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Extracellular Matrix of Fresh and Cryopreserved Porcine Aortic Tissues

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Abstract : The effect of cryopreservation on extracellular matrix was studied with the ultimate objective of permiting a prediction of the tendency of aorta conduit tissue to calcify following transplantation. Cryopreserved and fresh porcine aorta conduit tissues were extracted using guanidine-hydrochloride (Gdn-HCl) followed by sequential digestion of the tissues with collagenase, elastase, and papain. Glycosaminoglycans (GAGs) of the proteoglycans (PGs) were isolated and quantitated. Gdn-HCl extracted about 61% and 62% of the total GAG (proteoglycan) material from cryopreserved and fresh tissues, respectively. Collagenase-solubilized proteoglycans from Gdn-HCl extracted tissue represented 20% and 13%, respectively, of the total GAGs present in cryopreserved and fresh tissues. Subsequent elastase hydrolysis of collagenase-digested tissue released about 11% of total GAGs from cryopreserved tissue and 16% from fresh tissue. The remaining 8%, from cryopreserved tissue, and 9%, from fresh tissue, of the total GAGs were obtained after using a papain hydrolysis. There was essentially no difference between fresh and cryopreserved tissues in the relative distribution of proteoglycans in the extracts and digestions except in the initial digestion step where more proteoglycans were obtained from collagenase solubilization of cryopreserved tissue than fresh tissue ($p < 0.05$). The histologic status of the fresh and cryopreserved porcine aortic conduit did not differ markedly. The normal tissue architecture was not affected markedly by the cryopreservation procedure as neither alteration of elastic structure, fibrous proteins nor alteration of nuclear distribution or smooth muscle cell morphology was detected. Quantitative tissue mineral studies revealed that the mean calcium content of the cryopreserved aorta conduit tissue (165±3 μg/g wet tissue) was higher than that of the fresh tissue (105±4 μg/g wet tissue) ($p < 0.05$). The mean phosphorus content was 703±35 μ/g wet tissue from cryopreserved tissue and 720±26 μg/g wet tissue from fresh tissue. The study indicates that there is no significant alteration in the distribution of PGs in properly cryopreserved tissue, but the total calcium level appears to be increased in tissue cryopreserved by the cryopreservation process used in this study.

Key words : aorta conduit tissue, cryopreservation, mineral analyses, sequential extraction.

Calcification of transplanted heart valves impairs the durability of valves. Calcium deposition may lead to valve stenosis and mechanical fragility and results in degeneration and dysfunction. Although valved aortic homografts develop calcification much less frequently than xenograft, calcification of both aortic media and to a lesser exent the aortic valve leaflets leads to a functional compromise in late results (Miller and Shumway, 1987; Maxwell *et al.*, 1989). In addition to cell- and collagen-oriented calcification, such as in valves, aortic wall calcification occurs in close association with elastin as a prominent feature in experimental (subdermal and circulatory models) and clinical species (Gonzalez-Lavin *et al.*, 1988; Jonas *et al.*, 1988; Khatib and Lupinetti, 1990; Saravalli *et al.*, 1980). Urist and Adams (1967) demonstrated that the calcification of transplanted aorta in rats was localized mostly to the elastic structure after degradation or splitting of elastic fibers where an increased rate of calcium uptake was observed.

The pathophysiology of cardiovascular implant calcification is complex and poorly understood, and there are no satisfactory preventive measures or therapies to reverse degenerative calcification. However, pathological calcification in the calcific disease and the normal (physiological) calcification of skeletal and dental tissues share important features. Chief among the common elements in various types of cardiovascular implant calcification is cell-derived components, such as cellular debris and subcellular vesicle-like organelles,

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which serve as the initial locus of calcification in direct analogy to the matrix vesicles of endochondral skeletal and dental mineralization (Anderson, 1984). In addition, extracellular matrix proteoglycans (PGs) play an important role in the physiology of the vascular wall and calcification (Berenson *et al.*, 1973; Castellot *et al.*, 1982; Ruoslathi, 1988; Ruoslathi, 1989). PGs also have a close association with fibrous proteins and probably help to keep the collagenous and elastic elements firmly bound together. Little information describing the characteristics of individual proteoglycans and their precise location in the aortic tissue is available. However, arterial proteoglycans have physicochemical properties that are similar to those of hyaline cartilage, and recent advances in the study of cartilage mineralization indicate that matrix PGs have an inhibitory effect on cartilage calcification (Hirschman and Dziewiatkowski, 1966; Larsson *et al.*, 1973; Lohmander and Hjerpe, 1975; Mitchell *et al.*, 1982; Joseph, 1983). Calcification of cells and cell fragments is also due to a disruption of normal physiology for cellular calcium regulation (Han *et al.*, 1995). In damaged cells of cardiovascular implants, mechanisms for calcium exclusion are no longer functional (decreased efflux) (Schoen *et al.*, 1986), and the injured membrane is more permeable to calcium (increased calcium influx) (Yoon *et al.*, 1995). A net calcium influx reacts with phosphorus in the membrane of the cell and may contribute to the initiating mechanism of calcium phosphate crystallization in cardiovascular implants.

There has been little information describing the effect of cryopreservation on extracellular matrix in allograft heart valves. The present study focuses on the matrix components of the arterial conduit tissue since valved aortic homografts develop calcification (late after transplantation) significantly greater in the aortic wall than in the valve leaflets. The purpose of this study was to assess the distribution of proteoglycans in aorta conduit tissue, to evaluate the morphology of fresh and cryopreserved porcine aorta tissues, and to determine the calcium and phosphorus content of fresh and cryopreserved aorta conduit tissues. The ultimate objective of this research is to serve as a base-line study for subsequent studies into the effects of *in vivo* and *in vitro* warm ischemic times on cell death and attendant changes in extracellular matrix of the allograft. Are changes in proteoglycan and other matrix macromolecules in homograft heart valves associated with the tendency of such tissues to calcify following transplantation?

Materials and Methods

Preparation of tissues

The pig hearts were obtained from a local abattoir. Each heart was removed within 20 min of slaughter and immediately washed to remove residual blood. The hearts were packed in ice for transport to the laboratory. Fresh aorta conduit tissues are the tissues dissected from the hearts immediately on arrival at the laboratory. Cryopreserved tissues are the tissues taken through a standard preimplantation processing for cryopreservation as described by Lange and Hopkins (1989).

Isolation of proteoglycans

The procedure used for extraction of PGs from pig aorta with 4 M Gdn-HCl (three times) and hydrolysis of residual tissue by collagenase, elastase, and papain is

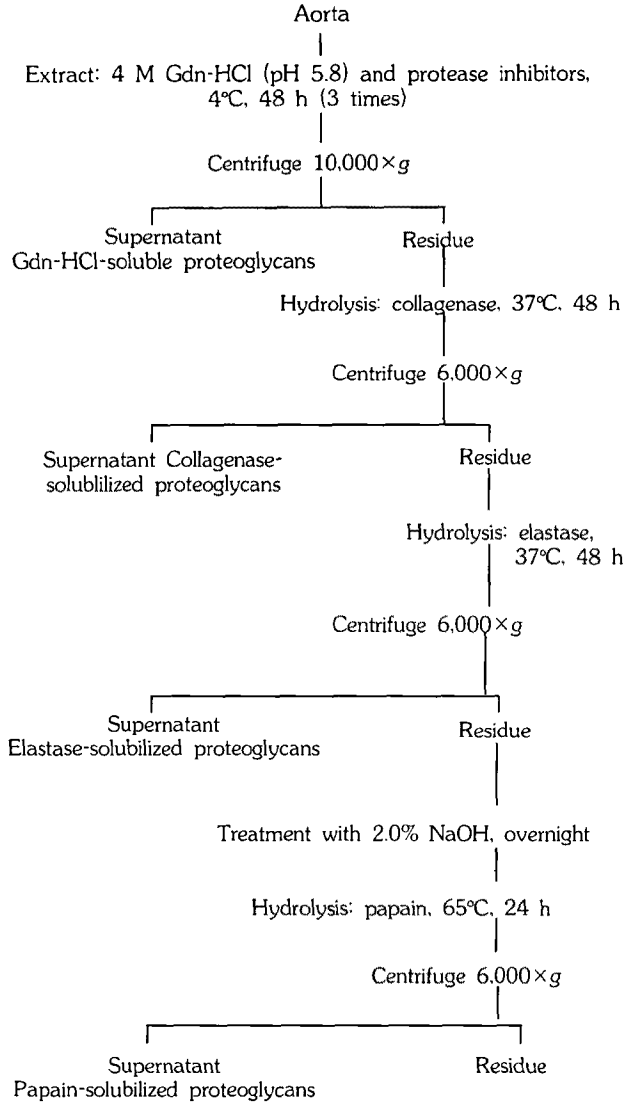

Fig. 1. Procedure for isolation of proteoglycans by sequential extraction of fresh and cryopreserved porcine aorta tissues with Gdn-HCl, collagenase, elastase, and papain.

shown in Fig. 1. Aorta conduit tissues were minced as finely as possible with dissecting scissors. Minced tissues were extracted with 4 M Gdn-HCl containing a number of protease inhibitors (0.1 M aminocaproic acid, 0.005 M benzamidine-HCl, 0.01 M EDTA, 0.005 M N-ethylmaleimide, 0.001 M iodoacetamide , and 0.001 M phenylmethylsulfonyl fluoride) for 48 h at 4°C (Lee *et al.*, 1995). Extracts were centrifuged at 10,000×*g* using a JA-20 rotor in a refrigerated centrifuge (Beckman Model J2-21) for 30 min, and the supernatants were dialyzed repeatedly (five times) against 200 ml of ultrapure water at 0°C with stirring. Porcine aorta conduit tissue was repeatedly (three times) extracted until no more appreciable quantities of hexuronic acid-positive material was obtained in the extraction solution. Gdn-HCl-extracted conduit tissue was washed three times with ultra-pure water and hydrolysed with collagenase from Clostridium histolytium (clostridiopeptidase A, EC 3.4.24.3, Sigma Chemical Co.; 1 mg/g wet tissue) in 10 ml of 10 mM $CaCl_2$/50 mM Tris-HCl buffer, pH 7.6. for 48 h at 37°C. All protease inhibitors, as previously described, were added to the sample to inhibit non-specific proteolysis (Jeon *et al.*, 1995). The hydrolysate was centrifuged at 6,000×*g* using a JA-20 rotor in a Beckman J2-21 centrifuge for 30 min, and supernatant (collagenase digest) was removed. The residue tissue, not digestible by collagenase, was washed three times with ultra-pure water and hydrolyzed with elastase (Type I, EC 3.4.21.36, Sigma Chemical Co.; 250 U/g wet tissue) for 48 h at 37°C in 0.2 M Tris-HCl buffer, pH 8.8, containing protease inhibitors. The supernatant (elastase digest) was removed after centrifugation of the digestion mixture at 6,000×*g* in a Beckman J2-21 centrifuge for 30 min. The pellet remaining after the digestion with elastase was washed with ultra-pure water and treated with 2% NaOH overnight at room temperature and further digested with papain (from papaya latex, EC 3.4.22.2, Sigma Chemical Co.; 1 mg/g wet tissue), after adjusting the pH to 6.4 with phosphoric acid. Digestion was performed at 65°C for 24 h in the presence of 0.01 M EDTA and 0.01 M cysteine hydrochloride.

Light microscopy

Small pieces of aorta, both fresh and cryopreserved, (1 mm×1 mm×1 mm) were fixed and sectioned for histology study. Semi-thin sections were stained with Richardson stain preparation (2 part Azure II, 1 part methylene blue, and 1 part sodium borate in deionized water) and studied by light microscopy (Nikon Biophot V series).

Calcium and phosphorus assays

Aortic conduit tissues to be used for mineral analyses were rinsed three times with 100 ml of 0.9% NaCl. Tissue (1 g) was placed in crucible (Fisher), and ashed in a muffle furnace (Sybron, Thermolyne 2000) at 550°C overnight. The ashed tissues were solubilized in 5 ml of 1 N HCl overnight and analyzed for calcium and phosphorus.

Analytical methods

Glycosaminoglycans were isolated from the extract by alkaline treatment as described by Carlson (1968). Uronic acid content in the proteoglycan fractions was determined by the method of Bitter and Muir (1962) with glucuronolactone (Sigma Chemical Co.) as a standard. The arsenazo III method (Bauer, 1981; Michaylova and Ilkova, 1971; Rowatt and Williams, 1989; Janssen and Helbing, 1991) was used for determination of total calcium content per gram of tissue. $CaCl_2$ solution (0.01 mM in 1 N HCl) was prepared as standard stock. The ascorbic acid method (Chen *et al.*, 1956) was used for the determination of tissue phosphorus in fresh and cryopreserved pig aorta conduit tissues. Statistical evaluations of significance were compared by Student's t-test. The significance level was set at 0.05.

Results

Distribution of proteoglycans in porcine aorta

Some proteoglycans in tissue are present in the soluble matrix while others are associated with other components, such as collagen and elastin. Proteoglycans in the soluble matrix were extracted by the use of a dissociative solvent (Gdn-HCl) by dissociating most non-covalent interactions between macromolecules. However, the proteoglycans bound to collagen and elastin require solubilization of fibrous components of the tissue for extraction. The quantitative distribution of proteoglycans in porcine aorta was thus studied using the sequential extraction of tissue.

Total uronic acid (as μg of the wet weight of tissue) and uronic acid distribution (as the % of total uronic acid) in the fresh and cryopreserved tissues obtained by sequential extractions of the tissues are shown in Table 1. There was no significant difference in total uronate between fresh and cryopreserved tissues. Based on analysis of the total uronate, 4 M Gdn-HCl solubilized 62.2% (in fresh tissue) and 61.2% (in cryopreserved tissue) of total tissue hexuronic acid content. Hydrolysis of the extraction residue with collagenase released 12.8% of the total uronate in fresh tissue and 20.1% of the total uronate in cryopreserved tissue. Subsequent elastase hydrolysis of the fresh and cryopreserved tissues released 16.0% and 10.5% of the total hex-

Table 1. Uronate distribution in sequential extracts of proteoglycans from fresh and cryopreserved porcine aorta conduit tissues. The aorta was sequentially extracted by 4 M Gdn-HCl (3X) and digested by collagenase, elastase, and papain.

			Fresh	Cryopreserved
Total UA[a] (μg/g wet tissue)			970±44	1051±25
UA distribution in different extraction steps (% of total UA)	Gdn-HCl extract	1st	50.6±0.5	47.7±2.6
		2nd	7.5±0.2	10.4±2.1
		3rd	4.1±0.6	3.1±0.4
	Collagenase solubilized		12.8±2.1	20.1±1.0
	Elastase solubilized		16.0±1.8	10.5±1.1
	Papain solubilized		9.1±0.4	8.2±0.9

[a] UA : uronic acid.
Values represent the mean±standard error of replicate assays, n=3.

uronic acid content, respectively. The remaining uronate-positive materials (9.1% from fresh and 8.2% from cryopreserved tissue) were obtained through papain hydrolysis. There was essentially no difference between fresh and cryopreserved tissues in the relative distribution of uronate-positive materials in the various tissue extracts.

Morphology of fresh and cryopreserved porcine aortic conduit tissues

Light microscopic studies revealed that the tunica media of porcine aortic conduit tissue contains an abundance of smooth muscle cells (represented by the nuclei); however, the most distinct feature of the tunica media is its large amount of elastin material. The elastin material is not present in the form of fibers, but rather as fenestrated membranes (Fig. 2A). Careful examination of Fig. 2A revealed what appears to be interruptions of some of the laminae. These interruptions are actually the fenestrations or openings in the elastin membrane. The smooth muscle cells of the media are arranged in a closely wound spiral between the elastic membranes, however, this arrangement is difficult to recognize in sectioned material.

A comparative histologic examination of tunica media from fresh and cryopreserved porcine aorta conduit specimens revealed a normal pattern of elastic tissue and distribution of the smooth muscle cells in the medial layer of the aorta (Fig. 2). The pattern of elastin distribution in fresh aortic tissue consisted of long, uniform, parallel laminae in a regular arrangement. The smooth muscle cells had prominent round nuclei, and were distributed between the elastic laminae as single cells (Fig. 2A). The normal structure of the media was not affected during tissue preimplantation processing since neither change of the elastic tissue nor evidence of altered distribution of smooth muscle cells was detected in the cryopreserved aorta conduit specimen (Fig. 2B).

The outermost layer of the porcine aorta, the tunica

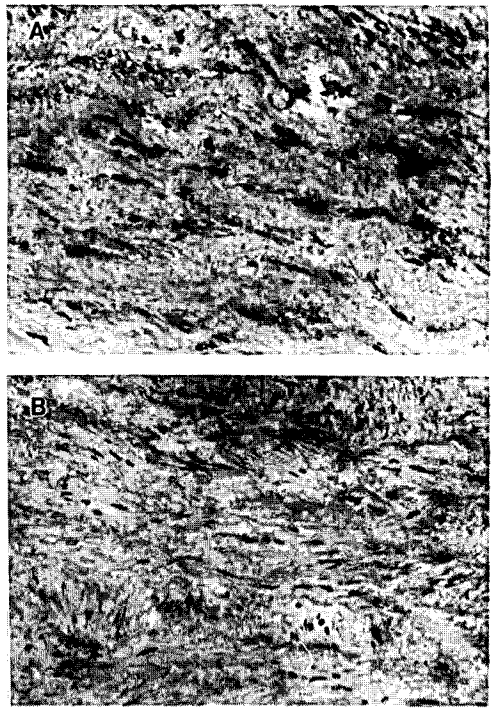

Fig. 2. Histologic sections of the tunica media of fresh (A) and cryopreserved porcine aortic conduit (B), showing normal elastic architecture (waves) and distribution of smooth muscle cell, nuclei, (Richardson stain; original magnification ×400).

adventitia, is shown in Fig. 3. The tunica adventitia consists mostly of collagenous fibers that course in longitudinal spirals. Their course, like the smooth muscle cells, however, is unrecognizable in individual tissue histology sections. The cells of the adventitia, represented by the nuclei seen in the adventitia in Fig. 3, are fibroblasts. There is no elastic laminae in the adventitia; but elastic fibers are present, even though they are relatively few. The presence of elastic fibers in histology sections used in this study was unrecognizable since the elastic fibers were not stained with Richardson stain preparation. A comparative histologic examination of tunica adventitia from fresh (Fig. 3A) and cryopreserved

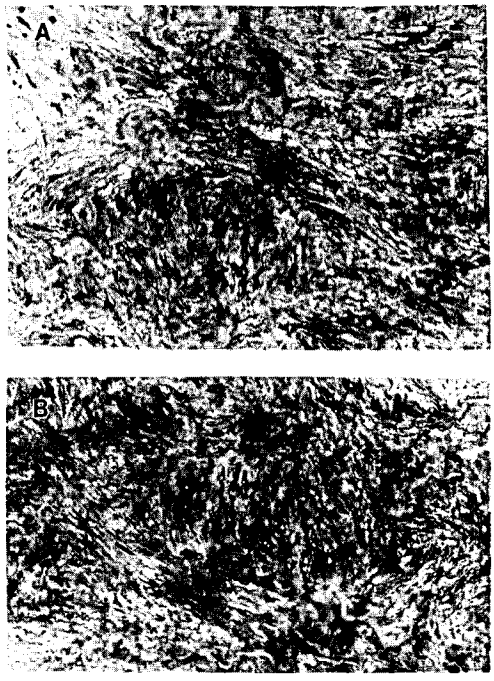

Fig. 3. Histologic sections of tunica adventitia of fresh (A) and cryopreserved porcine aortic conduit (B), showing the normal collagen structure and fibroblast distribution (Richardson stain; original magnification x400).

(Fig. 3B) porcine aortic conduits revealed no change in the structure of collagen or distribution of fibroblasts in tissue that has undergone preimplantation processing and cryopreservation (Fig. 3B).

Mineral analyses of porcine aorta tissue

The quantitative calcium levels for fresh and cryopreserved porcine aorta tissues is shown in Table 2. The average amount of chemically detectable calcium was 105.0±3.9 and 164.8±3.2 μg calcium/g of wet tissue in fresh and cryopreserved porcine aorta tissues, respectively. Thus, the calcium content in cryopreserved tissue was significantly ($p < 0.05$, Student's t-test) greater than that in fresh tissue. The increased total calcium level in cryopreserved tissue might be due to the higher calcium concentration of RPMI 1640 tissue culture medium (used for cryopreservation process) compared to the calcium content of porcine aorta tissue. The phosphorus content of fresh and cryopreserved porcine aorta conduit tissues is shown in Table 2. The tissues which were ashed and then hydrolyzed in 1 N HCl contained 719.8±28.5 μg phosphorus/g wet tissue in fresh and 703.1±39.2 μg phosphorus/g wet tissue in cryopreserved tissue, respectively. There was no difference in the phosphorus level between fresh and cryopreserved tissues.

Discussion

Table 2. The quantitative calcium and phosphorous contents of fresh and cryopreserved porcine aorta conduit tissues. Tissue for calcium and phosphorous analysis was rinsed with 0.9% NaCl and ashed overnight. The ashed sample was solubilized in 1N HCl and analyzed for calcium using the Arsenazo III assay method and for phosphorous using ascorbic acid assay method

	Calcium (μg/g wet tissue)	Phosphorous (μg/g wet tissue)
Fresh	105.0±3.9	719.8±28.5
Cryopreserved	164.8±3.2	703.1±39.2

Values represent the mean±standard error, n=9.

Aortic valve allografts have been used clinically for more than 25 years to repair complex heart malformations (Fontan *et al.*, 1984). The performance of fresh antibiotic sterilized-valvular allografts has been superior to that of mechanical or xenograft valves; however, severe limitations in the supply of allograft valves has restricted usage. The development of cryopreservation technologies has dramatically improved the availability of allograft valves, but issues of long-term durability remain. Allograft valve calcification remains a major concern for continued valve function and no effective means for inhibiting allograft calcificaion are currently available. The research undertaken in this study was designed to begin an alternative approach to the preparation of allograft valves and conduits that might possess a reduced tendency to calcify following transplantation.

An issue central to allograft heart valve calcification focuses on presumed mechanisms by which the heart valves might calcify. A great deal of research on bioprosthetic heart valve calcification has been conducted, but will not be discussed here because the author suggests that allograft valves calcify *via* mechanisms intrinsically different from those operating in bioprosthetic valves. Bioprosthetic valves are nonvital and are composed of variously "fixed" connective tissues. Allograft valves are frequently vital, *i.e.*, contain viable cells at the time of transplantation, and the macromolecules of extracellular matrix remain more or less in their native state. On histologic examination of this study, the normal tissue architecture was not affected markedly by the cryopreservation procedure, as neither alteration of elastic structure, fibrous proteins, nor altenation of nuclear distribution or smooth muscle cell morphology was detected.

Loss or alteration of extracellular macromolecules accompanies cartilage calcification or transformation of cartilage into bone. Lohmander and Hjerpe (1975) found that rib cartilage lost approximately half its con-

tent of proteoglycans with the onset of calcification. They suggested that the quantitative and qualitative change in the proteoglycans of cartilage during calcification resulted from the concerted actions of released lysosomal hydrolytic enzymes. The presence in cartilage of both protease and hyaluronidase has been demonstrated (Woessner, 1973). These enzymes may be released into the intercellular matrix directly from cells and/or from matrix vesicles during and after degeneration and disintegration of chondrocytes (Anderson, 1969; Thyberg and Friberg, 1970). Degeneration of chondrocytes is frequent in the hypertrophic and mineralizing parts of epipyseal cartilage (Thyberg and Friberg, 1970).

Allograft heart valves are analogous in many ways to mineralizing tissues. They typically contain a population of fibroblast cells with reduced viability at the time of transplantation, and it is generally accepted that donor cells in a homograft valve fail to persist much beyond transplantation, a characteristic not much different from chondrocyte degeneration in hypertrophic and mineralizing cartilage (Thyberg and Friberg, 1970). Remnants of dead cells mimic the matrix vesicles of endochondral skeletal and dental mineralization in serving as sites of nucleation of calcium to the phosphate-rich phospholipid in cell membranes (Anderson, 1984). Dead and dying cells in the homograft would certainly release hydrolytic enzymes that could serve to alter the proteoglycan component (as well as other macromolecular components) of the matrix. Such an alteration clearly mimics the changes in proteoglycan and other matrix macromolecules associated with cartilage mineralization.

The result of the sequential extraction of proteoglycans in the present study indicates that there is essentially no difference between cryopreserved and fresh tissues in the relative proportions of uronate-positive materials (proteoglycans) per unit weight of wet tissue extracted in the procedure, except perhaps for the amount of proteoglycans solubilized by collagenase from cryopreserved tissue. In the ultrastructural morphology study, dispersed collagen fibers were found in cryopreserved tissue (unpublished data), which may explain the result that more amounts of proteoglycans were extracted from the collagenase digestion of cryopreserved tissue than from fresh tissue. Because of the slightly more dispersed arrangement of collagen fibers in cryopreserved tissue, it might be easier to digest the collagen with enzyme treatment, which could then result in the extraction of more proteoglycan from cryopreserved tissue.

There is more biochemically detectable calcium in cryopreserved tissue than in fresh tissue. Cryopreserved aorta conduit tissue might be more likely to calcify after implantation because of this higher content of total calcium if this characteristic contributes to subsequent calcification. The phosphorus content of fresh and cryopreserved porcine aorta conduit tissues was similar, which suggests that cryopreservation processing does not influence the tissue phosphorus content. If the elastin structure, normally saturated with protein-calcium complexes, develops the capacity to calcify after degradation or the splitting of fibers and the increased rate of uptake of calcium ions, the implanted cryopreserved allograft might calcify because of a higher content of total calcium.

It must be emphasized that the data presented in this study were not completely intended to support proposed mechanisms for calcification of allograft valves. Rather, the proposed mechanisms formed the basis for conduct of the study. It was important to determine whether the cryopreservation of allograft valves altered the molecules of extracellular matrix. The results described herein document that cryopreservation does not alter extracellular matrix in cryopreserved porcine conduit tissues. These findings do not, however, alter the basis of the proposed hypothesis. The present studies were conducted with porcine tissues with very short warm-ischemic times, *i.e.*, in cases in which both fresh and cryopreserved valves might be expected to retain maximum cellular viability. With increasing warm ischemic times, fibroblast cell viability steadily declines (Hu *et al.*, 1990) and Hu (1992) demonstrated that porcine conduit tissues stimulated increased levels of alkaline phosphatase activities with increasing warm ischemic times in an *in vitro* cell culture bioassay system.

It has been suggested that an immunological basis exists for calcification, demonstrating a significantly more severe valvar calcification in allografts to transgenic rats compared with syngeneic rats (Khatib and Lupinetti, 1990). Gonzalez-Lavin *et al.* (1988) also demonstrated the importance of immunologic influences on allograft valve degeneration. They found that the calcium content of allograft valve leaflet and aortic conduits was greater when the transplant was performed between unrelated dogs than when the transplant was between littermates. It was initially believed that valve allografts may be immunologically privileged since the graft does not retain cellular viability following transplantation or the cells are rapidly replaced by those of recipient origin. However, it is now acknowledged that valve grafts are antigenic and provoke a host immune response (Heslop *et al.*, 1973; Gonzalez-Lavin *et al.*, 1988). Some investigators have proposed that ABO matching between donor and recipient should be performed if possible, or that it is necessary to consider immunosuppression, at least temporarily, after implantation of homovital grafts in growing individuals

(Gonzalez-Lavin *et al.*, 1988; Yankah *et al.*, 1988). Fresh aortic allografts are antigenic and their antigenicity is not altered by cryopreservation (Cochran and Kunzelman, 1989). Jonas *et al.* (1988) suggested that cryopreservation might enhance immunogenicity, since they observed more lymphocytic infiltration of the cryopreserved conduits relative to the fresh grafts in a sheep model. The cellular viability of cryopreserved allograft valves is superior to that of tissues preserved by other methods, and the antigenic effect may result from enhanced endothelial preservation, since the endothelium of vascular structures is the most immunostimulatory of all vascular components (Pober *et al.*, 1986). It has been speculated that persistence of the endothelium may be desirable in grafted valve leaflets to protect the physicochemical balance of the matrix and facilitate the retention of basic structural features until new fibrous connective tissue develops (Armiger *et al.*, 1985). However, since the endothelium is antigenic and incites a host immune response, its value is in question. Processing and storage techniques that result in gentle displacement of the graft endothelium may be optimal.

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