Published in final edited form as:

ACS Biomater Sci Eng. 2018 August 13; 4(8): 3006–3015. doi:10.1021/acsbiomaterials.8b00648.

Effect of Ice Nucleation and Cryoprotectants during High Subzero-Preservation in Endothelialized Microchannels

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Abstract

Cryopreservation is of significance in areas including tissue engineering, regenerative medicine, and organ transplantation. We investigated endothelial cell attachment and membrane integrity in a microvasculature model at high subzero temperatures in the presence of extracellular ice. The results show that in the presence of heterogeneous extracellular ice formation induced by ice nucleating bacteria, endothelial cells showed improved attachment at temperature minimums of -6°C . However, as temperatures decreased below -6°C , endothelial cells required additional cryoprotectants. The glucose analog, 3-*O*-methyl-D-glucose (3-OMG), rescued cell attachment optimally at 100 mM (cells/lane was 34, as compared to 36 for controls), while 2% and 5% polyethylene glycol (PEG) were equally effective at -10°C (88% and 86.4% intact membranes). Finally, endothelialized microchannels were stored for 72 h at -10°C in a preservation solution consisting of the University of Wisconsin (UW) solution, Snomax, 3-OMG, PEG, glycerol, and

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b00648. Experimental setup, characterization of endothelial barrier function and adhesion molecules, effect of intracellular and extracellular cryoprotectants, effect of long-term storage on endothelial cell attachment, and morphological differences between endothelial cells frozen to -10°C in the presence of snomax and 100 mM 3OMG versus 5% PEG, (PDF)

Notes

The authors declare the following competing financial interest(s): S.N.T., M.T., S.L.S., K.U., and L.W. are inventors on several provisional patents on the topic of cryopreservation of cells, tissues, and organs, including high subzero preservation. Further, K.U. has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. The interests of all researchers are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

trehalose, whereby cell attachment was not significantly different from unfrozen controls, although membrane integrity was compromised. These findings enrich our knowledge about the direct impact of extracellular ice on endothelial cells. Specifically, we show that, by controlling the ice nucleation temperature and uniformity, we can preserve cell attachment and membrane integrity. Further, we demonstrate the strength of leveraging endothelialized microchannels to fuel discoveries in cryopreservation of thick tissues and solid organs.

Graphical Abstract

Keywords

cryopreservation; endothelial cells; tissue engineering; microvasculature model

INTRODUCTION

Cell, tissue, and whole organ-based therapies have broad clinical and scientific importance. For the more established therapies already in routine clinic use, such as transfusion medicine and transplantation, the magnitude of the impact is already significant; 21 million blood components are transfused every year in the U.S.,¹ while more than 50 000 hematopoietic stem cell transplants² and 100 800 solid organ transplants³ are performed worldwide per year. At the same time, the fields of tissue engineering and regenerative medicine are rapidly advancing. From organs-on-chip^{4,5} to recellularized whole organs,⁶ these bold endeavors hold the promise of filling critical gaps in the availability of biologics for clinical purposes while also elevating biomedical research including drug development and disease modeling. Taken together, we are in an era poised for significant changes in healthcare and biomedical research fueled by a dramatic rise in medicinal products composed of living cells. However, even the relatively established biologic-based supply chains suffer from significant unmet needs. For example, lifesaving organs destined for transplantation only survive a short amount of time ex vivo (maximum 12 h for liver).⁷ Thus, given these significant bottlenecks in dissemination of therapies already in routine use, matched with the rapid advancements of technology composed of extremely sensitive living material, robust supportive networks focusing on preserving biologics from retrieval to user are an absolute need. If not adequately addressed, the full potential of these novel technologies will not be realized.

In some capacities, considerable progress has been made in the development of effective biopreservation and stabilization protocols (e.g., cryopreservation of cell lines); however, protocols of more complex systems have been met with limited success.⁸ Current clinical standards for organ preservation use hypothermic storage (+4 °C); however, this method only moderately slows down tissue deterioration. Conversely, vitrification—the transformation of a substance into a glass through extremely rapid freezing—holds great promise, although unsolved challenges have prevented translation to the clinic. For example, vitrification faces limitations in larger tissues because during devitrification extremely high heating rates are required in order to inhibit the growth of crystalline structures and prevent fracturing. Moreover, vitrification requires high molarity cryoprotectants⁷ which are not only highly toxic to cells but also cause osmotic damage. Our recent effort in supercooling was designed to build on the current clinical standard, and its success to achieve a tripling of the standard liver preservation duration is largely attributed to enabling high subzero storage temperatures (−6 °C), while retaining the preservation media in liquid state.⁹ However, there are issues in its scale-up for clinical use: the supercooled state is thermodynamically unstable, and the amount of liquid volume in human vital organs significantly increases the probability of freezing, as compared to organs of smaller research subjects.

Instead, new cryopreservation methods should look to nature for inspiration. Various species of vertebrates, such as *Rana sylvatica* (the wood frog), achieve a thermodynamically stable, noninjurious frozen state at high subzero storage temperatures ranging from −6 to −22 °C.^{10–13} Using cryomicroscopic analysis, researchers have detailed the mechanisms of ice propagation utilized by freeze-tolerant wood frogs in nature.¹⁴ In this study, micrographs of liver slices showed continuous ice formed along the vasculature with the parenchyma remaining ice-free and considerably shrunken because of cellular dehydration. In a subsequent study, researchers attempted to translate this strategy from nature to whole rat livers using slow freezing rates, and glycerol as the cryoprotectant,¹⁵ although the protocol was ultimately unsuccessful and all transplanted organs failed. While ice was indeed confined to the vasculature and viability of the hepatocytes appeared functional, as measured by bile production and histology, endothelial cells were rounded/detached, and neutrophils were seen in the sinusoidal space, suggesting freezing induced damage to the vasculature. These studies point out a critical challenge; endothelial cells are the most vulnerable cell type in our proposed partial freezing protocol since they will make direct contact with ice as it propagates throughout the organ. Moreover, endothelial cells have long been identified as having a particular sensitivity to reperfusion and the degree of endothelial injury has been shown to correlate with functional impairment of the graft following transplantation.^{16,17} Taken together, careful consideration of the impact of our storage and recovery protocols on endothelial cells will be critical to success.

In this study, we leverage tissue engineering techniques to emulate simple organ structures in a relatively high-throughput format to fuel new findings in cryobiology. The primary purpose is to demonstrate how bioengineering products, such as capillary-on-a-chip, can be leveraged to investigate the impact of new cryopreservation methods on endothelial cells adherent to a structural scaffold. In this capacity, we demonstrate a new cryopreservation protocol inspired by freeze-tolerant wood frogs in nature, which we call “partial freezing”. To achieve this, we address endothelial injury and ice dynamics using our capillary

constriction devices,¹⁸ which allow direct microscopic observation of normal, elongated cell morphology and continuous perfusion. Further, we test the effect of ice nucleation temperature, and the impact of intracellular and extracellular cryoprotective agents (CPAs) for maintaining endothelial morphology and membrane integrity as a function of high subzero temperatures ranging from -6 to -20 °C. We use Snomax, the freeze-dried form of *Pseudomonas syringae*, as a model ice nucleator in this study, as we have done before,¹⁹ and cryoprotectants including membrane-permeable 3-*O*-methyl-D-glucose (3-OMG)²⁰ and glycerol, as well as nonpermeable polyethylene glycol (PEG) and trehalose.⁹ In all cases, the efficacy of ice nucleators and CPAs are tested as a function of attachment/morphology and membrane integrity of endothelial cells that are coated in capillary constriction devices. In this way, we capture critical structural information, dynamically, and in a cost-effective, high-throughput format using bioengineering principles.

MATERIALS AND METHODS

Microfluidic Capillary Fabrication.

Our capillary model (Figures 1 and S1c) is based on our capillary constriction devices,¹⁸ which has also been described by others.^{21,22} Capillary constriction devices were fabricated using standard soft lithography techniques, as previously described¹⁸ and illustrated in Figure 1a/b. Briefly, mylar masks were designed in AutoCAD featuring devices with 16 parallel channels of the following dimensions: $30 \times 30 \times 5000$ - or $40 \times 40 \times 5000$ - μm (width by height by length). Each device was designed with one cylindrical inlet port (for cell seeding) and one outlet port (for media delivery) of 2.5 and 1.2 mm diameters, respectively. SU-8–50 negative photoresist (Microchem) was spun onto silicon wafers, and UV photolithography was conducted using the mask for patterning. The height of SU-8 features were verified to be within $\pm 10\%$ using a surface profilometer (Dektak ST System Profilometer; Veeco Instruments). A 10:1 (wt:wt) mixture of Polydimethylsiloxane (PDMS) prepolymer and cross-linker (Dow Corning) was poured onto silicon master molds before degassing. After curing at 65 °C for 24 h, devices were removed from the mold and punched (Harris Uni-Core biopsy punchers). Devices were then oxygen plasma treated at 300 mmTor O₂ at 50 W for 35 s, and bonded to glass slides (heated on a hot plate for 10 min @ 85 °C). Microchannels were rounded as described by others^{21,22} using uncured PDMS prepolymer mixed in equal parts with Xiameter PMX-200 1 cS silicone oil (Dow Corning). Prepolymer diluted in oil was loaded into devices while heating (100 °C for 10 s). Subsequently, negative pressure of 75 kPa was applied to the outlet using a vacuum pump for 2–3 min, before the pressure was reduced to 17 kPa on a hot plate at 100 °C for 5 min.

HUVEC Culture, Microchannel Coating, and Characterization.

Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in standard T25 or T75 flasks coated with 0.1% gelatin (catalog no. G9391; dissolved in sterile, distilled water). The gelatin solution (1.3–4 mL) was uniformly distributed in cell culture flasks and incubated at 37 °C. After 1 h, the remaining gelatin solution was aspirated, and flasks were equilibrated in HUVEC complete media in preparation for cell passage. HUVECs obtained from Lonza were maintained in EBM-2 basal media supplemented with EGM-2 bullet kit (CC-3156 and CC-4176) and subcultured every 3–4 days using 0.25% trypsin-EDTA. For all experiments,

HUVECs were between passage numbers 8–11. We performed barrier function assays on endothelial cells at passage 9 to confirm normal endothelial function. These assays were performed by seeding cells onto a fibronectin coated transwell removable insert (Cat# 353102) and culturing for 3 days before placing FITC-labeled dextran into the apical chamber (Figure S2a; 1:1000 v-v; Cat# for FITC-labeled Dextran 70, 40, and 10 kDa are D1818, D1844, D1821, respectively). After a 30 min incubation, 100 μL aliquots were sampled from the basal chamber (in triplicate) and read on a fluorescent spectrophotometer. Figure S2b illustrates the fluorescence signal of controls with no cells (coated with fibronectin only) versus those coated with fibronectin and cells, demonstrating low fluorescence signal in the basal chamber in the presence of endothelial cells. Finally, we stained endothelial cells with Hoescht (blue nuclear) and VE-Cadherin (green) in tissue culture plates (Figure S2c) and microchannels (Figure S 2d; microchannels were also stained with CD31) to confirm the presence of cell–cell adhesive structures which are typical of endothelial cells.²³

Prior to coating with endothelial cells, microchannels were sonicated briefly (3 min in isopropanol; Branson 200, Branson Ultrasonics), and then rinsed with PBS. Channels were then coated with 1 mg/mL fibronectin (dissolved in sterile PBS, total of 10 μL) and incubated for 60 min at 37 °C. After a priming step, HUVEC cells were trypsinized, counted, and introduced to the cell seeding port and incubated at 37 °C 5% CO₂ for 30 min (Figure 1a). Final constriction diameters of cellularized microchannels varied from ~10 to 20 μm across the lengths of individual channels because of the presence of cellular nuclei along channel walls. Coated microchannels were then fed under continuous gravity flow using a pipet tip containing media and inserted into the delivery port to generate flow in the reverse direction from loading (Figure 1a). Pipet tips (200 μL) filled with media provided an initial hydrostatic head of 2.5 cm H₂O. Cells incubated at 37 °C 5% CO₂ up to 3 days with daily replacement of tips with filled media before proceeding to experimentation. All microchannels were imaged prior to experimentation to ensure endothelial cells were uniformly coated and showed an elongated morphology characteristic of healthy, normal HUVECs (Figure 1b). Control, unfrozen microchannels were stained with Calcein green (1:1000), Hoescht (H3570; 1:1000 v-v), and propidium iodide (4 $\mu\text{g/mL}$) for 20 min and imaged, while microchannels imaged prior to experimentation/freezing were only stained with Calcein green (1:1000). These experimental microchannels were subsequently stained after experimentation/freezing with Hoescht (H3570; 1:1000 v-v), and propidium iodide (4 $\mu\text{g/mL}$), see further details below.

Cryostage Experiments.

Endothelial cell-coated microchannels were prepared, as described above, and placed on the silver block of a FDCS196 cryostage (Linkam Scientific Instruments Ltd., London, UK), the temperature of which was controlled by the TMS 94 temperature controller with the accuracy of ± 0.1 °C (Figure S1a/b). Microchannels bonded to glass slides, as imaged in Figure S1c, were cut into individual devices so they would fit on the silver block of the Linkam cryostage, as depicted in Figure S1b. A PixelINK PL-A662 camera (PixelINK, Ottawa, Canada) was used to visualize ice formation and propagation (imaged in Figure S1a). In all cases, ice nucleation temperature was recorded and ice propagation throughout

the device was confirmed by direct visual inspection (representative images showing ice formation are presented in Figure 1c and Figure S 1d). Prior to freezing, endothelial cell-coated microchannels in the 3-O-methyl-D-glucopyranose (3-OMG) experimental group were incubated with EBM-2 complete media supplemented with either 50 or 100 mM 3-OMG at 37 °C to promote intracellular transport of the glucose analog. Our previous efforts have shown supplementation of 3-OMG in the basal media and incubation at 37° is an effective method for intracellular delivery of 3-OMG.²⁰ Microchannels containing EBM-2 complete media was then changed for PBS containing 3-OMG (50 and 100 mM; catalog no. M4879), polyethylene glycol (PEG, 35 kDa; 2 and 5%; catalog no. 81310), and/or ice nucleators. Snomax (Snomax International, Englewood, CO, USA) was used as the model ice nucleator in this study (1 mg/mL). Microchannels were perfused with PBS containing ice nucleators and cryoprotectant agents for 10 min prior to beginning the cooling profile. During the freezing experiment, the temperature was first cooled at 10 °C/min to 4 °C. After thermal stabilization, a cooling rate of 1 °C/min was applied until reaching the holding temperature. For microchannels which did not contain ice nucleators, cells were cooled until ice nucleation/propagation was observed and then cooled to the closest round number. For microchannels which contained Snomax, ice was observed <−6 °C in all cases, and channels were cooled to −6, −10, or −20 °C. At the lowest holding temperature, microchannels were held for 3–5 min before a rapid rewarming at +30 °C/min (Figure 1c). After rewarming, microchannels were stained with Hoescht (nuclear) and propidium iodide (membrane integrity) dissolved in EBM-2 complete media for 20 min and imaged, in which the cell membrane integrity was used as a discriminator of live and dead cells.

Long-Term Storage Experiments.

Endothelial cell-coated microchannels were prepared, as described above, and placed in a controlled rate freezer (Kryo 560–16 Planar Controlled Rate Freezer). Microchannels were cooled at 1 °C/min down to −10 °C and held in the controlled rate freezer for 20–30 min before rapidly transferring to a freezer set at −10 °C for long-term storage (± 1 °C). Prior to freezing, microchannels were incubated for 1 h at 37 °C with EBM-2 complete media supplemented with 100 mM 3-OMG. Following 3-OMG cellular uptake, microchannels were cooled to 4 °C on a Teca plate cooler (ThermoElectric Cooling America Corporation, ACP-1200; ± 0.1 °C). After thermal stabilization, EBM-2 supplemented with 3-OMG was changed for prechilled storage solution. The base media for all storage solutions was the University of Wisconsin solution (Fisher Scientific, catalog no. NC0952695) supplemented with Trolox (10 mM; Cayman Chemical Company, catalog no. 10011659), 3-OMG (100 mM), PEG (2%), and Snomax (1 mg/mL). Further, the storage solution was supplemented with Glycerol (10%; Sigma-Aldrich, G7893) and Trehalose (30 mM; Sigma-Aldrich, catalog no. 90210). After a fixed storage duration of 72 h, microchannels were removed from the freezer and rapidly thawed in a warm media bath consisting of EBM-2 complete media supplemented with 100 mM 3-OMG, 50 mM Trehalose, and 2% PEG. This thawing solution was also used to wash out the storage solution by perfusing microchannels with a tip inserted into the media port, as described above. After ~1 h, the thawing solution was replaced with EBM-2 complete media and microchannels were left overnight (12–16 h) in an incubator at 37 °C. After this recovery period, microchannels were stained and imaged, as described above.

Quantification and Statistics.

Cell attachment values were quantified by counting the total number of Hoechst positive cells (i.e., total number of cell nuclei) and dividing by the total number of channels (each device has maximum of 16 channels). Membrane integrity was quantified by counting the number of propidium iodide positive cells divided by the total number of Hoechst positive cells. Cell attachment and membrane integrity are expressed as box-and-whiskers plots showing median, interquartile range, maxima, and minima ($n = 4-8$). Statistical testing used either the Student's *t* test or the one-way ANOVA and the Tukey posthoc functions from the GraphPad Prism software (San Diego, CA).

RESULTS

Effect of Ice Nucleation and Holding Temperature on Endothelial Cell Attachment in Endothelialized Microchannels.

We first tested the effect of ice nucleation temperature on endothelial cell attachment either in the presence or absence of a potent ice nucleator, Snomax (Figure 2). Without Snomax, endothelial cell attachment was significantly decreased (6.5 ± 3.4 cells/channel, $n = 8$), as compared to unfrozen controls (35.9 ± 2.9 cells/channel, $n = 9$; $p < 0.0001$; Figure 2a). However, when relatively higher and more consistent ice nucleation temperatures were initiated in the presence of Snomax, no significant difference was observed between control and microcapillaries with a T_{hold} of -6°C (37.2 ± 6.5 cells/channel; Figure 2a). When temperature was further decreased to -10 and -20°C (14.6 ± 14.7 and 9.3 ± 4.6 cells/channel, respectively, Figure 2a), cell attachment decreased significantly as compared to both controls and microcapillaries with a T_{hold} of -6°C ($p < 0.0001$ in all cases).

Conditions which did not have Snomax had nucleation temperatures ranging between -8.3 and -15.0°C (covering a range of 6.7°C), while microcapillaries with Snomax reliably nucleated between a much smaller temperature range of -5.1 and 6.0°C (covering a range of 0.9°C ; Figure 2a inset).

Effect of the Intracellular CPA, 3-OMG, as a Function of Holding Temperature on Endothelial Cell Attachment and Membrane Integrity in Endothelialized Microchannels.

We tested the contribution of intracellular CPAs to protect endothelial cells against freezing damage at two high subzero temperatures including -6 and -10°C in the presence of Snomax (Figure 3). Unfrozen controls with and without 3-OMG (100 mM) are presented in Figure S3 whereby there was no statistical significant difference ($p = 0.6995$). Endothelial cells frozen in the presence of 3-OMG were first exposed to a 1 h incubation with EBM-2 media containing either 50 or 100 mM 3-OMG to promote intracellular delivery of 3-OMG, which we have shown previously is sufficient for membrane transport.²⁴ Cell attachment with 50 mM 3-OMG was 33.2 ± 9.1 at $T_{\text{hold}} -6^\circ\text{C}$ versus 15.1 ± 8.2 at $T_{\text{hold}} -10^\circ\text{C}$, which was statistically significant (Figure 3a, $p = 0.0247$). Also, there was a statistically significant difference with respect to cell membrane integrity comparing -6 and -10°C holding temperatures whereby percent PI positive cells were $7.4 \pm 3.7\%$ versus $38.1 \pm 7.5\%$, respectively (Figure 3b, $p < 0.0001$). In contrast to the trend observed for 50 mM 3-OMG, there was no statistical difference between T_{hold} of -6°C and -10°C with 100 mM 3-OMG

which had 31.4 ± 6.6 and 33.6 ± 8.8 cells/lane following the freeze–thaw protocol, respectively (Figure 3a). However, cell membrane integrity assay showed that $20.5 \pm 12.4\%$ of the total cells were PI positive at $-10\text{ }^{\circ}\text{C}$ versus $7.3 \pm 2.8\%$ at $-6\text{ }^{\circ}\text{C}$, although this was not statistically significant ($p = 0.67$; Figure 3b).

Effect of the Extracellular CPA, PEG, as a Function of Holding Temperature on Endothelial Cell Attachment and Membrane Integrity in Endothelialized Microchannels.

We also tested the contribution of extracellular CPAs to protect endothelial cells from freezing damage. Unfrozen controls with and without PEG (2%) are presented in Figure S3 whereby there was no statistical significant difference ($p = 0.0611$). We observed no statistically significant difference when comparing 2% versus 5% PEG at either of the subzero temperatures tested for both cell attachment (cells/lane) and percent PI positive cells (% propidium iodide; Figure 4). With respect to cell attachment, values in the presence of 2% PEG were 43.1 ± 3.6 and 43.7 ± 6.9 , respectively, while values for 5% PEG were 38.8 ± 6.4 and 36.3 ± 8.1 at $T_{\text{hold}} -6$ and $-10\text{ }^{\circ}\text{C}$, respectively (Figure 4a). Membrane integrity quantified as percent PI positive cells (Figure 4b), showed values for 2% PEG were 14.1 ± 6.8 and 12.0 ± 11.3 , while values for 5% PEG were 9.4 ± 8.6 and 13.6 ± 15.9 at $T_{\text{hold}} -6$ and $-10\text{ }^{\circ}\text{C}$, respectively.

Endothelial Cell Attachment and Viability during Long-Term Storage in Endothelialized Microchannels.

We hypothesized that despite the benefits of 3-OMG and PEG for maintenance of endothelial morphology and viability during a brief freeze–thaw cycle, it would not be sufficient for long-term storage. Thus, we used the University of Wisconsin (UW) solution since it is the current clinical standard for solid organ preservation at hypothermic temperatures ($+4\text{ }^{\circ}\text{C}$) and we also showed its effectiveness at colder storage temperature ($-6\text{ }^{\circ}\text{C}$) using high subzero supercooling preservation.^{9,25} However, our preliminary data with UW as the base solution and supplemented with 1 mg/mL Snomax, 100 mM 3-OMG, and 2% PEG showed low endothelial cell attachment after 72 h of storage at $-10\text{ }^{\circ}\text{C}$ (15.46 ± 6.9 cells/lane; Figure S4). As a result, we further supplemented with 10% glycerol and 30 mM trehalose since these are well established and commonly used CPAs for cryopreservation. With this “complete” storage media including UW, 1 mg/mL Snomax, 100 mM 3-OMG, 2% PEG, 10% glycerol, and 30 mM trehalose, we stored microchannels at $-10\text{ }^{\circ}\text{C}$ for 72 h and measured endothelial cell attachment and viability after a recovery period of 12–16 h at $37\text{ }^{\circ}\text{C}$ (Figure 5). Under these conditions, endothelial cell attachment was 48.0 ± 9.1 at 72 h, as compared to unfrozen controls 43.7 ± 8.6 ($p = 0.4067$, two-tailed t test). While cell attachment was maintained after 72 h of storage, the percentage of PI positive cells was $68 \pm 24.7\%$, which was significantly increased as compared to unfrozen control values ($p < 0.0001$).

DISCUSSION

Our approach is based on achieving a thermodynamically stable, noninjurious partially frozen state (i.e., two-phase solid–liquid coexistence) at colder storage temperatures than can be realized with supercooling. Our recent effort in high subzero “supercooling” (ice-

free) achieved a tripling of the standard liver preservation duration due to low storage temperatures (-6°C) while retaining the preservation media in liquid state.⁹ These two principles promote a deeper metabolic stasis than can be achieved with standard hypothermic storage at $+4^{\circ}\text{C}$ and avoid complicated phase transitions, respectively. However, supercooling preservation is limited since the risk of accidental ice formation increases as a function of the minimum storage temperature and the freezing processes of highly supercooled solutions form sharper dendritic crystals, as compared to the equilibrium freezing processes.¹⁰ Leveraging our endothelial cell-coated micro-capillaries, we test a new high subzero preservation strategy which will store biologics in the frozen state. We call this preservation strategy “partial freezing” since ice crystals are restricted to extra-organ and vasculature spaces, and only some water is trapped as ice. To accomplish this, we look to nature for inspiration since diverse organisms have already mastered living in the presence of ice.¹⁴ Various species of vertebrates, such as *Rana sylvatica* (the wood frog), exhibits the ability to survive long periods of time in a partially frozen state with the whole animal, including every single organ demonstrating the ability to live in the presence of extracellular ice without injury.¹⁴ It has been shown that subarctic populations of wood frogs can tolerate temperatures close to -18°C , although temperature ranges can vary between -5 and -22°C .^{10–13}

Guided by strategies used in nature and literature,^{26–29} we hypothesized ice nucleation temperature would play a key role in freezing survival of endothelial cells. It is generally accepted that the minimization of cryoinjury may be achieved when ice nucleation occurs as near as possible to the equilibrium freezing point.²⁹ For example, a critical step for successful cryopreservation of embryos and oocytes for in vitro fertilization is control of ice nucleation whereby “seeding” is achieved by generating a cold spot on the outside of the cryocontainer.³⁰ Active ice nucleation is also a critical strategy for freeze-tolerant animals,^{10,27} although ice nucleating agents (INAs) in the blood and gut/skin induce controlled freezing of extracellular water at multiple nucleation sites.³¹ As the hemolymph gradually freezes, it is accompanied by an increase in the osmolality of the extracellular fluid, resulting in cellular dehydration as water is pulled from the intracellular environment.^{27,31} This provides further protection since the cellular fluids are no longer supercooled and ice nucleation in the intracellular environment is prevented.^{27,31}

In the present study, we employed the commercially available ice nucleator, Snomax, which is the freeze-dried form of *Pseudomonas syringae*.¹⁹ We chose Snomax since it is directly suspended in the media and would induce freezing at multiple nucleation points within endothelialized microchannels, as compared to external seeding with a cold spot. Since we aimed to understand the effect of ice nucleation temperature alone, we froze microchannels with Snomax dissolved in PBS. In the presence of Snomax, microchannels reliably froze between -5.1 to -6°C , as compared to -8.3 and -15.0°C without Snomax, and active ice nucleation significantly improved endothelial cell attachment at T_{hold} of -6°C (Figure 2). This positive effect of controlled ice nucleation on cell survival has also been demonstrated for diverse cell types in suspension,^{29,32,33} encapsulated cells,^{32,34} and more complex tissues adherent to a structural scaffold;³⁵ however, their relevance in endothelialized microchannels during high subzero freezing has never been described. While a holding temperature as high as -6°C cannot be achieved without the presence of foreign ice nucleators, as the

temperature minimum was further decreased from -6 down to -10 and -20 °C, endothelial cell attachment decreased significantly to levels similar to without Snomax. This data suggests that while active ice nucleation at multiple nucleation sites does influence endothelial cell attachment, to descend into colder storage temperatures of about -10 °C additional cryoprotectant agents are required (see Figure 3 and 4). This observation is also consistent with literature whereby cryoprotectant agents are a necessary supplement to controlled ice nucleation by reducing the risk of intracellular ice formation since water inside cells/tissues is replaced.^{32,33,36}

Another critical strategy used by freeze-tolerance organisms involves the synthesis of high amounts of low-molecular weight carbohydrates (glucose in wood frogs) in blood and tissues which provide colligative resistance to detrimental decreases in cell-volume, while also serving to stabilize the phospholipid bilayer of membranes and restrict the formation of intracellular ice.^{11,12,37} Our group has successfully employed an alternative to glucose; the nonmetabolizable glucose derivative 3-*O*-methyl-D-glucose (3-OMG) which accumulates naturally through glucose transporters. 3-OMG is a viable alternative to glucose since it is not as readily metabolized, ensuring it can accumulate in the intracellular environment. 3-OMG is nontoxic and our group has previously shown that the success of supercooling protocol relies in part on this cryoprotectant.⁹ Further, we have shown that endothelial cells stored in solutions containing polyethylene glycol (PEG) were significantly less damaged,^{38–40} while also showing positive benefits on hepatocytes stored at 4 °C⁴¹ and during our supercooling protocol of whole rat organs at -6 °C.⁹ The mechanism of action of PEG includes adsorption and stabilization of the membrane surface (>10 kDa), exerts an oncotic effect to prevent edema and reduce tissue water content, reduce oxidative stress, as well as depress the freezing point of solutions and promote cell dehydration.^{42,43} Taken together, we hypothesized intracellular saccharides such as 3-OMG and extracellular polyglycols such as polyethylene glycol would be particularly relevant for high subzero partial freezing.

To remove the variable of ice nucleation temperature on cell attachment, all conditions included Snomax. We observed a dose effect whereby 100 mM 3-OMG was required to effectively maintain endothelial cell attachment down to temperatures of -10 °C (Figure 3), also suggesting this intracellular cryoprotectant provides added support above and beyond that provided by Snomax alone. In contrast, endothelial cell attachment and membrane integrity with 50 mM 3-OMG was statistically different at -10 °C, as compared to -6 °C. While cell attachment was maintained down to -10 °C in the presence of 100 mM 3-OMG, there was an upward trend in the number of PI positive endothelial cells, although this was not statistically significant, suggesting that additional cryoprotectant agents may be required for long-term preservation of endothelial cells in microcapillaries. This upward trend in PI positive endothelial cells when frozen down to -10 °C in the presence of 100 mM 3-OMG is also reflected in endothelial cell morphology, as illustrated in Figure 3d (also see enlarged images of 100 mM 3-OMG condition frozen to $T_{\text{hold}} -10$ °C in Figures S5), whereby endothelial cells lose their elongated shape and become more circular.

Like conditions which characterized freezing damage on endothelial cells in the presence of 3-OMG and Snomax, we also measured the positive preservation features of polyethylene glycol (PEG). We chose the PEG of 35 kDa since this was also shown to be important for

our supercooling preservation approach, also at high subzero temperatures.⁹ As compared to 3-OMG, both 2% and 5% PEG were effective at preserving cell attachment at both -6 and -10 °C (Figure 4). Moreover, cell morphology was better maintained, as compared to 100 mM 3-OMG at the same temperature, at least upon visual inspection and qualitative observation of the elongated morphology (Figure 3d; also see enlarged images of 100 mM 3-OMG versus PEG conditions frozen to $T_{\text{hold}} -10$ °C in Figure S5). Finally, there were no statistically significant increases in cell death, although one biological replicate with 5% PEG down to -10 °C did show 36.3% of attached endothelial cells were positive for propidium iodine because of compromised cell membranes. In summary, conditions with 100 mM 3-OMG or 2% or 5% PEG showed the most promise.

For long-term preservation studies we used the University of Wisconsin (UW) solution as the base solution, and supplemented with optimal conditions as determined by brief freeze–thaw cycles described above. UW was selected since we have already demonstrated its application during supercooling preservation, and it contains CPAs which would provide additional support during freezing survival. For example, others have shown that tissue engineered vessels containing endothelial cells that were provided with 3% dextran or 5% hydroxyethyl starch (HES) had few focal leaks, maintained adhesion to the scaffold, and were typically viable for at least 2 weeks.⁴⁴ Authors suggested that plasma expanders such as dextran and HES stabilize microvessels via physical mechanisms that enhance VE-cadherin localization at junctions and thereby limit vascular leakiness.⁴⁴ However, when endothelialized microchannels were stored for 72 h with UW supplemented with Snomax, 3-OMG (100 mM), and PEG (2%) alone it was not sufficient to maintain endothelial attachment (Figure S4). In contrast, endothelial cell attachment after 72 h was maintained when this storage solution was further supplemented with glycerol (10%) and trehalose (30 mM) that are widely used in conventional cryopreservation (Figure 5). These CPAs would provide additional support to both the intracellular and extracellular environment, while also contributing to freezing point depression and an overall decrease in the amount of water trapped as ice.

These data demonstrating multiday storage of endothelialized microchannels challenge the paradigm that vitrification is the only solution for the long-term preservation of complex tissues including tissue-engineered constructs.⁴⁵ While it has been shown that isolated chondrocytes can be cryopreserved using standard freezing methods with high cell survival postthaw, chondrocytes embedded in the extracellular matrix using these freezing methods result in 80–100% cell death.^{45,46} Yet, rabbit cartilage can be preserved by vitrification with >80% cell viability,⁴⁷ suggesting completely avoiding ice formation for the preservation of more complex tissue structures is critical to success. Further evidence for the strength of ice-free approaches has been demonstrated using tissue engineered constructs such as bone whereby slow cooling approaches resulted in suboptimal cell viability,^{48,49} while it was concluded vitrification of osteoblast-biomaterial holds promise⁵⁰ (also reviewed in ref 45). However, it is critical to highlight and further discuss the relevance of the final storage/holding temperatures in the present study versus more classical methods of cryopreservation using slow cooling. Since our approach uses relatively high subzero temperatures (-10 °C) as compared to classical methods of cryopreservation (-80 °C or below), we overcome many of the mechanisms which cause cryoinjury including mechanical constraints, solute effects,

and catalysis of intracellular ice from growing extracellular ice crystals. Further, our method is less restricted by the high cooling and rewarming rates required for vitrification. Nonetheless, it should also be noted that our high subzero partial freezing approach does not replace the need for vitrification which offers the promise of longer-term, indefinite storage.

CONCLUSION

In summary, we demonstrate the strength of leveraging endothelialized microchannels to directly visualize and screen conditions in pursuit of cryopreservation of more complex biological systems, such as thick tissues and solid organs. For example, endothelial cells within microchannels can be cultured for several days and subjected to continuous flow allowing for an effective means of loading and removing cryoprotectants. Furthermore, our capillary constriction devices can be easily adapted to fit the cryomicroscope system, which allows for real-time monitoring of ice propagation and accurate control of temperatures. These lessons may also be used to develop new biopreservation methods for the transport and widespread dissemination of innovative technologies which contain living materials such as organ-on-a-chip systems and which offers an alternative to classical methods of cryopreservation at -80°C . Finally, we propose to further enhance our cryopreservation tools by including a separate cell chamber to mimic tissue parenchyma as well as test diverse types of endothelial cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Funding

This work was supported by NIH 1R01DK114506-01 and the Wang Family Foundation Grant. S.N.T. holds a Natural Sciences and Engineering Research Council (NSERC) of Canada Postdoctoral Fellowship.

We thank Octavio Hurtado and Dr. Maedeh Heidarpourroushan for microfabrication assistance. We also thank Lynne Stubblefield for administrative assistance.

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Figure 1. Microcapillary specifications, endothelial cell coating and imaging, and freezing/thawing protocol. (A) Representative illustration of the capillary constriction device. (B) Microcapillaries are coated with endothelial cells and stained/imaged (shown are bright field and fluorescent images using a 10 $\times$ objective). (C) The Linkam cryostage enables programmable freezing and thawing protocols, as well as direct visual observation of ice propagation.

Figure 2. Effect of ice nucleation temperature (T_{ice}) on endothelial cell attachment after exposure to a freeze–thaw protocol. (A) Cell attachment are expressed as box-and-whiskers plots showing median, interquartile range, maxima/minima, and all individual data points ($n = 4–5$) for control (no freeze/thaw), no Snomax (“no SM”), and with Snomax (“with SM”, 1 mg/mL) which were cooled to -6 , -10 , or -20 °C. No Snomax controls were cooled until ice nucleation and propagation throughout the device was observed, cooled to a round holding temperature, and thawed as per standard protocols. Data were analyzed using a one-way analysis of variance (ANOVA) with a posthoc Tukey test ($p < 0.05$); values that share the same letter notation are not significantly different from one another. Corresponding ice nucleation temperature with and without Snomax are shown as an inset. (B) Representative images of endothelial-cell lined microcapillaries before (pre-freeze) and after (post-freeze) a freeze–thaw cycle with or without Snomax and at three holding temperatures including -6 , -10 , and -20 °C. Images taken from the same channel are compared before (pre-freeze) and after (post-freeze) freeze/thaw.

Figure 3. Effect of intracellular cryoprotectants, 3-OMG, and holding temperature (T_{hold}) on endothelial cell attachment after exposure to a freeze–thaw protocol. (A) Cell attachment and (B) percent PI positive cells are expressed as box-and-whiskers plots showing median, interquartile range, maxima/minima, and all individual data points ($n = 4\text{--}5$) for two concentrations of 3-OMG (50 and 100 mM) and two holding temperatures (-6 and -10°C). Data were analyzed using a one-way analysis of variance (ANOVA) with a post hoc Tukey test ($p < 0.05$); values that share the same letter notation are not significantly different from one another. Representative images of 50 mM (C) and 100 mM (D) 3-OMG conditions. Endothelial cells are imaged in bright field and stained with Calcein green (live), Hoechst (nuclei, blue), and propidium iodine (dead, red). Images taken from the same channel are compared before (pre-freeze) and after (post-freeze) freeze/thaw.

Figure 4.

Effect of extracellular cryoprotectants, PEG, and holding temperature (T_{hold}) on endothelial cell attachment after exposure to a freeze–thaw protocol. (A) Cell attachment and (B) percent PI positive cells are expressed as box-and-whiskers plots showing median, interquartile range, maxima/minima, and all individual data points ($n = 4\text{--}5$) for two concentrations of PEG (2 and 5% w/v) and two holding temperatures (-6 and -10°C). Data were analyzed using a one-way analysis of variance (ANOVA) with a posthoc Tukey test ($p < 0.05$): no statistical significance was observed. Representative images of 2% (C) and 5% (D) PEG conditions. Endothelial cells are imaged in bright field and stained with calcein green (live), Hoechst (nuclei, blue), and propidium iodide (dead, red). Images taken from the same channel are compared before (pre-freeze) and after (post-freeze) freeze/thaw.

Figure 5.

Effect of long-term storage (72 h) on endothelial cell attachment with a holding temperature of -10°C . (A) Cell attachment are expressed as box-and-whiskers plots showing median, interquartile range, maxima/minima, and all individual data points ($n = 5-8$) for 72 h and unfrozen controls. Data were analyzed using two-tailed Student t test ($p < 0.05$). (B) Representative images of 72 h storage conditions. Endothelial cells are imaged in bright field and stained with calcein green (live), Hoechst (nuclei, blue), and propidium iodine (dead, red). Images taken from the same channel are compared before (“pre-freeze”) and after (“post-freeze”) freeze/thaw. The preservation solution consisted of University of Wisconsin (UW) solution, Snomax (1 mg/mL), 3-OMG (100 mM), PEG (2%), glycerol (10%), and trehalose (30 mM).