Cryobiology 71 (2015) 306–317

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Investigating membrane and mitochondrial cryobiological responses of HUVEC using interrupted cooling protocols

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ARTICLE INFO

Article history:
Received 5 May 2015
Revised 23 July 2015
Accepted 3 August 2015
Available online 5 August 2015

Keywords:
Cryopreservation
Cryoinjury
Cooling rate
Freezing damage
Flow cytometry
Membrane integrity
Mitochondrial membrane potential
Human umbilical vein endothelial cells (HUVEC)

ABSTRACT

The success of cryopreservation protocols is largely based on membrane integrity assessments after thawing, since membrane integrity can be considered to give an upper limit in assessment of cell viability and the plasma membrane is considered to be a primary site of cryoinjury. However, the exposure of cells to conditions associated with low temperatures can induce injury to cellular structure and function that may not be readily identified by membrane integrity alone. Interrupted cooling protocols (including interrupted slow cooling without a hold time (graded freezing), and interrupted rapid cooling with a hold time (two-step freezing)), can yield important information about cryoinjury by separating the damage that occurs upon cooling to (and possibly holding at) a critical intermediate temperature range from the damage that occurs upon plunging to the storage temperature (liquid nitrogen). In this study, we used interrupted cooling protocols in the absence of cryoprotectant to investigate the progression of damage to human umbilical vein endothelial cells (HUVEC), comparing an assessment of membrane integrity with a mitochondrial polarization assay. Additionally, the membrane integrity response of HUVEC to interrupted cooling was investigated as a function of cooling rate (for interrupted slow cooling) and hold time (for interrupted rapid cooling). A key finding of this work was that under slow cooling conditions which resulted in a large number of membrane intact cells immediately post thaw, mitochondria are predominantly in a non-functional depolarized state. This study, the first to look directly at mitochondrial polarization throughout interrupted cooling profiles and a detailed study of HUVEC response, highlights the complexity of the progression of cell damage, as the pattern and extent of cell injury throughout the preservation process differs by injury site.

1. Introduction

Cryopreservation is the use of low temperatures to preserve cells and tissues for extended periods of time. The success of cryopreservation protocols is dependent on minimizing the stresses that are associated with low temperature conditions. One of the most common models of cryoinjury is the two-factor hypothesis of freezing injury proposed by Mazur et al. that there are two independent mechanisms of freezing damage that impose stress on cells [23]. The first mechanism describes damage caused by cooling at relatively slow rates that prolong exposure time to increasing concentrations of solutes in the presence of extracellular ice [18,23]; the second mechanism describes the damage caused by cooling at relatively rapid rates that do not allow sufficient time for cells to lose the intracellular water necessary to maintain equilibrium with their surroundings [21,23] so that these cells become supercooled and the probability of forming intracellular ice (IIF) increases. The low temperature conditions that induce either of these mechanisms may be lethal to cells.

Protocols in cryopreservation involve cooling rates that are sufficiently rapid to avoid prolonged exposure to elevated concentrations of solutes yet sufficiently slow to reduce the probability of IIF [16,23]. However for most cells, cooling rates that cause slow and rapid cooling injury tend to overlap. In conventional cryopreservation investigations cells are typically assessed post-thaw, after completion of the freeze–thaw cycle. During cryopreservation processes cell damage occurs as the cell suspension traverses an intermediate zone of subzero temperatures (−15 to −60 °C) [22]. Although the survival of cells is readily apparent post-thaw, post-thaw assessments do not give insight into the cryobiological

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http://dx.doi.org/10.1016/j.cryobiol.2015.08.004
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response of cells within the important intermediate temperature zone. In addition, it has been shown that cell recovery can be improved by interrupting the cooling process within this intermediate range of subzero temperature [19]. There are currently two methods of interrupted cooling that are used to explore cryoinjury by separating the damage that occurs upon cooling to (and possibly holding at) the intermediate temperature range and the damage that occurs after plunging to the storage temperature (liquid nitrogen) that have the potential to both alleviate injury as well as reveal the response of cells throughout the cooling process. These methods are interrupted slow cooling without a hold time (graded freezing) [24], and interrupted rapid cooling with a hold time (two-step freezing) [8]. With fibroblasts as a cell model McGann used interrupted cooling to investigate cell damage at low temperatures during low rates of cooling [24]. More recently, Ross-Rodriguez et al. used interrupted cooling techniques to characterize the cryobiological responses of TF-1 hematopoietic stem cells [42–44].

Interrupted slow cooling uses relatively slow cooling rates to subject cells to intermediate subzero temperatures before being either: (a) directly thawed, or (b) plunged to the storage temperature in liquid nitrogen (−196 °C) before subsequent thawing. Relative slow cooling of cells allows them to remain in equilibrium with their surroundings during the presence of extracellular ice. The advantage of the interrupted slow cooling technique is the ability to conduct separate analyses of damage during initial slow cooling to the experimental temperature and damage after rapid cooling to the storage temperature (plunge into liquid nitrogen). At the time this method was established there was particular interest in the effect of cooling rate on various cell types [6,9,15]. McGann used interrupted slow cooling to examine the impact of manipulating cooling rate on cell survival over a range of subzero temperatures [24]. It was observed that the maximum recovery of cells (hamster fibroblasts) was obtained when slow cooling cells to higher subzero temperatures (−5 and −20 °C) before plunging them into liquid nitrogen [24]. Ross-Rodriguez et al. used a combined approach of simulations and experiments to investigate cell responses to interrupted slow cooling conditions with intracellular supercooling and osmolality as indicators of cell injury [44]. It was found that directly thawed TF-1 hematopoietic stem cells were damaged by solution effects alone, whereas plunged cells were observed to be damaged by IIF when rapidly cooled from high subzero temperatures (−3 to −6 °C) and by a combination of both solution effects and IIF when plunged from lower intermediate temperatures [44].

Interrupted rapid cooling procedures involve an initial uncontrolled cooling to a hold temperature, where the cells are held for a pre-designated time before being either: (a) directly thawed, or (b) plunged to the storage temperature (−196 °C) before subsequent warming. Interrupted rapid cooling has been described in earlier studies that involved a pre-freezing or stepwise cooling from intermediate temperatures prior to exposure to the storage temperature [5,19,52]. Farrant et al. showed that there are two stages of the cell response during two-step cooling: the initial occurrence of damage when cells are first subjected to the hold temperature and an incurred protection from further damage when cells are held at the intermediate hold temperature prior to plunging to the storage temperature [8]. This protection has been correlated with cell shrinkage during the hold time at the experimental temperature [11], and this decrease in cell volume is attributed to the osmotic removal of intracellular water that decreases the probability of IIF during the subsequent plunge [29]. The versatility of the two-step freezing method has been demonstrated in other studies exploring the effects of prolonged hold times [25], slower warming rates [26] and the differing actions of penetrating and non-penetrating cryoprotectantants [29].

The aforementioned cryopreservation studies primarily use membrane integrity assays to determine the state of post-thaw cells upon completion of the freeze–thaw cycle. This is largely because the plasma membrane has been considered one of the primary sites of cryoinjury [51], providing a simple and effective means to determine cell survival. Though membrane integrity assays have played an integral part as an upper limit in assessment of cellular viability, they do not provide insight into other important aspects of cell function that may also be susceptible to cryoinjury.

There has been a growing emphasis on studies investigating other sites of cryoinjury in addition to the plasma membrane. Cellular components such as lysosomes [35], mitochondria [13,45,46] and cytoplasmic granules [4] are adversely affected by freeze–thaw stress, causing structural and metabolic changes in cells. McGann et al. have conducted some extensive studies demonstrating cryoinjury to cytoplasmic and metabolic components of granulocytes [4,27,54], suggesting an additional mechanism of damage may be occurring when cooling these cells. Other studies have found a disconnect between membrane damage and metabolic activity in split-thickness skin, and isolated keratinocytes [56], as well as micropatterned canine kidney cells [53]. More recently, cryobiological investigations involving reproductive cells have used comparisons of membrane integrity and mitochondrial membrane potential post-thaw to evaluate the toxicity of cryoprotectant solutions in cephalopod [40] and farmed abalone spermatophores [17], as well as zebrafish follicles [12]. This comparison has also been used to assess the effects of osmotic stress during cryopreservation of koala sperm [13]. Mitochondria have been a particular point of cryopreservation interest as they are an important part of the functional capability of mammalian cells.

Determining mechanisms of cryoinjury by relating structure and function of cells is a reoccurring theme in cryopreservation studies. Sherman et al. had initially correlated observed structural changes in cells and tissue sections using electron microscopy with other parallel studies of functional integrity [48]. The "ultrastructural" changes observed in slowly cooled mouse kidney tissue [45] corresponded with reduced respiratory function of mitochondria [46]. However, these changes may not always be readily observed, as a follow-up study of cells in suspension showed that no "ultrastructural" changes were noted in cells prior to loss of sperm motility (the observed measure of cell survival), indicating that functional damage may be occurring without any physical manifestations before cell death [49].

The objective of the present study was to investigate the response of human umbilical vein endothelial cells (HUVECs) to both rapid and slow interrupted cooling strategies in the absence of cryoprotectant. The response of these cells was investigated as a function of cooling rate, hold time, and temperature, and the outcome was determined by comparing both physical (membrane integrity) and functional (mitochondrial membrane polarization) assessments. Revealing functional capacity of membrane-intact cells post-thaw provides a basis to be used in creating novel approaches to protect cells from the devastating effects of freezing during cryopreservation procedures.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells ((Lot# 0000120825) HUVEC; Lonza®, Walkersville, MD, USA) were cultured at 37 °C and 5% CO_2 in endothelial basal media (EBM-2) supplemented with a bullet kit (LONZA®) containing human fibroblast growth factor B,

hydrocortisone, vascular endothelial growth factor, ascorbic acid, heparin, human endothelial growth factor, and fetal bovine serum. For cell passage, cultures were incubated to approximately 40% confluence within the culture flask according to LONZA guidelines. For experiments, cultures were incubated to approximately 50% confluence, then harvested by exposure to trypsin–EDTA (Lonza®) for 2 min at 37 °C. Cell suspensions were centrifuged at 201 g for 5 min in an Eppendorf 5810R tabletop centrifuge (Eppendorf, Westbury NY, USA) and resuspended in endothelial growth media at a concentration of 4.0×10^6 cells/mL.

2.2. Interrupted cooling protocols

2.2.1. Graded freezing procedure (interrupted slow cooling without hold time)

The interrupted slow cooling procedure [24] was conducted as described in Ross-Rodriguez et al. [42]. A schematic representation of the interrupted slow cooling procedure is depicted in Fig. 1A. Aliquots (0.2 mL) of HUVEC cell suspension in endothelial growth media were transferred to 6×50 mm glass culture tubes (VWR, Edmonton, AB, Canada) and allowed to equilibrate at room temperature for 5 min. Positive controls were assessed at room temperature. Negative controls were plunged from room temperature directly into liquid nitrogen and assessed post-thaw. Experimental samples were transferred to a stirring methanol bath preset at −3 °C and allowed to equilibrate for 2 min before ice was nucleated with cold forceps. Post-nucleated samples were visually confirmed to have ice crystals present before cooling in a methanol bath set to 1.0 °C/min. The samples were monitored using a type-T thermocouple acquiring the data with Personal Daq View software (version 1.9; Omega Laval, QC, Canada). At each experimental temperature (−3, −6, −9, −12, −15, −20, −30 and −40 °C) one sample was directly thawed in a 37 °C water bath while the other was plunged into liquid nitrogen. The plunged samples were kept in liquid nitrogen for a minimum of 1 h before being thawed in a 37 °C water bath. Additional experiments were conducted using cooling rates of 0.1 and 0.2 °C/min. Duplicates of each sample were used for each experiment and were repeated at least in triplicate using cells from different passages.

2.2.2. Two-step freezing procedure (interrupted rapid cooling with a hold time)

The interrupted rapid cooling procedure [8] was conducted as described in Ross-Rodriguez et al. [42]. A schematic representation of the interrupted rapid cooling procedure is depicted in Fig. 1B.

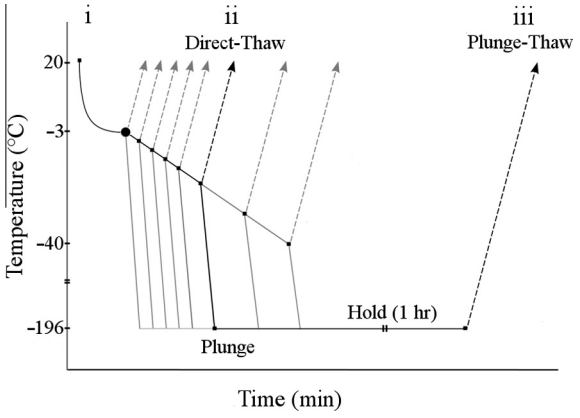

Fig. 1A. Schematic (axes not to scale) of interrupted slow cooling at a rate of 1.0 °C/min. (i) Pre-cooling of samples to −3 °C from room temperature and controlled ice nucleation (circle). (ii) Linear slow cooling (post-nucleation) to experimental temperatures and either directly thawing or plunging into liquid nitrogen (−196 °C). (iii) Subsequent thawing after storage time (>1 h).

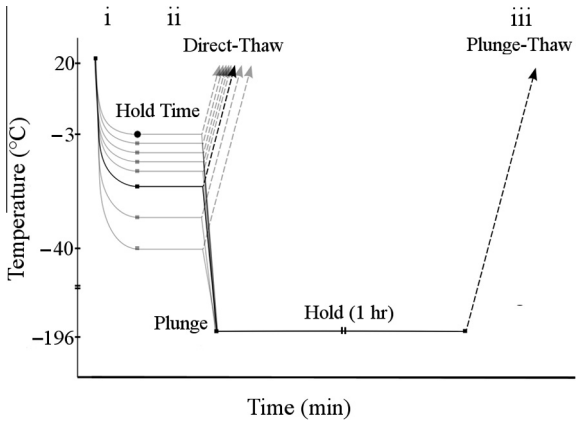

Fig. 1B. Schematic (axes not to scale) of interrupted rapid cooling with a hold time (3 min). (i) Pre-cooling of samples to the experimental temperature from room temperature and controlled ice nucleation. (ii) Hold time, followed by either direct thaw or plunge into liquid nitrogen (−196 °C). (iii) Subsequent thawing after storage time (>1 h).

Aliquots (0.2 mL) of HUVEC cell suspension in endothelial growth media were transferred to 6×50 mm glass culture tubes (VWR, Edmonton, AB, Canada) and allowed to equilibrate at room temperature for 5 min. Positive controls were left at room temperature. Negative controls were plunged from room temperature directly into liquid nitrogen. The experimental samples were transferred individually into a stirred methanol bath (FTS Systems Inc., Stone Ridge NY, USA) that was preset to −3, −6, −9, −12, −15, −20, −30, or −40 °C and equilibrated at that temperature for 2 min before ice was nucleated with cold forceps. After nucleation the samples were held at the experimental temperature for 3 min before either being thawed in a 37 °C water bath (VWR, Edmonton, AB, Canada) or plunged into liquid nitrogen. The plunged samples were kept in liquid nitrogen for a minimum of 1 h before being thawed in a 37 °C water bath. Additional experiments were conducted at these same temperatures for hold times of 5 and 10 min.

Additional samples were cooled to experimental hold temperatures of −9 or −12 °C, nucleated with cold forceps and held for 1, 2, 3, 4, 5, 7.5, 10, or 15 min. The samples were monitored using a type-T thermocouple acquiring the data with Personal Daq View software (version 1.9; Omega Laval, QC, Canada). Samples below −15 °C were prone to spontaneous nucleation prior to reaching the hold temperature; time zero was determined to be at completion of the 2 min equilibration period. For warming, samples were held in the 37 °C water bath until no ice was present. Duplicates of each sample were used for each experiment and were repeated at least in triplicate using cells from different passages.

2.3. Assessment of cell viability

2.3.1. Fluorescence microscopy (assessment of membrane integrity)

A dual fluorescent assay (SytoEB) using a combination of two fluorescent dyes, Syto13® (Molecular Probes, Eugene, OR, USA) and ethidium bromide (EB) (Sigma–Aldrich, Mississauga, ON, Canada) observed with a fluorescence microscope was used to assess HUVEC membrane integrity. Syto13 is a DNA/RNA binding stain that permeates all cells and fluoresces green on excitation by UV wavelengths. Ethidium bromide (EB) permeates cells with damaged plasma membranes, exhibiting red fluorescence when exposed to UV wavelengths. The combinations of these two dyes makes a binary assay with membrane intact cells exhibiting green fluorescence (Syto13) and membrane compromised cells

exhibiting red fluorescence (EB). The SytoEB assay was prepared using 1× phosphate buffered saline (PBS) and aliquots of Syto and EB diluted from stock solutions. The final dye solution was comprised of 25 μM of EB and 12.5 μM of Syto13, and 20 μL were added to a 0.2 mL aliquot of HUVEC in suspension, mixed and incubated for 2 min at room temperature.

Fluorescent cells were observed with a Leitz Dialux 22 fluorescence (440–480 nm) microscope (Leitz, Germany) with a dual band red/green filter set to detect emission of Syto13® (509 nm) and ethidium bromide (600 nm) and images were captured using a fitted Pixera viewfinder digital camera (Pixera Corporation, Los Gatos, CA, USA). An in-house custom counting program, Viability 3.2 (Locksley McGann, Edmonton AB, Canada) was used to assess cell membrane integrity from captured digital images. For each experimental sample the relative cell recovery was calculated by dividing the number of intact (green) cells by the total number of cells present including both compromised (red) and intact (green) cells.

2.3.2. Flow cytometry (assessment of mitochondrial membrane potential)

A 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye (Molecular Probes, Eugene, OR, USA) observed with a flow cytometer was used to assess HUVEC mitochondrial membrane potential. This cationic dye shows the extent of mitochondrial transmembrane potential. It shifts from green fluorescence (~525 nm) in low polarization states (non-functional mitochondria) to red fluorescence (~590 nm) in high polarization states (functioning mitochondria). This change in color is based on a concentration-dependent shift from monomers of the dye which fluoresce green to aggregates that fluoresce red [39]. Initially the dye is present as membrane permeable, cationic monomers (green) that congregate in cells due to negative intracellular potential. In healthy cells these monomers then accumulate in the mitochondrial matrix, drawn by the negative charge of the inner mitochondrial membrane and form aggregates (red), most likely due to mitochondrial transmembrane potential [14]. Therefore cells with depolarized mitochondria predominantly emit green fluorescence from monomers present in the cytoplasm, and cells with polarized mitochondria predominantly emit red fluorescence from J-aggregates of the dye.

The JC-1 assay was prepared starting with the stock solution by combining 5 mg of the JC-1 reagent with 5 mL of DMSO (Sigma–Aldrich) to a concentration of 1 mg/mL. 0.8 μL of JC-1 reagent/DMSO solution was added to 0.4 mL aliquots of HUVEC (final concentration: 2 μg/mL) and incubated for 30 min in an incubator at 37 °C and 5% CO_2. Flow cytometry assessment of HUVEC (0.2 mL) aliquots using an unmodified Coulter® EPICS® XL-MCL™ flow cytometer (Beckman-Coulter) equipped with a 488 nm argon laser has been previously described in Reardon et al. [37]. Emission of JC-1 monomers was detected using the FL1 (505–545 nm) bandpass filter and that of JC-1 aggregates was detected using the FL2 (560–590 nm) bandpass filter. Aliquots of HUVEC (0.4 mL) were loaded and run for a time interval of 2 min in Isoflow™ sheath fluid (Beckman-Coulter).

The data were analyzed with flow cytometry analysis software (Kaluza™ v1.2 from Beckman-Coulter). HUVEC were identified from debris using one parameter histograms of the intensity of green fluorescence on a log scale; a threshold was established to separate high fluorescent events (HUVEC) from low fluorescent events (sub-cellular debris) [37]. The mitochondrial membrane potential of events identified as cells was assessed using two parameter histograms of the intensity of red and green fluorescence on a log scale [37]. Cells with functional mitochondria were determined by identifying events with a high red/green ratio of fluorescence intensity and the number of functional cells was compared to the total number of cells in suspension.

2.4. Statistical analysis

Statistical comparisons were conducted using a one-way analysis of variance (ANOVA) ($p = 0.05$ level of significance) to determine significant differences in membrane integrity with respect to various cooling rates and hold times within groups of cells directly thawed, or plunged into liquid nitrogen and subsequently thawed. The non-parametric Mann–Whitney *U*-test was used for direct comparisons between directly thawed, and plunge-thawed cells from the same temperature when subjected to the same cooling rate ($p = 0.05$). The Mann–Whitney test was also used to determine differences between outcomes of membrane integrity and mitochondrial polarization of direct-thaw and plunge-thaw cells from the same corresponding temperature ($p = 0.05$). The mean values and standard error of the mean were also calculated and included in the result (mean ± sem).

3. Results and discussion

3.1. Part A. HUVEC membrane integrity responses to interrupted cooling (assessed with fluorescence microscopy)

3.1.1. Impact of varying cooling rate on the HUVEC membrane response to interrupted slow cooling

The membrane integrity of HUVEC during interrupted slow cooling is shown in Fig. 2 as a function of temperature for different cooling rates. All data were normalized to positive control samples (HUVEC at room temperature; 86.7 ± 2.1%). For directly thawed cells, a similar pattern of membrane integrity was observed at all cooling rates (0.1, 0.2, and 1.0 °C/min). High values of normalized membrane integrity (90 to 100%) were observed at higher subzero temperatures (−3 to −12 °C), and membrane integrity decreased with exposure to lower intermediate temperatures (−20 to −40 °C). Plunge-thawed samples also show a similar pattern of membrane integrity at all cooling rates (0.1, 0.2, and 1.0 °C/min), but unlike directly thawed cells, cells plunged from higher experimental temperatures initially show an increase in membrane integrity with plunge temperature (−3 to −15 °C) and then membrane integrity remains unchanged with further decreasing plunge temperatures (−20 to −40 °C). At all cooling rates (0.1, 0.2, and 1.0 °C/min), an increase in membrane intact cells was observed following plunge from lower temperatures (−15 to −30 °C) compared to plunging from the initial high subzero temperature (−3 °C).

It is generally recognized that slower cooling rates are associated with a reduction in cell volume and prolonged exposure to hypertonic conditions [23]. These hypertonic conditions have been found to cause solution effects injury and result in lowered numbers of cells with intact plasma membranes. Cell damage due to solution effects explains the decrease in membrane integrity with temperature that was observed in slowly cooled directly thawed cells. In contrast to directly thawed samples, plunge-thawed cells showed a different pattern of membrane damage when plunged into liquid nitrogen from high subzero temperatures, as they were subjected to much more rapid cooling conditions. It has been shown that rapid cooling rates are associated with IIF, and that the occurrence of IIF correlates with cell lethality [7,21,34]. IIF is the most likely explanation for the prominent decrease in membrane integrity upon plunging from high subzero temperatures (−3 to −12 °C), whereas there was a lesser degree of further membrane damage upon plunge from lower subzero temperatures (−15 to −40 °C).

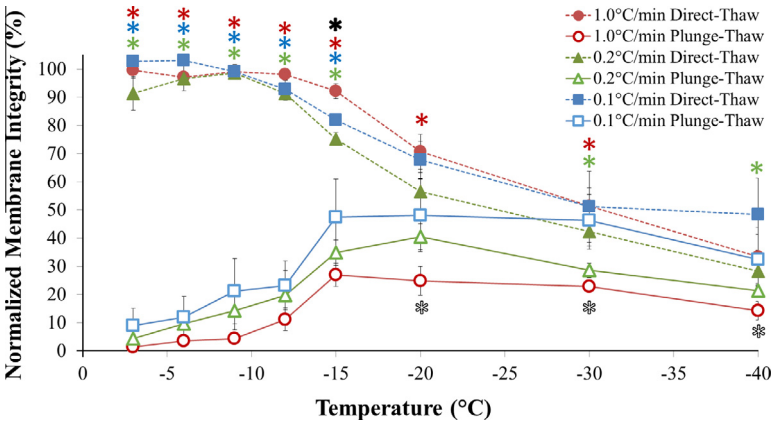

Fig. 2. Response of HUVEC to decreasing temperatures during interrupted slow cooling when subjected to different cooling rates. Membrane integrity of HUVEC (mean ± sem; normalized to controls; $n = 3$) cooled at rates of 0.1 °C/min (blue), 0.2 °C/min (green), and 1.0 °C/min (red) to various subzero temperatures, then either thawed directly (dashed lines, closed symbols) or plunged into liquid nitrogen (solid lines, open symbols) and subsequently thawed. ∗ (color) Signifies statistically significant difference in membrane integrity of plunge-thaw samples from respective direct-thaw samples ($p < 0.05$). ∗ (closed black) Signifies statistically significant differences in membrane integrity for various cooling rates in directly thawed samples ($p < 0.05$). ∗ (open black) Signifies statistically significant differences in membrane integrity for various cooling rates in plunge-thaw samples ($p < 0.05$).

In both directly thawed and plunge-thawed cells, a dependence of membrane integrity on cooling rate was observed during interrupted slow cooling (Fig. 2). The membrane integrity of cells directly thawed from higher subzero temperatures (−3 to −12 °C) was not strongly dependent on cooling rate; however, the membrane integrity of cells directly thawed from lower subzero temperatures (−15 to −40 °C) did show a dependence on cooling rate ($p < 0.05$). Slowly cooled HUVEC plunged into liquid nitrogen from experimental temperatures showed a more pronounced dependence on cooling rate than directly thawed cells ($p < 0.05$). It was observed that plunged samples cooled at a rate of 1.0 °C/min showed additional damage when plunged into liquid nitrogen from −30 °C; in contrast to cells cooled at a relatively slower rate (0.1 °C/min) that showed no additional damage between directly thawed and plunge-thaw samples. At −30 °C cells cooled slowly had more time to equilibrate to these conditions (270 min at 0.1 °C/min) than the time provided when cooled at a higher rate (27 min at 1.0 °C/min). As the temperature lowers, water is removed from the extracellular solution in the form of ice, increasing the concentration of solutes in the remaining unfrozen fraction. At slow cooling rates cells had sufficient time for water to efflux from the cell, allowing these cells to remain closer to osmotic equilibrium [21].

3.1.2. Investigating the HUVEC response to decreasing hold temperatures during interrupted rapid cooling

Fig. 3 shows the experimental results for HUVEC cooled rapidly to an experimental subzero temperature, and held for either 3, 5 or 10 min, before being directly thawed or plunged into liquid nitrogen and subsequently thawed. Directly thawed samples of HUVEC showed a trend of decreasing membrane integrity with decreasing experimental temperatures, including a particularly sharp decrease from −6 to −15 °C for all hold times (Fig. 3); followed by a gradual decline at hold temperatures below −15 °C (19.7 ± 2.8%) that continued at lower temperatures until there were no membrane intact cells left at the lowest hold temperature (−40 °C). Increasing the hold time to 5 and 10 min showed no further change ($p < 0.05$), indicating that damage most likely occurred during the initial equilibration period, from ice nucleation prior to the start of, or early in, the hold time (Fig. 1B, i). At hold temperatures below −15 °C, samples were prone to spontaneous ice nucleation prior to completion of the 2 min equilibration period;

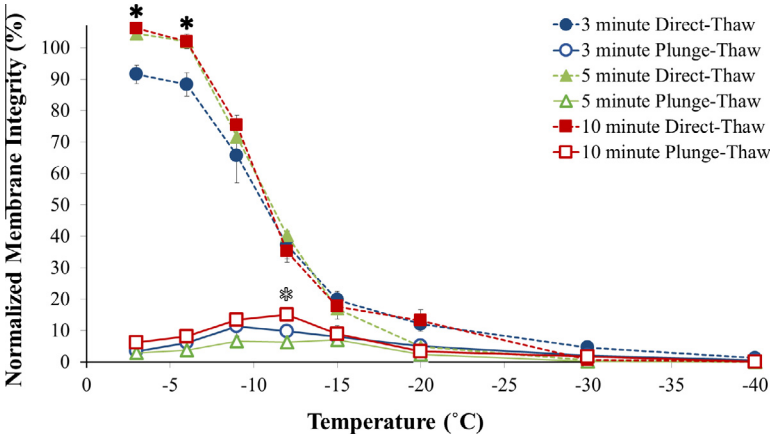

Fig. 3. Response of HUVEC to decreasing temperatures during interrupted rapid cooling when subjected to different hold times. Membrane integrity of HUVEC (mean ± sem; normalized to controls; $n = 3$) as a function of hold temperature for cells with hold times of 3 min (blue), 5 min (green), and 10 min (red) before being either thawed directly (dashed lines, closed symbols) or plunged into liquid nitrogen and subsequently thawed (solid lines, open symbols). ∗ (closed) Signifies statistically significant differences in membrane integrity for various hold times in directly thawed samples ($p < 0.05$). ∗ (open) Signifies statistically significant differences in membrane integrity for various hold times in plunge-thaw samples ($p < 0.05$).

spontaneous nucleation may have contributed to a loss of HUVEC membrane integrity prior to the start of the hold time.

Comparing the results for directly-thawed HUVEC after rapid cooling with those of slow cooling showed that cooling cells too rapidly results in a greater proportion of membrane damaged cells. At a high subzero temperature of −15 °C HUVEC cooled rapidly (two-step cooling) resulted in more than 80% membrane damaged cells (Fig. 3), compared to no more than 50% of cells that were damaged when slowly cooled (0.1 °C /min, graded freezing) to the same temperature (Fig. 2). The high rate of cooling associated with rapid cooling methods leads to the cytoplasm becoming increasingly supercooled and increases the probability of IIF [20].

HUVEC samples plunged into liquid nitrogen showed a slight increase in membrane integrity with decreasing hold temperature (−3 to −12 °C), that was followed by a decrease in membrane integrity with further decreasing temperatures (−15 to −40 °C) (Fig. 3). This indicated that some protection was conferred to these cells by being held for 3 min at high subzero temperatures (−3 to −12 °C) before plunging into liquid nitrogen. The longest experimental hold time of 10 min resulted in a slight but significant increase in the number of membrane intact cells to a maximum at −12 °C (15 ± 1.2%).

For hold temperatures of −15 °C and below, there was little to no difference in the membrane integrity of directly-thawed and plunge-thawed cells, as no additional damage was observed when these cells were plunged into liquid nitrogen from experimental hold temperatures (−15 to −40 °C). This suggests that the majority of cells at hold temperatures below −15 °C already have frozen intracellular water and that no additional intracellular freezing occurred upon plunging. The inability of these cells to efflux water may be explained by the hydraulic conductivity, since the measured value of water permeability for HUVEC has been found to be relatively low [41]. This low value of hydraulic conductivity may partly explain the susceptibility of HUVEC to IIF when subjected to rapid cooling.

3.1.3. A detailed investigation of HUVEC responses to changes in hold time at −9 °C and −12 °C for interrupted rapid cooling

Fig. 4 shows the membrane integrity of HUVEC after interrupted rapid cooling as a function of hold time (1 to 15 min) at hold temperatures of −9 and −12 °C. Directly thawed cells from both hold temperatures (−9 and −12 °C) showed a decrease in membrane integrity with increasing hold time. The correlation between decreased membrane integrity and increased hold time might suggest that these cells were being damaged by solution effects. However, when the same cell type was slowly cooled to −12 °C (90 min) the resultant membrane integrity was approximately 90% (Fig. 2) compared to the less than 50% membrane intact cells that were observed after rapid cooling (time 2 min) (Fig. 4). The prolonged exposure to increased solute concentrations during slow cooling (Fig. 2) showed that these cells were able to withstand conditions that are associated with solution effects injury. The observed decrease in membrane integrity under rapid cooling conditions (Fig. 4) is therefore more likely attributed to IIF, which is a stochastic process that takes time after extracellular ice nucleation.

Cells plunged into liquid nitrogen had more membrane damage compared to cells directly thawed from both hold temperatures (−9 and −12 °C) (Fig. 4). Plunge-thawed samples showed few membrane intact cells, with a slight increase (to a maximum of 11.8 ± 0.7%) with shorter hold times (from 1 to 5 min); this was followed by a plateau of membrane integrity with longer hold times (5 to 15 min) when held at −12 °C prior to plunging. The slight increase in membrane integrity with shorter hold times showed that lengthening the hold time (from 1 to 5 min) allowed cells to lose intracellular water and decreased the probability of IIF during the plunge, whereas longer hold times (from 5 to 15 min) did not allow these cells any further loss of intracellular water and the probability of IIF remained the same. Though it has been documented that the occurrence of intracellular ice correlates with membrane damage for cells in suspension [1,2,55] it is uncertain as to whether this damage is the cause of, or the result of, IIF. Acker and McGann were able to link membrane damage to IIF but could not establish a time course of membrane rupture from IIF [2,3]. Regardless of the mechanism, intracellular ice is most likely occurring as HUVEC are plunged into liquid nitrogen from −9 and −12 °C resulting in a low number of membrane intact cells.

A more distinct decrease in membrane integrity with hold time was observed in cells directly thawed from lower hold temperatures (−12 °C compared to −9 °C), whereas cells plunged into liquid nitrogen from both these hold temperatures showed no dependence of membrane integrity on hold temperature. A possible explanation for this occurrence is the existence of a subset of freeze resistant cells that may, for example, be attributed to cell volume as smaller cells contained less intracellular water and were

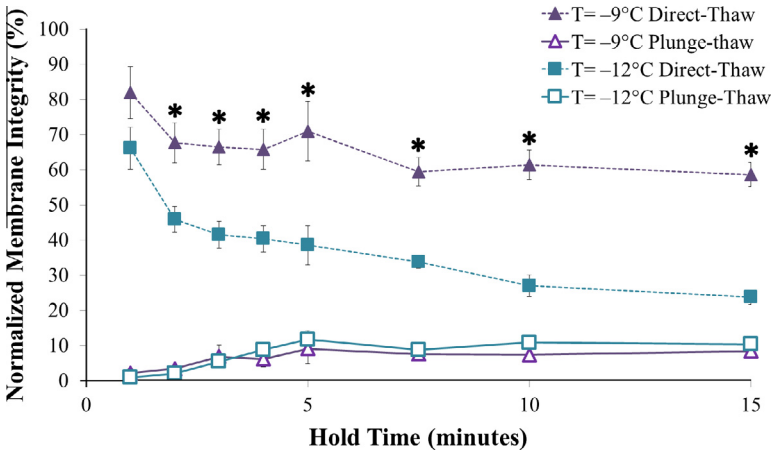

Fig. 4. Response of HUVEC to interrupted rapid cooling with increasing hold time when subjected to two different hold temperatures. Membrane integrity of HUVEC (mean ± sem; normalized to controls; $n = 6$) as a function of hold time for cells cooled rapidly to −9 °C (purple), and −12 °C (cyan) before being either thawed directly (dashed lines, closed symbols) or plunged into liquid nitrogen and subsequently thawed (solid lines, open symbols). ∗ Signifies statistically significant differences in membrane integrity between −9 and −12 °C in directly thawed samples ($p < 0.05$). There were no significant differences between −9 and −12 °C in plunge-thawed cells.

less likely to undergo IIF [21,33,36]. Another explanation may be the cells' present phase of the growth cycle, which may influence cell survival when subjected to decreasing temperatures, as McGann showed that fibroblasts in the M phase were more susceptible to freezing damage compared to other phases of the cell cycle [28].

3.1.4. A comparison of HUVEC membrane integrity responses to interrupted slow and rapid cooling strategies

The rate of cooling is an essential component of cryopreservation strategies; interrupted cooling of HUVEC in suspension generally resulted in higher values of membrane integrity when cooled at slower rates (Fig. 2). For these samples the membrane integrity of directly thawed cells was found to progressively decrease with temperature, in contrast to plunged cells where membrane integrity was observed to increase with lower experimental temperatures from which they were plunged. The increased membrane integrity that was observed during slow cooling (0.1 °C/min), as compared with 1.0 °C/min suggests HUVEC show some resistance to solution effects injury with respect to membrane integrity. The considerably decreased membrane integrity that was observed when these cells were rapidly cooled by plunging into liquid nitrogen from high subzero temperatures suggests that they may be susceptible to IIF injury.

HUVEC were observed to sustain less injury when cells were cooled with slow cooling protocols (graded freezing) compared to more rapid cooling protocols (two-step freezing), showing similar results to other interrupted cooling studies involving rapid [29] and slowly [24] cooled fibroblasts. However, results for both of these cell types are in contrast to results of cooling TF-1 hematopoietic stem cells that sustained less injury during two-step freezing [43] compared to slower cooling methods [44], revealing that preferred cooling strategies are cell-type specific, a specificity that should be accounted for when designing cryopreservation protocols.

In addition to these findings, it has also been suggested that freezing affects other components of cells in addition to the membrane [4,35,45,46]. The results of rapid cooling strategies (Figs. 3 and 4) were not high enough to justify further investigation. However, slow cooling graded freezing strategies showed relatively high values for membrane integrity (Fig. 2.) allowing for further examination of other aspects of cell injury under cryobiological conditions.

3.2. Part B. HUVEC mitochondrial membrane potential response to interrupted cooling (assessed with flow cytometry)

3.2.1. Controls and contour plots: Measurements of mitochondrial membrane potential with flow cytometry

The mitochondrial membrane potential assay JC-1 was used to assess HUVEC control samples at room temperature (Fig. 5). A flow cytometry scatterplot (Fig. 5A) shows each cell represented as a dot with a distinct measure of green (*x*-axis) and red (*y*-axis) fluorescence from the JC-1 dye. Recently, using membrane integrity assays, Reardon et al. showed that there are two populations present under control conditions: a large population representing the majority of cells (viable cells) and a smaller population with fewer cells (non-viable cells) [37]. Fig. 5 also shows two populations when visualized with JC-1: a large group of cells with polarized mitochondria, and a smaller group of cells with depolarized mitochondria. However, it is difficult to visually discriminate between these two populations within the scatterplot due to the high number of registered cells (Fig. 5A).

A contour plot was used as a visual representation of flow cytometry data in a two-parameter histogram (Fig. 5B). The contours within this figure outline regions that contain a number of densely packed events (cells), where the color of each contour indicates the density of events contained within it; high, moderate, and low density regions are denoted by red, green and blue contours respectively. Contour plots allow for ease of visualization in discriminating populations of cells assessed with JC-1, and distinguishing the population of cells with polarized mitochondria from the population with depolarized mitochondria.

Contour plots were used to assess both positive and negative HUVEC controls (Fig. 6), including control samples at room temperature (Fig. 6A) and negative control cells plunged directly into liquid nitrogen and subsequently thawed (Fig. 6B). Using these plots, gates were established to separate cells with polarized mitochondria (R1) that have a high red/green fluorescence ratio from cells with depolarized mitochondria (R2) that have a low red/green fluorescence ratio. Control samples showed a large population of cells with polarized mitochondria (83.3 ± 0.8%) and a smaller population of cells with depolarized mitochondria (16.7 ± 0.8%). The plunging of cells from room temperature into liquid nitrogen causes a decrease in the number of high red/green fluorescent cells (R1) and an increase in the number low red/green fluorescent cells (R2). This was an indication that this change in condition caused the mitochondria of the majority of cells in suspension to become

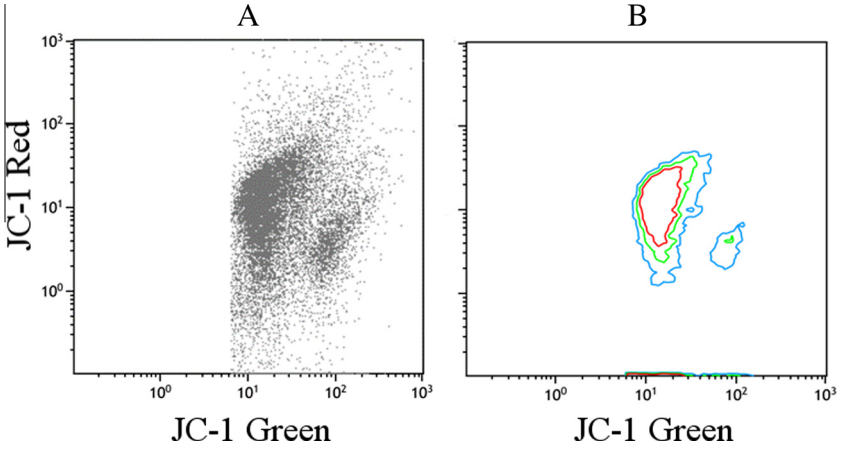

Fig. 5. A visual representation of HUVEC at room temperature measured with flow cytometry. (A) Scatterplot of all cells recorded by the flow cytometer. (B) Scatterplot converted to a contour plot of cells showing regions of high (red contour line), moderate (green contour), and low (blue contour), number of cells measured by flow cytometry.

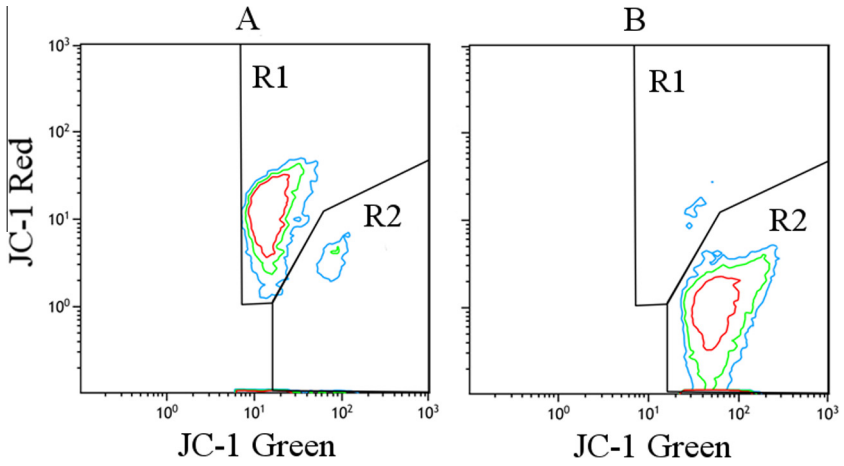

Fig. 6. Contour plots of HUVEC membrane integrity analysis with JC-1 with flow cytometry. A visual representation of cells containing polarized (R1) and depolarized (R2) mitochondria, as well as areas with a high (red contour), moderate (green contour), and low (blue contour) density of cells. (A) Control samples at room temperature. (B) Samples plunged in liquid nitrogen (−196 °C) and thawed rapidly.

depolarized, shifting them from R1 to R2. Based on the results of these controls, (and the mechanism of action of JC-1 [50]) regions R1 and R2 were used to assess the mitochondrial polarization of HUVEC during interrupted slow cooling.

3.2.2. Investigating the effect of decreasing temperature on the polarization state of HUVEC mitochondria during interrupted slow cooling

A series of contour plots shows the progression of the mitochondrial membrane potential of HUVECs when subjected to decreasing temperature and subsequently directly thawed (Fig. 7) or plunged into liquid nitrogen and then thawed (Fig. 8).

The mitochondrial polarization of cells directly thawed from decreasing experimental temperatures (−3 to −40 °C) during a single experiment is shown in Fig. 7. Directly thawing cells from the highest experimental temperature (−3 °C, top left panel of Fig. 7) resulted in the majority of cells containing polarized mitochondria; this population is shown as a large contour with high red/green fluorescence (R1). Cooling HUVEC at a relatively slow rate (0.2 °C/min) progressively decreased the size of the contour in R1 and simultaneously increased the size of the contour in R2 as cells were thawed from decreasing experimental temperatures (progressive panels in Fig. 7). This was an indication that when cooled slowly, as the temperature decreased, the number of HUVEC with depolarized mitochondria increased, shown as a shift in events from R1 to R2. Cooling these cells to temperatures lower than −20 °C resulted in very few cells with polarized mitochondria (<10%) (the last 3 panels of Fig. 7).

The mitochondrial polarization of cells plunged into liquid nitrogen from decreasing experimental temperatures (−3 to −40 °C) during a single experiment is shown in Fig. 8. Plunging cells from the highest experimental temperature (−3 °C, top left panel of Fig. 8) resulted in the majority of cells having depolarized mitochondria (R2). Post-thaw assessment of cells plunged from further decreased temperatures (−6 to −40 °C) and then thawed showed that these plunge temperatures had no further effect on the polarization state of cellular mitochondria as nearly all cells contained depolarized mitochondria regardless of whether they were plunged from high (−3 °C) or low (−40 °C) subzero temperatures.

Interrupted cooling protocols allow for separate observation and assessment of cryoinjury that occurs when cooling to the experimental temperature and when further cooling to the storage temperature. The progressive decrease in mitochondrial polarization that was observed in directly-thawed cells may be attributed to slow cooling injury from the relatively slow cooling rate (0.2 °C/min). After nucleation, the amount of extracellular ice increases with decreasing temperature, resulting in increased extracellular solutes; to maintain equilibrium at slower cooling rates cells efflux water, causing cells to shrink and lose volume; the culmination of these events increases exposure to intracellular solutes, and solution effects injury [18,32]. The plasma membrane has been the main focus around current theories of cell damage due to solution effects [10,18,31] but other sites within the cell such as mitochondria may also be affected. There were few cells with polarized mitochondria present when cells were plunged into liquid nitrogen from any of the experimental temperatures (−3 to −40 °C, Fig. 8) and then thawed. Rapid cooling rates do not give the cell enough time to lose intracellular water making them susceptible to the formation of intracellular ice [23].

3.3. Part C. Mitochondria and the membrane: A comparison of HUVEC physical and functional characteristics

Both the membrane integrity (SytoEB) and mitochondria polarization (JC-1) dyes were used in a previously conducted study that utilized flow cytometry to identify frozen-thawed cells [37]. A comparative assessment of positive and negative controls using both assays was conducted to ensure that the results obtained using the microscope would be the same as those obtained with flow cytometry. Under both control conditions, identical proportions of viable and non-viable cells were found using either instrument (data not shown) [38] that ensured accuracy of results when making comparisons between membrane integrity results with the fluorescence microscope and mitochondrial membrane potential results with the flow cytometer (Fig. 9).

3.3.1. Comparing the effects of slow cooling on HUVEC membrane integrity with the effects on mitochondrial membrane potential

In Fig. 9, for HUVEC subjected to interrupted slow cooling, membrane integrity results (part A) are plotted together with mitochondrial membrane potential results (part B). Directly-thawed HUVEC showed a significant decrease with decreasing temperature in both membrane integrity (−20 to −40 °C) and mitochondrial polarization (−12 to −40 °C) (Fig. 9). At higher experimental temperatures (−3 to −15 °C) there was no change

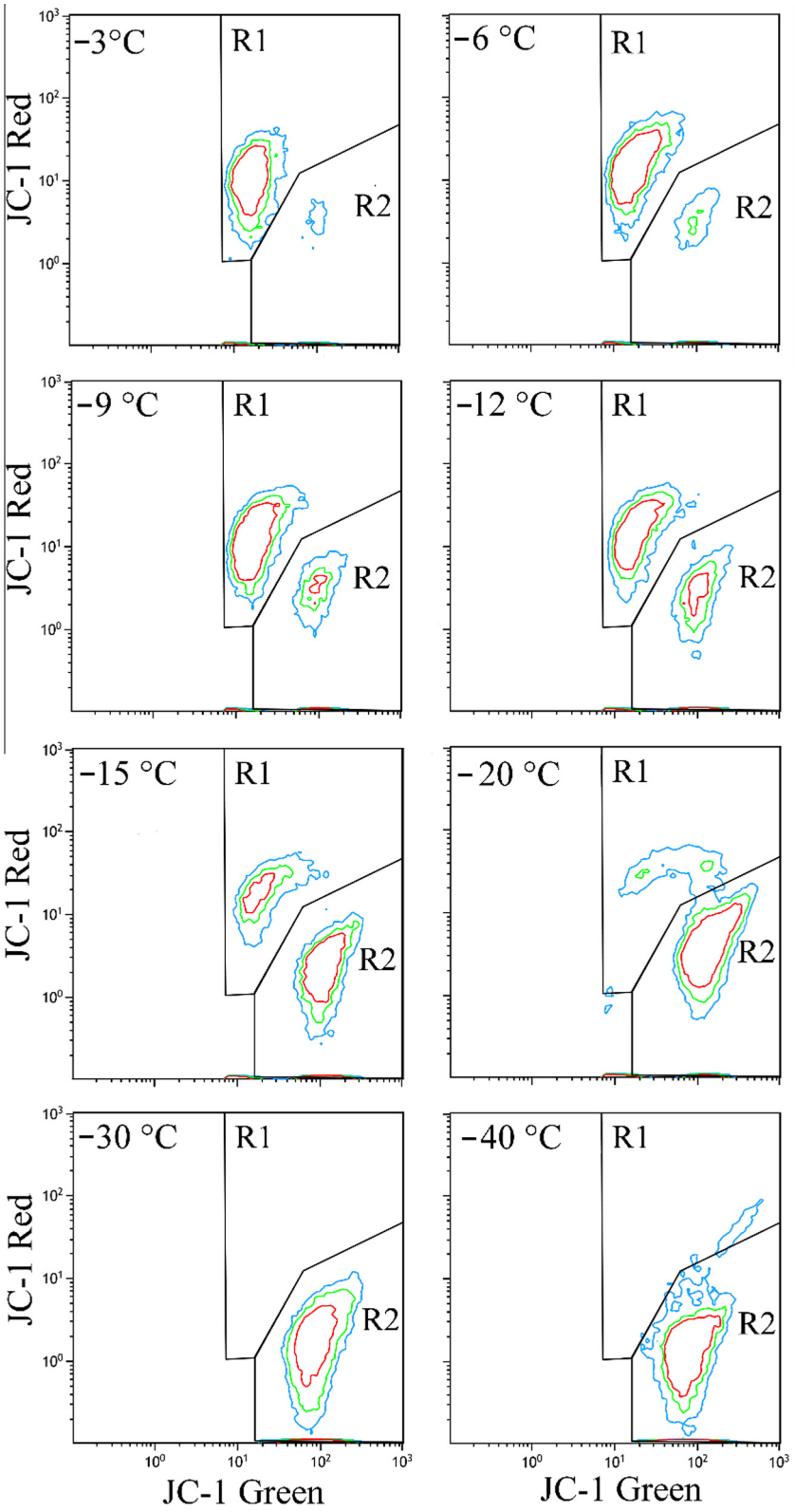

Fig. 7. Contour plots of mitochondrial polarization of HUVEC directly thawed from intermediate temperatures when cooled at 0.2 °C/min. Showing cells containing polarized (R1) and depolarized (R2) mitochondria, as well as areas with a high (red contour), moderate (green contour), and low (blue contour) number of cells measured by flow cytometry.

in membrane integrity, whereas mitochondrial polarization decreased at −12 °C and below. A greater number of cells exhibited depolarized mitochondria than exhibited loss of membrane integrity under these conditions, demonstrated by a 50% loss of mitochondrial polarization at −15 °C, and a 50% loss of membrane integrity at −30 °C. Progressive slow cooling had a more profound effect on mitochondrial membrane potential than on membrane integrity for directly thawed HUVEC. HUVEC plunged samples

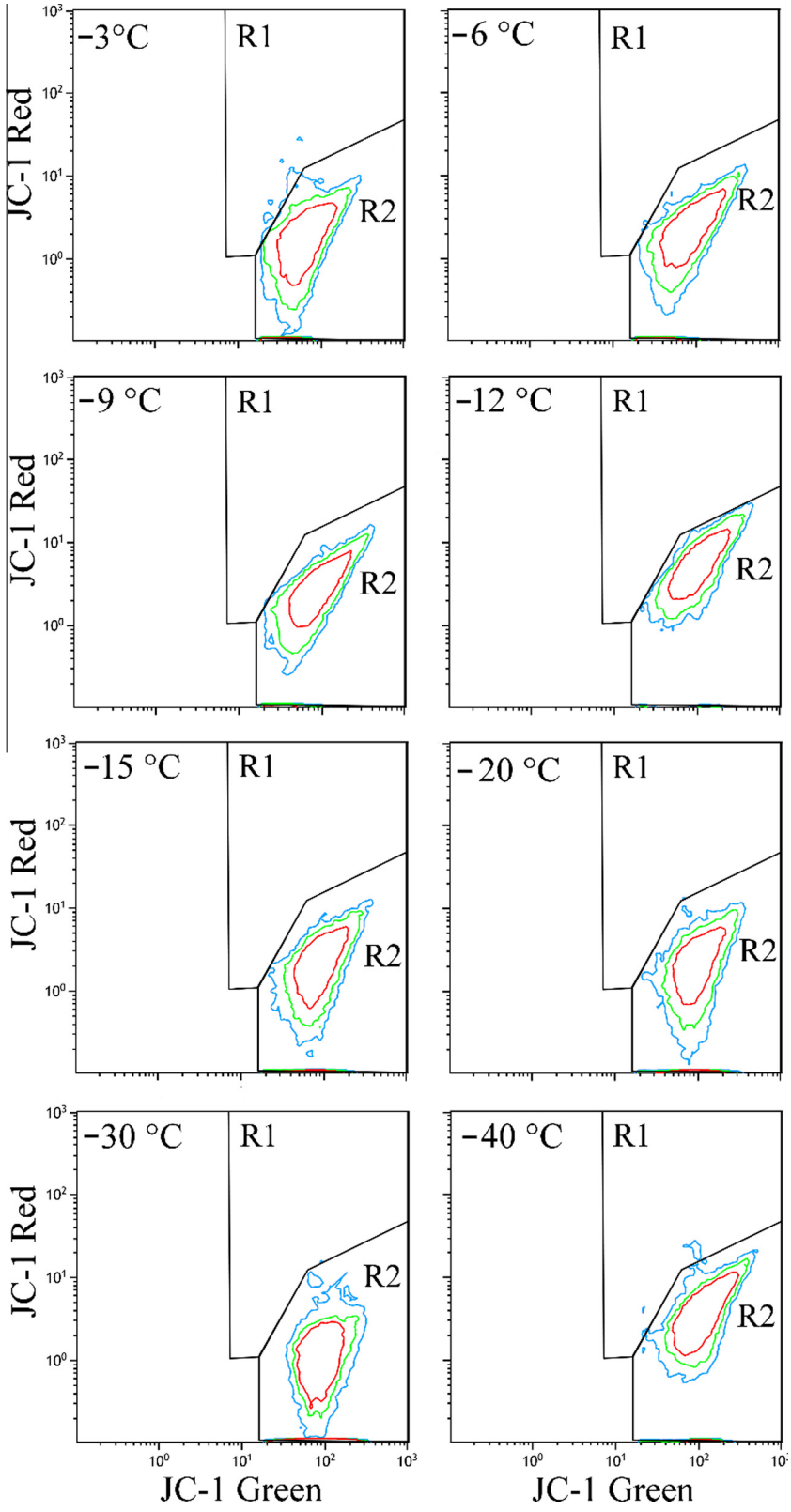

Fig. 8. Contour plots of mitochondrial polarization of HUVEC plunged into liquid nitrogen from different temperatures after cooling at 0.2 °C/min and then thawed. Showing cells containing polarized (R1) and depolarized (R2) mitochondria, as well as areas with a high (red contour), moderate (green contour), and low (blue contour) number of cells measured by flow cytometry.

showed a significant increase in the number of membrane intact cells at plunge temperatures from −15 to −20 °C, where mitochondrial polarization of identically-treated samples showed a low percentage of cells with polarized mitochondria (<10%) throughout the full range of experimental temperatures (−3 to −40 °C).

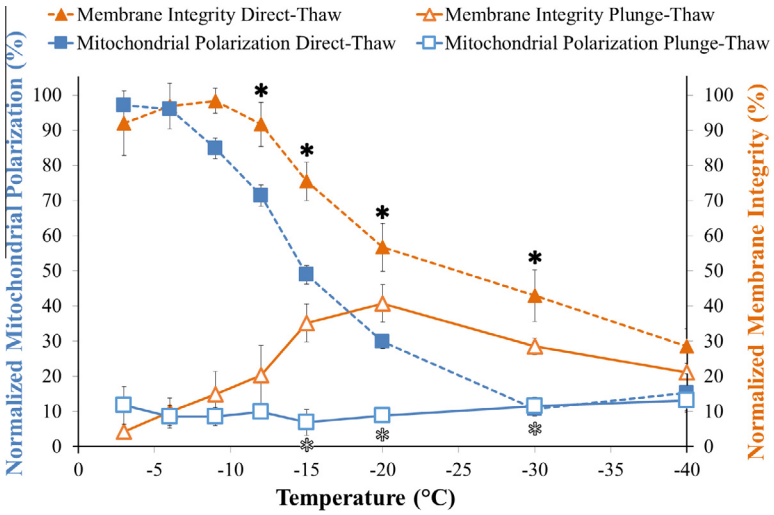

Fig. 9. Comparison of membrane integrity (SytoEB) and mitochondrial polarization (JC-1) analysis of HUVEC for interrupted slow cooling. Cells were cooled to various subzero temperatures at a rate of 0.2 °C/min (mean ± sem; normalized); then either thawed directly (dashed lines, closed symbols) or plunged into liquid nitrogen and subsequently thawed (solid lines, open symbols). ∗ (closed) Signifies a significant difference between membrane integrity and mitochondrial polarization of HUVEC directly thawed from the same temperature. ∗ (open) Signifies a significant difference between membrane integrity and mitochondrial polarization of HUVEC plunged into liquid nitrogen and subsequently thawed from the same temperature.

A comparison of mitochondrial and membrane responses to the same interrupted cooling protocol showed that cells were damaged to a different extent based on the site of injury that was assessed, with mitochondria more greatly affected compared to the plasma membrane of plunge-thawed cells. After normalization to controls, HUVEC slow cooled (0.2 °C/min) to −15, −20 and −30 °C showed a significant difference in the proportion of cells with polarized mitochondria and an intact plasma membrane, for both directly thawed cells, and cells plunged into liquid nitrogen from this temperature and subsequently thawed ($p < 0.05$). For example, HUVEC cooled to −20 °C and plunged into liquid nitrogen showed approximately 40% of cells with an intact plasma membrane; but few cells (<8%) contained polarized mitochondria. Previous studies have suggested that freezing and thawing have an indirect deleterious effect on cellular mitochondria and that these organelles may exhibit differential sensitivities to freezing conditions [45,46,53].

The difference between the cryobiological response of mitochondria and the plasma membrane may be explained by two separate mechanisms of damage affecting these sites of injury. McGann et al. proposed a general model of slow cooling injury in which lysosomes are initially adversely affected causing a release of hydrolytic enzymes that subsequently damage mitochondria and the plasma membrane [27]. Lysosomes had been identified as primary targets of cryoinjury [35], and the release of hydrolytic enzymes has the capacity to uncouple oxidative phosphorylation in mitochondria [30]. This model not only provides an explanation for indirect damage to mitochondria that manifested from slow cooling hypertonic conditions causing decreased mitochondrial polarization in cells that had maintained membrane integrity but also agrees with the phenomenon of latent cryoinjury that was described by Sherman in 8 h post-thawed cells [47].

This model has been generalized into the occurrence of two types of cell damage during slow cooling: a direct damage to the plasma membrane from solution effects injury, and an indirect damage that adversely affects components of the cytoplasm. Our results indicate that two types of damage are taking place (Fig. 9), that are affecting mitochondria and the plasma membrane differently during interrupted slow cooling. Other evidence of indirect slow-cooling damage has been demonstrated in granulocytes [4,54], fibroblasts [27], split-thickness skin and isolated keratinocytes [56].

4. Conclusions

This study investigated the HUVEC cryobiological response to two different interrupted cooling protocols. Interrupted slow cooling keeps the cells in equilibrium with their surroundings as the temperature decreases in the presence of extracellular ice and solutes become increasingly concentrated. Long exposure times leading to concentrated solutes during slow cooling may be damaging to cells in the form of solution effects injury, whereas interrupted rapid cooling has been found to confer a protection to cells when they are held at a designated experimental hold temperature for a period of time prior to plunging into liquid nitrogen. However, the high cooling rates associated with rapid cooling also leave cells susceptible to IIF, an inherently lethal event. For HUVEC, slower cooling methods (graded freezing) were shown to improve cell survival, in contrast to rapid cooling protocols (two-step freezing) that showed drastic decreases in membrane integrity. These results indicated that HUVEC had an observed resistance to solution effects injury under slow cooling conditions, but may be susceptible to IIF. This may be influenced by cell characteristics such as the hydraulic conductivity that allow HUVEC to sustain less injury compared to other cell types such as TF-1 hematopoietic stem cells under the same slow cooling conditions [42].

In addition to the results obtained from membrane integrity assays, slow cooling (graded freezing) protocols were also used to investigate the functional state of mitochondria in frozen-thawed HUVEC. Flow cytometry was used to identify and assess the mitochondrial polarization state of HUVEC showing a progressive increase in the number of cells with depolarized mitochondria with decreasing temperatures and that even fewer cells showed polarized mitochondria when plunged into liquid nitrogen. A comparison of mitochondrial polarization and membrane integrity showed that mitochondria were depolarized under cooling conditions where the plasma membrane was still intact. The differential cryobiological response between mitochondrial polarization and membrane integrity may be attributed to different types of damage at each of these sites. Direct damage to the plasma membrane may be attributed to solution effects injury, and indirect damage may be affecting the cytosolic components of cells such as HUVEC mitochondria.

Although the occurrence of indirect damage is well documented, there is only speculation on the mechanism of how this

damage occurs. Further studies on the details of damage to other organelles, cell processes and pathways during slow cooling would provide further insight into the mechanism of how slow cooling injury occurs and whether or not this injury is reversible or results in cell death. Investigation of the details of cryoinjury may lead to novel approaches and breakthroughs in future cryopreservation of cells and tissues.

Conflict of interest

None.

Acknowledgments

This research was funded by the Canadian Institutes of Health Research (MOP 86492 and MOP 133684), the Government of Alberta (Graduate Student Scholarship of Advanced Education and Technology) and the University of Alberta (Graduate Research Assistantship). J.A.W. Elliott holds a Canada Research Chair in Thermodynamics.

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