

**METHODS ARTICLE**

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# Combinations of Osmolytes, Including Monosaccharides, Disaccharides, and Sugar Alcohols Act in Concert During Cryopreservation to Improve Mesenchymal Stromal Cell Survival

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There is demand for non-dimethyl sulfoxide (DMSO) cryoprotective agents that maintain cell viability without causing poor postthaw function or systemic toxicity. The focus of this investigation involves expanding our understanding of multicomponent osmolyte solutions and their ability to preserve cell viability during freezing. Controlled cooling rate freezing, Raman microscopy, and differential scanning calorimetry (DSC) were utilized to evaluate the differences in recovery and ice crystal formation behavior for solutions containing multiple cryoprotectants, including sugars, sugar alcohols, and small molecule additives. Postthaw recovery of mesenchymal stem cells (MSCs) in solutions containing multiple osmolytes have been shown to be comparable or better than that of MSCs frozen in 10% DMSO at 1°C/min when the solution composition is optimized. Maximum postthaw recovery was observed in these multiple osmolyte solutions with incubation times of up to 2 h before freezing. Raman images demonstrate large ice crystal formation in cryopreserved cells incubated for shorter periods of time (~30 min), suggesting that longer permeation times are needed for these solutions. Recovery was dependent upon the concentration of each component in solution, and was not strongly correlated with osmolarity. It is noteworthy that the postthaw recovery varied significantly with the composition of solutions containing the same three components and this variation exhibited an inverted U-shape behavior, indicating that there may be a “sweet spot” for different combinations of osmolytes. Raman images of freezing behavior in different solution compositions were consistent with the observed postthaw recovery. Phase change behavior (solidification patterns and glass-forming tendency) did not differ for solutions with similar osmolarity, but differences in postthaw recovery suggest that biological, not physical, methods of protection are at play. Lastly, molecular substitution of glucose (a monosaccharide) for sucrose (a disaccharide) resulted in a significant drop in recovery. Taken together, the information from these studies increases our understanding of non-DMSO multicomponent cryoprotective solutions and the manner by which they enhance postthaw recovery.

**Keywords:** mesenchymal stromal cell, cryopreservation, osmolyte

## Background

**M**ESENCHYMAL STEM CELLS (MSCs) have generated much interest in the field of cell therapies and the unique biological properties of this cell type have led to the development of MSC-based therapies for a wide range of diseases, see Sharma *et al.*<sup>1</sup> for review. The ability to pre-

serve cells enables transportation of the cells from the site of manufacture to the site of administration and coordination of the therapy with patient availability. Conventional methods of cell freezing typically use the cryoprotective agent dimethyl sulfoxide (DMSO), which is associated with poor postthaw behavior<sup>2,3</sup> and with dangerous systemic side effects.<sup>4</sup>

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There is demand for non-DMSO cryoprotective agents that maintain cell viability without causing systemic toxicity or poor postthaw function. Single molecule replacement of DMSO has been explored, but results have been suboptimal. Multicomponent solutions have also been explored and factorial experiments to identify favorable combinations and freezing protocols have shown increased success.<sup>5,6</sup> Previously published work by this group<sup>7</sup> describes algorithm optimization of multicomponent cryopreservative solutions, including molecules that target different aspects of cell protection during freezing.

Diverse biological systems (plants, insects, etc.) survive high salt environments, dehydration, drought, freezing temperatures, and other stresses through the use of osmolytes.<sup>8</sup> It is common for multiple osmolytes to be present to stabilize the cells. For example, a mixture of five osmolytes has been used to stabilize the cells<sup>9</sup> in the human kidney *ex vivo*. Sugars, specifically trehalose, have been studied extensively for the osmoprotective effects they exert and have shown moderate cryoprotectivity at appropriate concentrations.<sup>10–12</sup> Polyols (such as glycerol) have been used as cryoprotectants for proteins, functionally stabilizing protein structure by maintaining hydrogen bonding in protein-bound water.<sup>13,14</sup> Amino acids have been utilized for liposomal cryopreservation,<sup>15,16</sup> and ampholytic amino acid polymers have demonstrated cryoprotection and result in cellular recoveries similar to DMSO.<sup>17,18</sup> Evidence from several groups indicates that the use of multiple cryoprotectants from the different families above, such as trehalose and proline,<sup>16</sup> or trehalose and glycerol,<sup>19</sup> exhibit improved cellular survival compared with either cryopreservative alone, suggesting that additive<sup>13</sup> or synergistic stabilizing effects are possible when multiple cryopreservatives are used.<sup>20</sup> However, the concentrations of these cryoprotectants dictate whether they will be stabilizing or destabilizing<sup>21,22</sup> and concentrations that result in stabilization optimums may differ when cryoprotectants are combined.<sup>23</sup>

In a previous study, we established our ability to optimize the composition of a multicomponent osmolyte solution<sup>7</sup> using a computational algorithm. The focus of this investigation involves expanding our understanding of multicomponent osmolyte solutions and their ability to preserve cell viability during freezing. The study characterizes interactions between solutes using surface plots to describe how concentrations of multiple components contribute to recovery. Differences in single cell response to freezing using different experimental parameters (incubation time, composition) were captured using low-temperature Raman spectroscopy. These studies will help expand our understanding of these solutions and the manner by which they enhance postthaw recovery.

## Materials and Methods

### Cell culture

Human H9 ESC-derived MSCs were isolated and generously gifted for this research by Trivedi and Hematti.<sup>24</sup> MSCs were cultured in alpha-MEM (Gibco) supplemented with nonessential amino acids (Gibco) and 10% FBS (qualified; Gibco) in a 37°C incubator at 5% CO<sub>2</sub>. Cell confluency was maintained between 20% and 80% and media were

changed every 3–4 days. Cells were used for experiments only between passages 8 and 12.

### Cell freezing

Cells diluted in Normosol-R™ blank solution (Hospira) were transferred to freezing vials and an equal volume of cryoprotectant solutions at 2× their final concentrations were added to the vials stepwise. Ten percent DMSO controls were also prepared, in which cells suspended in MSC media were added to vials and DMSO introduced in the same stepwise fashion. For incubation studies, these cell suspensions were incubated at room temperature for 0, 30 min, 1, 2, or 4 h before freezing according to the following protocol using a controlled rate freezer (Planer Series III Kryo 10):

1. Starting temp 20°C
2. –10°C/min to 0°C
3. Hold at 0°C for 15 min
4. –1°C/min to –8°C
5. –50°C/min to –45°C
6. +15°C/min to –12°C
7. –3°C/min to –100°C

The final 1× concentrations present with cells for each solution tested are listed in the Results section.

### Thawing

Frozen vials were thawed in a 37°C water bath. Vials were submerged halfway and agitated until only a miniscule ice pellet remained. Cells were assessed for viability immediately postthaw.

### Viability and functionality

Cells were assessed for viability at the conclusion of each experiment. Both preefreeze and postthaw vial counts were performed using Acridine Orange/Propidium Iodide (AO/PI). Briefly, cells were combined with AO/PI, loaded into a hemocytometer, and counted manually using a fluorescent microscope. A minimum of 200 total cells were counted for each sample.

Viability was calculated for all samples by dividing the number of live cells by the number of total cells. Recovery was calculated directly by dividing the number of live cells postthaw by the number of live cells preefreeze.

Cell surface phenotype of the cells was determined using flow cytometry both before and after freezing. Cells in all preefreeze and postthaw populations were >99% positive for MSC markers (CD73, CD90, CD105) and negative for hematopoietic marker CD45 (data not shown). These results are consistent with the MSC phenotype.<sup>25</sup>

### Osmolarity

Osmolarity of solutions was measured using an OSM-ETTE™ osmometer (Precision Systems) for each solution and all measurements were repeated in triplicate.

### Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a TA Differential Scanning Calorimeter Q1000.

Experimental solutions without cells were frozen to  $-150^{\circ}\text{C}$  using the following protocol:

1. Set starting temperature to  $20^{\circ}\text{C}$
2. Cool to  $-150^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$
3. Hold for 3 min at  $-150^{\circ}\text{C}$
4. Warm to  $20^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$

#### Confocal Raman system

Confocal Raman microspectroscopy measurements were conducted using a WITec Confocal Raman Microscope System Alpha 300R (WITec, Ulm, Germany) with a UHTS300 spectrometer and DV401 CCD detector with 600/mm grating. The WITec spectrometer was calibrated with a Mercury–Argon lamp. A wavelength of 532 nm Nd:YAG laser powered at 10 mw was used as an excitation source. The laser was transmitted to the microscopy in a single fiber. A 100 $\times$  air objective (NA 0.90; Nikon Instruments, Melville, NY) was used for focusing the 532-nm excitation laser to the sample. Samples were frozen using a controlled temperature stage described in more detail elsewhere.<sup>26</sup>

#### Raman measurement of frozen MSC cells

MSC cells were trypsinized and washed with DPBS solution before being suspended in experimental solutions. Roughly 1  $\mu\text{L}$  of cell suspension was placed on an aluminum sheet, covered with a piece of mica (Ted Pella, Redding, CA) and sealed with Kapton tape (DuPont, Wilmington, DE), to prevent evaporation/sublimation during each experiment. Cell suspensions were cooled to  $-6^{\circ}\text{C}$ , at which point the sample was seeded by a nitrogen-cooled needle. Subsequently, the solution was cooled down at  $3^{\circ}\text{C}/\text{min}$  to  $-50^{\circ}\text{C}$ . Ten Raman images of  $30 \times 30 \mu\text{m}$  were collected.

#### Raman image/spectral analysis

Spectrums at each pixel were analyzed using characteristic wave numbers of common intracellular and extracellular materials (Table 1), and were integrated with background subtraction to result in an image. Spectra for the osmolytes used in the investigation overlapped, so a broad peak centered at  $850 \text{ cm}^{-1}$  was used to generate Raman images for all osmolytes. Data analysis was performed by Windows-based Project FOUR software plus version 4.0.

TABLE 1. PEAK ASSIGNMENTS FOR MOLECULES OF INTEREST DETECTED USING RAMAN SPECTROSCOPY

Component	Frequency used for this study $\text{cm}^{-1}$	Ref.
Ice	3120 (OH stretching)	26
Amide I	1659 (C=O stretching)	27
Glycerol	851 (C-C stretching)	28
Sorbitol	878 (C-C=O stretching)	29
Glucose	840 (C-C stretching)	30
Sucrose	836 (C-C stretching)	30
Creatine	840 (C-N torsion)	31

#### Statistics

Averages plus or minus standard error of the mean are reported unless otherwise noted. Student's *t*-tests were performed to determine statistically significant differences ( $p < 0.05$ ) between samples tested in the osmolarity and sugar substitution studies.

#### Results

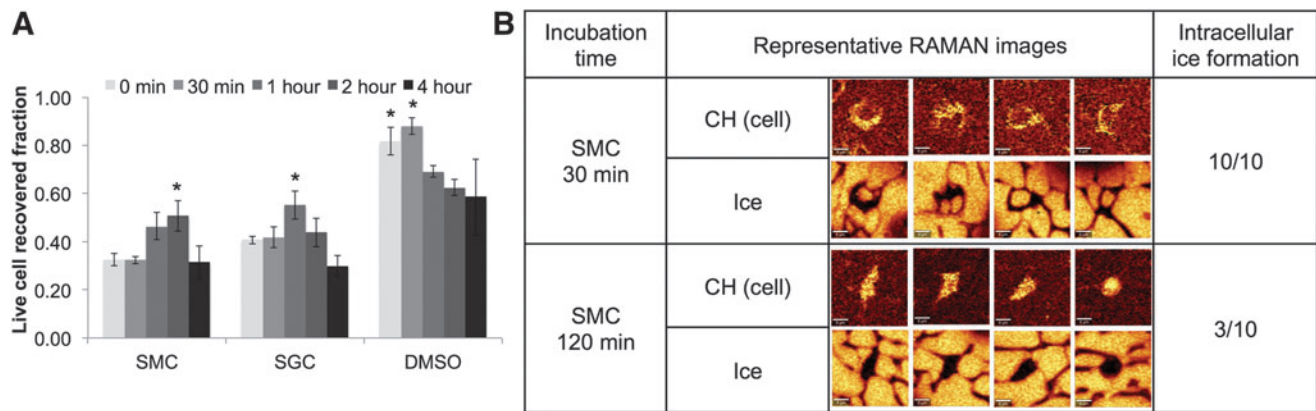
##### Permeation/partitioning of solute

Several of the osmolytes studied here are larger ( $> \sim 300 \text{ Da}$ ) and, as a result, may take longer to enter the cell. Other large molecules, including trehalose are known to have very limited penetration.<sup>32</sup> To determine the proper incubation time for the cells in the multicomponent osmolyte solutions, solutions of SMC (sucrose = 150 mM, mannitol = 125 mM, creatine = 12.5 mM) and SGC (sucrose = 150 mM, glycerol = 2.5%, creatine = 12.5 mM) were incubated with cells at room temperature in Normosol<sup>®</sup> for 0, 30 min, 1, 2, or 4 h before undergoing freezing at  $3^{\circ}\text{C}/\text{min}$ . Live cell recovery increased and experienced a maximum at 1 h for SGC samples (significantly greater than at least one other time point,  $p < 0.05$ ), and 2 h for SMC samples (significantly greater than at least one other time point,  $p < 0.05$ ). Longer incubation times (4 h) decreased the viability. As expected, incubation times of more than 30 min resulted in a decrease in viability for cells in DMSO.

Increased incubation times have a profound influence on cell response to freezing as imaged using Raman spectroscopy (Fig. 1B). MSCs were incubated for 30 and 120 min in the same SMC composition described above, frozen to  $-50^{\circ}\text{C}$ , and imaged using low-temperature Raman spectroscopy. Cells incubated for 30 min exhibited large internal ice crystals for 10/10 of the cells imaged. In contrast, cells incubated for 120 min exhibited ice in only 3/10 cells imaged. The formation of ice inside the cell is considered to be a damaging event and the ice formation observed here is consistent with the poorer postthaw viability observed in Figure 1A for cells incubated for only 30 min.

##### Influence of osmolarity and composition

The range of solution compositions studied is important. It is common for cryopreservation solutions to contain high concentrations of cryoprotective agents and therefore exhibit high solution osmolarity. For example, a 10% DMSO solution has an osmolarity of  $\sim 1400 \text{ mOsm}$ . MSCs suspended in different combinations of sucrose, mannitol, and creatine (SMC), and sucrose, glycerol, and creatine (SGC) were frozen at  $3^{\circ}\text{C}/\text{min}$ , thawed, and postthaw recovery measured (information regarding solution composition and corresponding recovery and osmolarity is listed in the Appendix [Tables A1 and A2]). The postthaw recovery of SGC and SMC were plotted as a function of total osmolarity for a range of different tested compositions (Fig. 2). For SMC solutions, the range of solution osmolarities is low ( $< 500 \text{ mOsm}$ ) and there is a weak negative correlation between osmolarity and postthaw recovery of MSCs (Fig. 2A,  $R^2 = 0.18393$ ). In contrast, SGC solutions were evaluated over a higher range of osmolarities ( $< 1200 \text{ mOsm}$ ) and exhibited a weak positive correlation with the compositions tested (Fig. 2B,  $R^2 = 0.10131$ ). These weak correlations



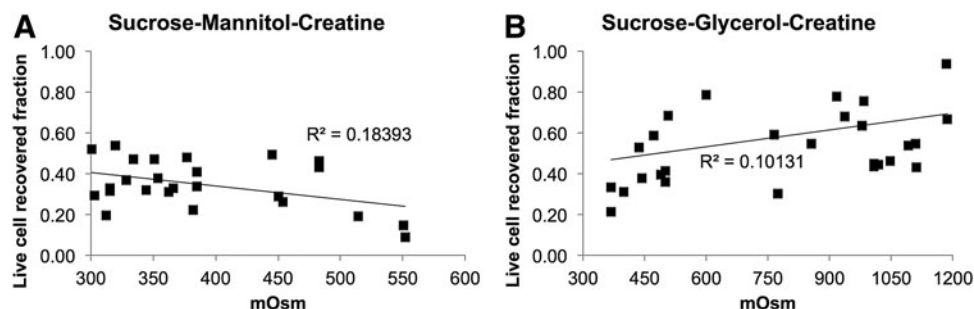
**FIG. 1.** Recovery reaches a maximum with appropriate incubation time for slow penetrating components. **(A)** Fraction of live cells recovered for DMSO-free solutions, SMC and SGC, and DMSO solutions as a function of incubation time before freezing at 3°C/min; significance markers indicate significantly greater recovery (*t*-test) than at least one other time point for the same solution. Only 1-h SGC and 2-h SMC statistically outperformed other time points ( $p < 0.05$ ). Both DMSO 0 h and DMSO 30 min outperformed other time points, but were statistically dissimilar from one another. **(B)** Raman images obtained of MSCs frozen at 3°C/min in SMC solution after 30 and 120 min of incubation before freezing. Raman images are rendered for both -CH and ice. DMSO, dimethyl sulfoxide; MSCs, mesenchymal stem cells. Color images available online at [www.liebertpub.com/tec](http://www.liebertpub.com/tec)

suggest that higher solution concentration does not necessarily correlate to higher levels of postthaw recovery.

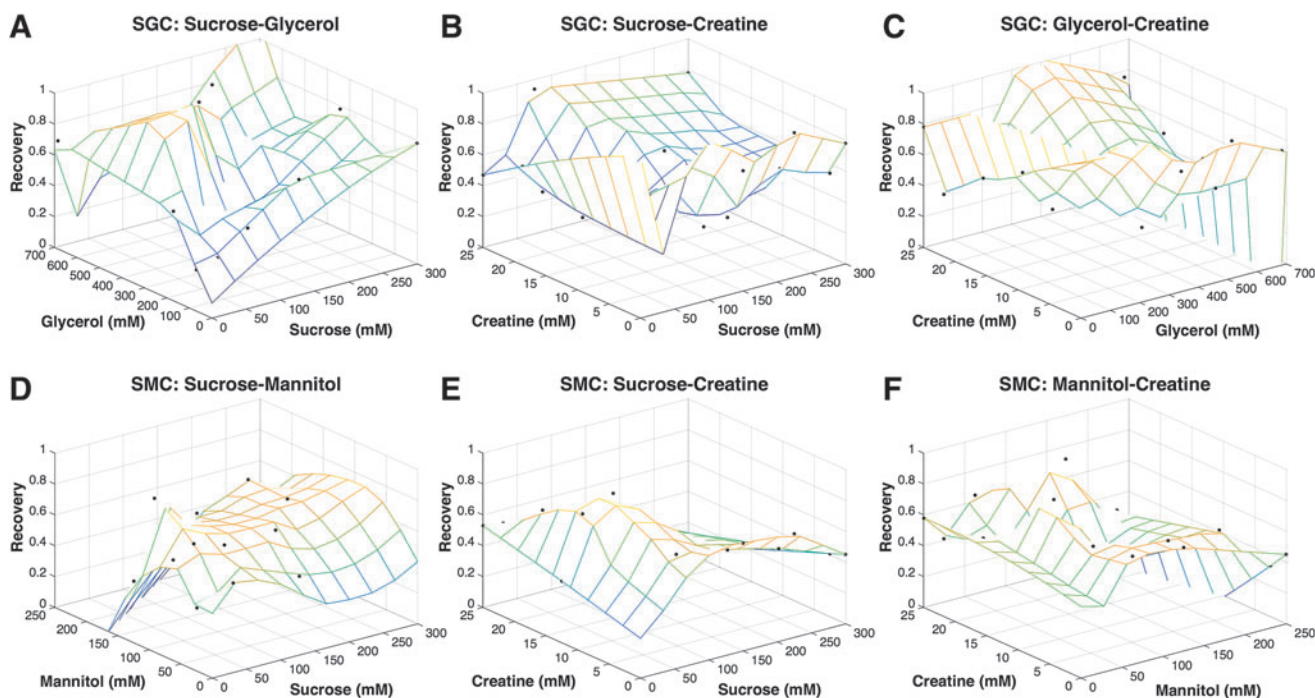
For the same three-component solution compositions used in Figure 2, MATLAB was used to generate scattered interpolant plots that mapped the postthaw recovery measured as a function of solution composition for two of the three components present in solution using 20–25 experimental points (recovery was averaged for vectors with same values for the two components being plotted). As described previously, the postthaw recovery varied significantly with composition for solutions containing the same three components (Fig. 3). It is noteworthy that the variation in the fraction of recovered cells postthaw exhibited an inverted U-shaped behavior. For example, the relationship between sucrose and creatine exhibits a maximum cell recovery for concentrations  $\sim 150$  mOsm of sucrose (Fig. 3B, E). The fraction of recovered cells diminishes for concentrations above and below that optimum. Something similar is observed in Figure 3D, where the optimum concentration of mannitol  $\sim 150$  mOsm and the fraction of recovered cells

increases with increasing sucrose concentration to the highest concentration tested (300 mOsm).

To understand differences in freezing response for different combinations of the same three osmolytes, freezing studies using two different compositions of SGC with similar total osmolarity (SGC-A: 150 mM Sucrose, 684 mM Glycerol, 25 mM Creatine and SGC-B: 300 mM Sucrose, 684 mM Glycerol, 12.5 mM Creatine) were repeated (Fig. 4). MSCs were frozen under the same conditions (1 h incubation and 3°C/min cooling rate) and imaged using low-temperature Raman confocal microscopy. Cells frozen in SGC-B exhibited ice inside the cells for 10/10 cells imaged and the formation of ice inside the cell is known to be damaging.<sup>33</sup> In contrast, cells cryopreserved in SGC-A exhibited intracellular ice formation (3/10 cells imaged) implying that 7/10 cells survived freezing. Postthaw recovery trends (average  $\pm$  SEM,  $n = 4$ ) for cells frozen in SGC-A ( $0.82 \pm 0.07$ ) and SGC-B ( $0.71 \pm 0.05$ ) using conventional controlled rate freezing were consistent with the ice formation trends observed using Raman, in that SGC-A had higher recovery and fewer cells with ice crystal formation than SGC-B.



**FIG. 2.** Postthaw recovery of MSCs cryopreserved in SGC and SMC as a function of total solution osmolarity. Linear best fit is given with correlation coefficient. **(A)** Recovery of cells has slight negative correlation for different osmolarity sucrose-mannitol-creatine solutions. **(B)** Recovery of cells has slight positive correlation for different osmolarity sucrose-glycerol-creatine solutions.



**FIG. 3.** Scattered interpolant meshgrid plots of recovery versus two of three components for SGC (A–C) and SMC (D–F) solutions. Color images available online at [www.liebertpub.com/tec](http://www.liebertpub.com/tec)

#### Solidification behavior

It is common to formulate cryopreservation solutions to result in full or partial vitrification (see Ref.<sup>34</sup> for review). The next phase of the investigation involved characterizing the solidification behavior of the solutions tested. SMC solutions with similar osmolality (SMC-A: 0 mM Sucrose, 125 mM Mannitol, 50 mM Creatine and SMC-B: 60 mM Sucrose, 50 mM Mannitol, 37.5 mM Creatine) and significantly different ( $p < 0.05$ ) recovery (recovery  $\pm$  SEM,  $n = 4$ ; SMC-A =  $0.73 \pm 0.03$ , SMC-B =  $0.62 \pm 0.02$ ) were seeded at  $-6^\circ\text{C}$ , cryopreserved without cells (solution only) at  $3^\circ\text{C}/\text{min}$ , and imaged using Raman microscopy. Representative images are given in Figure 5A. There is little difference in the macroscopic solidification patterns observed between the two solutions (in spite of the significant difference in postthaw viability observed between the two solutions). There is also no statistical difference in the area of ice crystals between the two solutions ( $p > 0.05$ ). As a control, a DPBS

solution was seeded at  $0^\circ\text{C}$ , cryopreserved at  $3^\circ\text{C}/\text{min}$ , and imaged using Raman microscopy. Only very small amount of solution was incorporated into ice and hydrohalite could be found in the narrow solution gap based on Raman spectra.

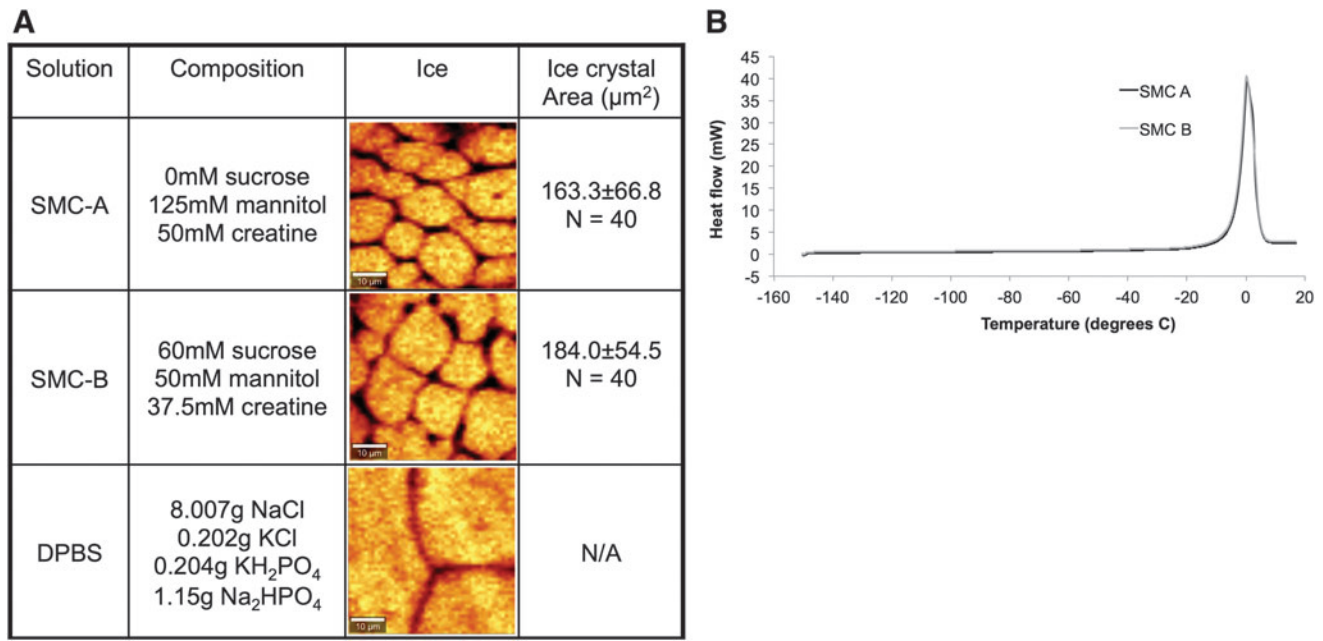
Additional studies were performed using DSC to determine whether the different compositions exhibited differences in glass-forming tendency (Fig. 5B). No change in the melting curves was observed for either pair, and no signs of full or partial vitrification of the solutions were observed using DSC.

#### Component substitution

There has been considerable interest in the use of sugars (specifically trehalose) for the preservation of cells.<sup>32</sup> The next phase of the investigation involved determining whether the structure of the sugar has a significant influence on postthaw recovery. Specifically, a monosaccharide, glucose, was substituted for a disaccharide, sucrose, at the same concentration for both SMC and SGC compositions (Table 2).

Solution	Composition	Ice	Osmolytes $780\text{--}920\text{cm}^{-1}$	CH(cell)	Intracellular ice formation
SGC-A	150mM Sucrose 684mM Glycerol 25mM Creatine				3/10
SGC-B	300mM Sucrose 684mM Glycerol 12.5mM Creatine				10/10

**FIG. 4.** Low-temperature Raman microscopy of MSCs cryopreserved at  $3^\circ\text{C}/\text{min}$  in SGC with two different compositions. Images are rendered on ice, osmolyte mixture, and  $-\text{CH}$ . The fraction of cells with ice is described for 10 cells measured. Color images available online at [www.liebertpub.com/tec](http://www.liebertpub.com/tec)



**FIG. 5.** Physical behavior of multicomponent solutions associated with different recovery. **(A)** Low-temperature Raman spectroscopy of two SMC solutions at  $-50^{\circ}\text{C}$ . Two different compositions with similar osmolality were studied: one associated with high recovery (SMC-A) and the other associated with lower recovery (SMC-B). The average ice crystal size for the two solutions was not statistically significantly different ( $p > 0.05$ ). **(B)** DSC thermograph for the same SMC solutions during warming. The melting curves are almost identical and display a large exotherm associated with melting of the solution, and no glass formation is observed for either solution. Color images available online at [www.liebertpub.com/tec](http://www.liebertpub.com/tec)

When a monosaccharide is substituted for a disaccharide for both of the solution compositions tested, the postthaw recovery of MSCs is reduced and those differences are statistically significant (Fig. 6A,  $p < 0.05$ ). Cells in SGC and GGC were cryopreserved and imaged using Raman spectroscopy. For cells cryopreserved in SGC, Raman images demonstrated that a small fraction of the cells exhibited ice during freezing (4/10) when compared with cells cryopreserved in a solution containing GGC exhibiting ice inside the cell during freezing (6/10) (Fig. 6B). Images of the cell freezing response are consistent with the controlled rate freezing experiments described in Figure 6A.

## Discussion

### Permeation/partitioning of solutes

Conventional cryoprotective agents (DMSO and glycerol) have small molecular weights (78 and 92 Da, respectively) and permeate the cell in a matter of minutes (see Ref. <sup>35</sup> for review). Little has been done to characterize the uptake of osmolytes with the exception of trehalose,<sup>36</sup> which appears to penetrate poorly into the cell. The results of this inves-

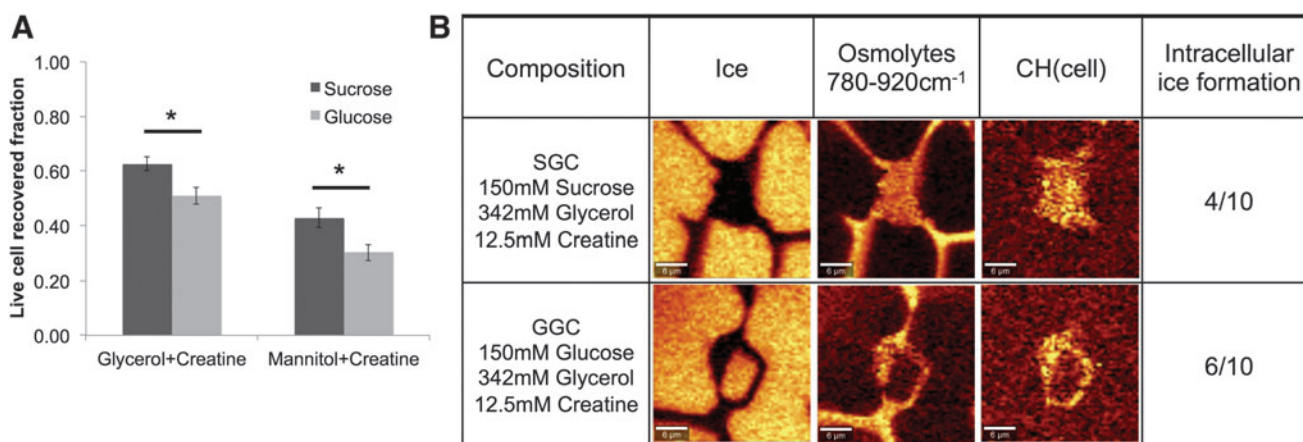
tigation suggest that longer incubation times may be needed for cells frozen with non-DMSO cryoprotectants to allow for penetration of larger components and elicit maximum cell recovery. Raman measurement of mannitol concentrations inside and outside the cell indicated that permeation for this molecule takes  $\sim 90$  min (data not shown), which is consistent with the results of this study. The observation that postthaw recovery increases and the prevalence of ice inside the cell decreases with increasing incubation time also suggests that the presence of osmolytes inside the cell is needed for protection against intracellular ice formation during freezing. This observation is consistent with previous studies of the protective effect of trehalose,<sup>37</sup> which demonstrated a similar outcome.

### Influence of osmolality and composition

It has been postulated that one mechanism of action for cryoprotective agents results from a colligative effect.<sup>38</sup> Specifically, higher concentrations of cryoprotectants reduce concentrations of damaging solutes and thereby protect the cell. In contrast, the outcome of this investigation suggests that there is little relationship between total osmolality of

TABLE 2. SOLUTION COMPOSITIONS FOR SUGAR SUBSTITUTION STUDIES IN FIGURES 5 AND 6

Solution name	Sugar (conc.)	Sugar alcohol (conc.)	Additive (conc.)
SMC	Sucrose (150 mM)	Mannitol (125 mM)	Creatine (12.5 mM)
GMC	Glucose (150 mM)	Mannitol (125 mM)	Creatine (12.5 mM)
SGC	Sucrose (150 mM)	Glycerol (2.5%)	Creatine (12.5 mM)
GGC	Glucose (150 mM)	Glycerol (2.5%)	Creatine (12.5 mM)



**FIG. 6.** Sugar substitution results in different recovery and ice formation behavior (A) postthaw recovery of MSCs cryopreserved in glycerol+creatine and mannitol+creatine solutions with either sucrose or glucose [ $n=9$ , error bars represent standard error of the mean, sucrose solutions had significantly higher recovery than glucose solutions ( $p<0.05$ )] (B) Low-temperature Raman microscopy of MSCs cryopreserved at  $3^{\circ}\text{C}/\text{min}$  in SGC and GGC concentrations listed. Images are rendered on ice, osmolyte mixture, and  $-\text{CH}$ . The fraction of cells with ice is consistent with cell recovery described in (A). Color images available online at [www.liebertpub.com/tec](http://www.liebertpub.com/tec)

the solution and postthaw recovery for multiosmolyte solutions (Fig. 2). Similarly, the solidification behavior of the solutions (crystallization patterns and DSC thermograms) for the different compositions tested does not exhibit significant differences from one SGC or SMC composition to the other. The results of this investigation (in particular Fig. 2) suggest that higher levels of cryoprotective agents are not necessarily better and that the colligative effect does not play a role in improving recovery with these solutions. However, combinations of additives are effective (Fig. 3), and optimized compositions may result in higher recovery. We are in the process of completing additional studies to prove that supposition.

Additionally, as shown in Figure 5, there was no change in the physical solution behavior at these low osmolarity solution concentrations below  $-30^{\circ}\text{C}$ . There is no eutectic peak indicating that a glass transition occurred in any of the samples at these concentrations (it would be expected around  $-80^{\circ}\text{C}$  if it existed). Because this physical change is absent, we hypothesize that the mechanism of protection for these solutions is biological. This suggests that there is a biological “sweet spot” solution composition that results in maximum cell recovery.

One reason these solution compositions may be successful could be the result of their interactions to stabilize proteins within the cell. Osmolytes are well known for stabilizing protein folding by providing a thermodynamically unfavorable environment for denatured proteins,<sup>39</sup> including during cryopreservation.<sup>40</sup> Additionally, mixtures of two different osmolyte monomers have been found to have better stabilization effects than that of a single type of monomer at the same molar concentrations.<sup>41</sup> Thus, literature supports the possibility that different osmolytes could stabilize different proteins and different parts of the cell to result in increased viability. Within mixtures of osmolytes, each osmolyte exerts independent protein stabilization effects.<sup>42</sup> This effect is due to the low binding affinity between osmolytes and proteins, resulting in neither competition nor cooperation between osmolytes.<sup>42</sup> Therefore, the activity of

each osmolyte should be considered individually without cooperation between them.

Different combinations of osmolytes may also stabilize the cell membrane. Both surface and internal membranes are main areas of injury regardless of cooling rate.<sup>43</sup> Normal cells have a layer of water surrounding the cell surface, which helps maintain surface protein folding.<sup>44</sup> During freezing, the concentration of liquid water is decreased, which can lead to destabilization of these proteins.<sup>44</sup> Combined with observations that membrane proteins are denatured and lost postthaw,<sup>44</sup> stabilization of proteins in the membrane is very important to the success of cryopreservation. Meryman proposed that conformational stability of surface macromolecules depends on the interactions of solute and water at the cell surface,<sup>44</sup> and osmolytes themselves have been postulated to replace water in the cell membrane during freezing.<sup>45</sup> Osmolytes also play a role in preferential exclusion, which has been associated with protein stabilization caused by increases in surface tension due to sugars and amino acids present in solution.<sup>46</sup> In the solutions used in this study, each component could differ in location (intracellular or extracellular), the macromolecules it stabilizes, and the surface area it stabilizes. These differences could help to protect multiple critical structures, thus decreasing the cumulative damage to the cell.

It is well established in literature that larger sugar osmolytes provide better stabilization.<sup>22,41,47</sup> due to their increased polar contact area.<sup>41</sup> Sugars can provide stabilization by replacing water surrounding membranes during dehydration.<sup>40,41</sup> The trend observed for improved disaccharide behavior may be due to insertion between the phospholipid heads of the lipid membrane, creating more space than monosaccharides for additional binding.<sup>47</sup> Binding of sugars to membranes can increase the rigidity of the membrane, which provides greater resistance to disruption.<sup>47</sup> Cells dehydrate at slow cooling rates, making stabilization with osmolytes important. During thawing, changes in the protein environment could induce denaturing,

but osmolytes, including sugars, can help with stabilization. This can reduce damage to membrane proteins, as well as internal proteins.

In a study that supplemented cryopreservation media with monosaccharides or disaccharides during freezing of boar sperm, glucose was found to be the most effective of the monosaccharides in maintaining postthaw sperm quality.<sup>48</sup> Gómez-Fernández *et al.* found that disaccharides containing the same monomers with different links had similar results.<sup>48</sup> Additionally, disaccharides are known for being better stabilizers than monosaccharides,<sup>41,48</sup> and this is supported by the results presented in Figure 6.

This work assesses postthaw recovery of cells frozen in non-DMSO solutions. Another pending publication from this group will address the postthaw functionality of MSCs in more depth, and includes data confirming that MSCs frozen in non-DMSO solutions maintain both multilineage differentiation ability, and appropriate surface marker expression (greater than 99% positive for CD73, CD90, and CD105 surface markers, and less than 1% positive for CD45 for both SMC and SGC frozen cells).

As the field of cryobiology expands to include new applications that require nontoxic alternatives to DMSO, it is important to understand the role of multicomponent solutions in preserving cell function. In this study, we demonstrate that incubation time must be sufficient for penetration of components, concentration of components (not total osmolarity) determines recovery, and molecular substitution results in changes in recovery. We hypothesize that these differences are biological, as we have found no evidence of physical changes in ice crystal formation between these solutions.

Ultimately, these observations increase our understanding of multicomponent solution behavior, and move the field closer to clinically acceptable DMSO alternatives for cryopreservation of MSCs and other cryosensitive cell types.

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### Disclosure Statement

No competing financial interests exist.

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## Appendix

TABLE A1. SMC COMPOSITION, RECOVERY, AND OSMOLARITY PLOTTED IN FIGURES 2 AND 3

<i>SMC Concentrations</i>				
<i>Sucrose (mM)</i>	<i>Mannitol (mM)</i>	<i>Creatine (mM)</i>	<i>Live cell recovered fraction</i>	<i>Osmolarity</i>
75	63	13	0.47	351
225	188	3	0.43	483
225	125	0	0.29	451
0	63	25	0.54	319
75	125	3	0.48	377
30	25	25	0.31	316
225	188	3	0.46	483
150	63	6	0.41	385
0	25	25	0.52	301
75	63	19	0.38	354
30	0	25	0.29	303
30	63	13	0.37	328
150	63	0	0.22	382
30	25	25	0.33	316
300	250	3	0.09	552
150	188	3	0.49	446
30	25	19	0.20	312
150	25	6	0.33	366
0	125	13	0.32	344
150	63	6	0.34	385
30	63	25	0.47	334
30	125	19	0.31	362
300	250	0	0.15	551
150	188	19	0.26	454
225	250	3	0.19	514

TABLE A2. SGC COMPOSITION, RECOVERY, AND OSMOLARITY PLOTTED IN FIGURES 2 AND 3

<i>SGC concentrations</i>				
<i>Sucrose (mM)</i>	<i>Glycerol (mM)</i>	<i>Creatine (mM)</i>	<i>Live cell recovered fraction</i>	<i>Osmolarity</i>
225	684	0	0.94	1184
300	342	0	0.78	917
30	342	25	1.02	672
300	0	25	0.78	600
300	171	19	0.59	765
0	684	19	0.64	978
75	68	19	0.53	437
30	171	25	0.36	501
75	68	25	0.38	443
225	684	3	0.67	1187
0	684	25	0.76	984
150	513	0	0.68	938
30	68	25	0.31	398
225	513	6	0.44	1019
75	684	13	0.46	1047
75	342	25	1.13	717
150	513	0	1.10	938
225	342	13	0.55	855
300	513	3	0.54	1091
150	684	3	0.43	1112
0	171	25	0.58	471
150	684	0	0.54	1109
0	68	25	0.33	368
150	342	6	0.30	773
30	684	19	0.45	1008
0	68	25	0.21	368
30	171	13	0.39	489
150	68	13	0.68	506
30	684	19	0.43	1008
30	171	25	0.41	501