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Cryopreservation of Adherent Smooth Muscle and Endothelial Cells with Disaccharides

Lia H. Campbell[1] and Kelvin G.M. Brockbank[1,2,3]
[1]*Cell & Tissue Systems, Inc., North Charleston, SC*
[2]*Georgia Tech / Emory Center for the Engineering of Living Tissues, The Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA*
[3]*Medical University of South Carolina, Department of Regenerative Medicine and Cell Biology, Charleston, SC*
USA

1. Introduction

There is a need for mammalian cell cryopreservation methods that either avoid or improve upon outcomes employing dimethyl sulfoxide (DMSO) as a cryoprotectant. DMSO was the second effective cryoprotectant to be discovered (Lovelock, 1959). Cell cryopreservation usually involves slow rate freezing with DMSO in culture medium and storage below -135°C for later use. Typically as long as there are enough cells surviving to start an expanding proliferating culture the yield of viable cells after thawing is not an important consideration. However, there are instances where cell yield and viability can be very important. Examples include minimization of expensive delays when starting cultures for bioreactor protein manufacturing runs and cellular therapies that involve administering cells into patients for treatment of various diseases, such as cancer. While some cells, for example fibroblasts, are easily cryopreserved other cell types like keratinocytes, hepatocytes, and cardiac myocytes do not freeze well and cell yields are often <50%. Furthermore, current opinion is that DMSO should be removed before cells are infused into patients (Caselli et al., 2009; Junior et al., 2008; Mueller et al., 2007; Otrock et al., 2008; Schlegel et al., 2009). The mechanism for DMSO cytotoxicity has not been determined, however, it is thought to modify membrane fluidity, induce cell differentiation, cause cytoplasmic microtubule changes and metal complexes (Barnett 1978; Katsuda et al., 1984, 1987; Miranda et al., 1978). DMSO also decreases expression of collagen mRNAs in a dose-dependent manner (Zeng et al., 2010).

One strategy for finding interesting new cryoprotectants and cryopreservation strategies is by evaluating what happens in nature (Brockbank et al., 2011). No examples of organisms synthesizing DMSO to survive freezing conditions have been found to date, however several creatures have been found that employ glycerol (Brockbank et al., 2011) the first effective cryoprotectant to be discovered (Polge, 1949). Nature has developed a wide variety of organisms and animals that tolerate low temperatures and dehydration stress by accumulation of large amounts of disaccharides, particularly trehalose, including plant seeds, bacteria, insects, yeast, brine shrimp, fungi and their spores, cysts of certain crustaceans, and some soil-dwelling animals. While the cryoprotective capabilities of

sucrose and trehalose has been known for years, conventional cryopreservation protocols have generally not employed them even though early work with them demonstrated their ability to protect proteins and membrane vesicles during freezing (Rudolf & Crowe, 1985; Crowe et al., 1990). Trehalose has both major advantages and disadvantages for potential preservation of mammalian cells. On the negative side mammalian cells do not have an active trehalose transport system for uptake of trehalose from the extracellular environment, while on the plus side if you can get it in mammalian cells it is not metabolized giving the opportunity for trehalose to be accumulated to potentially effective preservation concentrations. The purpose of the studies presented here were; 1) to assess or review alternative strategies for delivery of trehalose into mammalian cells, and; 2) to determine whether the benefits were specific to trehalose by investigating alternative sugars employing the same loading strategies.

2. Materials and methods

2.1 Cell culture

Cells used in these studies are described in Table 1. All were grown and maintained at 37^{o}C in 5% carbon dioxide.

Description	Acronym	Culture conditions
Rat aortic myofibroblast cells	A10 (ATCC# CRL-1476)	DMEM* (4.5 g/L) 10% FCS 1.0 mM sodium pyruvate
Bovine calf pulmonary artery endothelial cells	CPAE (ATCC# CCL-209)	EMEM** 10% FCS 1 mM sodium pyruvate 2 mM glutamine 1X non-essential amino acids
Rat aortic smooth muscle cells	A7R5 (ATCC# CRL-1444)	DMEM (4.5 g/L) 10% FCS 1.0 mM sodium pyruvate
Bovine corneal endothelial cells	BCE (ATCC# CRL-2048)	DMEM (4.5 g/L) 10% FCS 1 mM sodium pyruvate 4 mM glutamine

*Dulbecco's Modified Eagle's Medium
**Eagle's Minimum Essential Medium

Table 1. Cell types

2.2 Cell poration with H5

The pore-forming protein H5 was obtained from the lab of Hagan Bayley (Bayley, 1994). It is derived from the bacterial toxin α-hemolysin, which forms constitutively opened pores in cell membranes. The modified bacterial toxin has been engineered to form pores in the membrane that can be opened and closed by the addition of Zn^{+}. More specific details are

presented in the discussion. Cells were plated at 10,000-20,000 cells/well the night before in 96 well microtiter plates. The next day, the cells were washed with DMEM containing 1 mM EDTA for 2 minutes and then again with DMEM to remove the EDTA. 0.2M trehalose was added and incubated for 20 minutes at 37°C followed by the appropriate concentration of H5 for the respective cell type. Cells were porated and loaded with trehalose for 1 hour at 37°C before addition of DMEM with 25 µM $ZnSO_4$ or 10% serum to close the pores. Trehalose in DMEM was then added to the wells followed by cryopreservation using a controlled rate freezer (Planar) at ~-1.0°C/min from 4°C to -80°C with a programmed nucleation step at -5.0°C. Cryopreserved cells were stored overnight at <-135°C. The next day, the cells were placed at -20°C for 30 minutes followed by rapid thawing at 37°C (Campbell et al., 2003; Taylor et al., 2001). The cell cultures were washed twice and then placed at 37°C for 1 hour to recover under normothermic cell culture conditions before assessment of cell viability.

2.3 Pretreatment (Incubation) with trehalose

Cells were plated at 10,000-20,000 cells/well and placed in culture. The next day, the culture medium was replaced with EMEM or DMEM containing trehalose (0-0.6M) and cultured at 37°C for varying periods of time. After culture, the solution was replaced with fresh medium containing trehalose (0-0.6M) and the cells were cryopreserved using a controlled rate freezer as described for H5 above.

2.4 Cell poration with ATP

Cells were plated at 10,000-20,000 cells/well and placed in culture. The next day, the cells were washed with poration buffer (phosphate-buffered saline [PBS] with 1X essential amino acids, 1X Vitastock, 5.5 mM glucose) designed to optimize binding of ATP^{4-} to the receptor and facilitate formation of the pore. The cells were then placed in 50 µl poration buffer, pH of 7.45, with 0.2M trehalose. A stock solution of 100 mM ATP^{4-}, pH of 7.45, was made fresh and added to each well to achieve a final concentration of 5 mM. After addition of the ATP^{4-}, the cells were left at 37°C for 1 hour to allow sugar uptake. Following incubation, 200 µl of DMEM plus 1 mM $MgCl_2$ was then added to the cells at 37°C to close the pores. After 1 hour of recovery from the loading procedure cryopreservation was initiated.

2.5 Assessment of cell viability

Cell viability was determined using the non-invasive metabolic indicator alamarBlue™ (Trek Diagnostics). A volume of 20 µl was added to cells in 200 µl of DMEM (10%FCS) and the plate was incubated at 37°C for 3 hours. Plates were read using a fluorescent microplate reader (Molecular Dynamics) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Viability was measured before and after sugar loading, immediately after thawing and at several later time points post-thaw.

2.6 Statistical methods

All experiments were repeated at least four times with four replicates in each experiment. Statistical differences were assessed by two way analysis of variance. P-values <0.05 were regarded as significant.

3. Results

3.1 H5 poration

One of the first strategies for utilizing disaccharide sugars as cryoprotectants involved the use of a modified pore forming complex. In our initial studies we evaluated the H5 mutant α-hemolysin (Bayley, 1994) using two adherent cell lines, A10 and CPAE. The earlier studies had been done with cells in suspension (Eroglu et al., 2000). We also evaluated sucrose, another disaccharide sugar that is commonly found in nature, for its potential usefulness as a cryoprotectant. Using the protocol of Eroglu et al as a starting point, a protocol was established for adherent cells. Several parameters were evaluated and included the H5 concentration, time of poration, concentration of trehalose loaded, and time for loading trehalose. Conditions that worked best with adherent cells included 20 minutes for poration followed by 60 minutes for trehalose loading. The highest concentration of trehalose that caused the least drop in cell viability was 0.2M. The optimum H5 concentration varied according to cell type. The A10 smooth muscle cells were porated with 12.5 μg/mL of H5 while the endothelial CPAE cells were porated with 50 μg/mL. In contrast, the fibroblasts and keratinocytes in the literature were porated with 25 μg/mL (Eroglu et al., 2000). Other changes to the protocol were made that benefited viability for adherent cells specifically and included addition of trehalose prior to the addition of H5, the base solution used for poration, and the amount of EDTA (1 mM versus 10 mM) for the removal of Zn^{+} prior to poration. After cryopreservation, however, poor viability was obtained with both cell types. A10 cells demonstrated a viability of 5.57±0.17%. The endothelial cells demonstrated similar viabilities. These values were not as good as those observed when suspended cells were cryopreserved with sugars in the literature. However, it is our experience that adherent cells are generally more difficult to cryopreserve regardless of the cryoprotectant used.

3.2 Trehalose exposure without poration

When we started adding the trehalose to cells in the H5 experiments, control cells were exposed to trehalose by addition to the culture medium prior to cryopreservation. An unanticipated observation of cell survival was made with slow rate cryopreserved CPAE cells prompting further investigation. The cells exposed to trehalose overnight were observed to develop vacuoles (Fig 1) suggesting a possible pinocytotic uptake mechanism.

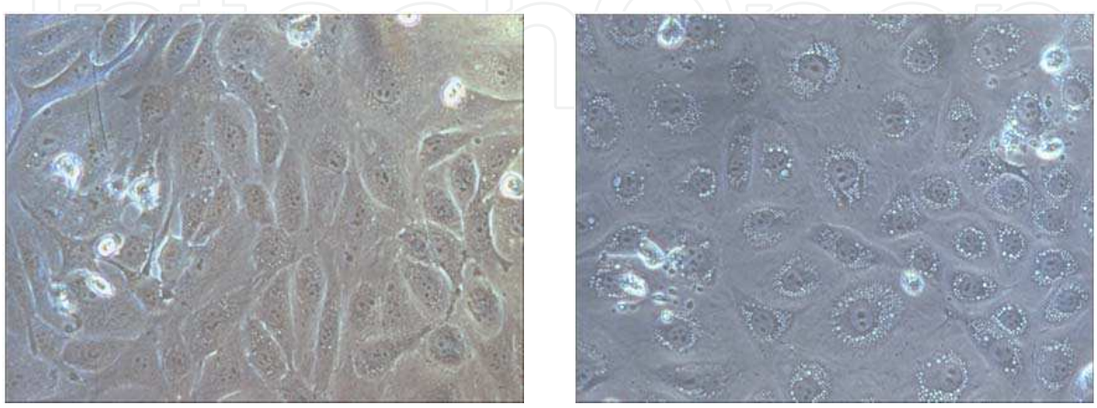


Fig. 1. CPAE cells after exposure to trehalose. Left: no sugar, Right: 0.1M trehalose. 40X magnification

After these observations were made, further experiments were designed to examine cell viability after extended trehalose exposure. CPAE cells were exposed to 0.2M trehalose in Dulbecco's Modified Eagle's Medium (DMEM) buffered with 25 mM Hepes for 0-72 hours at 37°C. After exposure the cells were left in 0.2M trehalose and cryopreserved at ~-1.0°C/min (Fig. 2). CPAE cell viability was observed immediately after thawing. An exposure time of 24 hours provided the best overall cell survival. Extracellular exposure alone during cryopreservation failed to produce any cell survival. In contrast, A10 smooth muscle cells generally did not survive cryopreservation after trehalose exposure as well as the CPAE endothelial cells. Examination of optimal concentrations of trehalose during incubation and during cryopreservation showed that a concentration of 0.1-0.2M trehalose for incubation produced the best viability with a similar concentration being required during the freezing process.

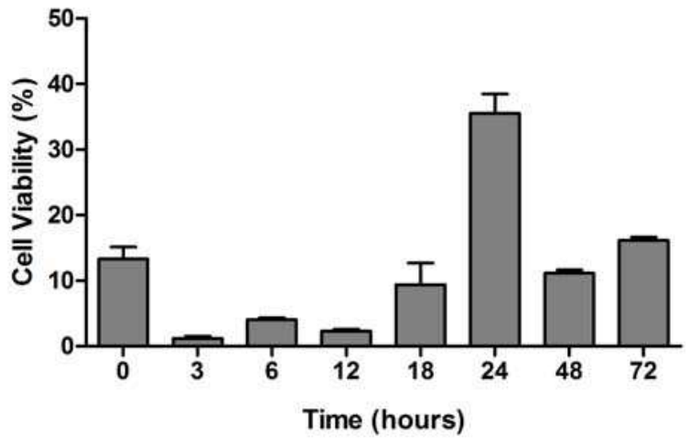


Fig. 2. Impact of cell culture time with trehalose on cryopreservation. CPAE cells were cultured with 0.2M trehalose for up to 72 hours followed by cryopreservation with 0.2M trehalose. Percent viability was calculated based on the pre-cryopreservation controls. ($p<0.05$)

Several other parameters were also examined to further improve cell viability. Other studies have shown that not only the concentration and choice of cryoprotectant but also the vehicle solution for the cryoprotectant can have a significant impact on cell viability after cryopreservation (Campbell & Brockbank, 2007; Mathew et al., 2004; Sosef et al., 2005). Initial experiments were performed using DMEM, however, it was observed that CPAE cells, which are grown in EMEM medium, actually preferred exposure to trehalose in EMEM medium. Further experiments examined the buffers used to maintain the pH of the system. Four cell lines were evaluated. CPAE cells demonstrated decreased viability when the zwitteronic buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used while the other 3 cell lines did not show decreased viability. Rather a combination of HEPES and sodium bicarbonate was preferred by the CPAE cells. This unusual choice of buffer prompted examination of solution pH during incubation, a pH of 7.4 was optimal for all the 4 cell lines tested. Once loaded with sugar, the cells could either be left in the extracellular sugar at another concentration or an alternative cryoprotectant for preservation.

These studies were then extended to include other sugars, sucrose, raffinose, and stachyose (Fig. 3). The potential cryoprotective benefits of these sugars were evaluated and it was found that stachyose was as good as trehalose using an identical protocol, sucrose was not quite as good and raffinose had very little benefit. All cell lines showed evidence of some cell survival days after cryopreservation and thawing. The second smooth muscle cell line, A7R5, demonstrated low levels of viability with stachyose. Both endothelial cell lines, CPAE and BCE, showed good viability after exposure and freezing with sucrose. Overall, the CPAE cell line had the best viability in these experiments. Use of an optimized protocol with trehalose produced excellent post cryopreservation results with 10-14mM intracellular trehalose (Campbell, 2011). Conditions included 24 hours of cell culture with 0.2M trehalose followed by cryopreservation with 0.2-0.4M trehalose in sodium bicarbonate buffered EMEM at pH 7.4 resulting in ~75% post-preservation cell viability (Campbell et al., 2011).These experiments confirmed that this technique is more effective for endothelial cells than smooth muscle cells and demonstrated that stachyose is effective for cryopreservation.

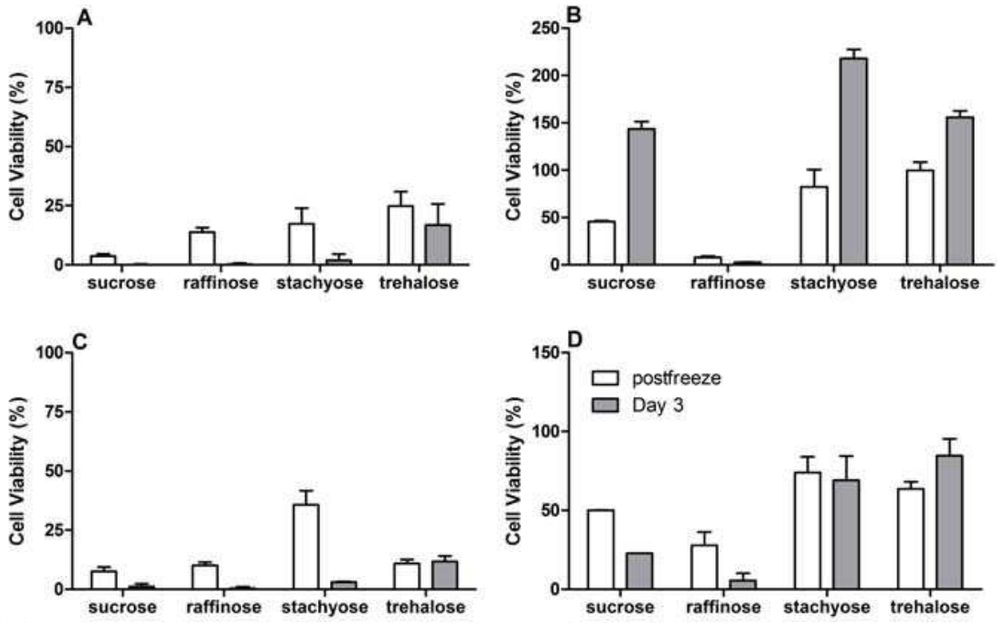


Fig. 3. Cell viability for A10 (A), CPAE (B), A7R5 (C) and BCE (D) cells after exposure and freezing in the presence of sugars.

3.3 ATP poration

In addition to the H5 mutant α-hemolysin poration strategy, we sought other poration techniques that could be used to permeate mammalian cells with disaccharides. Cells expressing the $P2_{X7}$ purinergic cell surface receptor, also known as the $P2_z$ receptor, may be permeabilized by the formation of a channel/pore that allows passage of molecules into and out of the cell when the active form of ATP (ATP^{4-}) binds to the receptor. Our initial studies focused on determination of whether or not the $P2_{X7}$ was expressed on smooth muscle and endothelial cells. Experiments using the ELICA assay demonstrated the presence of the $P2_{X7}$ receptor on both endothelial and smooth muscle cell lines to varying degrees (Fig 4). The smooth muscle cell lines demonstrated the greatest density of the receptor.

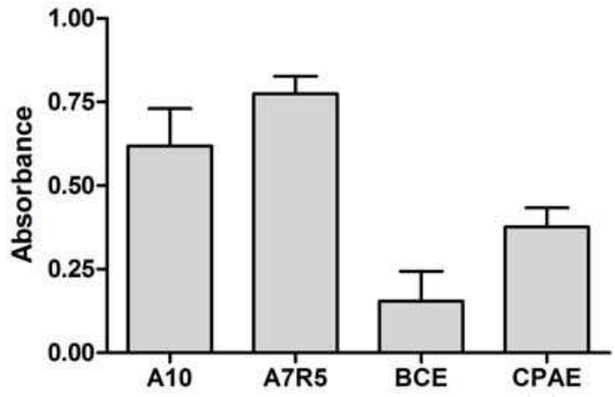


Fig. 4. Detection of the $P2_{X7}$ receptor by ELICA. Cells were probed for the presence of the $P2_{X7}$ receptor using antiserum specific for the receptor at a dilution of 1:25. The graph represents the average absorbance (±SEM) for 10 replicates.

ATP-permeabilized cells retained better viability than untreated cells both immediately after thawing and five days later (Fig 5). Immediate metabolic activity in A7R5 and CPAE cells demonstrated dependence upon increasing ATP concentrations, while for A10 and BCE cells immediate metabolic activity was increased at all ATP concentrations with only slight improvement at the higher concentrations tested. However, survival at five days demonstrated that intermediate concentrations of ATP (0.5-2.5mM) were best. Further cryopreservation studies were performed to optimize cell survival resulting in at least 25% cell survival for both endothelial cell lines but only low levels of survival for the smooth muscle cells.

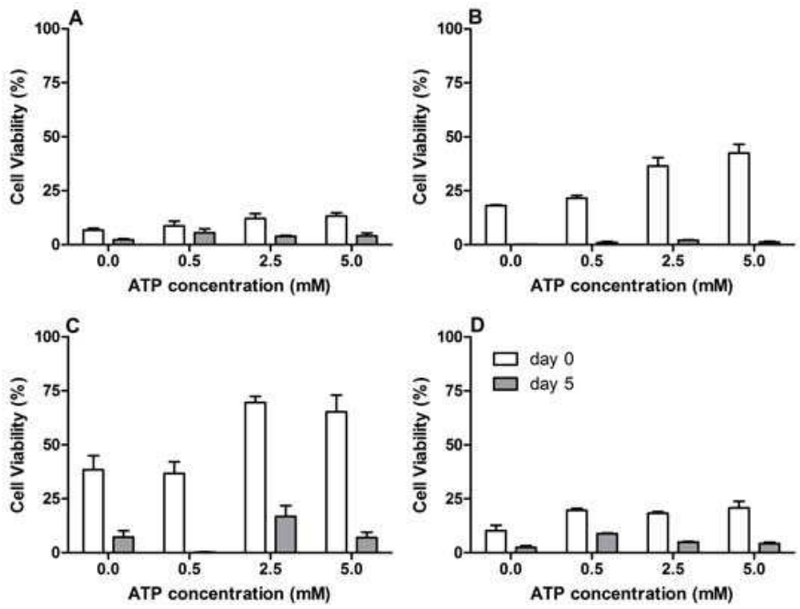


Fig. 5. Cell viability after poration and freezing with increasing concentrations of ATP. Cells were loaded with 0.2M trehalose using the $P2_{X7}$ receptor and the indicated concentrations of ATP. After poration and cryopreservation, cell viability was evaluated by alamarBlue. (A) A10, (B) A7R5, (C) CPAE, (D) BCE.

4. Discussion

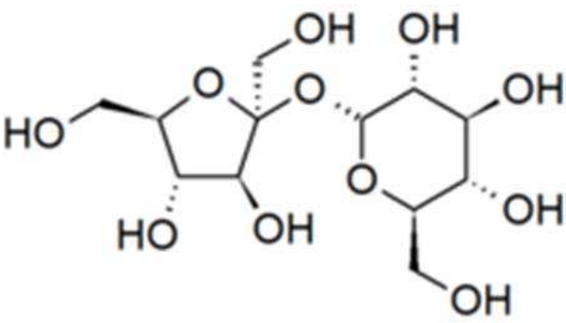
As cryopreservation has been applied to cells and tissues for clinical use, concerns about toxicity relating to the various cryoprotectants being used, particularly DMSO, have developed. Because of this, there has been renewed interest in finding less toxic cryoprotectants. The cryoprotective capabilities of some sugars, disaccharide sugars in particular, has been known for years and early work with them demonstrated their ability to protect proteins and membrane vesicles during freezing (Crowe et al., 1990; Rudolph & Crowe, 1985). Coupled with these early studies are observations made in nature regarding organisms that can survive extremes in temperature and desiccation due to their ability to accumulate large amounts of disaccharide sugars, specifically trehalose and sucrose, until more favorable conditions are available. The protective effects of trehalose and sucrose have been determined and may be classified under two general mechanisms: (1) "the water replacement hypothesis" or stabilization of biological membranes and proteins by direct interaction of sugars with polar residues through hydrogen bonding, and (2) stable glass formation (vitrification) by sugars in the dry state (Crowe et al., 1987, 1988, 1998, 2001; Slade & Levine, 1991).

Two primary stresses that destabilize membranes have been defined, fusion and lipid phase transition. Studies have shown that when the water that hydrates the phospholipid molecules of the membrane is removed, packing of the head groups increases. The result is an increase in van der Waals interactions and a dramatic increase in the phase transition temperature (T_m) (Crowe et al., 1987, 1988, 1990, 1991). At the phase transition the phospholipid bilayer shifts from a gel phase to a liquid crystalline phase, the state normally observed in fully hydrated cells. For example, the T_m of a cell membrane might be -10^{o}C when fully hydrated but when water is removed the T_m increases to over 100^{o}C. Thus, the membrane is in the gel phase at room temperature. As the membrane shifts between the gel phase and the liquid crystalline phase it becomes transiently leaky allowing its intracellular contents to leak out. Therefore it would be advantageous to avoid the lipid phase transition as this can compromise the health of a rehydrated cell. Addition of disaccharide sugars, in particular trehalose, depresses T_m allowing the membrane to remain in the liquid crystalline state even when dried, so that upon rehydration no phase transition takes place and no transient leaking. During cryopreservation water is not necessarily lost, but it undergoes a phase change forming ice as the temperature drops and depending upon the rate of cooling, the cells become more or less dehydrated rendering the cells vulnerable to damage by mechanisms similar to those proposed for desiccated cells.

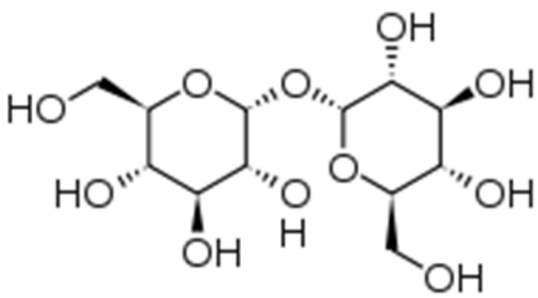
The stabilizing effect of these sugars has been shown in a number of model systems including liposomes, membranes, viral particles, and proteins. The mechanism by which disaccharide sugars are able to decrease the T_m for a given bilayer has been elucidated. Interactions take place between the sugars and the –OH groups of the phosphate in the phospholipid membrane preventing interaction or fusion of the head groups as the structural water is removed (Crowe et al., 1986, 1988, 1989a, 1989b). Although not as well understood, a similar mechanism of action stabilizes proteins during drying (Carpenter et al., 1986, 1987a, 1987b, 1989). Despite their protective qualities, the use of these sugars in mammalian cells has been somewhat limited mainly because mammalian cell membranes are impermeable to disaccharides or larger sugars and there is strong evidence that sugars need to be present on both sides of the cell membrane in order to be effective (Crowe et al.,

2001; Eroglu et al., 2000; Beattie et al., 1997). This is why, in addition to loading sugars, we added sugars to the cryopreservation solution just before initiating cooling.

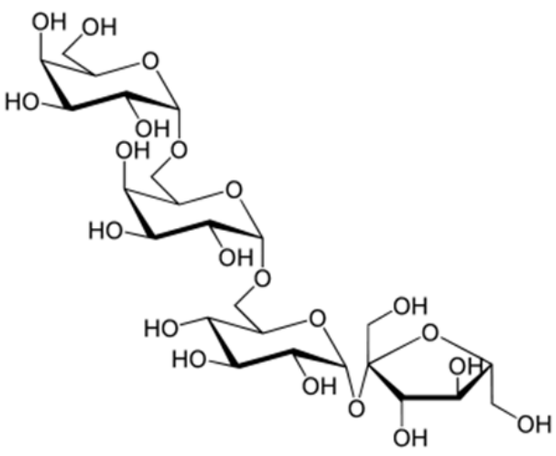
In addition to trehalose and sucrose, we were interested in other sugars that could be used as cryoprotectants avoiding monosacharides that would likely be degraded in the cell. Larger more complex sugars such as disaccharides or larger would be less likely to be degraded and utilized inside cells and might therefore be more stable as cryoprotectants. The comparative structures of the sugars we considered for preservation of mammalian cells are illustrated in Figure 6. Three other sugars were evaluated besides trehalose and included sucrose, raffinose and stachyose. Sucrose and trehalose are both non-reducing sugars, so they do not react with amino acids or proteins and should be relatively stable under low pH conditions and at temperature extremes. Raffinose is a trisaccharide and stachyose is a tetrasaccharide.



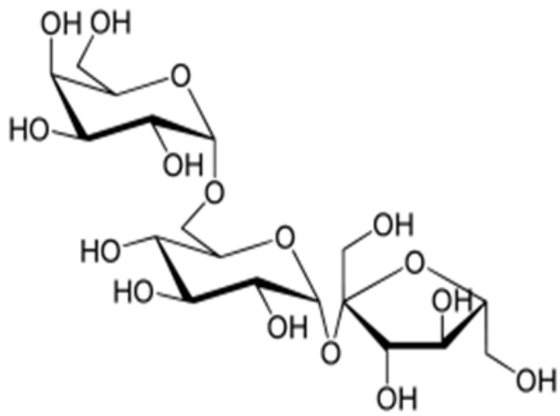
Sucrose (mw: 342.30)



Trehalose (mw: 342.30)



Stachyose (mw: 684.59)



Raffinose (mw: 504.44)

Fig. 6. Sugar structure

Before going further, it is important to point out that the cells we have employed were cryopreserved and thawed while adherent in 96-well plates using cooling and warming conditions defined and reported at the turn of the century (Campbell et al., 2003; Taylor et

al., 2001). We have since used these conditions to cryopreserve several adherent cell types (Campbell et al., 2007, 2010, 2011). Our rationale for using this adherent model was two-fold. First, due to our interest in regenerative medicine we thought that adherent cells more closely mimicked cells in tissue engineered devices. Second, we thought there might be a market for cells cryopreserved on plates for research and cytotoxicity testing, CryoPlate™. More recently another group has been using adherent cells for investigation of preservation by vitrification and drying and have reported on cryopreservation of adherent pluripotent stem cells (Katkov et al., 2006; Katkov et al., 2011;). Katkov et al. presented results for preservation of human embryonic stem cells in 4-well plates and pointed out several advantages of cryopreservation in adherent mode. These included elimination of possible bias due to selective pressure within a pluripotent stem cell line after cryopreservation and distribution of multiwell plates for immediate use for embryotoxicity and drug screening in pluripotent stem cell-based toxicology in vitro kits (Katkov et al., 2011).

There are several methods in the literature that could be employed for intracellular delivery of these sugars including those already discussed (Campbell et al., 2011; Table 2). Many drugs, therapeutic proteins and small molecules have unfavorable pharmacokinetic properties and do not readily cross cell membranes or other natural physiological barriers within the body. This has resulted in the search for and discovery of alternative methods to transport materials, like sugars, across mammalian cell membranes.

Some of these strategies have been presented in depth in the results sections. The first involved the use of the *Staphylococcus aureus* toxin, α-hemolysin. This toxin is produced as a monomer by the bacteria. It then oligomerizes to form pores on mammalian cell membranes. Hagan Bayley and his group modified the wild type α-hemolysin protein by replacing 4 native residues with histidines, termed H5. In addition to pore formation on cell membranes, the H5 mutant also enabled it to be opened and closed at will. When inserted into the membrane, it is open and molecules up to 3000 daltons are able to pass through. Then the pores are closed in the presence of Zn^{+}. To reopen the pore, addition of a chelating agent such as EDTA will remove the Zn^{+} and the pore is ready to be used again (Bayley, 1994; Walker et al., 1995). Early studies showed that H5 could create pores in mammalian cell membranes and that they could be used for efficient intracellular loading of trehalose (Eroglu et al, 2000; Acker et al., 2003). Our experiments with H5 worked well initially using adherent cells. The results demonstrated good poration and loading of trehalose into cells. However, after adherent cells were cryopreserved, their viability was not very good (<6%). At this point in our studies, several issues arose that prevented further studies using H5. First, the H5 pore was derived from the bacterial toxin α-hemolysin so there were concerns raised whether regulatory approval could be obtained if it was ever to be used clinically with human cells and tissues. There were some indications during these studies that the pores were shed from the membrane over time. However, H5 was still detectable in picogram quantities after 7 days in culture. Finally, as new batches of H5 were delivered the activity varied greatly and more H5 was required to achieve the same level of poration compared with earlier batches. Ultimately the batch variation was attributed to a protein stability issue. When these issues were not resolved other strategies for introducing trehalose into cells were explored.

An unexpected outcome of our H5 experiments was the development of a new, simple strategy for introduction of trehalose into cells which involved incubating cells in sugar for

extended periods of time at physiological temperature (Brockbank, 2007). One possible mechanism to explain this observation was that the trehalose is substituting for water molecules in the cell membranes keeping the membrane stable and preventing it from going through a phase transition (Crowe et al., 1988, 1989). A second mechanism is most likely an active uptake mechanism involving endocytosis similar to that proposed for loading of trehalose by Oliver et al (Oliver et al., 2004). Her results suggested that human MSCs are capable of loading trehalose from the extracellular space by a clathrin-dependent fluid-phase endocytotic mechanism that is microtubule-dependent but actin-independent (Oliver et al., 2004). Further research is required to elucidate the mechanism by which culture in the presence of trehalose facilitates cell cryopreservation and determine the degree of cell viability retention under different storage conditions.

The last method presented was poration using the $P2_z$ receptor and ATP. This was a somewhat unique strategy in that it took advantage of the cell's own machinery. It was shown that cells expressing the $P2_{X7}$ purinergic cell surface receptor, also known as the $P2_z$ receptor, could be permeabilized when the receptor binds to ATP^{4-}. The interaction with ATP resulted in the formation of a non-selective pore that allows molecules up to ~900 Daltons to pass through (Nihei et al., 2000). The $P2_{X7}$ receptor selectively binds to only ATP^{4-} whose presence in solution is dependent on temperature, pH, and the concentration of divalent cations such as Mg^{2+}. Closure of the pore after activation by ATP is achieved by simply removing ATP from the system or adding exogenous Mg^{2+} that has a high affinity for the active form of ATP, ATP^{4-}. The $P2_z$ receptor is found on a number of cell types including cells of hematopoietic origin (Nihei et al, 2000). There were several factors that likely affected the cell viability and survival of cells after ATP poration. First, is the density of the receptor on the cells which directly affects the amount of trehalose that can be loaded into the cells and how long it takes. Another factor is that poration with ATP tends to promote the detachment of adherent cells from their substrate. Part of the protocol requires a recovery period of 1 hour at 37°C to allow cells that may have been perturbed by the poration process the chance to settle back onto their substrate. Finally, cell loss is at least in part due to apoptosis. There is evidence in the literature that poration with ATP induces apoptosis in some cell types (Murgia et al., 1992).

In marked contrast the human stem cell line, TF-1, demonstrated excellent post cryopreservation survival (Buchanan et al., 2004; 2005). We have exposed TF-1 cells to ATP with trehalose for 1 hour followed by a 10-fold dilution of the ATP and inactivation of the active form of ATP (ATP^{4-}) by the addition of 1 mM $MgCl_2$ followed by a 1-hour recovery period at 37°C (Brockbank et al., 2011). When the cells were compared to cells cryopreserved with 10% DMSO, the DMSO group demonstrated greater initial viability close to 100% that steadily declined over days in culture post thaw. However by day 4 of culture post-cryopreservation cells cryopreserved in disaccharides were similar to the viability of cells cryopreserved in DMSO. Similarly colony forming assays with TF-1 cells demonstrated similar outcomes compared with DMSO. Furthermore, the use of disaccharides, trehalose and sucrose, appeared to result in similar results at both slow (1°C/min) and rapid (100°C/min) cooling rates. Buchanan et al (Buchanan et al., 2010) have extended these studies obtaining excellent TF-1 cell line and cord blood-derived multipotential hematopoietic progenitor cell survival after freeze drying and storage at room temperature for 4 weeks! It is studies such as Buchanan's that keep us optimistic that disaccharide introduction/preservation strategies can be developed for preservation of other mammalian

cell types. Further development work is required with the cell culture and $P2_{x7}$ methods with the promise of preservation by freezing and freeze-drying.

Existing Techniques	Description	Pitfalls	References
H5	Derived from α-hemolysin, which normally forms a constitutively opened pore in the membrane. Engineered to close in the presence of Zn+ or serum.	Derived from a bacterial toxin. Batch to batch variation and instability.	Acker et al. 2003 Bayley, 1994 Eroglu et al. 2000
ATP	The naturally occurring $p2_{x7}$ receptor forms a non-specific pore upon binding of ATP^{4-} able to allow molecules <900 daltons to pass through.	$P2_{x7}$ receptor found on some but not all cell types.	Buchanan et al. 2005
Culture methods	1) Prolonged incubation of cells in the presence of disaccharide sugars at 37°C. 2) Fluid phase endocytosis: disaccharide sugars are taken up by cells via a clathrin dependent endocytotic mechanism.	Works better with some cells but not others.	Brockbank et al. 2007 Oliver et al. 2004
Temperature manipulation	A shift in temperature can cause a lipid phase transition which temporarily changes the membrane permeability and allows molecules to pass through.	Has been demonstrated with pancreatic islets and kidney cells. Requires optimization by cell type.	Beattie et al. 1997 Mondal 2009

Table 2. Strategies for Loading Disaccharide Sugars

There are still other methods in the literature that could lead to intracellular delivery of disaccharides in addition to those already discussed (Campbell et al., 2011; Table 2). One method takes advantage of the lipid phase transition described above when the cell membrane is exposed to changes in temperature. As the membrane changes from the liquid crystalline phase to the gel phase it becomes leaky providing an opportunity to introduce molecules into the cell that would not normally cross like trehalose. Beattie used this method to cryopreserve pancreatic islets by introducing DMSO and trehalose into the islets during the thermotropic phase transition between 5 and 9°C. The islets were then cryopreserved in combination with DMSO and the viability of the islets after thawing was greater than when DMSO alone was used, 94% versus 58% (Beattie et al., 1997). In a related study, Mondal et al, cryopreserved kidney cells (MDBK) using 264 mM trehalose. The cells were suspended in trehalose with 20% fetal bovine serum in culture medium then incubated at 40°C for 1 hour before slow rate cooling for storage at -80°C. Viability was measured using Trypan Blue exclusion at 74% upon thawing (Mondal, 2009).

In another variation for loading molecules into cells, a number of proteins have been discovered that possess the ability to cross the cell membrane. These protein transduction domains (PTDs) generally correspond to portions of native proteins. Examples of PTDs include the Tat protein from the human immunodeficiency virus type I, the envelope glycoprotein E^{rns} from the pestivirus and the DNA binding domains of leucine zipper proteins such as c-fos, c-jun and yeast transcription factor GCN4 (Futaki et al., 2001, 2004; Langedijk, 2002; Langedijk et al., 2004; Lindgren et al., 2000; Richard et al., 2003; Vives et al., 1997). These PTDs are short cationic peptides that cross the cell membrane in a concentration-dependent manner that is independent of specific receptors or other transporters. The exact mechanism of translocation has not been defined. Enrichment of basic amino acids, particularly arginine and in some instances lysine, have been shown to be important for the translocation activity (Futaki et al., 2001, 2004; Vives et al., 1997). Some studies have suggested that endocytosis is involved (Lundberg & Johansson, 2002; Richard et al., 2003), however, the current theory includes interaction with glycosaminoglycans and uptake by a non-endocytotic mechanism that may involve the charged heads of the phospholipid groups within the cell membrane. (Langedijk, 2002; Langedijk et al., 2004; Mai et al., 2002).

While most of these peptides need to be cross linked to the molecule of interest, there are peptides that can move proteins and other peptides across the membrane without the requirement for cross-linking. Examples include Pep-1, a 21-residue peptide which contains three domains; a tryptophan rich region (5 residues) for targeting the membrane and forming hydrophobic interactions; a lysine rich domain to improve intracellular delivery whose design was taken from other nuclear localization sequences from other proteins like the simian virus 40 large T antigen, and, a spacer region with proline that provides flexibility and maintenance of the other two regions. When mixed with other peptides or proteins, Pep-1 rapidly associates and forms a complex with the protein of interest by noncovalent hydrophobic interactions to form a stable complex. Once in the cytoplasm the peptide dissociates from the protein that has been carried across the membrane causing little if any interference regarding the protein's final destination or function. The process occurs by an endocytosis independent mechanism (Morris et al., 1999, 2001). We anticipate that such peptides may eventually lead to methods for introduction of disaccharides into mammalian cells (Campbell et al., 2011).

Another alternative method is electroporation, also called electropermeabilization, which involves the application of an electric pulse that briefly permeabilizes the cell membrane. Since its introduction in the 1980's it has been primarily used to transfect mammalian cells and bacteria with genetic material. Initially electroporation tended to kill most cells. However, further work and development of the electroporation process, such as alternate electrical pulses like the square wave pulse, have refined the process so that better permeabilization and cell viability can be achieved (Gehl, 2003; Hapala, 1997; Heiser, 2000). The formation of pores, their size and the recovery of the membrane are important factors that influence the success of an electroporation protocol (Gehl, 2003; Hapala, 1997; Heiser, 2000). Most importantly, electroporation is applicable to all cell types.

It was hypothesized that trehalose provided protection during electropermeabilization in a manner similar to chelating agents such as EDTA or lipids like cholesterol (Katkov, 2002;

Mussauer et al., 2001). Effective electroporation protocols are a balance between how much material can be loaded into the cells and cell survival after membrane permeabilization. So, while it cannot be predicted how well certain cell types will respond to electroporation, there is ample evidence that electroporation can be used with a reasonably certainty of success. A short culture period may be all that is required to permit restabilization of membranes post-electroporation. Additionally, like trehalose which interacts with membranes under stressful conditions such as drying, other compounds, such as cholesterol and unsaturated fatty acids, can also interact with membranes and may facilitate resealing of the membranes increasing overall cell survival (Katkov, 2002). Efficient resealing of cell membranes after permeabilization is thought to be essential for promoting cell recovery (Gehl et al., 1999) and compounds such as Poloxamer 188 facilitate membrane resealing (Lee et al., 1992).

5. Conclusion

In conclusion, there are multiple potential ways to introduce trehalose into mammalian cells and in some cases excellent cell preservation can be achieved. However, it is clear that methods for each cell type will need to be diligently developed and many years of work remain before we can replace DMSO as the lead cryoprotectant. In the mean time, we must not forget that there are other relatively low molecular weight sugars available. Preliminary evidence suggests that with further work sucrose and stachyose may, in some cases, be equally effective for cell preservation.

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7. References

Acker, J.P., Lu, X.M., Young, V., Cheley, S., Bayley, H., Fowler, A. & Toner M. (2003). Measurement of trehalose loading of mammalian cells porated with a metal-actuated switchable pore. *Biotechnology and Bioengineering*, Vol.82, No.5, (June 2003), pp. 525-32, PMID: 12652476.

Barnett, R. (1978). The effects of dimethyl sulfoxide and glycerol on Na+. K+-ATPase and membrane structure. *Cryobiology*, Vol.15, (April 1978), p. 227, PMID: 149651..

Bayley, H. (1994). Triggers and Switches in a Self-Assembling Pore-Forming Protein. *Journal of Cellular Biochemistry*, Vol.56, (October 1994), pp. 177-82, PMID: 7829577.

Beattie, G.M., Crowe, J.H., Lopez A.D., Cirulle, V., Ricordi, C. & Hayek, A. (1997). Trehalose: A cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage, *Diabetes*, Vol.46, (March 1997), pp. 519-23, PMID: 9032112.

Brockbank, K.G.M., Campbell, L.H., Ratcliff, Kelly M. & Sarver, K.A. (2007, 2011). Method for treatment of cellular materials with sugars prior to preservation. U.S. Patents #7,270,946, #8,017,311.

Brockbank, K.G.M., Campbell, L.H., Greene, E.D., Brockbank, M.C.G. & Duman, J.G. (2011). Lessons Learned from Nature for Preservation of Mammalian Cells, Tissues and Organs, *In Vitro Cellular & Developmental Biology–Animal*, Vol.47, No.3, (March 2011), pp. 210-217, PMID: 21191664.

Buchanan, S.S., Gross, S.A., Acker, J.P., Toner, M., Carpenter, J.F., & Pyatt, D.W. (2004). Cryopreservation of Stem Cells Using Trehalose: Evaluation of the Method Using a Human Hematopoietic Cell Line, *Stem Cells and Development*, Vol.13, (June 2004), pp. 295–305, PMID: 15186725.

Buchanan, S.S.; Menze, M.A.; Hand, S.C.; Pyatt, D.W.& Carpenter, J.F. (2005). Cryopreservation of Human Hematopoietic Stem and Progenitor Cells Loaded with Trehalose: Transient Permeabilization via the ATP-Dependent P2Z Receptor Channel, *Cell Preservation Technology*, Vol. 3, No.4, (2005), pp. 212–22.

Buchanan, S.S., Pyatt, D.W. & Carpenter J.F. (2010). Preservation of Differentiation and Clonogenic Potential of Human Hematopoietic Stem and Progenitor Cells during Lyophilization and Ambient Storage, *PLoS ONE,* Vol.5, No.9, (September 2010), pp. e12518, PMID: 20824143.

Campbell, L.H., Taylor, M.J. & Brockbank, K.G.M. (2003) Two stage method for thawing cryopreserved cells, U.S. Patent #6,596,531.

Campbell, L.H., & Brockbank, K.G.M. (2007). Serum free solutions for the cryopreservation of cells, *In Vitro Cellular & Developmental Biology–Animal*, Vol.43, No.8-9, (September 2007), pp. 269-275, PMID: 17879124.

Campbell, L.H., Brockbank, K.G.M. (2011). Comparison of Electroporation and Chariot™ for Delivery of β-galactosidase into Mammalian Cells: Strategies to Use Trehalose in Cell Preservation, *In Vitro Cellular & Developmental Biology–Animal*, Vol.47, No.3, (March 2011), pp.195-199, PMID: 21184200.

Campbell, L.H., Sarver, K.A., Hylton, K.R., Leman, B.B., & Brockbank, K.G.M. (2011). Culturing with trehalose produces viable endothelial cells after cryopreservation, *Cryobiology*, Submitted, 2011.

Carpenter, J.F., Hand, S.C., Crowe, L.M., & Crowe, J.H. (1986). Cryoprotection of Phosphofructokinase with Organic Solutes: Characterization of Enhanced Protection in the Presence of Divalent Cations, *Archives of Biochemistry and Biophysics*, Vol.250, No.2, (November 1986), pp. 505-1, PMID: 2946263.

Carpenter, J.F., Crowe, L.M .& Crowe JH. (1987a). Stabilization of Phosphofructokinase with Sugars During Freeze-Drying: Characterization of Enhanced Protection in the Presence of Divalent Cations, *Biochimica et Biophysica Acta*, Vol.923, No.1, (January 1987), pp. 109-15, PMID: 2948571.

Carpenter, J.F., Martin, B., Crowe, L.M. & Crowe, J.H. (1987b). Stabilization of Phosphofructokinase During Air-Drying with Sugars and Sugar/Transition Metal Mixtures, *Cryobiology*, Vol.24, No.5, (October 1987), pp. 455-64, PMID: 2958239.

Carpenter, J.F. & Crowe, J.H. (1989). An Infrared Spectroscopic Study of the Interactions of Carbohydrates with Dried Proteins, *Biochemistry*, Vol.28, No.9, (May 1989), pp. 3916-22, PMID: 2526652.

Caselli, D., Tintori, V., Messeri, A., Frenos, S., Bambi, F. & Arico, M. (2009). Respiratory depression and somnolence in children receiving dimethyl sulfoxide and morphine during hematopoietic stem cell transplantation. *Haematologica,* Vol.94. No. 1, (January 2009), pp. 152–153, PMID: 19001279.

Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L. & Rudolph, A. (1986). Prevention of Fusion and Leakage in Freeze-Dried Liposomes by Carbohydrates". *Biochimica et Biophysica Acta,* Vol.861, (1986), pp. 131-40.

Crowe, J.H., Crowe, L.M., Carpenter, J.F. & Aurell Wistrom, C. (1987). Stabilization of Dry Phospholipid Bilayers and Proteins by Sugars, *The Biochemical Journal*, Vol.242, No.1, (February 1987), pp. 1-10, PMID: 2954537.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S. & Aurell Wistrom, C., Spargo, B.J. & Anchordoquy, T.J. (1988). Interactions of Sugars with Membranes, *Biochimica et Biophysica Acta,* Vol.947, No.2, (June 1988), pp. 367-84, PMID: 3285894.

Crowe, J.H., Crowe, L.M. & Hoekstra, F.A. (1989a). Phase Transitions and Permeability Changes in Dry Membranes During Rehydration, *Journal of Bioenergetics & Biomembranes*, Vol.21, No.1, (February 1989), pp. 77-91.

Crowe, J.H., McKersie, B.B. & Crowe, L.M. (1989b). Effects of Free Fatty Acids and Transition. Temperature on the Stability of Dry Liposomes, *Biochimica et Biophysica Acta*, Vol.979, No.1, (February 1989), pp. 7-10, PMID: 2917168.

Crowe, J.H., Carpenter, J.F., Crowe, L.M. & Anchordoguy, T.J. (1990). Are Freezing and Dehydration Similar Stress Vectors? A Comparison of Modes of Interaction of Stabilizing Solutes with Biomolecules, *Cryobiology*, Vol.27, No.3, (June 1990), pp. 219-31.

Crowe, J.H. & Crowe, L.M. (1991). Preservation of Liposomes by Freeze Drying, In: *Liposome Technology*, G. Gregoriadis, CRC Press, ISBN 0849340764, Boca Raton, FL.

Crowe, J.H., Carpenter, J.F. & Crowe, L.M. "(1998). The role of Vitrification in Anhydrobiosis, *Annual Review of Physiology*, Vol.60, (1998), pp. 73-103, PMID: 9558455.

Crowe, J.H., Crowe, L.M., Oliver, A.E., Tsvetkova, N., Wolkers, W. & Tablin, F. (2001). The Trehalose Myth Revisited: Introduction to a Symposium on Stabilization of Cells in the Dry State, *Cryobiology*, Vol.43, No.2, (September 2001), pp. 89-105, PMID: 11846464.

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H. & Toner, M. (2000). Intracellular trehalose improves the survival of cryopreserved mammalian cells, *Nature Biotechnology*, Vol.18, No.2, (February 2001), pp. 163-67, PMID: 10657121.

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda. K., Sugiura, Y. (2001). Arginine-rich Peptides: An Abundant Source of Membrane-Permeable Peptides having Potential as Carriers for Intracellular Protein Deliver, *Journal of Biological Chemistry,* Vol. 276, No.8, (February 2000), pp. 5836-40, PMID: 11084031.

Futaki, S., Goto, S., Suzuki, T., Nakase, I. & Sugiura, Y. (2004). Structural Variety of Membrane Permeable Peptides, *Current Protein & Peptide Science*, Vol.4, No.2, (April 2004), pp. 87-96, PMID: 12678848.

Gehl, J., Sorensen, T.H. & Nielsen, K. (1999). In Vivo Electroporation of Skeletal Muscle: Threshold, Efficacy and Relation to Electric Field Distribution, *Biochimica et Biophysica Acta*, Vol.1428, No.2, (August 1999), pp. 233-40, PMID: 10434041

Gehl, L. (2003). Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research, *Acta Physiologica Scandinavica*, Vol.177, No.4, (April 2003), pp. 437-47, PMID: 12648161

Hapala, I. (1997). Breaking the Barrier: Methods of reversible permeabilization of cellular membranes, *Critical Reviews in Biotechnology*, Vol.17, No.2, (1997), pp. 105-22, PMID: 9192473.

Heiser, W.C. (2000). Optimizing electroporation conditions for the transformation of mammalian cells, *Methods in Molecular Biology*, Vol.130, (2000), pp. 117-34, 2000. PMID: 10589426

Junior, A.M., Arrais, C.A., Saboya, R., Velasques, R.D., Junqueira, P.L. & Dulley, F.L. (2008). Neurotoxicity associated with dimethyl sulfoxide-preserved hematopoietic progenitor cell infusion. *Bone Marrow Transplantation*, Vol.41, No. 1, (January 2008), pp. 95–6, PMID: 17934528.

Katkov, I. (2002). Electroporation of cells in applications to cryobiology: summary of 20-year experience, *Problems of Cryobiology*, Vol.2, (2002), pp. 3-8.

Katkov, I., Isachenko, V., Isachenko, E., Kim, M., Lulat, A., Mackay, A. & Levine, F. (2006). Low- and high-temperature vitrification as a new approach to biostabilization of reproductive and progenitor cells, *International Journal of Refrigeration*, Vol.29, (February 2006), pp. 346-357.

Katkov, I., Kan, N., Cimadamore, F., Nelson, B., Snyder, E., Terskikh, A. (2011). DMSO-Free programmed cryopreservation of fully dissociated and adherent human induced pluripotent stem cells, *Stem Cells International*, Vol.2011, (June 2011), pp.1-8, PMID: 21716669.

Katsuda, S., Okada, Y., Nakanishi, I. & Tanaka, J. (1984). The influence of dimethyl sulfoxide on cell growth and ultrastructural features of cultured smooth muscle cells, *Journal Electron Microscopy*, Vol.33, No. 3, (July 1984), pp. 239-241, PMID: 6533232.

Katsuda, S., Okada, Y. & Nakanishi, I. (1987). Dimethyl sulfoxide induces microtubule formation in cultured arterial smooth muscle cells. *Cell Biology International Reports*, Vol.11, (February 1987), pp. 103-110, PMID: 3549003.

Langedijk, J.P.M. (2002), Translocation Activity of C-terminal Domain of Pestivirus E^{rns} and Ribotoxin L3 Loop, *Journal of Biological Chemistry*, Vol.277, No.7, (February 2002), pp. 5308-14, PMID: 11673454.

Langedijk, J.P.M., Olijhoek, T., Schut, D., Autar, R. & Meloen, R.H. (2004). New Transport Peptides Broaden the Horizon of Applications for Peptidic Pharmaceuticals, Molecular Diversity, Vol. 8, No.2, (2004), pp. 101-11, PMID: 15209161

Lee, R.C., River, L.P., Pan, F.S. & Ji, L. (1992). Surfactant-Induced Sealing of Electropermeabilized Skeletal Muscle Membranes In Vivo, *Proceedings of the*

National Academy of Sciences USA, Vol.89, No.10, (May 1992), pp. 4524-28, PMID: 1584787

Lindgren, M., Hällbrink, M., Prochiantz, A., Langel, Ü. (2000). Cell-penetrating Peptides, *Trends in Pharmacological Sciences*, Vol.21, No.3, (March 2000), pp. 99-103, PMID: 10689363.

Lovelock, J. & Bishop, M., (1959). Prevention of freezing damage to living cells by dimethyl sulfoxide. *Nature*, Vol.183, (May 1959), p. 1394, ISSN: 0022-202X.

Lundberg, M. & Johansson, M. (2002). Positively Charged DNA-Binding Proteins Cause Apparent Cell Membrane Translocation, *Biochemical and Biophysical Research Communications*, Vol.291, No.1, (February 2002), pp. 367-71, PMID: 11846414.

Mai, J.C., Shen, H., Watkins, S.C., Cheng, T. & Robbins, P.D. (2002). Efficiency of Protein Transduction is Cell Type-dependent and is Enhanced by Dextran Sulfate, *Journal of Biological Chemistry*, Vol.277, No.33, (August 2002), pp. 30208-18, PMID: 12034749.

Mathew, A.J., Baust, J.M., Van Buskirk, R.G. & Baust, J.G. (2004). Cell preservation in reparative and regenerative medicine: evolution of individualized solution composition, *Tissue Engineering*, Vol.10, No. 11, (November 2004), pp. 1662-71, PMID: 15684675.

Miranda, A.F., Nette, G., Khan, S., Brockbank, K.G.M. & Schonberg, M. (1978). Alteration of myoblast phenotype by dimethyl sulfoxide, *Proceedings of the National Academy of Sciences USA*, Vol.75, (August 1978), pp. 3826-3830, PMID: 278996.

Mondal, B. (2009). A simple method for cryopreservation of MDBK cells using trehalose and storage at −80°C, *Cell and Tissue Banking*, Vol.10, No.4, (November 2009), pp. 341-344, PMID: 19381873.

Morris, M.C., Robert-Hebmann, V., Chaloin, L., Mery, J., Heitz, F., Devaux, C., Goody, R.S. & Divita G. (1999). A New Potent HIV-1 reverse transcriptase Inhibitor. A Synthetic Peptide Derived from the Interface Subunit Domain, *Journal of Biological Chemistry*, Vol.274, No.35, (August 1999), pp. 24941-6, PMID: 10455170.

Morris, M.C., Depollier, J., Mery, J., Heitz, F., Divita, G. (2001). A Peptide Carrier for the Delivery of Biologically Active Proteins into Mammalian Cells, *Nature Biotechnology*, Vol.19, No. 12, (December 2001), pp. 1173-76, PMID: 11731788.

Mueller, L.P., Theurich, S., Christopeit, M., Grothe, W., Muetherig, A., Weber T, Guenther, S. & Behre, G. (2007). Neurotoxicity upon infusion of dimethyl sulfoxide-cryopreserved peripheral blood stem cells in patients with and without pre-existing cerebral disease. *European Journal of Haematology*, Vol.78, No.6, (June 2007), pp. 527–31, PMID: 17509106.

Murgia, M., Pizzo, P., Steinberg, T.H. & Di Virgilio, F. (1992). Characterization of the Cytotoxic Effect of Extracellular ATP in J774 Mouse Macrophages, *The Biochemical Journal,* Vol.288, (December 1992), pp. 897-901, PMID: 1472003.

Mussauer, H., Sukhorukov, V.L. & Zimmermann, U. (2001). Trehalose Improves Survival of Electrotransfected Mammalian Cells, *Cytometry*, Vol.45. No.3, (November 2001), pp. 161-9, PMID: 11746084

Nihei, O.K., Savino, W. & Alves, L.A. (2000). Procedures to Characterize and Study $P2_Z/P2_{X7}$ Purinoceptor: Flow Cytometry as a Promising Practical, Reliable Tool,

Memorias do Instituto Oswaldo Cruz, Vol.95, No.3, (May 2000), pp. 415-28, 2000, PMID: 10800201.

Oliver, A.E., Jamil, K., Crowe, J.H. & Tablin, F. (2004) Loading Human Mesenchymal Stem Cells with Trehalose by Fluid-Phase Endocytosis, *Cell Preservation Technology*, Vol.2, No.1, (2004) pp. 35-49.

Otrock, Z.K., Beydoun, A., Barada, W.M., Masroujeh, R., Hourani, R. & Bazarbachi A. (2008). Transient global amnesia associated with the infusion of DMSO-cryopreserved autologous blood stem cells. *Haematologica*, Vol.93, No.3, (March 2008), pp. 36–7, PMID: 18310533.

Polge, C., Smith, A.U. & Parkes A. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*. Vol.164, (October 1949), p. 666, ISSN: 0028-0836

Richard, J.P., Melikov, K., Vives, E., Ramos, C.,Birgit, V., Gait, M.J., Chernomordik, L.V. & Lebleu, B. (2003). Cell-penetrating Peptides: A Reevaluation of the Mechanism of Cellular Uptake, *Journal of Biological Chemistry*, Vol. 278, No.1, (January 2003), pp. 585-90, PMID: 12411431.

Rudolph, A.S. & Crowe, J.H. (1985). Membrane Stabilization During Freezing: The Role of Two Natural Cryoprotectants, Trehalose and Proline, *Cryobiology*, Vol.22, No.4, (August 1985), pp. 367-77, PMID: 4028782.

Schlegel, P.G., Wölfl, M., Schick, J., Winkler, B. & Eyrich, M. (2009). Transient loss of consciousness in pediatric recipients of dimethyl sulfoxide (DMSO)-cryopreserved peripheral blood stem cells independent of morphine co-medication, *Haematologica*, Vol.94, No.10, (October 2009), pp. 1473-5, PMID: 19608681.

Slade, L. & Levine, H. (1991). A food polymer science approach to structure-property relationships in aqueous food systems: non-equilibrium behavior of carbohydrate-water systems, *Advances in Experimental Medicine and Biology*, Vol.302, (1991), pp. 29-101, PMID: 17416335.

Sosef, M.N., Baust, J.M., Sugimachi, K., Fowler, A., Tompkins, R.G. & Toner, M. (2005) Cryopreservation of isolated primary rate hepatocytes: enhanced survival and long-term hepatospecific function, *Annals of Surgery*,.Vol.241, No.1, (January 2005), pp. 125-33, PMID: 15622000.

Taylor, M., Campbell, L., Rutledge, R., Brockbank, K. (2001). Comparison of Unisol with Euro-collins solution as a vehicle solution for cryoprotectants, *Transplantation Proceedings*, Vol.33, (February 2001), pp. 667-9, PMID : 11267013.

Vives, E., Brodin, P. & Lebleu, B. (1997). A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus, *Journal of Biological Chemistry*, Vol.272, No.25, (June 1997), pp. 16010-17, PMID: 9188504.

Walker, B., Braha, O., Cheley, S. & Bayley, H.(1995). An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch. *Chemistry & Biology*, Vol.2, No,2, (February 1995), pp. 99-105, PMID: 9383410.

Zeng, X., Zhao, C., Wang, H., Li, S., Deng, Y. & Li, Z. (2010). Dimethyl Sulfoxide Decrease Type-I and –III Collagen Synthesis in Human Hepatic Stellate Cells and Human

Foreskin Fibroblasts. *Advanced Science Letters*, Vol.3, No. 4, (December 2010), pp. 496–499.

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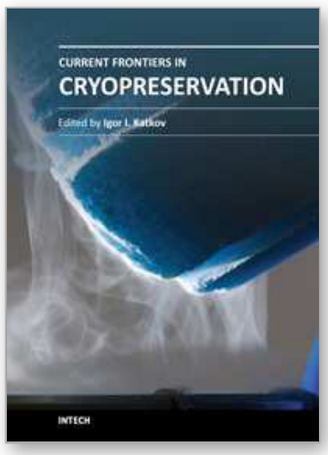

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
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