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Polyvinylpyrrolidone (PVP) Mitigates the Damaging Effects of Intracellular Ice Formation in Adult Stem Cells

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Abstract—The objective of this work was to assess the effect of 10% (w/v) polyvinylpyrrolidone (PVP) on the pattern of intracellular ice formation (IIF) in human adipose tissue derived adult stem cells (ASCs) in the absence of serum and other cryoprotective agents (CPAs). The freezing experiments were carried out using a fluorescence microscope equipped with a Linkam^{TM} cooling stage using two cooling protocols. Both the cooling protocols had a *common cooling ramp*: cells were cooled from 20 °C to −8 °C at 20 °C/min and then further cooled to −13 °C at 1 °C/min. At this point we employed either *cooling protocol 1*: the cells were cooled from −13 °C to −40 °C at a pre-determined cooling rate of 1, 5, 10, 20, or 40 °C/min and then thawed back to 20 °C at 20 °C/min; or *cooling protocol 2*: the cells were re-warmed from −13 °C to −5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20, or 40 °C/min to −40 °C. Almost all (>95%) of the ASCs frozen in 1× PBS and protocol 1 exhibited IIF. However, almost none (<5%) of the ASCs frozen in 1× PBS and protocol 2 exhibited IIF. Similarly, almost all (>95%) of the ASCs frozen in 10% PVP in PBS and protocol 1 exhibited IIF. However, ~0, ~40, ~47, ~67, and ~100% of the ASCs exhibited IIF when frozen in 10% PVP in PBS and utilizing protocol 2 at a cooling rate of 1, 5, 10, 20, or 40 °C/min, respectively.

Keywords—Cryomicroscopy, Kinetics of ice nucleation, Cryopreservation, Adipose tissue and cell viability.

INTRODUCTION

During freezing of cells in suspension, the two major phenomena that occur are cellular dehydration (loss of cell-water to the extra-cellular medium) and intracellular ice formation (IIF).[31] During cooling, water in the extra-cellular medium freezes first, increasing the concentration of solutes in the unfrozen fraction of the medium.[29,31] As a result of the osmotic pressure gradient thus created across the cell membrane between the cell cytosol and the extra-cellular medium, the cell starts losing water which in turn increases the solute concentration of the cytosol thereby depressing its freezing point.[11,29,31,49,50] If the cooling rates are relatively "slow," the cells have enough time to lose enough water and avoid intracellular freezing. However, too slow cooling rates may induce cell injury through "toxicity effects" or "solution effects."[11,29,31,49,50] If cooling rates are "fast," then the cells do not get enough time to lose water and the super-cooled intracellular water freezes causing IIF, which is known to be potentially lethal to cells.[11,29,31,49,50]

Often, chemicals known as cryoprotective agents (CPAs) are used as additives in the extra-cellular medium to protect against IIF and solution effects injury.[4,5,9,13,17,25,27,28,30,33,38–40,47,61] CPAs are typically classified on the basis of their ability to penetrate the cell membrane and enter the intracellular space, i.e. permeable (e.g. glycerol, dimethylsulfoxide, ethylene glycol) and impermeable (sucrose, trehalose, hydroxyl-ethyl-starch, polyvinylpyrrolidone).[4,5,9,13,17,25,27,28,30,33,38–40,47,61] Some cryoprotectants like dimethylsulfoxide (DMSO), glycerol, ethylene glycol, etc., are known to stabilize the plasma membrane and also increase the viscosity of cellular and extra-cellular water which reduces the rate of nucleation of damaging ice and associated crystal growth.[4,5,9,13,17,25,27,28,30,33,38–40,47,61] Polyvinylpyrrolidone (PVP), a high molecular weight and non-toxic polymer, has earlier been used as a CPA during freezing of mouse lymphocytes,[5] erythrocytes,[33] mouse bone marrow cells,[39,40] and Chinese hamster cells.[28] However, the exact mechanism and the nature of the cryoprotective ability of CPAs is still a matter of debate.[4,9,13,30,38,39,61] For example, Sputtek and colleagues[47] found that hydroxyethyl starch (HES) is highly effective as a CPA for erythrocyte freezing while

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Conner and Ashwood-Smith[9] found HES to be of limited value in cryopreserving nucleated cells. Farrant[13] suggested that the cryoprotective action of high molecular weight CPAs (e.g. HES and PVP) is related to their ability to enhance colligative properties and to confer protection to cells by lowering the external (damaging) salt concentrations. While Williams[61] suggested that the cryoprotective efficiency of polymers resides in their ability to alter the physical properties of solutions during freezing rather than directly altering and protecting the cell membrane freezing characteristics. Clearly, further studies are needed to elucidate the effect of CPAs on the freezing processes in cells, e.g. rate and nature of intracellular ice nucleation, the structure/shape/size of extracellular ice crystals.

Adult stem cells (ASCs) can differentiate into several other cell types of the body and studying them could provide valuable insights about early embryonic development, organ regeneration, and transplantation.[8,34,54,55,59] Fortunately, adipose tissue is an easy and abundant source for such adult stem cells and long-term storage (cryopreservation) procedures for these cells are currently being actively explored.[11,18–20,22,35,50–53,62] To alleviate the concerns with the *in vivo* use of commonly used cryoprotective chemicals (dimethylsulfoxide, glycerol), our laboratory has recently completed a series of studies on ASCs that demonstrate the utility of PVP as a CPA for ASCs.[51–53] Briefly, Thirumala *et al.*[53] found that the viability of ASCs frozen in PVP and Dulbecco's Modified Eagle Medium (DMEM) resembles an inverted U-shape (with 10% PVP being optimal). The use of 1 and 40% PVP in DMEM caused a dramatic loss in cell viability (~5%) whereas the use of 10% PVP produced a maximum viability of ~65%. A similar but opposite trend was observed for the % of cells exhibiting necrosis, i.e., the use of 1 and 40% PVP resulted in a dramatic increase in necrotic cells while the use of 10% PVP resulted in the lowest % of cells exhibiting necrosis. Similarly, the percentage of apoptotic cells decreased from ~23 to ~5% as the concentration of PVP in DMEM was increased from 1 to 40%. Although these results by Thirumala *et al.*[51–53] are interesting and useful in the development of cryopreservation protocols for ASCs, the possible mechanisms by which PVP confers its cryoprotective ability to ASCs during freezing are still unclear. We postulated that one such protective mechanism could be the ability of PVP to alter the characteristics of both extra- and intra-cellular ice formation during freezing of ASCs. Hence, the aim of this study was to assess the effect of PVP on the formation of extra- and intracellular ice during freezing in ASCs and the ability of PVP to possibly modify or mitigate the associated freezing injury.

MATERIALS AND METHODS

Isolation, Collection, and Culture of Cells

All human protocols were reviewed and approved by the Pennington Biomedical Research Centre Institutional Review Board. Unless otherwise stated, all reagents were obtained from Sigma Chemicals (St. Louis, MO). The method of culturing and harvesting adult stem cells has been described elsewhere.[11,20,49,51–53] Briefly, subcutaneous adipose tissue liposuction aspirates from three patients were provided by plastic surgeons in Baton Rouge, LA. These tissue samples (100–200 mL) were washed 3–4 times in phosphate buffered saline (PBS) pre-warmed to 37 °C, suspended in PBS supplemented with 1% bovine serum albumin and 0.1% collagenase (Type I, Worthington Biochemicals, Lakewood, NJ), and digested with gentle rocking for 45–60 min at 37 °C. The digests were centrifuged for 5 min at 1200 rpm ($300\times g$) at room temperature, re-suspended, and the centrifugation step repeated. The supernatant was aspirated and the pellet re-suspended in stromal medium (DMEM high glucose, 10% fetal bovine serum, 100 units penicillin/mL, 100 μg streptomycin/mL, and 25 μg amphotericin/mL). The cell suspension was plated at a density equivalent to 0.125 mL of liposuction tissue per sq. cm of surface area, using a 35 mL volume of stromal medium per T225 flask. Cells were cultured for 48 h in a 5% CO_2, humidified, 37 °C incubator. After 48 h, the adherent cells were rinsed once with pre-warmed PBS and re-immersed in fresh Stromal Medium. The cells were fed with fresh stromal medium every 2 days until they reached approximately 75–80% confluence. The medium was then aspirated; the cells were rinsed with pre-warmed PBS and harvested by digestion with 0.05% trypsin solution (5–8 mL per T225 flask) for 3–5 min at 37 °C. The cells were suspended in Stromal Medium, centrifuged for 5 min at 1200 rpm ($300\times g$), the pellet re-suspended in a volume of 10 mL of stromal medium, and the viable cell count determined by trypan blue exclusion. These cells were identified as Passage 0 (P0). The remaining cells were seeded in T225 flasks at a density of 5×10^3 cells per sq. cm. The cells were maintained in culture and passaged as described above to obtain Passage 1 (P1) ASCs and are the cells used in this study.

Cryomicroscopy Experiments

The cryomicroscopy experimental procedures are similar to those described extensively in the literature.[6,7,10,12,15,16,24,32,36,43,44,48,56–58] Briefly, P1 ASCs were detached using trypsin (ATCC, Manassa, VA) and transferred to a 1.5 mL centrifuge tube, spun

down for 5 min at 1200 rpm, and re-suspended in 50 μL of either 1× phosphate buffered saline (PBS) or 10% PVP (w/v) in 1× PBS solution. Following this, 2.5 μL of the sample was then transferred to the high thermal conductivity quartz crucible on the BCS-196 cryostage (Linkam Scientific^{TM}, Surrey, UK). The Linkam^{TM} cryostage is capable of controlled cooling and heating between −125 °C and +160 °C at cooling/heating rates ranging from 0.1 to 130 °C/min. A coverslip was then placed on top of the cell suspension within the quartz crucible to prevent leakage/media evaporation and to ensure proper visibility of the cells during the freezing process. The presence of a coverslip precluded the use of traditional extracellular ice nucleation techniques like the use of chilled needle and we had to resort to the use of a commercially available ice nucleating bacteria, *Pseudomonas syringae* (Snomax, NY). Both the PBS and the PBS + PVP solutions contained *P. syringae* concentrations of 1 mg/L. Note that the use of ice nucleating bacteria was to simply facilitate ice nucleation and was assumed to not directly affect the measurement of interest, i.e. IIF in ASCs. Independent differential scanning calorimetry experiments showed that with the presence of the bacteria, both solutions froze at approximately −9.5 °C, whereas without the bacteria, both of the solutions froze at approximately −18 °C. The Linkam^{TM} cryostage was attached to a microscope (Nikon Eclipse E600, Nikon Instruments, NY) fitted with a Photometrics Coolsnap cf camera (Hamamatsu, Photonics, Bridgewater, NJ). During the experiments a live video signal was sent from the camera to the attached DELL personal computer and the images recorded with the help of commercially available Metacam software (Universal Imaging Corp., Buckinghamshire, UK).

Description of the Cooling Protocols and Visualization of Intracellular Ice Formation

The protocols were so chosen that the following conditions could be related to the formation of IIF in ASCs: (i) formation (shape and size) of extracellular ice with and without PVP; (ii) re-equilibration at a high sub-zero temperature with minimal extracellular ice, i.e. the possibility of intracellular ice thawing upon heating to high sub-zero temperatures with and without PVP; and (iii) the possibility of re-nucleation or IIF on subsequent cooling (and the relation to adult stem cell membrane integrity) with and without PVP. Thus, ASCs were cooled on the Linkam^{TM} cryostage using two different temperature/time cooling protocols. Both the cooling protocols had a common cooling ramp: cells were cooled from 20 °C to −8 °C at 20 °C/min and then further cooled to −13 °C @ 1 °C/min. Except for the ice nucleating bacteria, no external seeding agents (e.g. chilled needle) were used. At this point we employed either *cooling protocol 1*: the cells were cooled from −13 °C to −40 °C at a pre-determined cooling rate of 1, 5, 10, 20, or 40 °C/min and then thawed back to 20 °C at 20 °C/min; or *cooling protocol 2*: the cells were re-warmed from −13 °C to −5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20, or 40 °C/min to −40 °C. The cells were then thawed back to 20 °C at a heating rate of 20 °C/min. During cooling the formation of extra- and intra-cellular ice was visually observed by the associated changes in crystalline structure and the darkening/flashing of cells, respectively.[6,7,15,16,24,56–58]

Viability Experiments

Post-freeze/thaw viability was determined by the ability of these cells to exclude a fluorescent dye, specifically propidium iodide (PI). The staining solutions (1.5 μg/mL of PI) were prepared, as described earlier,[18,46] and added to the frozen/thawed cells immediately after thawing and at room temperature. The cells that were unable to exclude the dye and positively stained with PI were considered to have experienced membrane damage during the freeze/thaw process while the remaining cells that were able to exclude PI and in the field of view were considered to be viable.

RESULTS AND DISCUSSION

Extra- and Intra-cellular Ice Formation (with and without PVP)

The extra-cellular solution remained unfrozen and super-cooled when cooled from +20 °C to −8 °C at a cooling rate of 20 °C/min, i.e. no extra-cellular ice formed during the initial part of the common cooling ramp. However, the extra-cellular ice was formed during the common cooling ramp between −10 °C and −11 °C, as shown in Fig. 1. Extra-cellular ice nucleation was rapid and was accompanied by the formation of intracellular ice in almost (>95%) all of the cells between −10 °C and −13 °C, as observed by ''darkening/flashing''. Thus, at the end of the common cooling ramp, the extra-cellular medium was completely frozen and nearly all the cells had undergone IIF; specifically 1015 cells exhibited IIF out of a total 1060 cells investigated as part of this study (or ~96%).

Cooling Protocol 1 (with and without PVP)

To reiterate, this protocol consisted of cooling the cells from −13 °C to −40 °C at a pre-determined

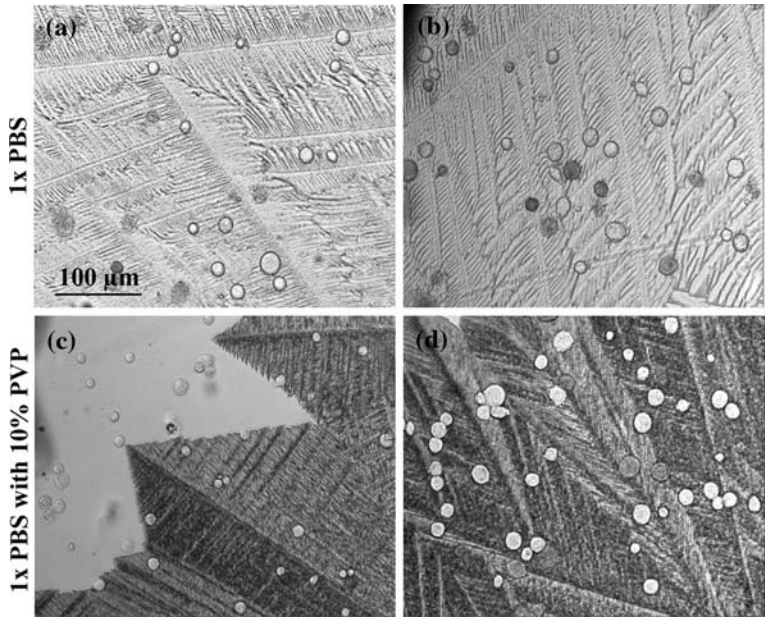

FIGURE 1. Cryomicroscopic images taken during the common cooling ramp demonstrating the nature of the extra-cellular ice formation (between −10 °C and −11 °C). (a, b) (row 1) The extra-cellular ice formation in 1× PBS; (c, d) (row 2) the extra-cellular ice formation in 1× PBS with 10% PVP. The appropriate scale bar (100 µm) is shown in (a) and is valid for all the figures.

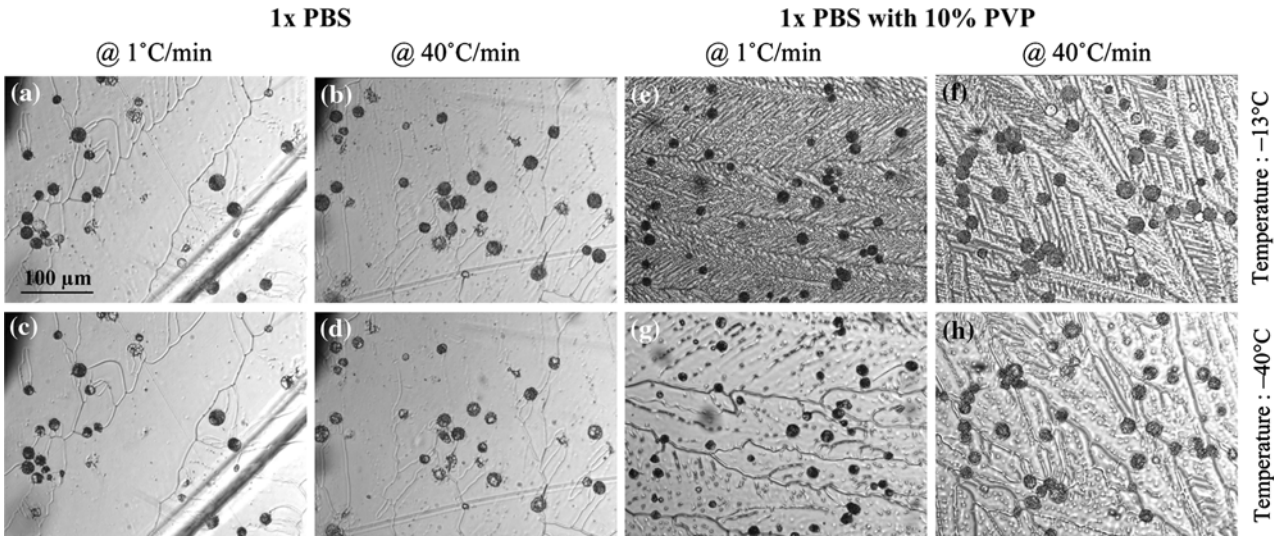

FIGURE 2. Cryomicroscopic images taken during cooling protocol 1 (either at −13 °C or at −40 °C) with 1× PBS (a–d; images in columns 1 and 2) and with 1× PBS with 10% PVP (e–h; images in columns 3 and 4). The images in row 1 represent the data at −13 °C while the corresponding images at −40 °C are shown in row 2. And, finally, columns 1 and 3 are images obtained at 1 °C/min while columns 2 and 4 represent images obtained at a cooling a rate of 40 °C/min. The appropriate scale bar (100 µm) is shown in (a) and is valid for all the figures.

cooling rate of 1, 5, 10, 20, or 40 °C/min (Fig. 2). Since the extra- and intra-cellular formation had already occurred, as described above in the common cooling ramp, no further changes in the extra- or intra-cellular space were observed at all the cooling rates investigated (comparing the two rows in Fig. 2). Consequently, the % of cells exhibiting IIF remained independent of the imposed cooling rate, i.e. no significant changes in the % of cells exhibiting IIF was noted between the various cooling rates. Thus, at the end of the

experiments with cooling protocol 1, the % of cells exhibiting IIF was >95% (i.e., same as that observed at the end of the common cooling ramp). Specifically, the % of cells exhibiting IIF at various cooling rates in PBS alone and frozen using cooling protocol 1 are 93.75, 94.2, 98.4, 100, and 100% for cells cooled at 1, 5, 10, 20, and 40 °C/min, from −13 °C to −40 °C, respectively. Similarly, the corresponding values for ASCs frozen in the presence of 10% PVP in PBS and frozen using cooling protocol 1 are 96.9, 100, 98, 100, and 100% for cells cooled at 1, 5, 10, 20, and 40 °C/min, from −13 °C to −40 °C, respectively.

Cooling Protocol 2 (in 1× PBS and without PVP)

The primary purpose of this protocol is to act as a control for the following experiments with PVP and to assess, if the observed intracellular ice was indeed damaging to ASC membrane integrity. To reiterate, this protocol consisted of re-warming the cells from −13 °C to −5 °C at 20 °C/min and then the cells were re-cooled at a pre-determined rate of 1, 5, 10, 20, or 40 °C/min to −40 °C (Fig. 3). During the re-warming of the cells/solution from −13 °C to −5 °C, the extra- and intra-cellular space was partially thawed and the ice formed within the cells was transformed into a transparent phase (as opposed to the opaque/dark phase), i.e., most probably due to the fact that the ice within the cells melted. However, further cooling of these cells from −5 °C to −40 °C at various cooling rates (1, 5, 10, 20, and 40 °C/min) *did not* result in re-nucleation or "darkening/flashing" in the cells, albeit with very few exceptions. Specifically, the % of cells exhibiting IIF at various cooling rates in PBS alone and frozen using cooling protocol 2 are 0, 3.2, 3.9, 0, and 4.6% for cells cooled at 1, 5, 10, 20, and 40 °C/min, from −5 °C to −40 °C, respectively.

To further investigate this phenomena, i.e., lack of IIF in the frozen-thawed cells, we measured the ability of these cells to exclude a fluorescent dye (PI), as previously described.[18,46] Briefly, the cells at the end of the initial cooling step in cooling protocol 2, i.e., the cells thawed from −13 °C to −5 °C were further thawed to 20 °C at 20 °C/min and exposed to PI solution. Approximately all of the cells (>90%) were unable to exclude the dye and hence had compromised membrane integrity. This suggests that the formation of intracellular ice during the common cooling ramp, between −10 °C and −13 °C, was extremely deleterious and damaging. Hence, these cells with compromised membranes did not and could not exhibit IIF on subsequent re-cooling and furthermore exhibited morphological distortions consistent with compromised membrane integrity (Figs. 3c, 3d, 3g, and 3h). As expected, these results are in agreement with earlier studies that suggest that IIF is damaging to cells[1,6,7,15,16,21,24,56–58] and in disagreement with other studies that suggest ice formation can be innocuous to cells.[2,3,26,37,41,42,45,60]

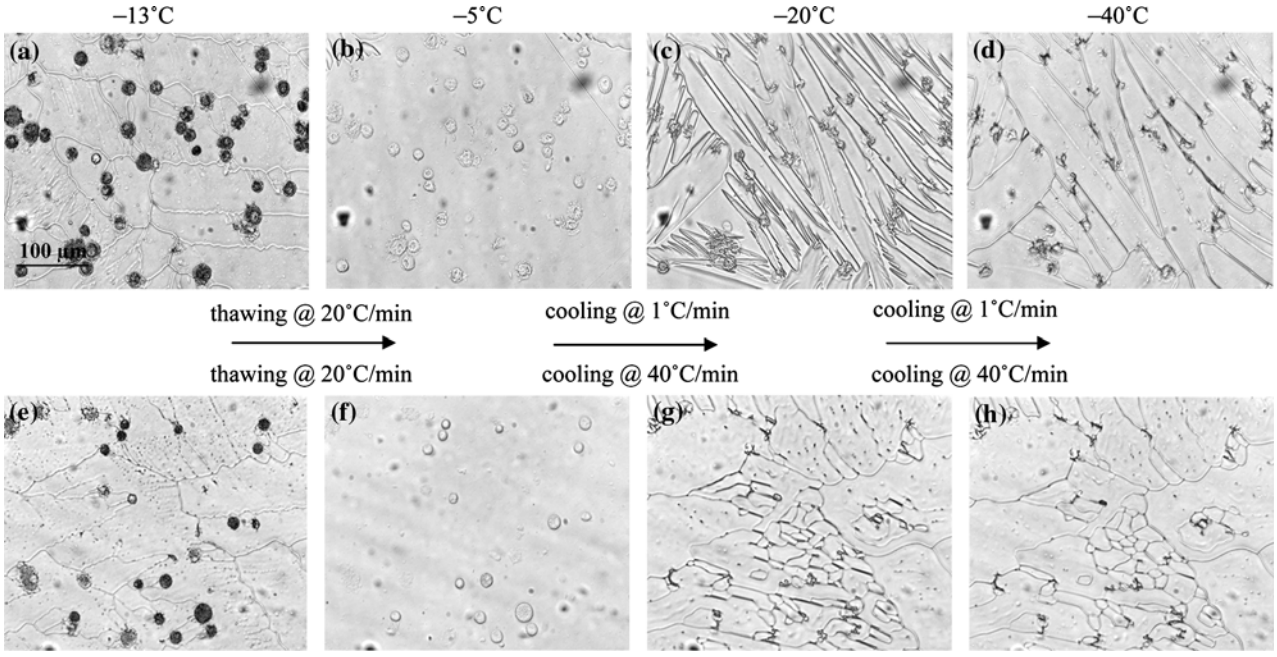

FIGURE 3. Cryomicroscopic images taken during cooling protocol 2 with 1× PBS. Images in columns 1–4 represent the data at −13 °C, −5 °C, −20 °C, and −40 °C, respectively. Images in rows 1 and 2 represent cells cooled at 1 °C/min and 40 °C/min (between −5 °C and −40 °C), respectively. The appropriate scale bar (100 μm) is shown in (a) and is valid for all the figures.

Cooling Protocol 2 (in 1× PBS and 10% PVP)

As before, during the re-warming of the cells/solution from −13 °C to −5 °C, the extra- and intra-cellular space was partially thawed and the ice formed within the cells was transformed into a transparent phase (Fig. 4). During further cooling, i.e. cooling from −5 °C to −40 °C, several phenomena were observed, namely: (i) trace amount of ice crystals present in the extra-cellular medium, acted as nucleating agents; (ii) consequently, ice nucleation started at a higher temperature (approximately −6 °C) and ice crystals formed were much larger compared to those obtained during extra-cellular ice nucleation in the common cooling ramp and/or cooling protocol 1. This is illustrated by a comparison of the images shown in Figs. 4a through 4h. This result is not surprising as it is well known that the size of ice crystals formed during nucleation is inversely correlated with the amount of super-cooling.[6,7,10,12,14–16,24,32,36,43,44,48,56–58]

Most importantly, further cooling of these cells from −5 °C to −40 °C at various cooling rates (1, 5, 10, 20, and 40 °C/min) *did* result in re-nucleation or "darkening/flashing" in most of the cells, i.e. most of the frozen-thawed cells exhibited IIF. Specifically, 0, ~40, ~47, ~67, and ~100% of the ASCs frozen in 10% PVP in PBS exhibited re-nucleation or "darkening/flashing" when cooled at 1, 5, 10, 20, and 40 °C/min, from −5 °C to −40 °C, respectively. The observed difference in the % of cells exhibiting IIF at different cooling rates can be attributed to the intrinsic statistical dependence of the formation of intracellular ice on the imposed cooling rate.[6,7,10,12,15,16,24,32,36,43,44,48,56–58] Basically, the imposed cooling rate of 1 °C/min was sufficiently "slow" to avoid IIF and hence all of the cells experienced water transport or cellular dehydration at this cooling rate. As the cooling rate increases, the fraction of cells experiencing IIF increases and, correspondingly, the fraction of cells experiencing water dehydration decreases. And at the "fast" rate of 40 °C/min, all the cells exhibit IIF. Finally, the maximum cumulative fraction of cells undergoing IIF as a function of sub-zero temperature and the imposed cooling rate, in the presence of PVP and cooled using cooling protocol 2, is shown in Fig. 5. Following prior protocols,[6,7,56–58] the cumulative fraction (%) of cells exhibiting IIF is presented as a population-based measurement; specifically, IIF data was obtained from 8 to 10 separate experiments for each cooling rate with approximately 10 cells in the field of view per experiment or the data from ~80 to 100 cells was obtained and pooled as a single population per cooling rate. Since the data is a representation of the cumulative fraction of cells experiencing IIF from all the experiments (or from a population of cells), typically no error bars are shown within Fig. 5.[6,7,56–58] However, it is theoretically possible to calculate the % of cells exhibiting IIF from each individual experiment and, consequently, the standard deviation in the % of cells exhibiting IIF between individual experiments

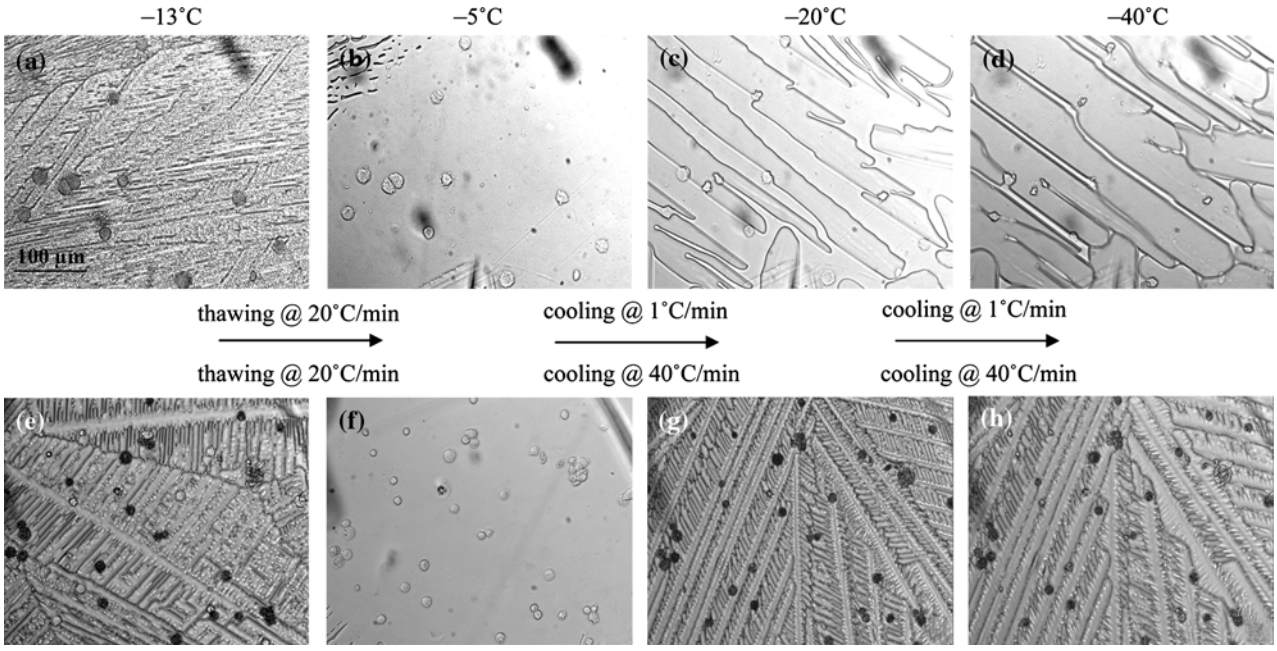

FIGURE 4. Cryomicroscopic images taken during cooling protocol 2 with 10% PVP in 1× PBS. Images in columns 1–4 represent the data at −13 °C, −5 °C, −20 °C, and −40 °C, respectively. Images in rows 1 and 2 represent cells cooled at 1 °C/min and 40 °C/min (between −5 °C and −40 °C), respectively. The appropriate scale bar (100 μm) is shown in (a) and is valid for all the figures.

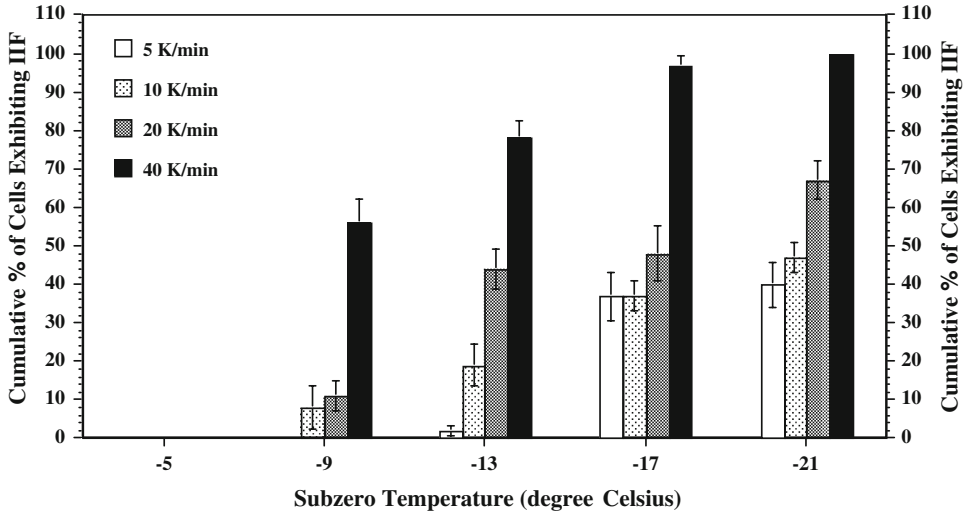

FIGURE 5. The maximum cumulative fraction of cells exhibiting IIF when frozen with 10% PVP in 1× PBS using cooling protocol 2 is shown for different (5, 10, 20, and 40 °C/min) cooling rates. The cooling rates were imposed between −5 °C and −40 °C (see text for further details). Note that the ice nucleation data from several different experiments was pooled to calculate the cumulative fraction (%) of cells undergoing IIF (~10 cells per field of view with 8 to 10 separate experiments per cooling rate or a total of ~80 to 100 cells per cooling rate). However, it is theoretically possible to calculate the % of cells exhibiting IIF from each individual experiment and, consequently, the standard deviation in the % of cells exhibiting IIF, as shown in the figure. The sub-zero temperatures are shown on the *x*-axis while the cumulative % of cells exhibiting IIF is shown on the *y*-axis.

(and different fields of view). Thus, we have included error bars with Fig. 5. It is hoped that the inclusion of this atypical data would give an idea to the interested reader of the repeatability of the results presented, as well as whether statistically significant differences are present between groups.

As before, we investigated the membrane integrity of the cells at the end of the initial cooling step in cooling protocol 2, i.e., the cells thawed from −13 °C to −5 °C at 20 °C/min, frozen with 10% PVP in PBS. Based on our earlier observations of these cells to re-nucleate and exhibit IIF, we fully expected that these viability numbers would be significantly higher than that obtained for cells frozen in the absence of PVP. Indeed, this was found to be the case (90% vs. 10%). Thus, suggesting that IIF in ASCs in the presence of PVP is innocuous and conversely, IIF in ASCs is cytotoxic in the absence of PVP. This led us to infer that the presence of PVP has a significant role in protecting ASCs during the formation of intracellular ice that accompanies extra-cellular ice nucleation in the common ramp. This is an intriguing result that suggests a possible cryoprotective mechanism of PVP on ASCs, i.e. the presence of PVP alters the nature (shape and size), and correspondingly the damaging nature of intracellular ice crystals.[9,13,25,30,39,61]

The equilibrium melting point of 1× PBS is approximately −0.6 °C and that of 10% PVP in 1× PBS is approximately −1 °C.[23] However, with the use of a finite cooling rate instead of infinitesimally small cooling rate coupled with the lack of external seeding, e.g. with a chilled needle, the solutions exhibit supercooling to temperatures as low as −10 °C. Obviously, once nucleation occurs in such a highly unstable and super-cooled medium, the process of ice formation is very rapid and ice freeze-front covers the field of view almost instantaneously.[24,43,44,56–58] It has been postulated that the probability of ice formation is significantly increased if the extra-cellular medium freezes after a high degree of super-cooling or at a low subzero temperature, mainly because of two reasons: (i) the high degree of cooling rates experienced by the sample due to the thermal fluctuations caused by the release of latent heat of the extra-cellular medium and (ii) due to the high amount of intracellular supercooled water retained within the cells due to the lack of cellular dehydration.[24,32,43,44,48,56–58] Thus, during the common cooling ramp, a significant % (or ~96%) of the cells exhibit IIF.

Possible Cryoprotective Mechanisms of PVP

McGann[30] hypothesized that non-permeating CPAs, like hydroxy-ethyl-starch (HES) and PVP, have the effect of "squeezing out" intracellular water from cell when the extra-cellular solution freezes. The precipitation of pure ice from the extra-cellular solution has the effect of increasing the concentration of the dissolved non-permeating CPA in the unfrozen fractions, which in turn dehydrates the cell.[30] It is entirely plausible that during the freezing of the extra-cellular solution (between −10 °C and −11 °C) in the common

cooling ramp the presence of PVP leads to significant cellular dehydration and the associated loss of intracellular water (as opposed to those cells cooled in the absence of PVP). Clearly, the lower volume of intracellular water will lead to smaller intracellular ice crystals (and possibly less damaging or innocuous) when, and if, IIF does occur. To check this hypothesis further, we suspended the cells in a solution of 40% (w/v) PVP in 1× PBS. Two such samples were frozen under the cryomicroscope from 20 °C to −40 °C (at either 1 or 40 °C/min) and thawed back to 20 °C (at 20 °C/min). Briefly, in neither of these cases did we notice any of the cells experiencing/exhibiting IIF (data not shown). Intriguingly, a recent study by Thirumala *et al.*[53] showed that more than 90% of the ASCs were necrotic when frozen/thawed in the presence of 40% PVP. These observations together, i.e. lack of IIF in ASCs and the high % of necrotic ASCs post-thaw when cooled with 40% PVP, suggest that at higher concentrations of PVP, the cells are experiencing excessive cellular dehydration (and the associated membrane damage, solute effects injury) as opposed to damaging IIF, lending credence to the hypothesis proposed earlier by McGann.[30] And finally, it is important to note that there exist differences between the temperature gradients experienced by ASCs cooled using the Linkam™ cryostage (present study) and ASCs frozen overnight in an ethanol-jacketed container placed in a −80 °C freezer (earlier studies by Thirumala *et al.*[51–53]). Obviously, these variations in the temperature gradients need to be further investigated with respect to the formation of IIF in ASCs, with and without PVP.

CONCLUSION

The main objective of this work was to assess the effect of 10% (w/v) PVP on the pattern of IIF in ASCs in the absence of serum and other cryoprotective agents (CPAs). All of the cells were cooled in a common cooling ramp from 20 °C to −8 °C at 20 °C/min and then further cooled to −13 °C at 1 °C/min (during which the extra-cellular medium froze very rapidly and was accompanied by the formation of intra-cellular ice in ~90% of the cells). Almost none (<5%) of the ASCs frozen in 1× PBS and protocol 2 (re-warmed from −13 °C to −5 °C at 20 °C/min and then re-cooled at a pre-determined rate of 1, 5, 10, 20, or 40 °C/min to −40 °C) exhibited IIF. The lack of IIF in cells cooled in 1× PBS and protocol 2 was due to the initial loss of cell viability that was associated with the formation of extra-cellular ice and associated IIF in the common cooling ramp. Intriguingly, ~0, ~40, ~47, ~67, and ~100% of the ASCs frozen in 10% PVP in PBS and protocol 2 exhibited IIF at a cooling rate of 1, 5, 10, 20, or 40 °C/min, respectively. The observed increase in the % of ASCs exhibiting IIF when frozen in 10% PVP and protocol 2 is presumably due to PVP mitigating the damaging effects of IIF during the initial common cooling ramp. The results of this study can hence be used to construct cryopreservation protocols for ASCs without the use of serum and DMSO.

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