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Cryopreservation of Mammalian Oocytes by Using Sugars: Intra- and extracellular raffinose with small amounts of dimethylsulfoxide yields high cryosurvival, fertilization, and development rates

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Abstract

Accumulation of intra- and extracellular sugars such as trehalose, glucose, and raffinose is central to survival strategies of a variety of organisms coping with extreme conditions including freezing and almost complete drying. The objective of the present study was to investigate the potential application of intra- and extracellular raffinose in combination with low concentrations of dimethylsulfoxide (Me₂SO) to mammalian oocyte cryopreservation. To this end, the fertilization and embryonic development of cryopreserved metaphase II (M II) mouse oocytes were studied in comparison to unfrozen controls. For cryopreservation, M II oocytes were microinjected with 0.1 M raffinose, and then cooled to -196°C in the presence of either 0.3 M raffinose and 0.5 M Me₂SO (cryopreservation group 1) or 0.3 M raffinose and 1.0 M Me₂SO (cryopreservation group 2). The control groups included untreated oocytes (untreated control) and oocytes microinjected with raffinose, but not frozen (injection control). The post-thaw survival rates were 83.9% and 80.6% for the cryopreservation group 1 and 2, respectively. The fertilization and blastocyst rates in the cryopreservation group 1 (90.0% and 77.8%, respectively) and 2 (94.6% and 72.5%, respectively) were also high and similar to the ones of the injection controls (97.8% and 78.5%, respectively) and untreated controls (98.8% and 83.6%, respectively). These results are consistent with the findings of our earlier studies and support the use of sugars as intra- and extracellular cryoprotectants. Furthermore, the results of the present study indicate that the presence of intra- and extracellular sugars alleviates high concentrations of conventional penetrating cryoprotectants, and thus minimizes their toxicity.

Keywords

Oocyte; cryopreservation; freezing; raffinose; sugar; microinjection; dimethylsulfoxide; glass transition temperature

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INTRODUCTION

Oocyte cryopreservation is needed to preserve future fertility of young cancer patients anticipating loss of their ovarian function as a result of radiation and chemotherapy. Current statistics suggest that 1 in 52 females under the age of 40 are diagnosed with cancer [35]. Furthermore, oocyte cryopreservation may also avoid many legal and ethical issues that are associated with human embryo freezing. Other applications of oocyte cryopreservation include banking of livestock oocytes for commercial and research purposes, as well as conservation of genetic material of endangered species.

While embryo freezing became a routine practice after the first successful cryopreservation of mouse embryos in the 1970s, oocyte cryopreservation has proven to be challenging. Although the first successful cryopreservation of human oocytes was achieved in the mid 1980s[8], it took a decade and the use of intracytoplasmic sperm injection (ICSI) to reproduce the initial success of human oocyte cryopreservation [39;56;74;77]. In recent years, both slow cooling [5;6;10;55] and vitrification techniques [2;40;46;78] have yielded encouraging results. However, the overall success rate of human oocyte cryopreservation is still unsatisfactory while vitrification techniques requiring direct contact between oocytes and liquid nitrogen pose a biosafety risk [4;73]. Therefore, further research is needed for more reliable protocols, as well as for alternative approaches.

In nature, a variety of organisms from some frogs and tardigrades to bacteria, yeast, and plant seeds efficiently use sugars such as glucose, trehalose, and raffinose to survive extreme drying and freezing [17;57]. Inspired by these survival schemes in nature, we have focused our efforts on sugar-based preservation methods. The protective effect of sugars has also been shown in a number of model systems including liposomes, membranes, and proteins during both air and freeze drying [14;15;16;18;59]. Until recently, sugars have been used as extracellular additives [44;48] due to impermeability of mammalian cell membranes to sugars. In recent years, several groups overcame the permeability barrier using different approaches such as thermotropic lipid-phase transition [3], reversible poration by a genetically engineered protein [23], transfection [30] and ATP poration [20], and demonstrated the beneficial effect of intracellular sugars with somatic cells. To introduce sugars into mammalian oocytes, we developed a quantitative microinjection technique because the aforementioned approaches were not practical for the oocytes due to their small surface-to-volume ratio [22]. Our study with mouse pronuclear eggs revealed the non-toxicity of microinjected trehalose at its effective concentrations up to 0.15M [22]. We were also able to obtain improved cryosurvival after microinjection of trehalose into failed-to-fertilize human oocytes [25]. More recently, using M II mouse oocytes, we have systematically addressed the cryoprotective effect of intra- and extracellular trehalose as a sole cryoprotectant as well as in combination with a conventional penetrating cryoprotectant Me₂SO and shown that the combination of intra- and extracellular trehalose with small amounts of Me₂SO results in high cryosurvival, fertilization and development rates [21]. Moreover, we have demonstrated the proof-of-principle by obtaining healthy offspring from cryopreserved mouse oocytes [21].

The aim of this study was to address whether another sugar, raffinose, can afford a similar protection when present intra- and extracellularly and combined with a conventional penetrating cryoprotective agent (CPA) (i.e., Me₂SO). In addition, the present study also addresses how different concentrations of Me₂SO affect the cryopreservation outcome in the presence of intra- and extracellular raffinose.

MATERIALS AND METHODS

Reagents and Media

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. A concentrated raffinose (Fluka) solution (i.e., 0.5 M) was prepared for microinjection into oocytes by dissolving pure raffinose in 15 mM HEPES (pH 7.4) that was prepared in ultrapure water. The concentrated raffinose solution was filtered using 0.22 μ m membrane filters, aliquoted, and then stored at -80°C. HEPES-buffered Hypermedium[21;22] containing 4 mg/ml bovine serum albumin (BSA) and 50 mg/ml gentamycin was used for manipulation of oocytes and embryos under air. Further, this medium was supplemented with various concentrations (i.e., 0.1 M and 0.3 M) of raffinose and galactose for manipulations related to cryopreservation as explained subsequently. Microinjection was also performed in this medium after supplementation with 0.10 M raffinose. For *in vitro* fertilization (IVF) and subsequent culture of non-injected and microinjected oocytes, Hypermedium with an osmolality of 315 to 320 mOsm was used to partially compensate the increased intracellular osmolality of raffinose-injected oocytes [22]. Our previous experiments revealed that the Hypermedium supports mouse embryonic development at least as well as other commonly used culture media [22]. Furthermore, our studies showed that fertilized mouse eggs cultured in Hypermedium can develop into healthy mice when transferred to pseudo pregnant females[21;22]. Before culturing oocytes and embryos, drops of the Hypermedium were overlaid by embryo-tested mineral oil and equilibrated overnight under a humidified atmosphere of 5% CO₂ in air at 37°C.

Oocyte Isolation

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Medical College of Georgia. Metaphase II (M II) oocytes were obtained from 4-8 week-old B6D2F1 (C57BL/6NCr1 X DBA/2NCr1; Charles River Laboratories, Wilmington, MA) hybrid mice. Superovulation was induced by a combination of 5 IU pregnant mare serum and 2.5 IU human chorionic gonadotropins (PG 600, Intervet, Millsboro, DE) followed by 7.5 IU human chorionic gonadotropin (hCG) alone 48-49 hours later. Both hormone solutions are given intraperitoneally. To collect M II oocytes, the oviducts were excised from euthanized mice 13 to 14 hours after hCG injection and oocyte-cumulus masses were released from the ampulla. To remove cumulus cells, the oocyte-cumulus masses were exposed to 120 IU /ml of bovine testis hyaluronidase (Type IV-S) at ambient temperature for 3-4 min. Next, the oocytes were washed in HEPES-buffered Hypermedium twice and then transferred to the Hypermedium for recovery before experimentation. For each experiment, M II oocytes were typically isolated from three or more female mice, pooled, and then randomly distributed among the experimental groups.

Microinjection of Raffinose

Quantitative microinjection of sugars was described in detail elsewhere [22]. Briefly, microinjection and holding pipettes were manufactured from 1-mm borosilicate thin-wall (B100-75-10, Sutter, Novato, CA) and thick-wall (B100-50-10, Sutter) glass capillaries, respectively. Both types of pipettes were pulled using a horizontal micropipette puller (Model P-97, Sutter). The holding pipettes were broken off at an external diameter of 70-80 μ m, and then fire-polished using an MF-900 microforge (Narishige, East Meadow, NY). To obtain a sharp tip with an inside diameter of 0.5 to 0.7 μ m, the injection pipettes were beveled at an angle of 40° on a modified Sutter micropipette beveler (BV-10, Sutter) and their tip diameter was determined by bubble pressure measurement in order to calibrate them. To keep the pipette tip clear of cell debris during microinjection, the injection pipettes were exposed to vapors of hexamethyldisilazene in a desiccator for 6 to 12 hours. Microinjection was accomplished using two identical sets of MMN-1 coarse mechanical and MMO-202D fine hydraulic

micromanipulators (Narishige) mounted on an inverted Diaphot 300 Microscope (Nikon, Melville, NY). To minimize vibration during microinjection, the micromanipulation system was setup on an antivibration table (TCM, Peabody, MA). A modified Stoelting piezo injector (PM-20, Stoelting, Chicago, IL) was used to facilitate puncture of the plasma membrane by a controlled sudden movement of the injection pipette. To deliver predetermined amounts of intracellular raffinose, a PLI-100 Pico-Injector (Medical Systems Co., Greenvale, NY) was used, and intracellular raffinose concentrations were confirmed by volumetric response of microinjected oocytes as described previously [22]. Oocytes that were degenerated after microinjection were easily identifiable using morphological criteria i.e., non-translucent appearance of cytoplasm, lack of integrity of the plasma membrane and the zona pellucida, and the size of the perivitelline space) and excluded from the experiments.

Experimental Groups and Oocyte Cryopreservation

To examine the cryoprotective effect of intra- and extracellular raffinose, approximately 0.1 M raffinose was microinjected into M II mouse oocytes based on our previous results [21; 22] while the extracellular concentration of raffinose was set to 0.3 M due to its solubility limitation. To test the hypothesis that the presence of an intra- and extracellular sugar obviates the use of high concentrations of conventional CPAs, and thus minimizes CPA toxicity [24; 33;37;42;75] without compromising the cryopreservation outcome, 1/3 (0.5 M) and 2/3 of the typical Me₂SO concentration (1.5 M) employed in slow-freezing protocols were also included in the cryopreservation medium. Some experimental groups (i.e., 0.5 M Me₂SO alone, extracellular raffinose+Me₂SO, and intra- and extracellular raffinose without Me₂SO) have been omitted in the present study to avoid the unnecessary use of mice based on our previous data showing that (i) 0.5M DMSO alone or in combination with an extracellular sugar yields either a poor survival rate or a poor blastocyst rate, respectively; and (ii) the presence of an intra- and extracellular sugar provides excellent survival and fertilization rates, but the subsequent development to the blastocyst stage remains unsatisfactory [21]. The control groups included untreated controls and injection controls that were microinjected with the same amount of raffinose but not frozen.

For cryopreservation, raffinose-injected M II oocytes were exposed first to 1:1 dilutions of cryopreservation media containing either 0.3 M raffinose+ 0.5 M Me₂SO or 0.3 M raffinose +1.0 M Me₂SO for 5 minutes, and then to the undiluted respective cryopreservation media for 10 minutes. Both exposures were performed at ambient temperature (~23°C), and during the final exposure time, the oocytes were aspirated into ¼ cc plastic straws (TS Scientific, Perkasi, PA). Next, the straws containing oocytes were placed in a programmable freezer (Planer Kryo 10, Planer PLC, Middlesex, UK) at 0°C and cooled to -6°C at a rate of 2°C/min. After seeding of extracellular ice and holding at -6°C for 10 min, the straws were cooled first to -60°C at 1°C/min and then to -70°C at 5°C/min where they were plunged into liquid nitrogen. For thawing, the straws containing oocytes were transferred to the controlled-rate freezer at -40°C after being held in air for 20 seconds and then slowly warmed up to 0°C at 5°C/min to reduce osmotic stresses. After thawing, the oocytes were released into HEPES-buffered Hypermedium containing 0.1 M galactose at ambient temperature where they were held for 7 min. Next, the oocytes were transferred to a fresh drop of Hypermedium plus 0.1 M galactose for 7 to 10 min before being transferred to the culture medium for a recovery period of 1h. Following the recovery period at 37°C, the post-thaw survival was assessed using the aforementioned morphological criteria and the rate of the cryosurvival was calculated based on the number of frozen oocytes. Subsequently, both cryopreserved and control oocytes were inseminated as described next to evaluate fertilization and embryonic development.

In Vitro Fertilization and Embryo Culture

In vitro fertilization (IVF) and culture of inseminated oocytes were carried out in Hypermedium at 37°C under a humidified atmosphere of 5% CO₂ in air. For IVF, sperm were obtained from the cauda epididymides of a mature (4-6 months old) BDF1 male (C57BL/6NCrl X DBA/2NCrl; Charles River Laboratories). The cauda epididymides were dissected and placed in a large drop (0.4 ml) of pre-equilibrated BSA-free Hypermedium. Sperm were released into the medium by gently puncturing the epididymides (5-7 times) with a hypodermic needle and allowed to disperse for 15 minutes at 37°C. After dispersion, sperm concentration was determined using hemocytometer. To give a final concentration of $1-2 \times 10^6$ sperm/ml, an appropriate volume of the sperm suspension was added to each insemination drop containing Hypermedium with BSA supplementation. The insemination drops were then incubated for 1-2 h in order to capacitate the sperm before introducing control and microinjected oocytes. After 5 to 6 h of incubation with sperm, all oocytes were washed twice in the Hypermedium, and then cultured in fresh drops of the same medium. Cleavage to the two-cell stage was examined after overnight culture while development to the blastocyst stage was evaluated after 5 days of culture. Fertilization and blastocyst rates were calculated based on the number of surviving and fertilized oocytes, respectively.

Statistical Analysis

The data were analyzed by ANOVA /Tukey's multiple comparison test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Before ANOVA, arcsine transformation was performed on proportional data. Differences between the groups were considered statistically significant when the p-value was less than 0.05. Experiments in each series were repeated at least three times. Data reported are means of survival, fertilization, and development rates with error bars representing standard error of mean.

RESULTS

To evaluate the cryoprotective effect of intra- and extracellular raffinose in combination with 0.5 M (cryopreservation group 1) and 1.0 M Me₂SO (cryopreservation group 2), a total of 348 M II oocytes were used in two cryopreservation and two control groups. Of these 348 oocytes, 88 oocytes were used as untreated controls while the remaining 260 were microinjected with ~0.1 M raffinose. Two hundred fifteen oocytes (83%) survived the microinjection procedure and were distributed between the injection control group (97), cryopreservation group 1 (62), and cryopreservation group 2 (56). The results of these experiments are summarized in Fig. 1. The cryosurvivals of the M II oocytes microinjected with ~0.1 M raffinose and subjected to cryopreservation in the presence of either 0.3 M extracellular raffinose+0.5 M Me₂SO (cryopreservation group 1) or 0.3 M extracellular raffinose+1.0 M Me₂SO (cryopreservation group 2) were similar with 83.9% and 80.6%, respectively. Likewise, the fertilization and embryonic development rates in the cryopreservation group 1 (90.0% and 77.8% respectively) and 2 (94.6% and 72.5% respectively) were comparable and statistically similar to those of the injection controls (97.8% and 78.5% respectively) and untreated controls (98.8% and 83.6% respectively), demonstrating a striking cryoprotective effect of intra- and extracellular raffinose with small amounts of Me₂SO.

DISCUSSION

This study demonstrates that raffinose, a non-reducing trisaccharide, affords a remarkable protection against cryopreservation-associated stresses when present intra- and extracellularly along with small amounts of Me₂SO. Furthermore, the results of the present study also indicate that the presence of intra- and extracellular raffinose greatly reduces the necessary concentration of a conventional penetrating CPA for an optimal cryoprotection, and

thus alleviates CPA toxicity. Considering the role of sugars in survival strategies of a diversity of organisms (e.g., certain frogs, nematodes, tardigrades, insects, bacteria, yeasts, and as well as plant seeds) under extreme drying and freezing conditions [17;57], the results presented here are in accord with the findings of previous studies revealing the beneficial effect of intra- and extracellular trehalose in cryoprotection of mammalian oocytes [21;25] and somatic cells [3; 23].

The ultimate goal of any cryopreservation technique is to safely bring cells below the glass transition temperature where the cells and surrounding medium vitrify (i.e., convert to a glassy state). Consequently, a high glass transition temperature can greatly facilitate any cryopreservation procedure. As shown in Table 1, sugars are more effective glass formers than conventional penetrating CPAs such as Me₂SO, ethylene glycol (EG), and 1,2-propanediol (PROH). Further, there seems to be a strong correlation between the glass transition temperature and molecular weight (MW), particularly at low MW ranges (Fig. 2). Hence, non-reducing disaccharides (e.g., trehalose) and trisaccharides (e.g., raffinose) represent better alternatives to the conventional CPAs in this respect. In addition, sugars also afford their protection (i) by stabilizing lipid membranes as a result of direct interactions with polar residues through hydrogen bonding (so-called “water replacement hypothesis”) [14;15;19]; (ii) by preventing aggregations of intracellular proteins [71;72]; and (iii) by acting as osmolytes against osmotic stresses [65;68], as well as, against chemical [67] and hypoxic stresses[9]. The direct interactions are particularly important in case of extreme cell dehydration because sugars such as trehalose hydrogen bond to proteins and lipid head groups, and serve as a replacement for the lost water in the dehydrated state [14;15;19]. For such direct interactions with polar lipid head groups, sugar molecules must be sufficiently small, yet they also have a high glass transition temperature. Trehalose fits well into this category as experimentally shown [18; 19]. In contrast, smaller sugars such as glucose or their polymers such as dextran cannot provide optimal protection due to their poor glass forming properties or inability to directly interact with polar lipid head groups through hydrogen bonding, respectively [13]. Considering these aspects, the rationale beyond the selection of raffinose for testing in the present study was the following: (i) as trehalose, raffinose is a non-reducing sugar, (ii) a good glass former as shown Table 1 and Fig. 2, and (iii) small enough (595 Da) to directly interact with biomolecules. In fact, it has been shown that raffinose hydrogen bonds to the lipid head groups although less stably than trehalose [18]. Nevertheless, unlike extreme drying, freezing does not result in removal of almost all water molecules in and around the cell. Therefore, cell freezing may not require optimal direct interactions between the lipid head groups and a protective sugar molecule to prevent the phase transition of the cell membrane from a liquid crystalline to a gel-like state, which would lead to a leaky membrane and cell death. Indeed, the high cryosurvival, fertilization, and blastocyst rates obtained in the present study using raffinose support this notion.

It is important to note that there is an ongoing debate on the glass transition of maximally freeze-concentrated solutions (T_g') and the corresponding solute concentration (C_g') [1;45; 60;62;66]. The presence of ice complicates measurements of subzero thermal events, and hence interpretation of resulting thermograms. Some studies suggested that incipient ice melting above the T_g' produces a thermal event that might have been misinterpreted as T_g' [1;60;61]. Yet others explained these thermal events by two distinct glass transitions based on modulated temperature differential scanning calorimetry (MT-DSC) and scanning electron microscopy studies[27] while earlier studies identified another thermal event around T_g' called “ante-melting”[47;58]. The controversy surrounding T_g' has extensively been discussed in the literature, but no consensus has been reached so far [28;38;43]. Readers may check relevant publications for further detail [1;28;38;43;45;61;66]. While it is important to understand the exact nature of these subzero thermal events, these issues are beyond the scope of this study. Nevertheless, it can be pointed out that from a practical standpoint, these thermal events (T_g'

and incipient ice melting in its vicinity) mark a temperature zone of extreme viscosity, below which a stable storage of biological samples/food products is possible. In the present study, efforts have been made to cite the glass transition temperature of freeze-concentrated CPA solutions from diverse studies. Also, a long general term (i.e., “glass transition temperature of freeze-concentrated solutions”) has been used in this study instead of T_g because the maximal freeze-concentration is hard to achieve and was not the primary objective of some of the cited studies while the freeze-concentration to different extents is always an essential part of slow cooling protocols.

For oocyte cryopreservation, conventional penetrating CPAs are typically used at moderately high concentrations (i.e., 1.5 M) in case of slow cooling and at very high concentrations (e.g., 6 M) to achieve vitrification. An early study by Schroeder et al. [63] represents an exception to this by demonstrating reasonably successful cryopreservation of mouse oocytes using a slow cooling protocol and 1.0 M DMSO. In the present study, the combination of intra- and extracellular raffinose with 1/3 (0.5 M) and 2/3 (1.0M) of typical conventional CPA (Me_2SO) concentration (i.e., 1.5 M) employed in slow cooling protocols yielded cryosurvival, fertilization and embryonic developmental rates that are similar to those of unfrozen controls and comparable to the best results in the literature achieved by using much higher cryoprotectant concentrations [7;11;41;53;69]. This indicates the outstanding efficiency of sugars along with small amounts of a conventional CPA in cryopreserving mammalian oocytes. Further, the results of this study show that in the presence of intra- and extracellular raffinose, the lower concentration (i.e., 0.5 M) of Me_2SO , which alone cannot provide an adequate cryoprotection, is sufficient for an excellent cryoprotection. The use of lower concentrations of penetrating CPAs is advantageous in terms of avoiding/reducing the potential toxicity of such CPAs [24;33;37;42;75]. Another advantage of the use of lower CPA concentrations is lesser depression of the elevated glass transition temperature based on the presence of sugar molecules. Indeed, our recent study shows that the presence of 0.5 M Me_2SO depresses the glass transition temperature of 0.5 M trehalose/medium mixture from $\sim 33^\circ\text{C}$ to $\sim 42^\circ\text{C}$ as shown also in Table 1. Yet even the depressed glass transition temperature is much higher than that of conventional penetrating CPAs (Table 1) and can greatly facilitate safer cooling and warming of oocytes. Moreover, such a high glass transition temperature would in theory allow the cryostorage of oocytes at a -80°C freezer and transport on dry ice if the intracellular glass transition also occurs at a similarly high subzero temperature. Considering the highly concentrated state of macromolecules and sugar/CPA in the oocyte cytoplasm as a result of freezing-induced cell dehydration, it is highly likely that this will be the case.

It should also be noted that the impermeability of mammalian cell membrane to sugars such as trehalose and raffinose represents a practical disadvantage for their use as an intracellular cryoprotectant. Design and development of cell-permeable sugars, which requires further comprehensive research, would be an ideal solution to this issue. Nevertheless, microinjection that is routinely used in fertility clinics can also be used with relative ease to introduce sugars into oocytes as demonstrated in several studies [21;22;25] while the results of this work and a recent study [21] suggest that a second microinjection (i.e., ICSI) may not be necessary to fertilize oocytes cryopreserved using sugars.

During the course of our studies involving mouse and human oocytes, we noticed that human M II oocytes are easier to microinject and more tolerant to microinjection-associated stresses than mouse MII oocytes. When microinjected properly, all human oocytes survive the microinjection procedure [25] that can be carried out using a setup typically found in fertility clinics. In contrast, successful microinjection of sugars into mouse M II oocytes requires a piezo injector and siliconized microinjection pipettes with a tip diameter less than $1\ \mu\text{m}$. The tip of non-siliconized microinjection pipettes quickly becomes sticky after injecting a few oocytes and accumulates debris, which in turn lead to degeneration of microinjected oocytes.

Using properly siliconized pipettes and a piezo injector, we were able to obtain a post-injection survival rate of 96% in our previous study [21]. In the present study, a new batch of hexamethyldisilazene was used. However the new batch was not as effective as the previous one in keeping the pipette tip clear of debris. Consequently, the oocyte survival following the microinjection (83%) was lower in the present study. This indicates the importance of a properly prepared microinjection pipette for successful injection of mouse M II oocytes.

Taken together, the results of the present study are consistent with the survival schemes in nature and support the use of sugars as intra and extracellular protectants for oocyte cryopreservation.

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Figure 1. Cryosurvival, fertilization, and embryonic development of mouse oocytes after cooling to -196°C . The survival rates represent mean $\pm$ s.e.m. There was no significant difference between the groups ($p > 0.05$).

Figure 2. Molecular weight (MW) dependence of glass transition temperatures. Mean $\pm$ s.e.m of the glass transition data in Table 1 was plotted against the reciprocal MW.

Table 1

Glass transition temperature of freeze-concentrated solutions of some sugars and conventional penetrating CPAs^a

Molecule	MW	Glass Transition Temperature and Related References	
Water	18	-135°C	[29;36]
Glycerol	92	-84°C	[34]
		-87°C	[47;70]
		-93°C	[26]
		-95°C	[1]
		-97 to -101°C	[50]
Ethylene glycol	62	-115°C	[51]
1,2-Propanediol	76	-101°C	[51]
		-104°C	[54;76]
Dimethylsulfoxide	78	-102°C	[51]
Glucose	180	-43°C	[45]
		-53°C	[60]
Galactose	180	-42°C	[45]
		-51°C	[60]
		-58°C	[64]
Sucrose	342	-32°C	[21;31;45]
		-40°C	[1;61]
Trehalose	378	-22°C	[49]
		-28°C	[21]
		-30°C	[12;45;52]
		-32°C	[32]
Raffinose	595	-27°C	[45]
0.5 M Trehalose+0.5 M Dimethylsulfoxide		-42°C	[21]

^a Note that cooling rates in some of the cited references do probably not result in maximally freeze-concentrated solutes, but might be close. Reported values are the midpoint of the glass transition. Also, some of the measurements were performed in the presence of buffered salt solutions/media instead of binary solutions.