
Summary of second harmonic generated signal intensities. Collagenous structures in heart valve tissues were observed by spectral fingerprinting after VS83 preservation at −80 or −135 °C. Data presented as the mean ± 1 standard deviation, $n = 4$, of individual sample mean maximal intensity emission values detected at peak emission wavelengths of 414–425 nm. There were no significant differences [39]
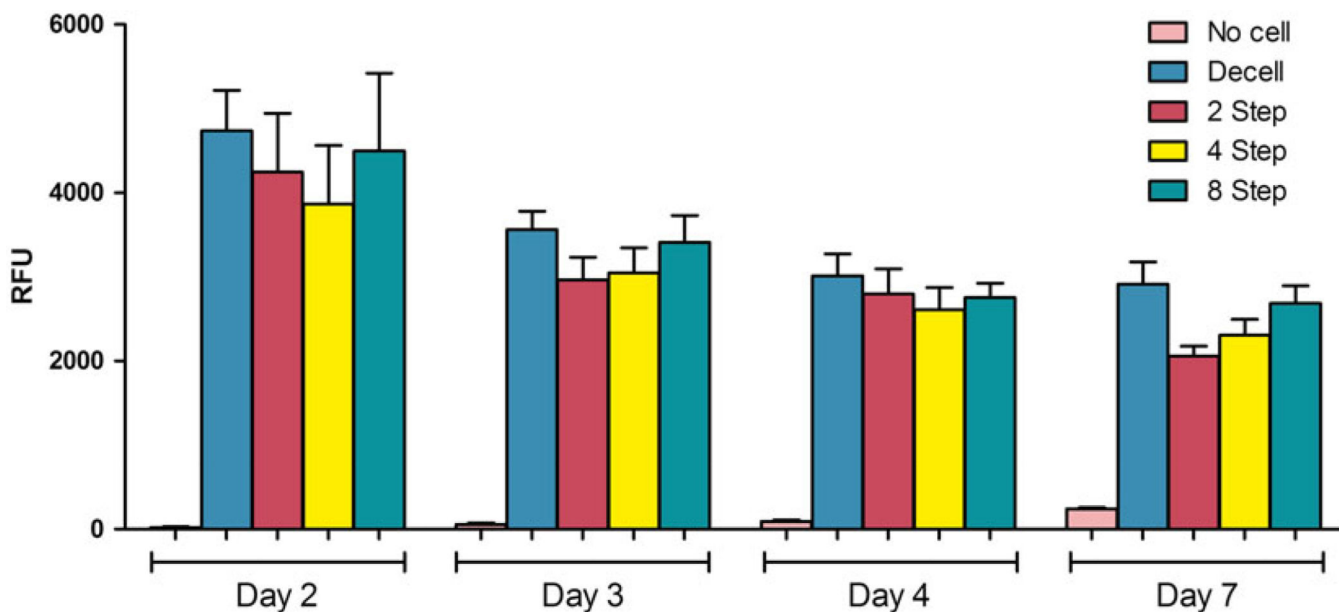
Fig. 8.
Assessment of VS83-preserved cytotoxicity in vitro. Human umbilical vein-derived endothelial cells were cultured with ice-free preserved vessels for 7 days in non-tissue culture grade tubes. The constructs were tested on days 2, 3, 4, and 7 using the resazurin reduction assay. There was a gradual decrease over time in culture for all groups, no significant differences except for comparison of the no cell group with the HUVEC groups ($p < 0.05$). No cell = ice-free preserved vessels without added endothelial cells, Decell = decellularized vessels plus cells. Rewarmed ice-free preserved vessels were subjected to 2, 4, and 8 wash steps. Note: The no cell and 2–8 step groups were not decellularized. The gradually increasing viability in the no cell group is due to proliferation of a small number of smooth muscle cells, which were used to engineer the vessel, surviving the ice-free preservation process
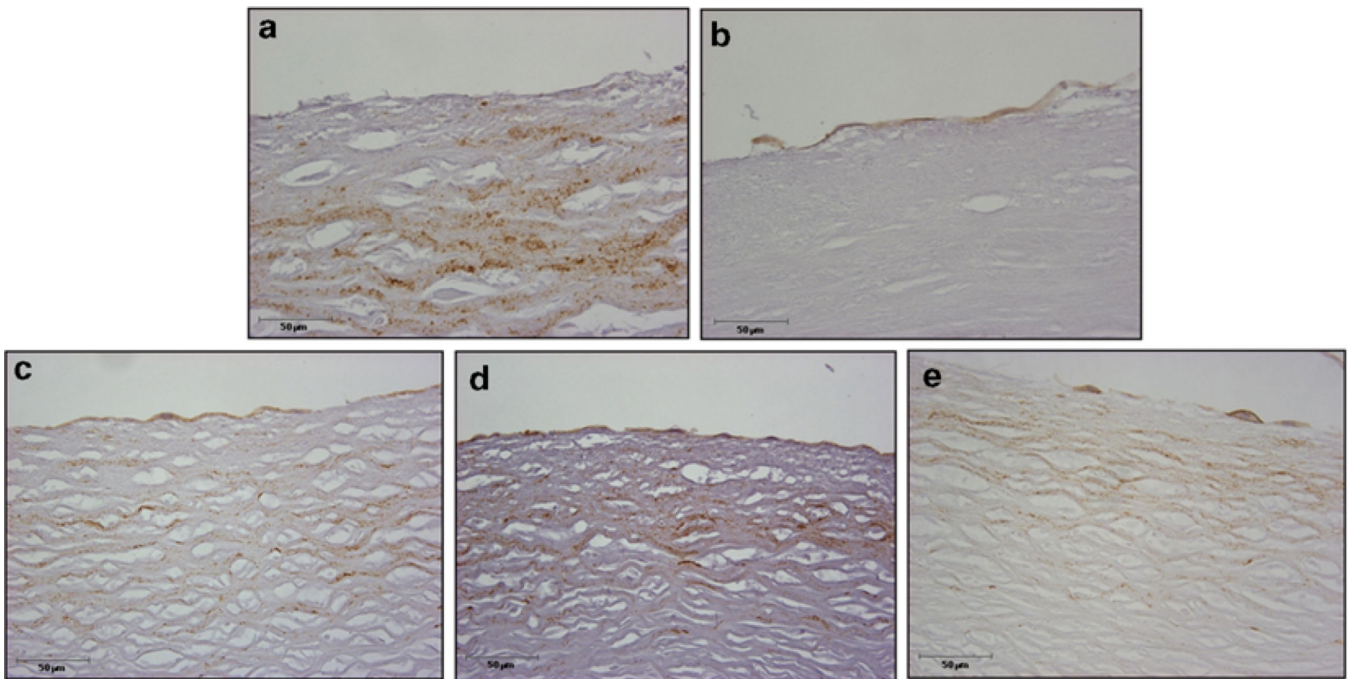
Fig. 9.
Endothelial cell-vessel co-culture histology after 7 days. Human umbilical vein-derived endothelial cells were cultured with ice-free preserved vessels for 7 days in non-tissue culture grade tubes. The tissues were then fixed, embedded, sectioned, and stained for the CD31 endothelial cell marker and photographed at 40× magnification. (**a**) No cell = ice-free preserved vessel without added endothelial cells, (**b**) control decellularized vessel plus endothelial cells, and ice-free preserved vessel with either (**c**) 2 wash steps, (**d**) 4 wash steps, or (**e**) 8 wash steps. No qualitative differences were observed between the decellularized and IFC groups. Note: CD31-positive endothelial cells at the upper edge of the tissue in **b–e**. The interstitial staining is not associated with endothelial cells since it is also seen in **a**, the "no cell" added group

Table 1

Viability post-VS55 heart valve leaflet preservation

Post-recovery time incubation (days)	0	1	2
Relative florescent units/mg dry weight[a] (mean, $N = 6$)	2,047	2,318	2,743
Results in % of fresh untreated controls	80	90	107

[a]Resazurin metabolic assay [45]

Table 2

Pathology overview

Group	Description	Implant pathology[a]	AI at 6 months[b]
1	Conventional freezing method	Mild diffuse leaflet fibrosis. 3/4 exhibited focal nodular vegetation (organized thrombi) and 1/4 exhibited calcified foci. 1/4 exhibited a basal dehiscence and 1/4 a paravalvular aneurysm. 1/4 conduits mineralized	1–2+, 1–2+, 2+, 2–3+
2	VS83 stored at –80 °C	Implants all exhibited mild diffuse fibrosis; 2 of 4 conduits were mineralized	–2+, none,1+, trace-1+
3	VS83 stored in vapor-phase nitrogen	Diffuse and focal fibrosis, 2 of 4 exhibited thrombi and 1 of 4 exhibited focal nodular vegetation on leaflets. 3 of 4 conduits were mineralized	None, 2–3+, 2–3+, 2–3+

[a] Leaflets in all groups were devoid of cells except for recipient overgrowth that appears to be a chronic low-grade inflammatory response with a marked tendency for all leaflets to exhibit some thinning with thicker fibrous overgrowth at the base of the cusps resulting in leaflet thickening and stiffening

[b] Aortic Insufficiency(AI) was assessed by echocardiographic evaluation just prior to sacrifice

Table 3

VS formulation, quantities

	VS55	**VS83**
5× Euro-Collins (mL)	200	200
HEPES buffer (g)	2.39	2.39
Propylene glycol[a] (g)	168.38	252.57
Formamide[a] (g)	139.56	209.34
Me$_2$SO[a] (g)	242.14	363.21
Water (mL)	to 1 L	to 1 L
pH (units)	7.9–8.1	7.9–8.1
Osmolality[b] (mOsm/kg)	460 ± 5	670 ± 5

[a]Liquids measured by weight

[b]20× dilution = 50 μL in 950 μL of distilled water