Opinion

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An alternative strategy for reducing tissue immunogenicity using ice-free cryopreservation

Opinion

The costs of regenerative medicine tissue therapies are very high when individualized autologous tissues are employed. In contrast allogeneic tissues are cost effective because many grafts can be manufactured simultaneously, but the effectiveness of such tissues is often limited by the recipient's immune response. Currently the most common means of ameliorating the recipient's immune response is to remove the primary source of immune stimulation, antigens, by decellularization. Typically decellularization procedures use combinations of enzymes and detergents with extensive post-treatment washing resulting in significant manufacturing costs and risks of inducing matrix damage. We have developed a novel low cost tissue cryopreservation method called ice-free cryopreservation[1–4] without the risks of ice-induced matrix damage associated with conventional cryopreservation. The potential impact of this cryopreservation technology is reduction of the complexity and cost of the processing required for production of allogeneic human matrices for regenerative medicine therapeutic products. Rather than decellularizing tissues and then storing them, the preservation/storage step alone will be used to minimize graft immunoreactivity. This technology has a wide range of surgical applications for treatment of natural and tissue engineered allogeneic tissues employed for patch grafts (throughout the body), orthopedic soft tissue grafts (ligaments and tendons) and cardiovascular tissue grafts (blood vessels and heart valves).

Natural and engineered allogeneic tissues potentially impact huge orthopedic, urinary, cardiac and vascular surgery applications.[5] The potential worldwide market for vascular grafts alone was predicted to be 1,657,000units valued at $2,588M in 2013.[5] The potential for skin grafts is even larger with an estimated potential market of 2,200,000units worldwide. Vascular grafting is a commonly performed surgical procedure in the repair or replacement of diseased coronary artery and peripheral vessels to restore normal blood flow patterns. Autologous internal thoracic arteries and saphenous veins are the gold standards for vascular grafting but they are limited by donor vessel shortages and donor site morbidity.[6,7] Synthetic grafts composed of polymers such as Dacron and expanded polytetrafluoroethylene (e-PTFE) have had success only in high-flow large-diameter vessels. In small diameter (<6mm) vessels, these grafts exhibit low patency rates and fail by thrombosis due to the lack of an adequate endothelium, restenosis due to inflammatory responses and compliance mismatch, and infection.[8] A variety of approaches are being explored for development of tissue engineered vascular grafts for vascular bypass, including synthetic grafts and scaffolds that can induce population by autologous cells; biological grafts; autologous cell-culture derived grafts; and genetically engineered grafts.[5,9] A significant issue for commercialization of some of these personalized grafts is cost. For example Cytograft's autologous product in clinical trials is anticipated to cost over $15,000.[5,10,11] Ice-free cryopreservation has the following advantages over current freezing and decellularization tissue processing strategies, including:

a. Long term preservation capability at -80°C;[12]

b. Reduced risk of microbial contamination, due to outgrowth of low level contaminants that may occur over months and years at 4°C;

c. Similarly, a decreased risk of material deterioration over years;

d. No ice formation resulting in extracellular matrix preservation and retention of biomaterial properties;[1]

e. Simplification of the manufacturing process (no decellularization required) resulting in reduced costs with lower regulatory hurdles for allograft blood vessels from donors, and;

f. No significant recipient allogeneic immune response.

We also believe that current decellularization techniques are inadequate resulting in subtle material changes, undetectable by traditional implant biomaterial test methods, which will lead to poor long-term performance in patients. The main approach for reduction of allogeneic tissue immunogenicity to date has involved detergent/enzyme decellularization strategies. The current most commonly employed techniques are variants of the SynerGraft decellularization method, which also uses freezing methods, for cardiovascular tissue decellularization (Table 1). Cryopreserved allografts have especially benefited children with congenital heart disease since the use of alternative mechanical and xenogeneic tissue valves have historically been limited in this patient population. In recent years decellularization technologies have been applied to allogeneic donor-derived heart valves in order to minimize the immune reaction to allogeneic valve tissues observed in young patients.[13–15] Both human and "processed" xenograft scaffolds seem to be more proinflammatory when their cells are disrupted and cellular debris, cytokines, and other inflammatory moieties are not thoroughly removed from the matrix.[16,17] Rieder et al.[16] demonstrated that the lowest level of stimulation was with thoroughly "decellularized" human tissues. Decellularized porcine leaflets stimulated greater macrophage responses than extracts of human native pulmonary cusps that had not been decellularized. This observation, combined with the poor performance of decellularized xenografts in patients[18] led to the selection of human allograft tissues–not xenografts–as the tissue source for decellularization technologies. The major US allograft heart valve processors are focusing on development of decellularization methods and clinical experience is accumulating. Early reports on these valves suggested that they are biomechanically and hydrodynamically equivalent to allografts processed by standard methods without decellularization.[19,20] Short-

Volume 3 Issue 3 - 2016

Kelvin GM Brockbank
Tissue Testing Technologies LLC, USA

Correspondence: Kelvin GM Brockbank, Tissue Testing Technologies LLC, 2231 Technical Parkway, Suite A, North Charleston, South Carolina, USA, Tel 8437226756, Fax 8437226657, Email kgbrockbankassoc@aol.com

Received: May 04, 2016 | **Published:** May 09, 2016

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term clinical follow up shows excellent hemodynamics and durability as well as low incidence of complications.[21,22] However mid-term patient data has more recently demonstrated that decellularization did not significantly improve outcome in terms of pressure gradients and structural deterioration compared to non-decellularized allografts.[23] These clinical results, with a mean follow-up of 52months, question the effectiveness of current decellularization practices. It is likely that the detergent/enzyme treatment methods employed damage tissue matrices, despite the fact that traditional biomechanics studies do not reveal damage prior to implant, such that the benefits of decreased immunogenicity are not realized. Konuma et al.[24] studied SynerGraft decellularized valves versus conventional frozen valves performance and revealed no difference with regard to regurgitation, stenosis, or reintervention. There are also publications indicating concerns regarding the impact of freezing methods;[25–28] on valves and the combination of freezing and decellularization.[29]

Table 1 Competitive advantages of ice-free cryopreservation

Advantage	Ice-free cryopreservation	Frozen cryopreservation	Decellularization
Immunogenicity	Reduced	No impact, immunogenic	Reduced
Extracellular matrix	Preserved	Damaged by ice	Damaged
Process complexity	Simple, rapid	Complex, long	Complex
Storage	-80°C (-20°C & +4°C, possible?)	Below -135C, risk of cracking	Variable*, +4°C
Shipping	Cheap, dry ice, (-20°C & +4°C?)	Nitrogen dry shipper, expensive	Variable*, +4°C
Rewarming	2 steps, rapid	Similar	Similar
Preparation in surgery	3-4 wash steps	2-3 wash step	Variable
Hemocompatibility	Excellent	Excellent	Reduced
Regulations	Low, minimal manipulation	Low, minimal manipulation	High, device/biologic

***Note**: freeze drying possible but it results in more matrix damage due to ice, Humacyte

The simplicity, versatility, and scalability of our proposed ice-free cryopreservation approach (Table 1) will allow cost effective long-term storage and shipping, rapid clinical translation and market penetration for treated products. If successful, our work will have a far-reaching clinical impact on surgical repairs by providing unprecedented access to low cost tissue equivalents with retention of structural and mechanical properties while minimizing immunogenicity. It is anticipated that tissues treated with our technology should have long-term efficacy and stability *in vivo*.

Human tissue processors either freeze with cryoprotectants, freeze without cryoprotectants, decellularize or combine freezing methods with decellularization. Some tissue engineering groups are storing in salt solutions.

We have previously demonstrated that ice-free cryopreserved heart valve leaflets have better compatibility with human blood than either chemically treated or decellularized leaflets.[30] Decellularized tissue demonstrated significantly higher thrombin/antithrombin-III-complex levels than ice-free cryopreserved leaflets. Glutaraldehyde treated leaflets demonstrated significant increases in poly-morphonuclear neutrophil-elastase and the terminal complement complex SC5b-9. Glutaraldehyde treatment is the main method used for animal products but it is not utilized for allogeneic tissues.

We use methods based on ice-free cryopreservation strategies developed for extracellular matrix preservation that avoids documented extracellular matrix damage caused by ice formation.[26–28] This is done employing high concentrations of cryoprotectants combined with rapid cooling rates. When 55-70% cryoprotectants are added and removed in multiple steps tissue cell preservation occurs as long as the tissue is stored colder than the glass transition temperature in vapor phase nitrogen or a mechanical freezer. In contrast, when 83% cryoprotectants are added and removed in single steps the cells are killed due to the cytotoxicity of the cryoprotectants and the high concentration of cryoprotectants permits storage at -80°C above the glass transition temperature of the cryoprotectant formulation with retention of matrix structure and biomaterial properties without ice formation.[1] We decided to call this preservation method ice-free cryopreservation rather than vitrification because technically the tissue is not vitrified when stored at -80°C above the glass phase transition temperature. At -80°C the cryoprotectant solution is a very viscous fluid that can be deformed by manual pressure and, unlike frozen or vitrified materials, it can't crack.

We have performed two published studies to look at immunoreactivity of ice-free cryopreservation of tissues, the first was in an *in vivo* sheep allograft model[12] and the second was in a xenogeneic model *in vitro*.[31] In the first study we concluded that ice-free cryopreservation of heart valves avoids any ice formation, tissue-glass cracking and preserves the extracellular matrix. Ice-free cryopreservation significantly decreased T-cell infiltration of the stroma with less leaflet thickening that translated into superior hemodynamics. The observation that explant leaflets are cell free after 7months *in vivo* leads us to believe that decellularization may no longer be needed for tissue engineering allogeneic matrices if ice-free cryopreservation treatment of allogeneic tissues results in a cell free matrix in patients. This study is currently being repeated with a larger number of recipient sheep. The second study, utilized xenogeneic tissue combined with human peripheral blood mononuclear cells, together with anti-CD3 co-stimulation. Proliferation profiles of immune cell subpopulations were analyzed by flow cytometer. Ice-free cryopreserved leaflets showed significantly reduced proliferation

Citation: Brockbank KGM. An alternative strategy for reducing tissue immunogenicity using ice-free cryopreservation. *MOJ Cell Sci Rep.* 2016;3(3):76–79. DOI: 10.15406/mojcsr.2016.03.00058

of T cells, especially effector memory T cells, in comparison with fresh or frozen tissue. Moreover, the cytokine levels for interferon-γ (IFNγ), tumor necrosis factor α, and interleukin-10 were significantly reduced in the ice-free group compared with the traditionally frozen with dimethylsulfoxide control group. However, no difference between treatment groups in the expression of the α-Gal antigen was observed.[31] The cryopreserved by freezing data is in agreement with the literature suggesting that cryopreserved cells and tissues (using dimethylsulfoxide), are less immunogenic. Historically such studies have not been very reproducible suggesting that we are on the edge of an interesting phenomenon but all the variables have not yet been identified.

The mechanism of action of ice-free cryopreservation on the immune response is the subject of ongoing investigation. Ice-free cryopreservation may be reducing immunogenic triggers, such as the presence of viable endothelial cells, or by modification of co-stimulation by the matrix via integrin molecules. Another possibility is activation of monocytes via the release of damage associated molecular pattern molecules, molecules that can initiate and perpetuate immune response in the noninfectious inflammatory response. Ice-free cryopreservation treatment might reduce the release or the availability of damage-associated molecular pattern molecules, such as DNA fragments or necrotic cell debris, which may be present in frozen cryopreserved tissues.

We concluded that the immune cell responses towards allogeneic and xenogeneic tissues were reduced by ice-free cryopreservation. It is possible that our ice-free cryopreservation strategy may be effective for treatment of xenogeneic materials, however considering that the α-Gal antigen was still detectable, our data to date is not adequate to support this outcome. Therefore we stay focused on treatment of allogeneic tissues in our future research and development.

Acknowledgements

None.

Conflict of interest

The author declares no conflict of interest.

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Citation: Brockbank KGM. An alternative strategy for reducing tissue immunogenicity using ice-free cryopreservation. *MOJ Cell Sci Rep*. 2016;3(3):76–79.
DOI: 10.15406/mojcsr.2016.03.00058