Guidance for Removal of Fetal Bovine Serum from Cryopreserved Heart Valve Processing

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Key Words

Cryopreservation • Heart valves • Fetal bovine serum • Extracellular matrix • Viability

Abstract

Bovine serum is commonly used in cryopreservation of allogeneic heart valves; however, bovine serum carries a risk of product adulteration by contamination with bovine-derived infectious agents. In this study, we compared fresh and cryopreserved porcine valves that were processed by 1 of 4 cryopreservation formulations, 3 of which were serum-free and 1 that utilized bovine serum with 1.4 M dimethylsulfoxide. In the first serum-free group, bovine serum was simply removed from the cryopreservation formulation. The second serum-free formulation had a higher cryoprotectant concentration, i.e. 2 M dimethylsulfoxide, in combination with a serum-free solution. A colloid, dextran 40, was added to the third serum-free group with 2 M dimethylsulfoxide due to theoretical concerns that removal of serum might increase the incidence of tissue cracking. Upon rewarming, the valves were inspected and subjected to a battery of tests. Gross pathology revealed conduit cracking in 1 of 98 frozen heart valves. Viability data for the cryopreserved groups versus the

fresh group demonstrated a loss of viability in half of the comparisons ($p < 0.05$). No significant differences were observed between any of the cryopreserved groups, with or without bovine serum. Neither routine histology, autofluorescence-based multiphoton imaging nor semiquantitative second-harmonic generation microscopy of extracellular matrix components revealed any statistically significant differences. Biomechanics analyses also revealed no significant differences. Our results demonstrate that bovine serum can be safely removed from heart valve processing and that a colloid to prevent cracking was not required. This study provides guidance for the assessment of changes in cryopreservation procedures for tissues. Copyright © 2010 S. Karger AG, Basel

Abbreviations used in this paper

DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix
FBS	fetal bovine serum
RFU	relative fluorescent units
SHG	second-harmonic generation

Introduction

Three types of heart valves are employed for the replacement of defective patient aortic and pulmonary valves, namely mechanical valves, xenogeneic tissue valves and allogeneic human valves (allografts or homografts) derived from human donors postmortem. Mechanical and xenogeneic valves are not employed in children due to the anticoagulant therapies required and/or calcification, respectively. Allografts have especially benefited children with congenital heart disease and they are also used in young adults, women of child-bearing age, patients with infective endocarditis and older patients with memory problems who may not be compliant with the anticoagulation medications required for mechanical valves. Transplantation of allograft heart valves was first introduced clinically in 1962 [Ross, 1962]. They have demonstrated exceptionally good initial hemodynamic characteristics and hardly any thromboembolic events without the need for anticoagulation as well as better resistance to endocarditis compared to bioprosthetic or mechanical valve substitutes [O'Brien et al., 1987; Tuna et al., 1990]. Initially, the valves were collected and immediately transplanted as so-called homovitals [Gonzalez-Lavin et al., 1988]. Due to logistical issues, grafts were subsequently stored at 4°C in tissue culture medium with antibiotics for up to 6 weeks prior to implantation [reviewed in Jonas et al., 1988]. Eventually, in order to enable long-term storage and improve safety by means of donor screening, microbiological assessment and virology profiling, cryopreservation with controlled-rate freezing and storage in vapor phase nitrogen was introduced [O'Brien et al., 1987; reviewed in Brockbank, 2007]. For the last 20 years, cryopreservation has been the worldwide choice for the long-term storage of human heart valves.

Bovine serum is commonly used in cryopreservation of tissues, including cardiovascular tissues. However, bovine serum is both expensive and carries a risk, albeit low with screened isolated donor herds, of product adulteration by contamination with bovine-derived infectious agents. Nakayama et al. [1994] demonstrated that bovine serum may not be necessary for retention of cell viability for cryopreserved aortic valve tissues. Similarly, we have previously demonstrated that bovine serum can be removed from cryopreservation formulations for adherent cell cultures with retention of cell viability, providing that appropriate formulation validation is performed [Campbell and Brockbank, 2007]. In this study, we compared serum-free cryopreservation with a heart valve cryopreservation method utilizing bovine serum.

Materials and Methods

Animal Model

No animals were sacrificed for these studies. Bona fide excess tissue was employed. Bona fide excess tissue is a term used to describe animal-derived materials obtained from animals after they have been sacrificed for other uses. In this case, pig hearts were procured from a local slaughter house in line with the current standards of the American Association of Tissue Banks for human cardiac tissue recovery, ischemia and preservation [Pearson et al., 2008]. The hearts were rinsed and transported on ice in lactated Ringer's solution. The pulmonary heart valves were then dissected under aseptic conditions, treated with antibiotics by means of Dulbecco's modified Eagle medium (DMEM; Mediatech, Herndon, Va., USA) containing 4.5 g/l glucose and 1% penicillin-streptomycin (Sigma, St. Louis, Mo., USA) at 4°C and prepared for control rate cryopreservation in bags with 80–100 ml of cryopreservation formulation (table 1). We employed a simpler antibiotic cocktail than those typically employed clinically. The choice of antibiotics should not affect the results. Cryopreservation was performed at a controlled rate of 1°C/min to –80°C, and then the valves were stored below –130°C [McNally et al., 1990]. Rewarming was performed in 2 steps, i.e. warming to –100°C in ambient air and warming to approximately +4°C in a 37°C water bath until all ice had visibly disappeared. The cryoprotectant formulation was then removed and the valves were washed in ice-cold DMEM culture medium with 0.5 M mannitol for 15 min 0.25 M mannitol for 15 min and then mannitol-free DMEM for 15 min. Finally, the DMEM was exchanged for fresh DMEM and the tissues were prepared for testing.

Test Methods

All valves were visually inspected for holes or cracks after rewarming and then subjected to viability, morphological or biomechanical assessment.

Alamar Blue™ Assay

Cell survival was assessed using a metabolic assay (Alamar Blue assay). The Alamar Blue assay incorporates a water-soluble fluorometric viability oxidation-reduction indicator which detects metabolic activity by both fluorescing and changing color in response to chemical reduction of the growth medium [O'Brien et al., 2000]. Tissue samples were incubated for 3 h with Alamar Blue working solution, and aliquots of medium were then placed in microtiter plate wells and read on a microtiter plate spectrofluorometer at a wavelength of 590 nm. The data were normalized to the dry weight of the tissue samples and expressed as the mean $\pm$ 1 SE Alamar Blue fluorescence intensity per milligram dry weight of 3 or more replicate samples of tissues (leaflet, artery and muscle) from each valve.

Morphological Methods

Ultrastructural analyses were performed by employing autofluorescence-induced multiphoton imaging and second-harmonic generation (SHG) microscopy [reviewed in Schenke-Layland, 2008]. Briefly, multiphoton imaging and SHG microscopy were performed using a Zeiss LSM 510 META NLO femtosecond laser scanning system described previously [Schenke-Layland et al., 2007]. All observations were made using unprocessed, untreated, intact tissues. Extracellular matrix (ECM) structure-dependent

Fig. 1. Leaflet biomechanical testing methods. **A** Preconditioning of the leaflet sample by cycling through several physiological loading cycles. Low load modulus is measured at this step. **B** The leaflet section is then elongated to result in a stress of 400 kPa and held at constant deformation for 1,000 s while recording the decay in stress. **C** Measurement of ultimate properties by pulling the leaflet at a constant strain rate until failure.

Table 1. Control and experimental tissue groups

Treatment groups	Preservation solution composition
Fresh control	stored overnight in DMEM cell culture medium at 4°C
Frozen with bovine serum	1.4 M DMSO in DMEM cell culture medium plus 10% FBS
Frozen serum-free group 1	1.4 M DMSO in DMEM cell culture medium
Frozen serum-free group 2	2.0 M DMSO in Unisol solution [Taylor et al., 2001]
Frozen serum-free group 3	2.0 M DMSO in Unisol solution plus 6% dextran 40

autofluorescence was induced using a wavelength of 760 nm, depicting elastin-containing fibers, and SHG was induced using a wavelength of 840 nm, showing collagen structures, as described previously in more detail [König et al., 2005; Schenke-Layland et al., 2007]. Noninvasive optical horizontal sections of four different areas of each of the specimens were taken at depths of 10 and 20 μm on each side of the leaflet, the intimal surface of the pulmonary artery and the associated cardiac muscle. A semiquantitative scale was employed to assess SHG intensities as a measure of tissue structure integrity as previously described [Schenke-Layland, 2008].

Routine histopathology was performed using formalin-fixed, paraffin-embedded 5- μm sections. Mounted sections were stained with hematoxylin and eosin or Movat's pentachrome [Schenke-Layland et al., 2007] to evaluate potential cryopreservation-induced changes by light microscopy. The slides were prepared and stained at the Translational Pathology Core Laboratory at the University of California Los Angeles. A semiquantitative scale was employed to assess possible ice damage, seen as white spaces in the tissues.

Biomechanics Methods

Biomechanics analyses were performed at CryoLife Inc. (Kennesaw, Ga., USA) using an Instron Model 5565 materials tester. The methods were previously employed in studies performed for

regulatory purposes and were based upon published testing and calculation methods [Lee and Boughner, 1981; Lee et al., 1984; Vesely et al., 1990]. Cryopreserved valves were thawed and the cryoprotectants removed as described. A hoop section of pulmonary artery was removed from the valve and an average width and thickness were determined. The valve was then opened by dividing along a commissural line to reveal the leaflets. All leaflets were grossly inspected and a single leaflet was resected. A 5-mm-wide 'dog bone' test segment was removed from one leaflet of each valve with the longitudinal axis of the sample oriented circumferentially with respect to the leaflet. The thickness of the sample was measured using a digital micrometer and conductivity gauge.

The artery sections were mounted on a customized load frame and pretensioned. The gauge length was recorded. The conduit loop was then pulled under constant elongation until failure occurred while simultaneously recording the resulting load. Force/elongation data were converted to stress/strain, and the stress and strain were determined at failure and tensile modulus. Testing of conduit samples was not performed under submerged/physiologic conditions.

Leaflet samples were mounted onto the load frame equipped with a constant-temperature bath (Hank's buffered salt solution at 37°C), and all testing was performed with the leaflet section submerged. Prior to testing, the leaflet was preconditioned by cycling through several physiological loading cycles at 400 kPa

Fig. 2. Summary of viability data obtained using the Alamar Blue assay. All data are displayed as the mean $\pm$ SE ($n > 4-6$). RFU = Relative fluorescent units. * $p < 0.05$ versus fresh control samples.

(fig. 1A). The sample was pretensioned and the gauge length recorded. The leaflet section was then elongated to obtain a stress of 400 kPa and held at constant deformation for 1,000 s while recording the stress decay (fig. 1B). The sample was then unloaded for 10 min. Following recovery, the leaflet was again pretensioned, the gauge length was measured and then it was pulled under constant elongation until failure occurred while simultaneously recording the resulting load (fig. 1C). The data collected during the above loading scenarios permitted determination of leaflet extensibility, physiological load modulus, stress relaxation rate, ultimate tensile strength, elongation and modulus.

Statistical Methods

All testing, except the viability tests, were performed using blinded samples. The individuals performing the analyses did not know to which group each sample belonged. The code was not revealed until all test results were finalized. Viability ($n = 4-6$) and morphology data ($n = 4$) were analyzed using a *t* test. Biomechanical group differences for each of the 17 outcome variables were measured with a one-way analysis of variance employing an n of 7–10. All data were considered statistically significant at $p < 0.05$.

Results

Gross pathology revealed a single conduit crack in 1 heart valve-associated pulmonary artery out of the 98 frozen heart valves in this study.

Statistical comparisons of the viability data for cryopreserved groups versus the fresh heart valve data demonstrated a statistically significant loss of viability in half of the comparisons ($p < 0.05$) and a trend for all cryopreserved means to be lower (fig. 2). There were no significant differences between any of the cryopreserved groups with or without bovine serum.

Neither autofluorescence-based multiphoton imaging nor semiquantitative SHG microscopy of ECM components revealed any statistically significant differences between the fresh controls or serum-containing cryopreserved controls and the serum-free cryopreserved groups (fig. 3, table 2). Matrix damage was observed in most groups, including the fresh control group.

Histopathology review demonstrated no qualitative differences in the tissues of the experimental groups compared to either fresh controls or bovine serum-containing cryopreserved controls (table 3). Semiquantitative analyses were performed on the leaflet sections. The serum-free samples treated with Unisol appeared to have more white space, possibly due to ice, but similar white space was observed in fresh controls. Occasional tissue damage foci were observed in cryopreserved tissue sections, but not in the fresh tissue sections (table 3, fig. 4). There was no difference seen between the bovine serum-containing cryopreserved group and the serum-free cryopreserved group with DMEM (table 3).

Biomechanical analyses had an acceptable power of 86.5%. The biomechanics data p values ranged from 0.103 for the low modulus thickness to 0.990 for the ultimate tensile modulus. Because all the p values were greater than the critical value for significance (0.05), no post hoc testing was conducted. The p values indicated that the bovine serum-containing cryopreserved group and the fresh control group were not significantly different from any of the cryopreserved groups without bovine serum (table 4). The leaflet and conduit ultimate load data ($n = 7-10$) are shown as representative data sets (fig. 5).

Fig. 3. **A** Cross-section of a fresh intact heart valve leaflet, stained with Movat's pentachrome stain. **B–F** Multiphoton-induced autofluorescence images of the leaflet inflow side of a fresh specimen (**B**) and specimens cryopreserved in serum (control; **C**), serum-free DMEM (**D**), serum-free Unisol (**E**) and serum-free Unisol +

dextran 40 (**F**). No significant differences were observed between the samples with regard to the ECM components such as elastic fibers (green, 760 nm) and collagen bundles (red, 840 nm). Scale bars = 50 μ m.

Discussion

In this study, we compared 3 serum-free experimental groups with a currently practiced heart valve cryopreservation method using bovine serum and fresh control groups (table 1). In the first serum-free group, we simply removed bovine serum from the cryopreservation formulation. The second serum-free formulation employed had a higher cryoprotectant concentration in combination with a serum-free solution, Unisol, based upon cryopreservation studies of two cell types, vascular smooth muscle and corneal endothelial cells [Campbell and Brockbank, 2007]. A colloid, consisting of 6% dextran 40 (molecular weight 40,000), was added to the third serum-free group, which was otherwise the same formulation as

group 2, due to theoretical concerns that removal of bovine serum might increase the incidence of tissue cracking. Upon thawing, we only observed a single cracking incident and that was in the serum-free group with dextran 40. Serum removal did not cause a high incidence of tissue cracking.

Historically, leaflet cell viability was considered important because it was thought that the cells survived implantation and maintained a normal leaflet ECM structure. The present results indicate that 67–79, 35–82 and 48–63% of leaflet, conduit and muscle cell viability, respectively, remained after cryopreservation in the presence and absence of bovine serum (fig. 2). Significant differences were observed between fresh controls and cryopreserved tissues. There were no significant differ-

Fig. 4. A–C Histology of the worst areas seen in fresh tissue, demonstrating white spaces (arrows, **A**), bovine serum-containing cryopreserved controls with tissue breaks (arrows, **B**) and serum-free cryopreserved tissue in DMEM with a single focal area of tissue damage (arrows, **C**). **D** Fresh tissue without a white space.

E Serum-free cryopreserved tissue in Unisol with white spaces (arrows). **F** Serum-free cryopreserved tissue in Unisol + dextran 40 with a white space (arrow). The damage seen in **B** and **C** may be due to induced artifacts or, more likely, ice damage. Hematoxylin and eosin stain. Scale bar = 100 μ m.

Table 2. Overview of multiphoton imaging and SHG microscopy

Group	Quantitative (SHG) collagen relative intensity		Semiquantitative elastin/collagen
Fresh control	ventricularis:	8.01 ± 1.87	+++, +++, +, ++++ (mean +++)
	arterialis:	6.87 ± 1.91	
Frozen DMEM + bovine serum	ventricularis:	10.00 ± 4.16	++++, ++, +++, + (mean ++/+++)
	arterialis:	6.81 ± 1.91	
Frozen serum-free group 1 (DMEM)	ventricularis:	8.41 ± 2.78	+, +++, +, ++ (mean ++)
	arterialis:	7.47 ± 1.44	
Frozen serum-free group 2 (Unisol)	ventricularis:	8.21 ± 1.54	++++, +, +++, + (mean ++)
	arterialis:	10.96 ± 4.57	
Frozen serum-free group 3 (Unisol + colloid)	ventricularis:	5.35 ± 1.29	+++, +++++, +++, ++/+++ (mean ++++)
	arterialis:	10.33 ± 3.44	

Qualitative review indicates that there are no differences between serum-free and bovine serum groups with generally good elastin/collagen preservation with some negative comments. Semiquantitative analysis was based on generalized review of 8 locations (4 on each surface) for each leaflet: + = best; ++ = good; +++ = good with negative comment; ++++ = not good, damaged to bad.

Fig. 5. Leaflet and conduit ultimate load. All data are displayed as means $\pm$ SE (n = 7–10). No significant differences were observed.

Table 3. Overview of leaflet damage

Group	White space	Focal disruption
Fresh control	+/+/+/+/+/+	no
Frozen DMEM + bovine serum	+/+/+/+/+/+	yes, several in 1 sample
Frozen serum-free group 1 (DMEM)	+/+/+/+/+/+	yes, 1 in 1 sample
Frozen serum-free group 2 (Unisol)	+/+/+/+/+/+/+	no
Frozen serum-free group 3 (Unisol + colloid)	+/+/+/+/+/+	yes, 1 in 1 sample

Leaflet white space: + = none; ++ = very little; +++ = variable (<25%); ++++ = >25%.

ences in cell viability between cryopreserved groups with or without bovine serum. Currently, however, cell viability in allograft heart valves is not considered to be a significant issue after implantation because of studies demonstrating that cell viability is lost by apoptotic pathways over a few days after implantation [Hilbert et al., 1999]. We retained cell viability studies in this investigation because they are still considered a measure of quality, atraumatic valve processing, even though we know the valve cells do not survive in patients. Furthermore, this study provides information of value for preservation of other tissue types where cell survival might be a requirement.

No significant differences were observed between any of the cryopreserved groups, with or without bovine serum, for any of the tests in this study. This included extensive biomechanical testing using established methods for heart valve tissue testing [Lee et al., 1984; Vesely et al., 1990] in which the biomechanical properties of the cryo-

preserved groups were also not significantly different compared with fresh untreated controls (table 4, fig. 5). There have been a few reports of cryopreservation-induced changes in cardiovascular tissue biomechanical properties. Cryopreservation resulted in significantly increased strain in aortic heart valve leaflet matrices when pressurized [Narine et al., 2006], and cryopreservation has also been reported to significantly affect the overall tensile strength and elasticity of aortic arteries [Thakrar et al., 2006]. In two studies of tissue engineered blood vessel cryopreservation, increased tissue strength was observed following cryopreservation [Elder et al., 2005; Dahl et al., 2006]. Cryopreservation by freezing has also been reported to predispose both syngeneic and allogeneic rat aortic heart valve leaflets to accelerated injury and destruction [Legare, 2000]. Cryopreservation by vitrification has been shown to better preserve biomechanical properties and ECM structure compared with cryopreservation by freezing [Thakrar et al., 2006; Schenke-

Table 4. Overview of biomechanics analysis results

Analysis	Mean $\pm$ SD	p value
Low modulus		
Thickness, mm	0.250 $\pm$ 0.058	0.103
Modulus, MPa	7.825 $\pm$ 3.095	0.303
Stress relaxation		
Stress remaining (10 s), %	84.782 $\pm$ 2.763	0.765
Stress remaining (1,000 s), %	59.011 $\pm$ 8.389	0.258
Relaxation slope (first 10 s)	9.783 $\pm$ 1.638	0.802
Relaxation slope (overall)	14.102 $\pm$ 4.831	0.220
Ultimate tensile		
Thickness, mm	0.249 $\pm$ 0.060	0.107
Ultimate extension, mm	4.140 $\pm$ 0.890	0.584
Ultimate load, gf	896.468 $\pm$ 308.723	0.386
Ultimate stress, MPa	7.514 $\pm$ 3.253	0.986
Ultimate strain, %	82.808 $\pm$ 17.819	0.586
Modulus, MPa	31.135 $\pm$ 13.784	0.990
Conduit ultimate tensile		
Ultimate extension, mm	34.510 $\pm$ 3.524	0.474
Ultimate load, gf	1,636.440 $\pm$ 431.509	0.782
Ultimate stress, MPa	1.010 $\pm$ 0.499	0.346
Ultimate strain, %	322.810 $\pm$ 83.940	0.509
Modulus, MPa	1.610 $\pm$ 0.791	0.274

Layland et al., 2007]. However, the excellent long-term outcomes of cryopreserved heart valves in patients suggest that this is not an issue in the clinic [reviewed in Brockbank, 2007].

Occasional tissue damage foci were observed in cryopreserved tissue histology sections (table 3, fig. 4). They may have been due to either ice formation or sectioning artifacts. However, no similar damaged foci were observed in fresh tissue sections that were not subjected to cryopreservation, so it is likely that the damaged foci were indeed due to ice formation. There is extensive literature demonstrating a correlation between the formation of ice crystals and structural or functional damage to tissues and organs [Pegg, 2010]. We have previously documented ice formation in cryopreserved heart valves [Brockbank et al., 2000] and demonstrated ECM damage by laser scanning microscopy in frozen cryopreserved heart valves [Schenke-Layland et al., 2006, 2007]. In the present study, all 4 of the cryopreserved groups and the fresh control group had at least 1 sample that exhibited damaged ECM structures (table 2). We have not observed ECM damage in fresh untreated porcine controls previously [Schenke-Layland et al., 2006, 2007], and in the present case, the damage was not due to ice since the untreated control sample was not frozen. All the hearts used as a

source of valves and the valves themselves were treated in a similar manner using American Association of Tissue Banks standards for guidance [Pearson et al., 2008], so it is also unlikely that the ECM damage in the fresh untreated control was due to postmortem warm or cold ischemia after heart recovery. Furthermore, individual valves in 3 of the 4 cryopreserved groups had excellent ECM preservation (table 2), in contrast with earlier observations [Schenke-Layland et al., 2006, 2007] but in agreement with a recent human heart valve study [Gerson et al., 2009]. It is clear that further laser scanning microscopy studies of fresh and cryopreserved heart valves are needed.

Despite the fact that fetal bovine serum has been used for cell and tissue cryopreservation for many years, it is time to remove it from cryopreservation formulations. The same issues that have driven the development of serum-free media in recent years for other purposes, in particular better definition of cell culture medium for controlled cell growth and differentiation, drive removal of fetal bovine serum from cryopreservation formulations. Sera batches contain uncontrolled, variable amounts of stimulatory and inhibitory molecules that combine to have unpredictable effects on cells. Difficulties in dealing with variability between lots of serum and the availability of serum when it is needed have been reasonably well managed. However, with cell-based medical products, such as human allogeneic cryopreserved heart valves, the risk of transmission of potentially infectious agents to humans has become a real concern [Nuttall et al., 1977; Erickson et al., 1989, 1991; Levings and Wessman, 1991; Froud, 1999; Jayme, 1999; Jennings, 1999; Merten, 1999; Wessman and Levings, 1999; Zabal et al., 2000; Makoschey et al., 2003; Weber, 2004]. Whereas most agents do not cross species barriers, there have been instances where a prion, for instance, has crossed over and infected a new species. An example of this is the ability of the bovine spongiform encephalopathy prion to infect humans with a variant of Creutzfeldt-Jakob disease [Will et al., 1996; Brown et al., 2001; Croes and van Duijn, 2003; Irani and Johnson, 2003; Ironside and Head, 2003; Trevitt and Singh, 2003; Ward et al., 2003; Bradley, 2004]. Guidelines have been put in place to reduce the risk of possible transmission from cell-based products to humans [Food and Drug Administration, 1998; Asher, 1999; Committee for Veterinary Medicinal Products, 2001; Food and Drug Administration, 2001] and involve restriction of the sources of fetal bovine serum, as well as guidelines for production and handling [Committee for Veterinary Medicinal Products, 2001; Food and Drug Administration,

2001]. With these guidelines in place, it is felt that the risk of transmission of bovine disease agents to humans is minimal. However, the best approach, when possible, is to remove all possible sources of contamination.

For cryopreserved human tissue products, this means the removal of animal-derived serum from the entire production process. There have been sporadic reports in the literature indicating that bovine serum is not necessary for tissue cryopreservation [Nakayama et al., 1994; Reuther et al., 2006], but these reports did not result in changes in clinical practice. Nakayama et al. [1994] demonstrated that bovine serum is not necessary for the cryopreservation of aortic valve tissues, and Reuther et al. [2006] reported that bovine serum can be removed for cryopreservation of iliac cancellous bone with minor losses of osteoblast viability. Nakayama et al. [1994] assessed fibroblast viability of porcine aortic valves cryopreserved with and without bovine serum using tritiated proline autoradiography. No significant difference was observed between the two groups at any time. These results suggested that fibroblasts were adequately preserved even after 3 months of cryopreservation. In addition, it was shown that the addition of bovine serum to the medium did not further improve their viability. Thus, from the perspective of fibroblast viability, the authors [Nakayama et al., 1994] concluded that it was not necessary to use fetal bovine serum for the cryopreservation of aortic valve tissues. The present study extends these observa-

tions and demonstrates that fetal bovine serum can be removed from heart valve processing without significant impact on either cell viability, tissue structure or biomechanical properties. With the exception of some reduction in viability compared with fresh control tissue, all other tests showed that there was no difference between fresh and cryopreserved tissues. In addition, there was no difference between the various cryopreservation formulations employed in this study. This animal model study also provides guidance for assessment of changes in cryopreservation procedures for tissues intended for clinical use.

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