

# Advances in Viable Ice-free Cryopreservation of Heart Valves

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## ABSTRACT

Investigation of heart valve cryopreservation has been employed as a model for development of new methods of tissue preservation based upon vitrification and nanowarming using Fe nanoparticles. Cryoprotectant cytotoxicity can be reduced by performing the last cryoprotectant/nanoparticle exposure step below zero degrees centigrade at -10C. Tissue viability outcomes can be improved by supplementation of cryoprotectant formulations with disaccharides and nanowarming can rewarm such complex tissues with retention of cell viability from storage temperatures below -135°C to -25°C in 80-100 seconds. It is anticipated that ice-free tissue cryopreservation methods for tissues up to 50 mLs can be developed that do not require the use of nanowarming, since we are already close to achieving this with heart valves at 30 mL volumes. However, at larger volumes nanowarming will likely continue to be the best warming method for retention of tissue cell viability. Further studies to optimize cryopreservation of cardiac muscle, the somewhat fibrous muscle band at the base of heart valves, and pulmonary and aortic arteries need to be performed since it is clear that different heart valve components vary in their preservation requirements. It is anticipated that other complex tissues may also have components with different cryopreservation requirements including nanowarming.

**Keywords:** Cryopreservation; Nanowarming; Nanoparticles; Cytotoxicity

## INTRODUCTION

We have developed methods for ice-free cryopreservation of tissues that retain heart valve extracellular matrix and leaflet cell viability is an option depending upon the cryoprotectant formulation employed [1]. The non-viable methods result in minimization of recipient immune reaction and good allograft function in large animals. However, there is a huge unfulfilled military and civilian need for viable banked natural and bioengineered tissues and organs for replacement of disease or trauma-induced damage to body parts [2-3]. Investigation of heart valve cryopreservation methods with retention of cell viability in the leaflets, associated artery and fibrous muscle band has been employed as a model for development of new methods of tissue preservation based upon vitrification.

Vitrification, cryopreserved storage in a “glassy” rather than crystalline phase, is an important enabling approach for tissue banking and regenerative medicine, offering the ability to store and transport body parts for a variety of biomedical uses. Unfortunately, practical application of vitrification has been limited to cells and thin tissues in small volumes of cryoprotectant solutions due to diffusive (heat and mass transfer) and phase change limitations

that preclude use in bulk systems such as organs and larger tissues. Greg Fahy developed a vitrification cryoprotectant formulation for tissues and organs in the 1980's consisting of 55% cryoprotectants in Euro Collins organ preservation solution [4]. In the year 2000, our successful VS55 ice-free cryopreservation of rabbit blood vessels with retention of cell viability and tissue contractile functions was published [5] but in the ensuing years we could not scale up to volumes > 3-4mLs. In the past 5 years two breakthrough strategies were developed to circumvent this problem. First, the use of radiofrequency (RF) excited magnetic nanoparticles (mNPs) in cryoprotectant solution to aid in rewarming of tissues developed by the Bischof laboratory at the University of Minnesota. This approach relies on uniform heat generation in close proximity to or within the biomaterial and overcomes the fundamental limitations experienced with boundary or microwave heating in the past. The most important opportunity for this new warming technology is rapid rewarming after ice-free cryopreservation of large tissues and in the future organs. Organ cryopreservation is currently limited by cracking during storage or rewarming and ice formation due to devitrification during warming in a liquid bath. Furthermore, faster rewarming rates may reduce the risks of exposure to the high concentrations of cytotoxic cryoprotective agents needed to avoid

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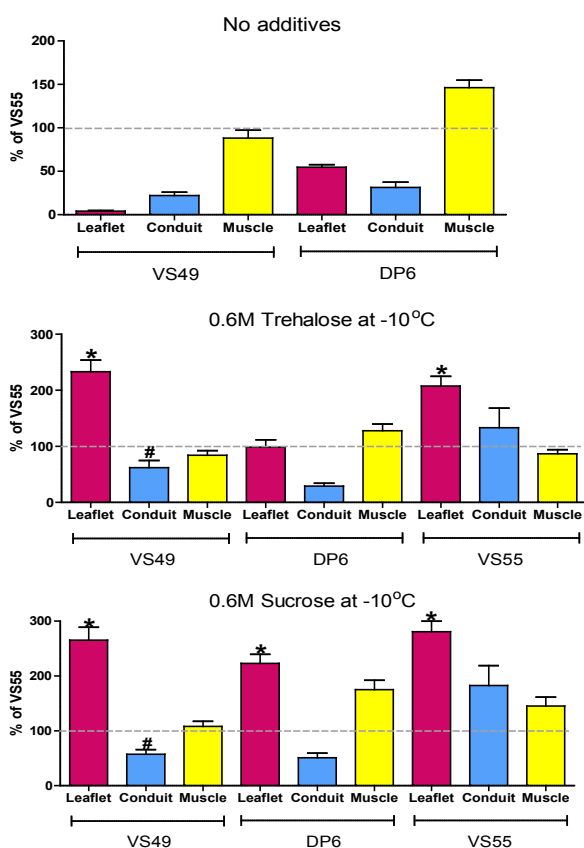
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devitrification. The second strategy, developed by the Brockbank laboratory at Tissue Testing Technologies, was to make the lead ice-free vitrification solution, VS55, less likely to devitrify during rewarming by supplementation with disaccharide sugars.

Our studies with RF coated Fe mNPs in collaboration with the Bischof laboratory at the University of Minnesota were published in 2017 [6]. However in the same time frame we discovered the benefits of two disaccharides in combination with VS49, DP6 and VS55 vitrification formulations (Brockbank patent pending, Figure 1, see Table I for formulations). The impact of sucrose on prevention of devitrification was demonstrated by differential scanning calorimetry [7]. At that time VS55 was our best ice-free vitrification formulation, while a dilute version of this formulation, VS49, that does not vitrify was used as a negative control. Similarly, DP6 is also a poor solution for vitrification in which formamide has been removed and equimolar concentrations of DMSO and propanediol are employed to produce a 6M cryoprotectant formulation. The viability outcomes are poor for the leaflet and conduit components of heart valves preserved in these negative control solutions (Figure 1, top panel). Addition of 0.6M disaccharides prevents visible ice formation in both DP6 and VS49 formulations and we observed some of the highest post-vitrification viability results to date in DP6, VS49 and VS55 plus 0.6M disaccharides (Figure 1). The

**Table 1:** Vitrification Formulations (in Euro Collins Solution).

Components	DP6	VS49	VS55
Dimethylsulfoxide (DMSO) (M)	3.0	2.7	3.1
Propanediol (M)	3.0	1.9	2.2
Formamide (M)		2.7	3.1



**Figure 1:** Comparison of VS49, DP6 and VS55 ± 0.6M trehalose or 0.6M sucrose. The results are expressed in percent of the VS55 vitrified group. The last cryoprotectant addition step ± sugars was added at -10°C. The data is expressed as the mean ± 1SE, n=6 samples, \*indicates P<0.05 significance by one way ANOVA. DP6 consistently has good muscle preservation, while VS55 has the best overall tissue viability.

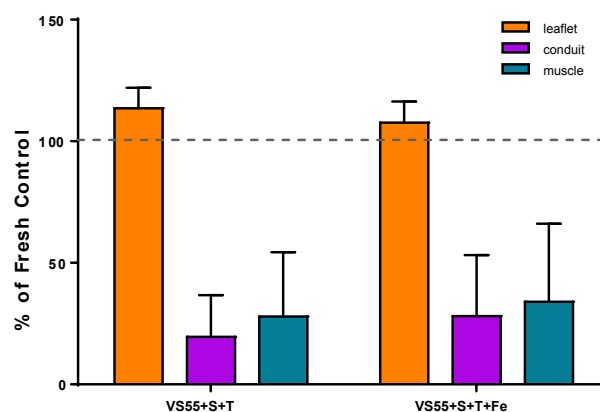
presence of 0.6M trehalose and particularly 0.6M sucrose increased post-rewarming leaflet viability 2 to 3-fold achieving statistical significance, compared with the best outcomes we could achieve employing VS55 without disaccharides (Figure 1, middle and lower panels, p<0.05 by T-test). A significant increase was also observed for pulmonary artery conduit components of the heart valves (Figure 1). Sucrose combined with VS55 yielded the highest post-cryopreservation viabilities.

We published our follow up experiments in which we combined our sucrose VS55 cryopreservation strategy with nanowarming in a review of ice-free heart valve cryopreservation in 2021 [8]. The combination of 0.3 M sucrose and 0.3 M trehalose produced similar results comparing convection with nanowarming (using Ferrotec EMG308 at 7.69 mg Fe/mL) for leaflets but the conduit and muscle band viabilities were significantly higher post-nanowarming (Figure 2). The thicker less cryoprotectant permeable tissue components, conduit and muscle band, are probably less permeated by the cryoprotectants than the leaflets. Therefore, these tissues benefit from the rapid warming possible using nanowarming preventing ice formation due to devitrification during warming. Work in progress is focused on improved cryoprotectant loading strategies for large arteries and the muscle band at the base of pulmonary heart valves.

In conclusion results of studies to improve cell viability in pulmonary heart valves demonstrate that:

- Cryoprotectant cytotoxicity can be reduced by performing the last cryoprotectant/nanoparticle exposure step below zero degrees centigrade at -10°C (Figure 1). Colder temperatures can also be used.
- Tissue viability outcomes can be improved by supplementation of cryoprotectant formulations with disaccharides (Figure 1).
- Nanowarming can rewarm such complex tissues with retention of cell viability from nitrogen storage temperatures below -135°C to -25°C in 80-100 seconds (Figure 2).

It is anticipated that ice-free tissue cryopreservation methods for tissues up to 50 mLs in volume, tissue and surrounding



**Figure 2:** Ice-free vitrification of porcine pulmonary heart valves with VS55 supplemented with 0.3M sucrose and 0.3M trehalose in a total volume of 30 mLs. The tissue components were rewarmed by convection boundary warming or by nanowarming using EMG308 Fe nanoparticles and viability assessed using alamarBlue. There was no significant statistical difference between nanowarmed and convection warmed leaflets. However, nanowarmed conduit and muscle band were both significantly higher (p<0.05, t-test). Data redrawn from results published in Brock bank et al., 2021.

solution combined, can be developed that do not require the use of nanowarming, since we are already close to achieving this with heart valves at 30 mL volumes (Figure 2). However, at larger volumes nanowarming will likely continue to be the best warming method for retention of tissue cell viability. Further studies to optimize cryopreservation of cardiac muscle, the somewhat fibrous muscle band at the base of heart valves, and pulmonary and aortic arteries needs to be performed since it is clear that different heart valve components vary in their preservation requirements. It is anticipated that other complex tissues may also have components with different cryopreservation requirements. Biomaterial testing and *in vivo* large animal tissue transplant studies employing ice-free cryopreservation methods and nanowarming with retention of cell viability are in progress.

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