Impact of Cryopreservation on Extracellular Matrix Structures of Heart Valve Leaflets

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Background. **Transplantation of cryopreserved allografts represents a well-established valve replacement option. Despite their clinical use for more than 40 years, the integrity of the extracellular matrix (ECM) of these valves after thawing has not been determined. The purpose of this study was to investigate and compare ECM structures of fresh and cryopreserved porcine heart valve leaflets with special emphasis on the condition of collagenous and elastic fibers.**

Methods. **Pulmonary valves were excised from unprocessed porcine hearts under sterile conditions. After treatment with antibiotics, the valves were incubated in a cryoprotective solution, cryopreserved stepwise, and stored at –196°C for 1 week. Two groups of heart valves (fresh untreated and thawed cryopreserved [each, n = 8]) were analyzed using biochemical (collagen, elastin, desmosine), histologic (hematoxylin-eosin, Movat-pentachrome, resorcin-fuchsin), and immunohistochemical (antibodies against collagen I, III, IV, and elastin) methods. Near-infrared femtosecond multiphoton laser scanning microscopy and second harmonic generation were used for high-resolution three-dimensional imaging of ECM structures.**

Results. **Biochemical testing demonstrated similar amounts of collagen and desmosine, but a minor loss of elastin in the cryopreserved specimens. Conventional histology revealed almost comparable cell and ECM formations in fresh and cryopreserved valve leaflets. In contrast, laser-induced autofluorescence imaging showed substantial ultrastructural deterioration and disintegration of most collagenous structures. Second harmonic generation was not inducible.**

Conclusions. **Conventional cryopreservation of heart valves is accompanied by serious alterations and destruction of leaflet ECM structures, specifically demonstrated by multiphoton imaging. Further in-depth studies to clarify the impact of alternative cryopreservation techniques proposed for clinical use, such as vitrification, are crucial.**

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Despite advantages such as superior hemodynamic properties, resistance to infections, and low incidence of thromboembolic complications, the long-term durability of cryopreserved allogeneic homografts remains limited, especially in children, young adults, and patients with terminal renal failure. In the majority of cases, tissue deterioration, manifested as structural and calcific degeneration of the valves, leads to graft dysfunction, and eventually reoperations are required [1, 2].

The identification and understanding of the possibly multifactorial mechanisms of cryopreserved homograft failure has been the subject of studies for several decades. These investigations were concentrated on the relevance of cellular viability [3–5], the role of immune responses [6], and biochemical aspects of extracellular matrix (ECM) elements such as proteoglycans [7], elastin [8], or collagen [4, 9–11]. Others focused on the impact of damage caused by ice formation after cryopreservation [12] of human, porcine, ovine, and rat aortic or pulmonary heart valves. Neither the relative contributions of cellular viability, immune responses, and ECM durability nor the immediate effects of cryopreservation on structural properties of the amorphous and fibrillar valve matrix have yet been clearly identified.

The valvular ECM contains a variety of structures, underlies and surrounds the interstitial cells, and performs many essential functions, including mechanical support and physical strength [13–15]. Moreover, the ECM exerts profound influences on cell adherence, migration, and differentiation as well as the pattern of gene expression of the cells in contact with it [16, 17]. Since the quality of the structural matrix at implantation may predetermine durability or failure of a cryopreserved heart valve, our goal was to carefully examine state and quality of the major ECM elements (collagenous bundles

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and elastin-containing fibers) based on different techniques before implantation. Besides biochemical assays and conventional matrix visualization methods such as histology and immunohistochemical staining, near-infrared (NIR) femtosecond multiphoton laser scanning imaging and second harmonic generation (SHG) microscopy have been applied in this study as novel non-contact optical technology for three-dimensional resolved ECM component imaging and heart valve tissue state diagnosis [18, 19].

Material and Methods

Tissue Preparation and Cryopreservation

Hearts of 9- to 12-week-old pigs (weight, 25 to 35 kg) were obtained from a local slaughterhouse and immediately transferred to the laboratory. Pulmonary valves were excised under sterile conditions and gently rinsed free of blood in sterile phosphate-buffered saline (PBS [1x; Invitrogen, Carlsbad, California]). Eight specimens of the untreated, fresh valves were directly processed for histology, immunohistochemistry, NIR multiphoton imaging, and biochemistry. The remaining valves (n = 8) were incubated each in 100 mL of a solution containing a combination of antibiotics (1.2 mg amikacin; 3 mg flucytosin; 1.2 mg vancomycin; 0.3 mg ciprofloxacin; 1.2 mg metronidazol in 1 mL aqua ad injection) and medium 199 (M199; Invitrogen) at 4°C for 24 hours. After this sterilization, each valve was placed separately in cryopreservation bags containing 100 mL of a cryoprotective solution (M199; 10% dimethyl sulfoxide [DMSO (Me_2SO)]; Sigma, St. Louis, Missouri), and immediately controlled-rate frozen at –1°C per minute for 60 minutes, down to –40°C, and at 5°C per minute until a temperature of –196°C was attained (Ice Cube 1810CD; SY-LAB GmbH, Purkersdorf, Austria). The valves were stored in the vapor phase of liquid nitrogen at approximately –196°C (Cryo 200; Thermo Forma, Marietta, Ohio). After 1 week of storage, the valves were fast thawed using a 37°C water bath (total thawing time approximately 10 minutes).

Histology, Immunohistochemistry, and Transmission Confocal Laser Scanning Microscopy

Samples of each heart valve (each, n = 8) were processed as described before [19, 20]. All specimens were treated and sectioned similarly. Each leaflet considered for histologic or immunohistochemical analysis was processed in serial sections. To determine general cellular and tissue morphology, representative sections of the paraffin-embedded samples were stained with hematoxylin-eosin (HE). A modified Movat-pentachrome stain [21] was used to demonstrate ECM components such as collagen, elastin, and proteoglycans/glycosaminoglycans (GAGs). For an improved histologic imaging of the elastic fiber system, tissue slides were prepared and stained with resorcin-fuchsin [22]. After staining, all sections were dehydrated in ethanol (Mallinckrodt Baker, Deventer, Netherlands), cleared in xylene (Merck KGaA, Darmstadt, Germany), mounted using Entellan (Merck), analyzed, and documented using routine bright-field light microscopy (Axiovert S 100 System; Carl Zeiss, Jena, Germany).

Immunohistochemical analysis was carried out using cryostat sections and standard indirect immunoperoxidase (strept)avidin-biotin techniques. The primary antibodies used in this study were as follows: a monoclonal mouse antibody to elastin (1:50; Acris Antibodies GmbH, Hiddenhausen, Germany), a polyclonal rabbit antibody to collagen type I (1:50; Acris), a polyclonal rabbit antibody to collagen type III (1:50; Acris), and a polyclonal rabbit antibody to collagen type IV (1:50; Acris). The (strept)avidin-biotin-complex technique was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California). Secondary antibodies were a biotin-labeled anti-mouse antibody (elastin) and a biotin-labeled anti-rabbit antibody (collagens [each 1:200; DakoCytomation GmbH, Hamburg, Germany]). The immunobound peroxidase activity was also visualized using the chromogenic substrate Jenchrom px blue (MoBiTech GmbH, Göttingen, Germany) according to the manufacturer's instructions. Negative controls were performed without primary antibodies. All sections were analyzed and documented using a routine bright-field microscope (Axiovert S 100 System; Zeiss) or a confocal laser scanning microscope system (LSM 310; Zeiss). Jenchrom px blue-stained sections were additionally examined by the use of the transmission mode of the LSM 310, equipped with a helium-neon gas laser (excitation wavelength 543 nm) and an argon ion laser (excitation wavelength 488 nm). Further processing of the stored transmission images was performed using digital image analysis based on features provided by the Carl Zeiss LSM 310 software. After subtraction of the background based on electronic noise, room light, or dust on the optical system, the image underwent gray value inversion [23], which enhanced the ability to assess the images by eye.

Multiphoton Imaging

Shortly after dissection, fresh and thawed cryopreserved leaflet specimens (each, n = 8) were examined using a NIR femtosecond laser scanning microscope system, as described previously [24]. Extracellular-matrix-dependent autofluorescence and SHG were induced using wavelengths of 760 nm and 840 nm. Non-invasive serial optical horizontal sections from both the inflow (ventricularis) and the outflow side (arterialis-fibrosa) of the different leaflet specimens were taken in z-steps of 5 μm and 10 μm. Induction of SHG radiation, which can be detected at half of the incident laser wavelength, was demonstrated with a filter FB420-10 (central wavelength, 420 nm; full width half maximum, 10 nm [Thorlabs, Newton, New Jersey]) in front of the detector. A 700 nm short pass filter (E700SP; Chroma Technology, Brattleboro, Vermont) was used to block ultraviolet radiation (transmission