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Xeno-immunogenicity of ice-free cryopreserved porcine leaflets

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ABSTRACT

Background: Undesirable processes of inflammation, calcification, or immune-mediated reactions are limiting factors in long-term survival of heart valves in patients. In this study, we target the modulatory effects of ice-free cryopreservation (IFC) of xenogeneic heart valve leaflet matrices, without decellularization, on the adaptive human immune responses *in vitro*.
Methods: We tested porcine leaflet matrices from fresh untreated, conventionally cryopreserved (CFC), and IFC pulmonary valves by culturing them with human blood mononuclear cells for 5 d *in vitro*. No other tissue treatment protocols to modify possible immune responses were used. Matrices alone or in addition with a low-dose second stimulus were analyzed for induction of proliferation and cytokine release by flow cytometry-based techniques. Evaluation of the α-Gal epitope expression was performed by immunohistochemistry with fluorochrome-labeled B4 isolectin.
Results: None of the tested leaflet treatment groups directly triggered the proliferation of immune cells. But when tested in combination with a second trigger by anti-CD3, IFC valves showed significantly reduced proliferation of T cells, especially effector memory T cells, in comparison with fresh or CFC tissue. Moreover, the cytokine levels for interferon-γ (IFNγ), tumor necrosis factor α, and interleukin-10 were reduced for the IFC-treated group being significantly different compared with the CFC group. However, no difference between treatment groups in the expression of the α-Gal antigen was observed.
Conclusions: IFC of xenogeneic tissue might be an appropriate treatment method or processing step to prevent responses of the adaptive immune system.

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1. Introduction

Cryopreservation of cardiovascular structures by freezing with dimethyl sulfoxide (DMSO) is a common method of tissue banking for clinical practice. However, it is well known that traditional cryopreservation methods manifest several disadvantages. Mainly, the long-term function of heart valves is limited by inflammation as well as cellular and humoral immune responses causing subsequent deterioration and destruction of the tissue. Previous works indicated that the process is linked to the induction of T cell-mediated responses [1,2].

One option to overcome the immunogenicity problem is the generation of decellularized tissue, where calcification may be prevented and the ability for autologous recellularization has been observed [3]. Moreover, in a rat model less infiltration of CD3+CD8+ cells was detected [4]. Early studies with Synergraft and Matrix P demonstrated that incomplete decellularization was associated with strong foreign body reactions, inflammation, an immune response and graft failure [5]. Subsequently SynerGraft (CryoLife Inc., Kennesaw, GA) valve allografts with more complete decellularization have demonstrated good midterm performance [6].

Recently, a new method of ice-free cryopreservation (IFC) was developed that enables long-term storage at −80°C without any decellularization [7]. It could be demonstrated that the conventional freezing of tissue causes more destruction of the extracellular matrix (ECM) structure than by ice-free preservation of the tissue [8,9]. The first *in vivo* application of this cryopreservation method in an allogeneic sheep model showed that the implantation of ice-free tissue resulted at explant in a cell-free matrix with preserved ECM components, elastin and collagen, good hemodynamic performance, and reduced T cell infiltration in the valve stroma [10,11]. *In vitro* IFC tissues displayed reduced cell viability compared with fresh or conventional frozen cryopreservation (CFC) tissue, but exhibit the same hemocompatibility features [12,13].

The objective of this study was to determine the human immunological responses *in vitro* to xenogeneic frozen and IFC porcine heart valve leaflets in comparison with fresh xenogeneic leaflets. The primary focus was on the capacity of cryopreserved porcine leaflets to induce human peripheral blood immune cell proliferation and cytokine release.

2. Materials and methods

2.1. Tissue preparation and cryopreservation

Pig hearts ("German landrace" pigs; equal gender distribution) were obtained as bona fide excess tissue from local slaughterhouses with an age of 5–6 mo and a weight of approximately 120 kg. The tissues were collected and processed according the Standards of the American Association of Tissue Banks [14]. Cooled pig hearts were transported to the laboratory. Pulmonary valves were immediately excised aseptically and rinsed three times in cold phosphate-buffered saline (PBS; Lonza, Cologne, Germany). Thereafter, all valves were placed in 80 mL pyruvate-free Dulbecco Modified Eagle Medium with 4.5 g/L glucose, 2 mM L-glutamine (DMEM; Lonza) and antibiotic cocktail with 15 mg/L amikacin, 37.5 mg/L flucytosine, 15 mg/L vancomycin, 3.75 mg/L ciprofloxacin, and 15 mg/L metronidazole for 24 h storage at 4°C. Heart valves treated with antibiotics were used in part as fresh control tissue or were randomly allocated for the IFC or CFC preservation groups.

CFC heart valves were prepared by placing them in freezing media consisting of DMEM with 10% human serum albumin (HSA; Baxter Deutschland GmbH, Unterschleißheim, Germany) and 10% DMSO (Sigma, Taufkirchen, Germany) for 30 min at 4°C before controlled rate freezing (Ice-Cube 14S; SY-LAB Geräte GmbH, Neupurkersdorf, Austria) at 1°C per min to −80°C. The valves were then stored in the vapor phase above liquid nitrogen. After a storage time of 1 wk, the tissue was thawed in a 37°C water bath. The valve tissue was then removed from the bag aseptically and washed subsequently for 5 min each in 4°C cold DMEM with 0.5 M mannitol (Sigma–Aldrich) followed by DMEM with 0.25 M mannitol and then DMEM alone.

IFC of heart valves was achieved by placing them in sterile polyethylene bags (Ampac Flexible SealPack; Fisher Scientific, Pittsburg, PA) containing 80 mL of an 83% 12.6 mol/L cryoprotectant formulation (VS83; made up of 4.65 mol/L formamide, 4.65 mol/L DMSO, and 3.31 mol/L 1,2-propanediol [all Sigma–Aldrich] in Euro-Collins solution) as described previously [15]. The valve tissue was then incubated on a shaker for at least 1 h at room temperature before cooling. The cooling process was achieved by placing the bags for 10 min in a precooled bath of 2-methylbutane (Sigma) (<−100°C) and then stored at −80°C for at least 1 wk. Rewarming of the tissue was performed by submersion of each bag containing valves in a 37°C water bath, followed by five washing steps for 5 min with room temperature Euro-Collins solution.

Leaflet discs were prepared, using a 5 mm diameter punch (Stiefel Laboratorium GmbH, Offenbach, Germany), from rewarmed and fresh-untreated control leaflets. The leaflet discs were placed in PBS with antibiotic mixture as described previously at 4°C until the performance of immunologic tests.

2.2. 5,6-carboxyfluorescein diacetate N-succinimidyl ester-based proliferation assay

The ability of fresh, CFC, and IFC leaflets to influence human immune cell responses was tested by a 5,6-carboxyfluorescein diacetate N-succinimidyl ester (CFSE)-based proliferation assay for 5 d. The assay was performed with human peripheral blood mononuclear cells (PBMCs) prepared from healthy volunteers (both genders) after informed consent (approval of the local research Ethics Committee EA2/139/10) or from buffy coats bought from the German Red Cross (DRK; Berlin, Germany) by density centrifugation using Biocoll separation solution (Biochrom AG, Berlin, Germany) as described elsewhere [16]. Briefly, isolated PBMCs were labeled at a density of 10^7 cells/mL with 2.5 μM 5.6-CFDA-SE (CFSE; MoBiTec, Göttingen, Germany) for 3 min in the dark at room temperature. Labeled cells were seeded at a concentration of 3×10^5 cells