# Influence of Cryopreservation on Structural, Chemical, and Immunoenzymatic Properties of Aortic Valve Allografts

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## ABSTRACT

**Objectives.** The problems in preparing (including cryopreservation) and implanting aortic valve allografts (AVAs) is widely elaborated, but some issues need explanation.

**Material and Methods.** Twenty AVAs cryopreserved in dimethylsulphoxide/RPMI solution under −160°C for 1–15 years and 3 controls stored at +4°C up to 2 weeks, from 19 male and 4 female donors, aged 20–51, ±30.8 years, were examined using light (LM), digital (DM), and scanning electron microscopy (SEM), energy dispersion X-ray spectroscopy (EDS), and enzyme-linked immunosorbent assay immunoenzymatic tests (PECAM1, CD34).

**Results.** All AVAs were macroscopically correct. LM revealed normal structure of leaflets but massive endothelial decellularization (±59 cells remained on the surface of 5 mm scraps). DM and SEM demonstrated generally normal collagen structures, but local alterations, probably influenced by freezing-thawing (gaps, separated plates) or being initial phase of native degeneration (grains). EDS detected a little elevated calcium amount in 1 specimen only. The mean PECAM1 and CD34 concentrations were at similar low level in all probes.

**Conclusions.** Fresh and cryopreservation technologies did not significantly influence the basic properties of AVA leaflets; however, massive endothelial decellularization was present in both groups. Therefore, no endocardial cell activity nor signs of inflammation were observed. These results were independent of donors' age and sex, processing technology, and time of storage of cryopreserved AVAs.

AORTIC valve allografts (AVAs), introduced into clinical use by Ross in 1962 and applied since 1980 in our department, still remain a good substitute in patients with severe damage of the aortic valve and surrounding tissues, especially due endocarditis [1–6]. They may be prepared as fresh, requiring implantation within 4 weeks, or using deep freezing technology, which allows for durable storage.

## MATERIAL AND METHODS

We examined 23 AVAs (20 frozen and 3 fresh), prepared and stored in our laboratory after generally accepted protocols [3,7,8], which had been procured during forensic autopsies from 4 female and 19 male donors, aged 20–51, ±30.8 years, who died of brain trauma (11), hanging (5), stroke (3), suffocation (2), drowning (1), and alcohol abuse (1). The AVAs underwent estimation of morphology and function, antibiotic decontamination at +4°C for 24 hours, and microbiologic and serologic control. Fresh AVAs were stored in calf serum at +4°C, and examined after 2–14 days.

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AVA for cryopreservation, treated with 10% dimethylsulphoxide (DMSO) and RPMI1640 medium, were initially cooled to −40°C in a SY-LAB 14 S-A freezer (Neupurkersdorf, Austria) and subsequently stored in vapors of liquid nitrogen at −160°C for 1–15, ±10 years. They were thawed in water baths for 30 minutes at + 40°C and rinsed to remove the excess of DMSO. All specimens were divided into 3 equal parts, containing 1 complete leaflet and an amount of aortic tissue, for examination in adequate laboratories. Hematoxylin-eosin dyed scraps were estimated using light microscopy (LM) at 10x magnification (Olympus BX43, Tokyo, Japan), with counting of endothelial cells on 5 mm distance. Probes for immunoenzymatic investigations were homogenized with T-PER (Thermo Fisher Scientific, Waltham, Mass, United States) and Micro-Disembramator S (Sartorius, Goettingen, Germany) for 3 minutes at 1500 rpm, and centrifuged for 5 minutes at 1000 g (Sigma Centrifuge, Osterode am Harz, Germany). PECAM1 (platelet/endothelial cell adhesive molecule) and CD34 (hemopoetic progenitor cells) were determined by enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp, Wuhan, China). Morphologic examinations were performed with a Motic Model 07-100477 digital microscope (DM) and an FEI Quanta 200 FEG scanning electron microscope (SEM) in low vacuum with a cathodic current of 10 kV. Qualitative chemical analyses were performed with energy dispersion X-ray spectroscopy (EDS), a periphery of SEM.

## RESULTS

All specimens were macroscopically normal. Cryopreserved tissues were a little more flexible. LM revealed an unchanged structure of leaflets, but massive endothelial loss in all cases (Fig 1A,B).

The average quantity of cells was 59 (11–277). Two frozen probes yielded 273 and 277 cells, respectively, but the remaining probes did not exceed 95, and the fresh yielded only 20.

In all probes, ELISA demonstrated low protein concentrations, marked in ng/mL. PECAM1 analyses of leaflets showed content of 0.982–4.583, ±1.88 (fresh AVAs: 1.989, 0.877–2.561, ±1.989; frozen: 1.721 0.982–4.58, ±1.721), while in aortic tissue 1.537–6.966, ±3.429 (in fresh: 1.87–3.735, ±3.0 vs 3.487 1.537–6.566, ±3.487 in frozen). CD34 concentration in the leaflets was 0.144–1.237, ±0.537 (in fresh: 0.144–0.762, ±0.35, and in frozen: 0.144–1.237, ±0.822), as well as in aortic tissue 0.211–1.574, ±0.799 (fresh: 0.643 0.16–1.166, ±0.643; frozen AVA: 0.211–1.574, ±0.822), respectively.

The DM and SEM images presented in general normal leaflet and collagen structures (Fig 1C,E,G,H). Collagen fibers were smooth, regular, and of stable thickness. However, there were apparent small local alterations within collagen (10–40 μm in diameter), described as micrograins or bead formations: solitary, in chains, or as focal concentrations, located on normal collagen (Fig 1D).

Some cryopreserved probes showed delicate gaps between collagen layers (Fig 1F), or separation of thin plates. Rarely, small superficial cracks or scratches occurred on the surface. EDS did not detect calcium mineralization; in 3 probes the calcium amount was only traceable, but one (fresh, M, 34) showed energetic spectra of an elevated calcium level in the material from a single grain, not formed as concentration but reckoned as built into proteins.

## DISCUSSION

The applied procedures for AVA procurement, selection, preparation, and storage may be recognized as proper. Our study shows adequate preservation of tissues, including collagen structures, as compared with fresh AVA. The tissue properties and ELISA results occurred not dependent to the donors' demography, method of preparation, and time of cryopreservation. Deep-freezing technology used with DMSO significantly prevents tissue damage and water crystallization during the initial phases of cooling [3,9]. At the low temperatures, the time-related changes are not expected because of stable conditions and practically absent metabolism. Despite the beneficial role of freezing, some tissue changes occur [3,9]. Our study also presents some alterations, as local fissures, empties, separated plates, which may be related with freezing and thawing. It should be noted that in some AVAs, injuries probably occurred due to contact with instruments, therefore the "no-touch" technique of preparation and implantation is strongly recommended. All of the above-mentioned lesions may be important factors influencing further graft degeneration and mineralization, in addition to inflammation [10]. However, these alterations, found at the microscopic level, may not affect graft pathology during relatively longer time periods, as it implies over 11 years of observation of a cohort of patients after cryopreserved AVA implantation. In general, echocardiography revealed no evident calcifications. Only 1.5% of patients needed reoperation because of severe AVA failure and mineralization. The micrograins may be perceived as primary changes that have occurred already in young individuals, and because of some lipid content are considered as early atherosclerosis-binded degeneration. Moreover, an elevated amount of calcium, as seen in one probe, may also suggest them as possible further centers of mineralization [10]. Some papers have indicated that lipopolysaccharides stimulation of interstitial cells may activate inflammation and osteogenesis in AVA [11].

Massive endothelial cell loss, a meaningful phenomenon, appeared in all specimens. We suggest that it occurs early posthumously, independently of preparation. Decellularization is considered beneficial for preventing further graft degeneration and calcification, due to significant reduction of proinflammatory, prothrombotic, and immunologic activity of endothelial cells, and increasing re-endothelialization [3,12–14]. Therefore, many technologies were developed for allografts and xenografts decellularization, and found their clinical application [3,13–16]. The expression of multifunctional factors such as PECAM1 and CD34, strongly connected with endothelial cells, may have implications for cardiovascular physiology, pathology,