

Rates of Cycling Cells in Cryopreserved Valvular Homograft: A Preliminary Study

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Abstract: Some investigators claim that the viability of cryopreserved human valvular homograft is necessary for the duration of implanted homograft. In this preliminary study, the percentage of cycling cells in cryopreserved valvular homografts was evaluated with the use of monoclonal Ki-67 antibody. Three human aortic valves were harvested from multiorgan donors and cryopreserved. Sections of 5 μm in thickness were stained with monoclonal Ki-67 antibody. The proportion of endothelial cells with Ki-67 positive nuclei was $1.80 \pm 0.20\%$. No differences in distribution were observed from basal to marginal sites. Few fibroblasts showed Ki-67-immunopositivity ($0.10 \pm 0.06\%$) while the Ki-67 immunostaining was $0.80 \pm 0.20\%$ in myocytes. Our preliminary study shows that cryopreserved valvular homograft cells are not only viable but they also have the potential to replicate. These data can lead to the hypothesis that valvular cells could actively replicate even after implantation, permitting cellular renewal and regeneration of extracellular matrix's components. **Key Words:** Homograft—Cryopreservation—Proliferation rate.

Cryopreserved human valvular homografts represent an alternative to mechanical and biological prostheses in patients who undergo valvular replacement. Whereas the viability of a cryopreserved human homograft is claimed to be necessary for the vitality and duration of the implanted homograft, this very characteristic has been considered the reason for the immune rejection that is mainly responsible for homograft degeneration (1). Although several previously published studies have evaluated the viability

of cryopreserved homografts with contrasting results, the main focus of these studies was on cellular synthesis (2). However, one of the most important aspects of valvular viability is cell proliferation. This preliminary study was designed to evaluate the percentage of cycling cells in cryopreserved valvular homografts with the use of monoclonal antibody against Ki-67 protein, a proliferation marker, in order to understand if cells can replicate after cryopreservation and thereby permit cellular renewal after post valve implantation.

MATERIALS AND METHODS

Three human aortic valves were harvested from multiorgan donors whose hearts were not suitable for transplant (two males and one female, mean age: 42.7 ± 8.4 years). The tissues were placed in a cold balanced salt solution (Eurocollins at 4°C) and transported to the laboratory. Mean cold ischemic time was 56 ± 12 min. The explanted aortic roots were first incubated in the balanced salt solution containing antibiotics (Roswell Park Memorial Institute [RPMI] media 1640 with L-Glutamine tamponed until a pH of 7.2–7.4; cefoxitin 240 $\mu\text{g}/\text{mL}$, lincomycin 120 $\mu\text{g}/\text{mL}$, polymyxin B 100 $\mu\text{g}/\text{mL}$, vancomycin 50 $\mu\text{g}/\text{mL}$) at 4°C for 24 h and then placed into hemofreeze bags containing a cryopreservation solution (100 mL RPMI media 1640 with L-Glutamine tamponed until a pH of 7.2–7.4 with 10% dimethyl sulfoxide [DMSO] as cryoprotectant). The specimens were cooled in a controlled-rate freezer (Kryo 10–16 series III, Planer, London, England) that decreased the temperature at a rate of $1^\circ\text{C}/\text{min}$ down to a temperature of -80°C and finally maintained in liquid nitrogen vapors (-180°C). The mean cryopreservation time was 36.2 ± 18.3 months. The specimens were analyzed 4 h after thawing.

Tissue analysis

Aortic valves were sectioned longitudinally. Sections of 5 μm in thickness obtained with cryostat (1720 Digital, Leica, Bensheim, Germany) were stained with hematoxylin and eosin as well as monoclonal Ki-67 antibody (DAKO, Glostrup, Denmark). An avidin-biotin technique with biotinylated secondary antibody was used after incubation at room temperature for 2 h. Binding was revealed using a peroxidase-conjugate streptavidin (BioGenex, San Ramon, CA, USA) and peroxidase was revealed by 3,3'-diaminobenzidine. Light microscopic examination was performed under standard conditions. A minimum of 1000 cells were scored in each experiment and scoring of Ki-67 positive nuclei was

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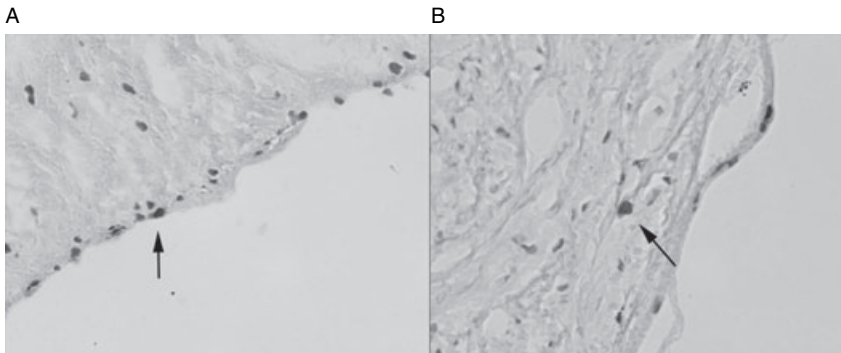


FIG. 1. Ki-67 labeling of cycling cells in cryopreserved homograft. (A,B) Brown dark spots represent localization of Ki-67 immunostaining.

reported as percentage of total cells. To obtain a negative control, the primary antibody was routinely omitted.

RESULTS

No significant microscopic alterations were found in aortic leaflets or small fragments of cardiac muscle. Nuclei were regular in shape and dimension without chromatin condensation or fragmentation. Cytoplasm and cellular contour were similar to those generally observed in paraffin-embedded material.

The proportion of endothelial cells with Ki-67 positive nuclei was $1.80 \pm 0.20\%$ (Fig. 1). The highest percentage of positive Ki-67 immunostaining was observed in endothelial cells along the aortic lumen, often crowded in small groups, and in capillary endothelial cells localized in the peripheral aortic side of the leaflet. No differences in distribution were observed from basal to marginal sites.

Few fibroblasts showed Ki-67 immunopositivity ($0.10 \pm 0.06\%$) while Ki-67 immunostaining was $0.80 \pm 0.20\%$ in myocytes.

DISCUSSION

Our preliminary study shows that cryopreserved valvular homograft cells are not only viable but also that the valvular homograft cells do have a potential to replicate. These data are important, as they lead to the hypothesis that valvular cells could actively replicate after implantation. Tissue chimerism, the concomitant presence of persisting donor cells with the penetrating recipient cells, was previously demonstrated (3) in explanted homografts even years after implantation. However, at the time, donor cell vitality was not correlated to the replication capability.

We found different potential proliferation rates among the different cell populations with fibroblasts having lower proliferating rates than endothelial cells. This could either be related to the physiological differences, as fibroblasts by nature are slow dividing

(4), or this could just be an effect of cryopreservation. Fibroblasts are essential for extracellular matrix replacement and a low proliferation rate could lead to a progressive decrease in number and subsequent degeneration of extracellular matrix. A persisting higher number of proliferative fibroblasts could be associated with longer duration of homograft.

Proliferative index of valvular homograft cells was never clearly evaluated. Several different methods were used to evaluate the vitality of homograft after cryopreservation but none of them was a specific indicator of cell proliferation. Ki-67 is an essential element of the outer dense fibrillar compartment of the nucleolus and it is expressed in all phases of the cell cycle except G0. Expression of Ki-67 is a requirement for the cell to undergo cell division and hence the Ki-67 antigen represents the preferable method to evaluate the percentage of cycling cells and potential cellular proliferation rates (5). To our knowledge, this is the first report on Ki-67 immunostaining of cryopreserved aortic valve.

This study represents a preliminary evaluation of the growth fraction of valvular homograft cell populations that we undertook in order to investigate the role of donor cells in homograft duration. Higher numbers of samples are required to confirm these data and understand the eventual role of gender, cryopreservation time, and other parameters on changing proliferation rates. However, this study is the first demonstration of persisting cycling cells in valvular homograft after cryopreservation.

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An In Vitro and In Vivo Study of the Detection and Reversal of Venous Collapse During Extracorporeal Life Support

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Abstract: The objective of this study was to investigate venous collapse (VC) related to venous drainage during the use of an extracorporeal life support circuit. A mock circulation was built containing a centrifugal pump and a collapsible vena cava model to simulate VC under controlled conditions. Animal experiments were performed for in vivo verification. Changing pump speed had a different impact on flow during a collapsed and a distended caval vein in both models. Flow measurement in combination with pump speed interventions allows for the detection and quantitative assessment of the degree of VC. Additionally, it was verified that a quick reversal of a VC situation could be achieved by a two-step pump speed intervention, which also proved to be more effective than a straightforward decrease in pump speed. **Key Words:** Venous collapse—Venous drainage—Cardiopulmonary bypass—Ventricular assist device—Hemodynamic monitoring—Rotary blood pump.

The growing field of indications to install extracorporeal life support (ELS) (1–5) has introduced the need for guidelines to choose an optimal pump flow rate. As current ELS systems become smaller with low priming volume and small or absent reservoirs,

instantaneous pump flow adaptations are more critical. Whereas a low flow rate sometimes can be recognized by, for example, a low arterial pressure and decreased blood gas values, a high flow rate may result in a too high negative pressure at the pump inlet, which may be related to a venous collapse (VC) and a drop in pumping efficiency (6–10). Apart from the hemodynamic consequences, blood damage and tissue aspiration have been reported (11–15). Prevention is difficult, because VC situations often develop gradually and will therefore not always be noticed in time. Detection of VC can be performed adequately using Doppler imaging techniques (16) or by monitoring pump parameters (17–22). However, such techniques may prove too complex or are still under development. Simple manual pump speed adjustments and simultaneous observation of flow changes by perfusionists may indicate an acute VC, however, a quantitative evaluation and a method for early detection of VC have not been reported earlier. The use of a roller pump in combination with a bladder bag inserted into the venous line, prevents excessive drainage by an immediate pump shut down (23,24). However, the device does not solve the VC and requires immediate attendance of a perfusionist or ELS-trained nurse. To allow the number of indications for ELS to grow, pump speed adjustments should be automated to improve safety.

We investigated the potential of pump speed manipulations during centrifugal pump-based life support for the early detection of VC and the quantitative influence on hemodynamics for a proper VC reversal. For this purpose, VC was simulated in a mock circulation and in an animal protocol.

MATERIAL AND METHODS

Mock circulation

Figure 1 shows a scheme of the water-filled mock circulation. It consists of a venous reservoir with a collapsible vena cava model, an ELS system, and an arterial reservoir. The venous filling pressure and extravascular pressure can be set by adjusting the height of the corresponding reservoirs. A rubber tube clamped into a transparent plastic housing functions as a collapsible vena cava model. The arterial reservoir is closed to the atmosphere and functions as a vascular compliance, which can be set by adjusting the entrapped air volume. An adjustable tube clamp mimics the peripheral vascular resistance. A rising tube placed behind the tube clamp simulates the capillary perfusion pressure and is set to +15 mm Hg.

An adult 30-inch 23 Fr femoral venous cannula (Medtronic Inc., Minneapolis, MN, USA) was used

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