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The Effects of Solutes in the Cryopreservation of Adherent Neuroblastoma (Neuro-2a) Cells

Trisha L. Bailey

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THE EFFECTS OF SOLUTES IN THE CRYOPRESERVATION OF ADHERENT NEUROBLASTOMA (NEURO-2A) CELLS

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BY

Trisha L. Bailey

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THE EFFECTS OF SOLUTES IN THE CRYOPRESERVATION OF ADHERENT NEUROBLASTOMA (NEURO-2A) CELLS

by

Trisha L. Bailey

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ABSTRACT

A simple method to cryopreserve adherent monolayers of neuronal cells is currently not available, but the development of this technique could facilitate numerous applications in the field of biomedical engineering, cell line development, and drug screening. However, complex tissues of some exceptional animals survive freezing in nature. These animals are known to accumulate several small molecular weight solutes prior to freezing. Following a similar strategy, we investigated the effects of osmolytes such as trehalose, proline, and sucrose as additives to the traditional cryoprotectant dimethyl sulfoxide (Me2SO) in modulating the cryopreservation outcome of mouse neuroblastoma (Neuro-2a) cells. Neuro-2a cells adhered to cell culture plates were incubated for 24 h at varying concentrations of trehalose, proline, sucrose and combinations of these compounds. Cells were cryopreserved for 24 h and cell viability post-freezing and thawing was quantified by trypan blue exclusion assay. On average, only 13.5% of adherent cells survived freezing in the presence of 10% Me2SO alone (control). Pre-incubation of cells with medium containing both trehalose and proline severely decreased cell proliferation, but increased cell recovery by 288% (overall recovery of 52.5%). Our results suggest that pre-incubation of Neuro-2a cells with trehalose and proline in combination provides cell protection resulting in increased cell survival post-freezing.

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Firstly, I would like to express my sincere gratitude to my advisors Dr. Michael Menze and Dr. Britto Nathan for their continuous support of my master's study and research, as well as their patience, motivation, and immense knowledge. Their guidance has made my research and writing of this thesis possible. I could not have imagined having better advisors and mentors for my master's study.

Besides my advisors, I would like to thank Dr. Gary A. Bulla as a member of my thesis committee. Not only for his encouragement and helpfulness but his insightful questions and encouragement helped to stimulate the production of the highest viability results we obtained.

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INTRODUCTION

Tissue engineering, gene therapy, and cellular implantation all rely on the ability to store and transport cells and tissues in order to be clinically successful [1]. Mammalian cells (such as Chinese hamster ovary (CHO) and nonsecreting murine myeloma (NS0)) are widely used in the biopharmaceutical industry for the production of recombinant therapeutic proteins [2]. For each product, an independent cell line must be developed. Given that any in vitro culture will undergo phenotype and genetic changes when propagated for long periods of time, it is neither possible nor practical to maintain a constantly growing culture of cells in order to supply this material reproducibly [3]. In drug screening, fresh neurons, cryopreserved neurons and sliced fresh neural tissues have been used. However, a laborious procedure for inducing differentiation so that the cells extend their neurites and build a neuronal network is usually needed prior to screening when neuronal cells in suspension without neurites are used, whether fresh or cryopreserved cells [4]. The ability to cryopreserve neuronal monolayered cells would facilitate drug development by providing phenotypically identical cells and proteins for assays as well as potentially preserve neurite outgrowth.

The main limitations in cryopreservation are ice formation and osmotic stress due to the addition of cryoprotective agents and the increasing concentration of solutes in the remaining water phase during the freezing process. However, it appears that certain cells are not damaged by exposure to cold temperatures. Extensive evidence indicates that ice-formation inside and/or outside of the cell leads to the damage that cells experience when exposed to freezing temperatures, and not the cold temperature by itself [5]. Intracellular ice can form when the cooling rate is intermediately high and the cell cannot maintain

osmotic equilibrium with the environment [6]. However, extracellular ice always forms prior to intracellular ice and as extracellular ice forms, a significant increase in the concentration of solutes occurs in the remaining liquid water fraction. If the cooling rate is too low, this can lead to extreme dehydration that may result in irreversible membrane alterations and cell death. Even with an ideal cooling rate, the formation of extracellular ice results in an increased extracellular osmotic pressure that creates an osmotic gradient across the plasma membrane, which provides the driving force for an efflux of water from the cell [7]. However, the optimal cooling rate can vary substantially among cell types and depends on the water permeability of the plasma membrane [5]. This hyperosmotic stress may leads to cell death via the induction of autophagy and apoptosis processes, as seen in recombinant Chinese hamster ovary (rCHO) cells and in human neuroblastoma cells [8], [9]. In addition, cells at low temperatures must cope with a reduction in the molecular kinetic energy of the environment, which lowers the rate of many physical processes [10].

Nature employs a variety of compounds and strategies to enhance the survival of ectothermic animals during extreme environmental conditions [11]. Anhydrobiosis ("life without water") is found throughout all biological domains, for example in several species of eubacteria, archea, some fungi, certain invertebrate species and "resurrection plants". All these organisms accumulate the disaccharide trehalose [12]. For many years, trehalose was considered to be a rare sugar because it had only been isolated from resurrection plants, yeast and the larvae of certain insects. Today this sugar has been found in a wide number of organisms, although in many cases in low concentrations [13]. Both trehalose and proline were found to be more effective in preventing membrane

mixing between small unilamellar vesicles than the standard protectants, glycerol and dimethylsulfoxide [14]. Increased stress protection was observed in yeast cells under the artificial condition of proline accumulation and it has been demonstrated that proline acts as an osmoprotectant and that overproduction of proline results in increased tolerance to osmotic stress of transgenic tobacco plants [15], [16].

Since cells in freeze-tolerant organisms experience dehydration in highly organized tissues, we hypothesized that the protective osmolytes trehalose and proline would be useful in the cryopreservation of neuronal cell monolayers. We have demonstrated in this study that incubation of confluent neuronal monolayers with a combinatorial 112.5 mM trehalose and proline solution prior to freezing increases cell viability to roughly 53% after thawing and recovery.

LITERATURE REVIEW

Current approaches in organ, tissue, and cell preservation. The general principles of organ preservation, static cold storage in a solution to reduce metabolic activity [17], were developed over 30 years ago and are still standard protocol today. Developed in the 1980s, University of Wisconsin (UW) solution remains the gold standard in preservation fluid [18]. The principal cryoprotectants of the UW solution are lactobionate, a large molecular weight anion impermeable to most membranes and thought to suppress hypothermia-induced cell swelling, raffinose, a trisaccharide, and dexamethasone, a corticosteroid. However, UW solution has limitations, principally its cost and its incomplete cell protection [19].

Tissues are more challenging to cryopreserve than cellular systems in suspension because both cellular integrity and structure of the extracellular matrix must be preserved, which is complicated by the intimate relationship between both [20]. There is evidence that the core response of cells to cryopreservation is different if the cells are part of a tissue and the scale-up of procedures from a microscopic cellular level to a macroscopic tissue scale will introduce new modes of injury specific to tissue freezing [21]. Most studies of freezing injury have been carried out with fairly dilute cell suspensions, whereas densely packed cells, such as those seen in tissues, are more likely to be damaged by mechanical stresses due to the space within which they are sequestered changing shape as a result of recrystallization of the ice that form their boundaries [22].

For the cryopreservation of cells, the standard protocol calls for freezing in a solution containing 5-10% of the cryoprotective agent (CPA) dimethyl sulfoxide (Me2SO), which is able to enter cells and at least partly reduce injury by moderating the

increase in solute concentration during freezing [19], [23], [24]. Me2SO is a small amphiphilic molecule with a hydrophilic sulfoxide and two hydrophobic methyl groups. Inclusion of Me2SO protects cells in solution but this method alone does not work well for cell monolayers as adherent attached human embryonic stem cells yield extremely low survival rates of >5% and this has been shown to be due to apoptosis rather than necrosis from freeze-thaw injury [25], [26]. Additionally, there is a concern with using Me2SO due to its toxicity at high concentrations and/or room temperature [27], [28].

Compared to cryopreservation of suspended cells, protocols for adherent cell monolayers are significantly lacking. Although CPA requirements for adherent cells are beginning to be investigated [29], [30], there are two principal approaches to the cryopreservation of cell monolayers independent of CPAs. The first involves growing the cells on glass slides coated in an extracellular matrix (i.e. collagen) which are then removed from the culture dish and exposed to CPA solutions in cryogenic vials. The vials are then cooled at a controlled rate to the desired temperature [31], [32]. The second approach uses a passive freezing device to cryopreserve cells directly in the culture dishes in which they are grown after exposing cells to a CPA [33]–[35]. While both methods result in varying degrees of success, protocols to preserve cell monolayers are still highly underdeveloped.

Ice and Osmotic Stress. The main limitations in the cryopreservation of biological materials are ice and hyperosmotic stress due to the precipitation of water as ice. It appears to be the case that cells are not damaged by exposure to cold temperatures. Extensive evidence indicates that ice-formation inside or outside of the cell leads to the damage that cells experience when exposed to cold, not the cold temperature itself [1].

Cells at low temperatures must also cope with the reduced molecular kinetic energy of the environment and the consequent lower rate of many physical processes [10]. This reduced kinetic energy can limit processes such as the ion pumps as dissipation of ion gradients may occur when energy flow is restricted to the point that ion transport cannot keep up with passive ion leak. This is known to unavoidably trigger the initiation of apoptosis in mammalian species [36].

Another result of reduced ion transport is that intracellular ice can form when the cooling rate is sufficiently high that the cell cannot maintain osmotic equilibrium with the environment [6]. Intracellular ice formation (IIF) may occur when rates are substantially higher than 1 °C/min but are lower than those seen in the ultra-rapid rates used in vitirification [6], [37]. A highly controlled rate of freezing is essential to ensure movement of water across the plasma membrane in order for osmotic dehydration to reach equilibrium with intracellular and extracellular contents to prevent IIF [38], [39]. It has been demonstrated that the formation of intracellular ice may be less harmful to cells in monolayers than for cells frozen in suspension [40] and IIF may be preceded by damage to the plasma membrane thus IIF would be a result of cell injury and not the cause [7].

Furthermore, as extracellular ice precipitates, a significant increase in the concentration of all other solutes occurs in the remaining liquid water fraction. This can lead to excessive dehydration that may result in irreversible membrane alteration and cell death. The formation of extracellular ice, therefore, results in an increased osmotic pressure in the extracellular space that creates an osmotic gradient across the plasma membrane, which provides a driving force for the efflux of water from the cell [7].

However, the presence or absence of extracellular ice determines the rate of vitrification for cells. Low rates of cooling coupled with dehydration, driven by extracellular freezing, allows vitrification to occur before intracellular ice can form. The interior of the cell is crowded and similar in viscosity to a colloid, as the interior dehydrates, there is a sharp increase in viscosity, which moves the interior closer to the glass transition temperature [10].

There must exist a limit to cell dehydration, as a defining feature of apoptosis in cells is the apoptotic volume decrease or AVD, which has been considered a passive component of the cell death process. Most cells have inherent volume regulatory increase (RVI) mechanisms to contest an imposed loss in cell size, thus cells exposed to severe shrinkage during freezing will have their apoptotic pathways turned on when thawed [41]. Autophagy as well as apoptosis was observed in rCHO cells subjected to hyperosmolality and also induced apoptosis and tau phosphorylation in human neuroblastoma cells [9], [42]. Hyperosmotic stress has been shown to induce metacaspase- and mitochondria-dependent apoptosis in *Saccharomyces cerevisiae* and is thought to kills cells by triggering different molecular pathways, which converge at mitochondria where pro- and anti-apoptotic members of the Bcl-2 family exert their control [43], [44]. Additionally, hyperosmotic stress has also been implicated in cell cycle arrest, DNA damage, oxidative stress, inhibition of transcription and translation, and mitochondrial depolarization [45].

Nature's Lessons in Cryoprotection. Nature has provided some organisms with mechanisms to survive desiccation, exposure to sub-zero temperatures, or both. In the arctic springtail *Megaphorura arctica*, where the body shrivels to a fraction of its former

volume as the total water content of the animal reduces from 70% to 40%, there is an accompanying production of trehalose as a cryoprotectant, with temperatures of between 0°C and -2°C appearing to act as the tipping point for trehalose synthesis [46]. The soil-dwelling nematodes *Aphelenchus avenae* are able to survive complete dehydration due to their ability to produce large amounts of trehalose and the amount of trehalose present was strongly correlated with the ability of the nematodes to survive dehydration [47]. Encysted embryos of the brine shrimp *Artemia franciscana* enter an ametabolic state termed cryptobiosis when they undergo desiccation as a normal and necessary part of their life history and trehalose has been shown to be the major stored carbohydrate [48].

Proposed Mechanisms of Action: Trehalose. The specific mechanism by which trehalose protects cellular structures is still debated. The water replacement hypothesis, first proposed by James Clegg in 1967 [48], suggests that the hydroxyl groups of sugars, such as trehalose, can substitute for the hydrogen bonding of water to polar residues, such that the hydration effect of water can be imitated by sugars during the dry state. Reduction in intracellular water content during freezing rates of around 1 ° C/min may be 'buffered' by the sugar. It has been shown that during desiccation in the presence of trehalose, the hydration effect of water is compensated by the presence of sugar, thus preserving the phospholipid bilayer. However, without trehalose, desiccation leads to heterogeneities in phospholipid packing and reduced acyl chain density, thereby destabilizing the membrane and resulting in damage upon the influx of water [49].

Cells almost universally respond to the stress of long-term hyperosmolality, such as those seen during freezing, by accumulating compatible organic osmolytes [50]. When the freshwater prawn *Macrobrachium rosenbergii* was subjected to a hyperosmotic

environment there was shown to be an increase in proline synthesis [51]. PUT1-disrupted *Saccharomyces cerevisiae,* supplemented with external proline, accumulated higher levels of proline in cells and conferred a higher tolerance to freezing and desiccation stresses [52]. Additionally, increased proline transport is suggested to generate or maintain an osmoprotective transmembrane proline gradient in the bacterium *Escherichia coli* [53].

Proposed Mechanisms of Action: Proline. Proline is a non-essential amino acid with the unique characteristic of being a secondary amine. Proline is membrane permeable regardless of pH [54], and transfer may also occur by either a classical Na-dependent A-type system with a wide substrate specificity or by the combination of Na-dependent PHE (phenylalanine preferring) and IMINO (proline, alpha-methylaminoisobutyric acid preferring) systems [55]. The brain-specific high affinity L-proline transporter (PROT) has been proposed to provide an intracellular pool of L-proline which may serve a metabolic or osmotic role in neurons [56]. The sodium-dependent neutral amino acid transporter-2 is a member of the system A-type transporters which mediate the uptake of neutral amino acids in order to regulate cell volume [57] and there is a direct link between the stimulation of this transporter and recovery of cell volume [58]. Proline has also been implicated as a survival factor that protects the cell against apoptosis and maintains the progression of the cell cycle through a signal recognition function of the transporters that may take part in the control of cell cycle progression and programmed cell death [59]. Even more striking, when a high level of proline was incorporated into the tissues of *Drosophila melanogaster*, a chill susceptible insect, it was able to survive when 50% of its body water was frozen [60].

Metabolic Preconditioning. One protective mechanism used by natural systems that undergo seasonal exposure to environmental stresses include downregulation of metabolism to enter a hypometabolic state (diapause) [6] and metabolic depression and cell stasis are often prerequisites to survival for animals whose evolutionary history has provided natural adaptations to desiccation, freezing temperatures, and anoxia [61]. As such, a requirement for freezing survival in both *Caenorhabditis elegans* [49] and *Drosophila melanogaster* [60] was a reduction in metabolic activity. It has also been shown that pretreatment with 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), a compound with the potential to mimic a number of the metabolic features associated with diapause, promoted a significant increase in post-freezing hepatocyte cell survivability [62]. There is evidence for both a rapid cold-hardening process as well as a slower hardening response. The slow response can take anywhere from days to months and is often linked to entry into a state of quiescence or diapause [63], [64]. In addition, the downregulated metabolism characteristic of larval diapause can also contribute directly to supercooling capacity [65]. For example, the stag beetle *Ceruchus piceus* (Lucanidae) removes hemolymph lipoproteins with ice nucleating activity, allowing them to supercool significantly without the production of antifreeze [66]. There are two distinct threshold limits for life: T_M is the threshold for metabolism and T_S is the threshold for survival. Between T_M and T_S the organism is in a state of suspended animation [10]. It is therefore possible that a reduction in metabolism is a necessary and inherent step for the suspension of life processes.

Extrapolating this information, the ideal CPA solution would appear to be low cost, non-toxic, and include non-permeating and permeating molecules capable of

suppressing swelling, stabilizing cellular membranes, reducing metabolic activity, and mitigating apoptotic pathways.

METHODOLOGY

Chemicals. Low endotoxin α,α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL). L-proline and sucrose were obtained from Thermo Fisher Scientific Inc., (Waltham, MA). Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA) to an electrical resistance of 18 mΩ.

Cell culture. Mouse neuroblastoma cells (Neuro-2a) were obtained from the American Type Culture Collection (Manassas, VA) and grown in 75 cm^2 cell culture flasks (Corning Incorporated, Corning, NY). Standard cell culture medium was composed of Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) supplemented with 5.5% fetal bovine serum (FBS) purchased from Atlanta Biologicals Inc. (Flowery Branch, GA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (PSA) (MP Biomedicals, Santa Anna, CA). Neuro-2a cells were maintained in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C and the culture medium was renewed every 3–4 days. The cells were subcultured every 7 days or before reaching 90% confluency. To subculture, cells were dissociated using 0.25% trypsin plus 1 mM EDTA in balanced salt solution (Invitrogen, Carlsbad, CA) and reseeded at $1.5X10^6$ cells per 75 cm^2 cell culture flasks.

Solution preparation. Solutions for cell incubation experiments were prepared by dissolving the individual compounds in Opti-MEM I supplemented with 5.5% FBS and 1X PSA (solutions used as freezing buffers did not contain PSA).

Cryopreservation of Neuro-2a cell monolayers. Cells to be frozen in the monolayer format were seeded at $0.5X10^6$ cells per well in 500 μL of cell culture medium in 24-well plates (Corning Incorporated, Corning, NY) coated with collagen. To promote attachment

of cells, collagen I from rat tail (Invitrogen, Carlsbad, CA) was diluted to 50 μg/mL in 200 mM acetic acid (Fischer Scientific, Fair Lawn, NJ) and added to each well of the cell culture plate at 5 μg collagen cm^2. Plates were incubated with the dissolved collagen for 1 h, then the collagen solution was removed and the plates were rinsed two times with 200 μL Dulbecco's phosphate buffered saline to remove any residual acetic acid solution. The collagen treated plates were allowed to dry for 1 h in a laminar flow hood and stored for less than 1 week at 4 °C prior to use. Before experimental treatments, cells were allowed to attach for 2 h to the collagen coated plates in a humidified atmosphere of 6.5% CO_2 and 93.5% air at 37 °C. The medium was exchanged against medium that was or was not supplemented with solutes as indicated in the figures and tables. Control cells received no additional solutes and experimental cells were incubated with concentrations of the individual solutes ranging from 25 to 200 mM for 24 h. Following the incubation period the culture medium was removed and cells were exposed for 10 min to different concentrations of solutes dissolved in OptiMEM I supplemented with 5.5 % FBS and 10% Me2SO. After 10 min the freezing solutions were removed and the plates placed inside a CoolCell® MP plate (BioCision, LLC, Larkspur, CA), transferred to a -80 °C freezer and frozen at a rate of 1 °C/min. After 24 h at -80 °C, cells were rapidly thawed by addition of cell culture medium warmed to 37 °C. Cells were placed in a humidified atmosphere for 24 h and then dissociated using 0.25% trypsin plus 1 mM EDTA in balanced salt solution. The number of viable cells was then determined by counting with a hemocytometer (Hauser and Son, Philadelphia, PN) after 1:1 dilution of the sample with 0.4% trypan blue solution (Sigma–Aldrich, St. Louis, MO). The percentage of recovered cells was calculated by dividing the number of cells with intact membranes

after freezing and thawing by the number of cells present prior to freezing (i.e. after application of pre-treatments), multiplied by 100.

Impact of solutes on cell proliferation. To assess the effect of the solutes on cell proliferation, cells were seeded at a density of $5X10^4$ cells per 60 mm culture dish (Corning Incorporated, Corning, NY) or 5 – $10X10^3$ cells per well in 24-well plates in fully supplemented cell culture medium containing individual solutes in concentrations ranging from 0 to 200 mM. Cells were incubated for 1 to 6 days followed by dissociation using 0.25% trypsin plus 1 mM EDTA in balanced salt solution, and the number of viable cells was determined by counting with a hemocytometer after 1:1 dilution of the sample with 0.4% trypan blue solution. For cell proliferation recovery assays, cells were incubated in medium containing solutes for 3 days, followed by replacing the medium with fresh fully supplemented cell culture medium without any additional solutes present on the second half of day 3. Fold increase of cells was calculated by dividing the number of cells with intact cell membranes by the number of cells initially plated.

Statistical analyses. Data were analyzed with a one-way analysis of variance (ANOVA) on ranks followed by comparison of experimental groups with the appropriate control group (Holm–Sidak method) followed by Tukey's post hoc test. Excel 2013 (Microsoft, Redmond, WA) and R (R Foundation for Statistical Computing, Vienna, Austria) were used for the analyses. Data sets are presented as mean ± (SEM).

RESULTS

Cryopreservation of Neuro-2a cell monolayers is improved in the presence of trehalose. Me2SO is widely used in the cryopreservation of cells in suspension, however, protection of confluent Neuro-2a cell monolayers using Me2SO as the single cryoprotective agent (CPA) was poor. After thawing and a recovery period of 24 h, trypan blue exclusion assays indicated that only 8.3 ± 2.6 (SEM)% of the cells present before freezing were recovered (Fig 1A). Pre-incubation with trehalose concentrations ranging from 100 to 200 mM slightly improved recovery of cells frozen as monolayers compared to controls (Fig 1A, $n = 4$, $P = 0.18$), and pre-incubation with 100 mM trehalose outperformed all other concentrations evaluated resulting in 24.9 ± 6.2 (SEM)% of cells recovered after freezing. To investigate if the addition of trehalose to the freezing solution would further improve cryopreservation outcome, the same series of experiments were repeated in the presence of 100 mM trehalose added to the freezing solution containing 10% Me2SO (Fig 1B). The inclusion of trehalose in the freezing buffer significantly improved recovery of cells frozen as monolayers compared to cells frozen without trehalose in the freezing buffer (Fig 1B, $n = 4$, $P = 0.001$). Outperforming all other conditions, 100 mM trehalose used during the pre-incubation step and added to the freezing buffer resulted in 35.6 ± 3.4 (SEM)% of cells recovered after cryopreservation.

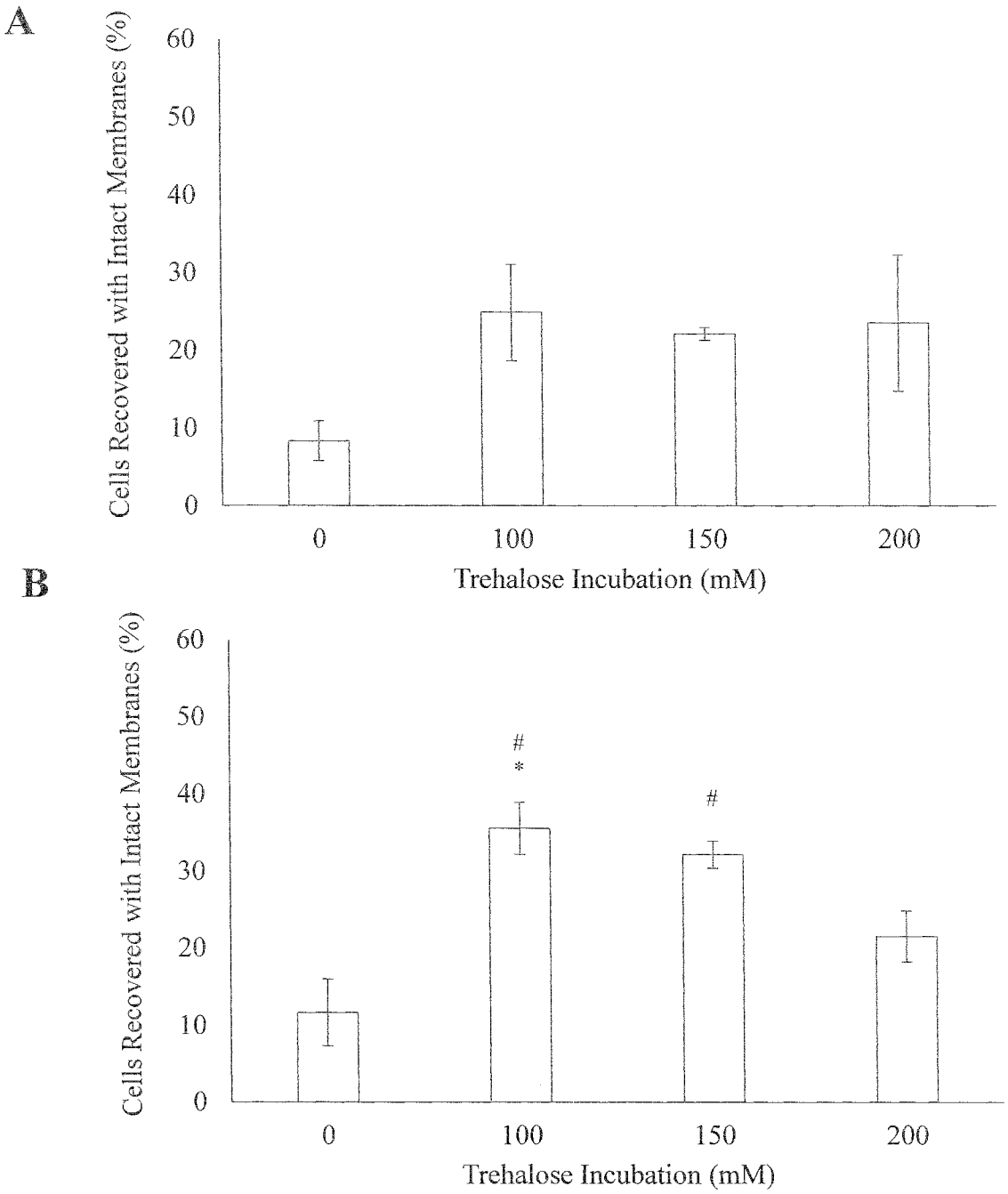

FIGURE 1. Effect of trehalose on recovery of Neuro-2a cells with intact membranes after freezing at -80 °C for 24 h. Cells were incubated for 24 h with the indicated concentrations of trehalose. Trypan blue exclusion assay was used to estimate the recovered cells with intact membranes at 24 h post-thaw. (*A*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO prior to freezing. (*B*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO + 100 mM trehalose prior to freezing. The data represent the mean ± SEM of 4 independent experiments with 2 nested replicates each (# $P < 0.05$ compared to 0 mM trehalose with 10% Me2SO exposure; * $P < 0.05$ compared to 0 mM trehalose with 10% Me2SO + 100 mM trehalose exposure).

Cryopreservation of Neuro-2a monolayers is improved in the presence of proline. Since proline accumulates under water stress in some organisms [16], [67], we next investigated the effect of proline on the cryopreservation of Neuro-2a cell monolayers. Contrary to our results with trehalose, these experiments revealed that pre-incubation with proline concentrations ranging from 50 to 200 mM did not improve the recovery of cells frozen as monolayers compared to controls if proline was absent in the Me2SO-based freezing solution (Fig 2A, $n = 3$, $P = 0.16$). However, proline incubation combined with proline added to the freezing solution in addition to 10% Me2SO significantly improved the recovery of cells frozen as monolayers compared to cells frozen without the addition of proline to the freezing buffer (Fig 2B, $n = 3$, $P = 0.005$). The largest amount of viable cells were recovered after using 100 mM proline in the pre-incubation step and freezing buffer resulting in 35.5 ± 4.5 (SEM)% of the cells recovered after cryopreservation followed by 24 h of cell culture under standard conditions.

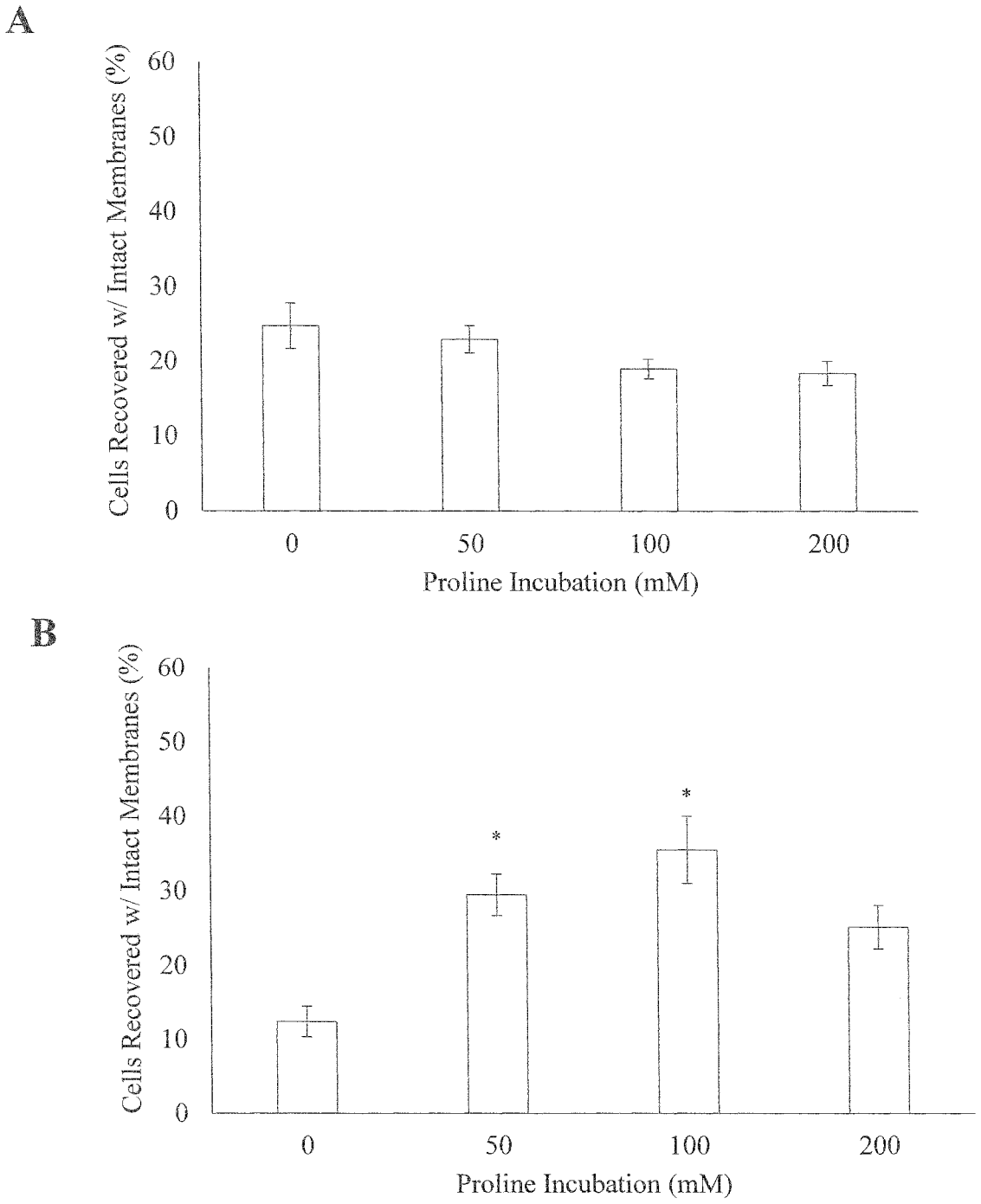

FIGURE 2. Effect of proline on recovery of Neuro-2a cells with intact membranes after freezing at -80 °C for 24 h. Cells were incubated for 24 h in indicated concentrations of proline. Recovered cells with intact membranes were evaluated 24 h after thawing via trypan blue exclusion assay. (*A*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO prior to freezing. (*B*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO + 100 mM proline prior to freezing. The data represent the mean ± SEM of 3 independent experiments with 3 nested replicates each (* $P < 0.05$ compared to 0mM with 10% Me2SO + 100 mM proline exposure).

Cryopreservation of Neuro-2a monolayers is greatly improved after concurrent incubation with proline and trehalose. In order to evaluate if the protective effects we observed with proline or trehalose alone were additive, we combined trehalose and proline in a 1:1 ratio to evaluate protection during freezing. These experiments revealed that concurrent pre-incubation of Neuro-2a cells with trehalose and proline at concentrations ranging from 87.5 to 112.5 mM showed significant improvement in recovery of cells frozen as monolayers compared to controls (Fig 3A; $n = 3$, $P = 0.0002$). Furthermore, the inclusion of trehalose and proline in the freezing buffer significantly improved recovery of cells frozen as monolayers compared to cells frozen without both compounds. The addition of 112.5 mM trehalose plus 112.5 mM proline to the incubation and freezing buffer recovered 52.5 ± 4.1 (SEM)% of cells after cryopreservation and 24 h of standard cell culture (Fig 3B, $n = 3$, $P = 9.25 \cdot 10^{-9}$).

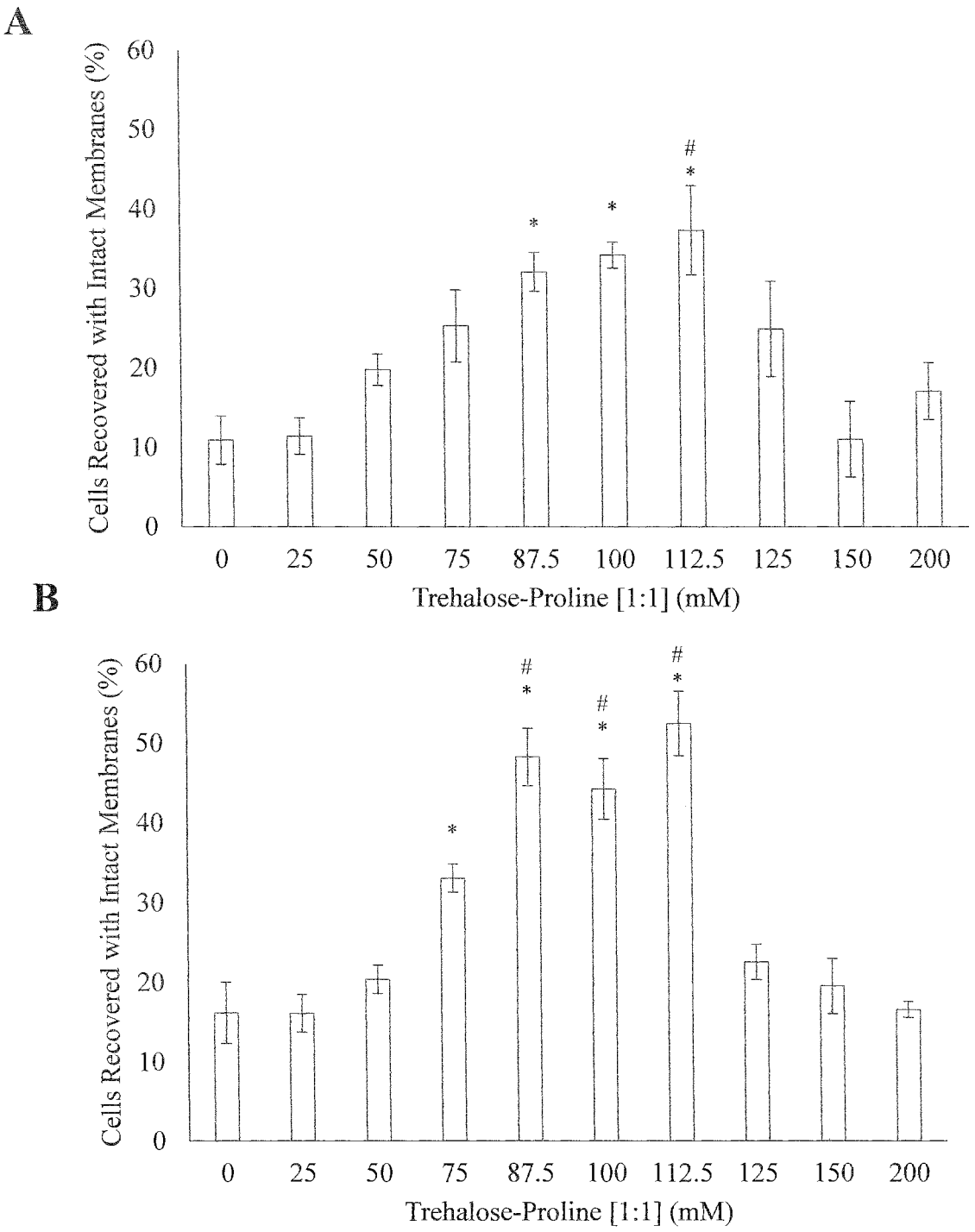

FIGURE 3. Combined effect of trehalose and proline on recovery of Neuro-2a cells after freezing at -80 °C for 24 h. Cells were incubated for 24 h in indicated concentrations of trehalose and proline. (*A*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO prior to freezing. (*B*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO + concentration of trehalose and proline in the freezing solution that matched the pre-incubation conditions (*$P < 0.05$ compared to 0 mM with 10% Me2SO; # $P < 0.05$ compared to 0 mM with 10% Me2SO + 25 mM trehalose and proline exposure). The data represent the mean ± SEM of 3 independent experiments with 2 nested replicates each.

Cryopreservation of Neuro-2a monolayers after incubation with sucrose. In order to further evaluate if the protective effects we previously observed were specific to the compounds themselves or due to a general cellular stress response caused by the hyperosmotic treatment, we replaced trehalose or proline with sucrose in a 1:1 ratio to evaluate protection during freezing. These cell counts revealed that pre-incubation with sucrose solutions, either solely or in combination with trehalose or proline, provided no significant improvement in recovery of cells frozen as monolayers compared to controls (Fig 4A, $n = 3$, $P = 0.67$). Furthermore, we saw no improvement of recovered cells with sucrose also present in the freezing buffer (Fig 4B, $n = 3$, $P = 0.39$). These results clearly demonstrate that the observed improvement in cryogenic performance was specifically due to the cryoprotective effects of trehalose and proline.

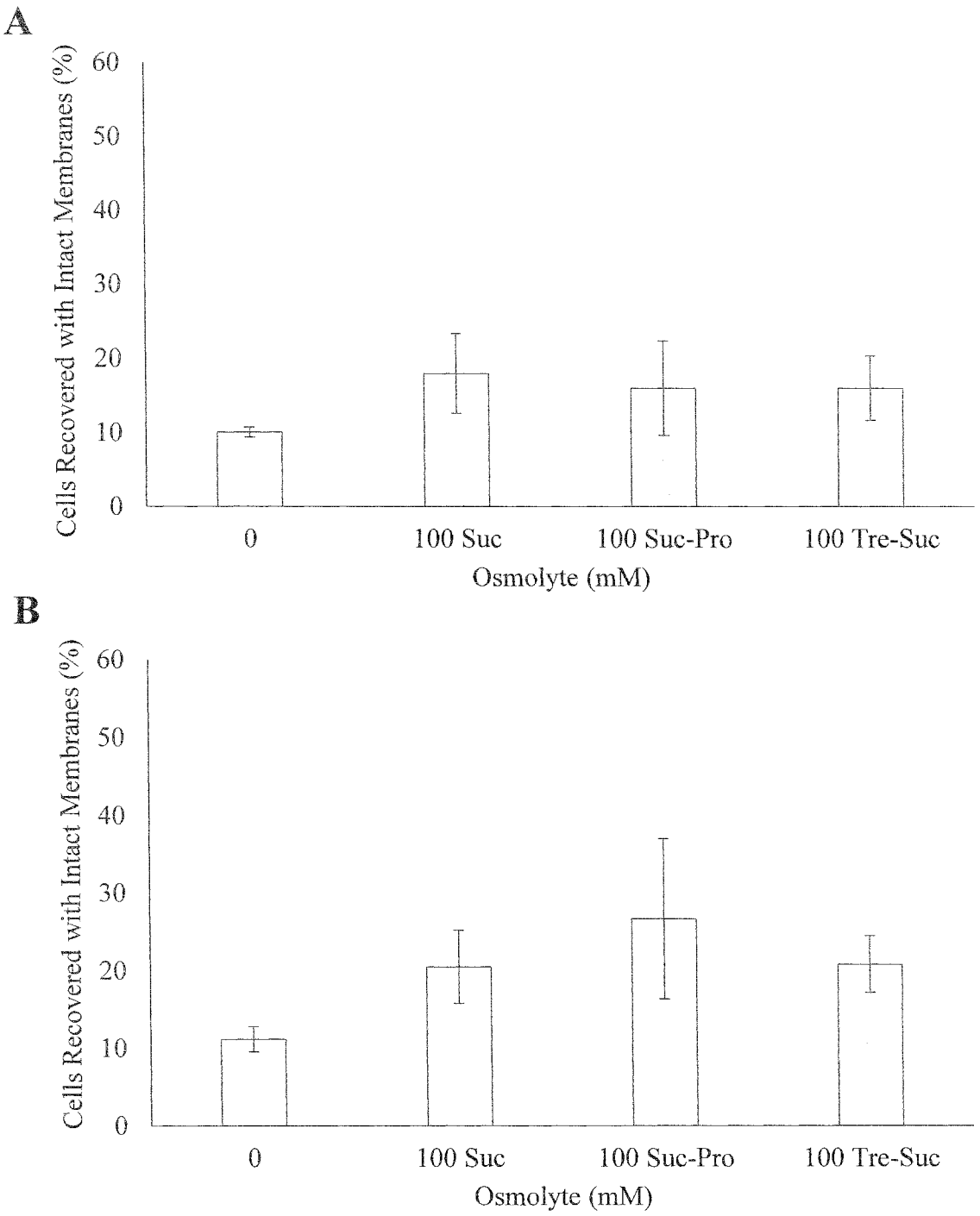

FIGURE 4. Effect of sucrose on Neuro-2a cells with intact membranes after freezing at -80 °C for 24 h. (*A*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO prior to freezing (Suc = sucrose; Pro = proline; Tre = trehalose). (*B*) Cells recovered with intact membranes after 10 min exposure to 10% Me2SO + concentration of trehalose and proline in the freezing solution that matched the pre-incubation conditions. The data represent the mean ± SEM of 3 independent experiments with 2 nested replicates each.

Growth of Neuro-2a cells in the presence of solutes. To determine the effects of our pre-incubation conditions on cell proliferation and viability we assessed the growth rate of Neuro-2a cells over a period of 6 days in the presence and absence of the solutes trehalose, proline, and sucrose. Cell counts were significantly reduced from untreated control cells on day 6 under all treatment conditions (Table 1, $n = 3$, $P = 7.9 \cdot 10^{-16}$). Cells incubated in 100 mM trehalose plus 100 mM proline had a drastically reduced growth rate compared to all other treatments with only a 4.8-fold increase in cell numbers compared to the 55.9-fold increase seen in control cells on day 6. To establish whether the cells were irreversible damaged after 3 days in the presence of solutes or if they were able to recover, we switched all conditions back to standard culture conditions at day 3 and assessed cell proliferation for an additional 3 days. We found that proliferation rates increased back to control levels, or slightly above, by day 6 for all conditions investigated (Table 2, $n = 3$, $P = 1.3 \cdot 10^{-5}$). To further assess that the cells were in fact growing slower and not simply dying faster, we evaluated the number of dead cells on day 3, 6, and on day 6 in the sample of recovering cells. We found that only in the presence of 150 mM trehalose plus 150 mM proline on day 3 and 6 were the amount of dead cells significantly increased above controls (Table 3, $n = 3$, $P = 3.2 \cdot 10^{-7}$).

Table 1. Average fold change in growth per day in presence of proline, trehalose, and sucrose solutions. The data represent the mean ± SEM of three independent experiments (* $P < 0.01$ significant from control).

Treatment	Cell Proliferation Increase (Fold)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	1.1±0.1	2.2±0.8	7.6±0.5	18.8±1.0	44.3±2.1	55.9±2.0
50 mM Trehalose-Proline	1.1±0.06	2.5±0.1	4.5±0.3*	14.4±1.5*	32.8±8.0	67.6±1.9*
100 mM Trehalose	0.7±0.01	1.5±0.5	5.3±0.7*	8.5±0.3*	16.1±1.0*	23.4±2.7*
100 mM Proline	0.5±0.02*	1.3±0.3	1.9±0.3*	5.4±0.1*	10.9±0.9*	31.3±3.2*
100 mM Trehalose-Proline	0.5±0.07*	0.6±0.1*	1.7±0.1*	2.7±0.7*	3.1±1.1*	4.8±1.8*
100 mM Sucrose-Proline	0.6±0.04*	1.1±0.03*	1.5±0.05*	5.3±1.4*	4.4±1.1*	13.1±1.8*
100 mM Trehalose-Sucrose	0.5±0.06*	0.6±0.01*	0.8±0.05*	0.9±0.5*	2.1±0.9*	5.9±0.8*
150 mM Trehalose-Proline	0.5±0.09*	0.6±0.1*	0.5±0.2*	1.1±0.2*	1.2 ±0.2*	0.8±0.2*
200 mM Trehalose	0.5±0.05*	0.6±0.09*	2.4±0.9*	8.8±0.3*	12.9±1.3*	12.0±1.8*
200 mM Proline	0.8±0.03	1.0±0.1*	1.1±0.2*	3.0±0.4*	6.7±1.1*	10.7±1.7*

Table 2. Average fold change in growth per day in presence of proline, trehalose, and sucrose solutions after 3 days followed by switch to solute-free cell medium. The data represent the mean ± SEM of three independent experiments.

Treatment	Cell Proliferation Increase (Fold)		
	Day 4	Day 5	Day 6
Control	2.7±0.2	7.5±2.3	12.0±3.5
50 mM Trehalose-Proline	3.8±0.5	7.7±1.5	9.9±1.2
100 mM Trehalose	4.0±1.2	12.1±3.5	19.7±5.7
100 mM Proline	2.2±0.7	7.5±2.4	16.2±4.7
100 mM Trehalose-Proline	2.1±0.6	5.8±1.7	18.7±5.5
100 mM Sucrose-Proline	3.5±0.9	9.4±2.4	20.0±3.8
100 mM Trehalose-Sucrose	1.1±0.3	3.9±0.5	7.3±1.7
150 mM Trehalose-Proline	1.5±0.5	3.4±0.7	6.5±1.3
200 mM Trehalose	2.0±0.2	7.8±0.6	13.4±0.6
200 mM Proline	5.4±1.3	13.6±2.8	14.2±0.8

Table 3. Average percentage of dead cells in presence of proline, trehalose, and sucrose solutions. Recovery cell proliferation measured for 3 days post incubation in indicated solutions for 3 days. The data represent the mean ± SEM of three independent experiments (* $P < 0.01$ significant from control).

Treatment	Dead Cells (%)		
	Day 3	Day 6	Day 6-R
Control	4.7±1.1	7.3±1.2	6.1±1.2
50 mM Trehalose-Proline	8.1±0.2	4.1±0.2	5.4±1.3
100 mM Trehalose-Proline	14.0±0.7	9.2±0.8	5.0±0.6
100 mM Sucrose-Proline	10.6±1.1	9.1±1.3	8.6±2.0
100 mM Trehalose-Sucrose	8.8±2.6	11.7±2.6	4.4±0.1
150 mM Trehalose-Proline	26.3±4.2*	34.9±4.6*	6.3±0.8
200 mM Trehalose	15.3±4.8	18.8±0.4	11.3±0.6
200 mM Proline	12.6±2.5	9.0±1.1	4.7±0.4

DISCUSSION

Since cells in some freeze-tolerant organisms experience subzero conditions in highly organized tissues, we hypothesized that protective sugars and compatible osmolytes, such as trehalose and proline, would be beneficial in the cryopreservation of cell monolayers. We have demonstrated in this study that the addition of trehalose and proline to confluent Neuro-2a monolayers increases recovery of viable cells by 288% after freezing and thawing.

We have shown that adherent Neuro-2a cells treated solely with Me2SO only had an average recovery of viable cells of 13.5%. This result is consistent with previous reports showing Me2SO yields extremely low survival rates of >5% for adherent human embryonic stem cells [68], [69]. CPA formulations containing 5 – 10% Me2SO are the standard protocol utilized in freezing cells in solution due to the ability of the CPA to enter cells and reduce cryoinjury by moderating the increase in solute concentration during freezing [19], [23], [24]. However, our results reinforce that treatment with Me2SO alone does not sufficiently protect adherent cells during the freezing process. We found that cells cultured in presence of 100 mM trehalose for 24 h and frozen in cell culture medium supplemented with both 100 mM trehalose and 10% Me2SO increased recovery of viable cells to 35% (Fig 1). In opposition to the results obtained with HepG2 cells [30], we saw that inclusion of the trehalose with Me2SO transiently prior to freezing improved viability. One main difference between the Neuro-2a and HepG2 cell lines is the presence of junctions in the hepatic cells and previous research has shown that these gap junctions facilitate intracellular ice formation [70]. However, it has more recently been shown that cells lacking junctions freeze at a higher temperature and the penetration

of extracellular ice into the paracellular space between cells is correlated with the presence of intracellular ice, and intracellular ice appeared to start most often from the location in the cell next to this paracellular ice [71]. Regardless of the mechanism, Neuro-2a cells do not typically form junctions between themselves and this may explain the differing results obtained for each line.

Trehalose has been shown to be the major protectant in most organisms able to survive harsh environmental conditions such as severe dehydration, and the sugar accumulates at the onset of water stress in several species such as the arctic springtail *Megaphorura arctica* and the soil-dwelling nematodes *Aphelenchus avenae* [46], [47], [72]. Additionally, trehalose has been shown to be the major stored carbohydrate in encysted embryos of the brine shrimp *Artemia franciscana* which undergo desiccation and experience subzero temperatures as a normal part of their life history [73], [74]. Trehalose is only permeable to the plasma membrane in the presence of a specific sugar transporter [75]–[77] which is not present in mammalian cells. However, in order to impart protection to cells, trehalose must be present on both sides of the membrane [78]. Despite the absence of transporters, intracellular trehalose can be accumulated via endocytosis by incubating mammalian cells in medium containing the sugar [79], [80]. The specific mechanism by which trehalose protects cellular structures is still debated. The water replacement hypothesis suggests that the hydroxyl groups of trehalose can substitute for the hydrogen bonding of water [72], [73], [81]. It has been shown that during desiccation, the loss of the hydration effect of water is compensated by the presence of trehalose, thus preserving the phospholipid bilayer. However, without trehalose, desiccation leads to heterogeneities in phospholipid packing and reduced acyl

chain density, thereby destabilizing the membrane and resulting in damage upon the influx of water [82]. Our results show that trehalose affords protection to Neuro-2a cells frozen as adherent monolayers.

Cells almost universally respond to the stress of hyperosmolality, such as that experienced during freezing, by accumulating compatible organic osmolytes [50]. An increase in proline synthesis was observed when the freshwater prawn *Macrobrachium rosenbergii* was subjected to a hyperosmotic environment [51], and increased proline transport is suggested to generate an osmoprotective proline gradient in the bacterium *Escherichia coli* [83]. Consistent with these findings, we found that preconditioning Neuro-2a cell monolayers with 100 mM proline exerted protective effects in the same range as trehalose if cells were frozen in the presence of 100 mM proline in addition to 10% Me2SO (Fig 2). In contrast to trehalose, proline is membrane permeable [54] and the brain-specific high-affinity L-proline transporter (PROT) has been proposed to provide an intracellular pool of proline which may serve an osmotic role in neurons [56]. Additionally, proline has also been implicated as a survival factor that protects the cell against apoptosis and maintains progression of the cell cycle [59]. Even more striking, when a high level of proline was incorporated into the tissues of *Drosophila melanogaster*, a chill susceptible insect, the flies were able to survive when 50% of its body water was frozen [60]. We have shown in this study that proline affords cryoprotection to adherent Neuro-2a cell monolayers resulting in viability similar to that seen with trehalose (~35%).

In our study, the effects of trehalose and proline were partially additive, and we recovered the highest amount of viable cells (~53%) after freezing and thawing when

cells were incubated in a solution composed of both 112.5 mM trehalose and 112.5 mM proline (Fig 3). According to the two-factor hypothesis of cryo-injury [21], [37], [84], [85], the ice formation characteristics tied to suboptimal cooling rate can cause 'solution effects injury' as the extracellular environment progressively becomes concentrated at cryogenic temperatures. Furthermore, there are strong indications that the nature of extracellular ice formation can have a strong bearing on the phenomenon of intracellular ice formation (IIF) at supraoptimal cooling rates [86]. While CPAs are known to play an important role in modulating cryogenic injury, the actual mechanism of action remains an active area of research in cryobiology. One mechanism by which CPAs exert cryoprotective properties might be via an increase in the total concentration of all solutes, which may reduce the amount of ice formed at any temperature [22]. In order to ensure our response was specific to the combination of trehalose and proline and not just an effect of raising the overall osmolarity, we also incubated our cells with combinations of sucrose and proline or trehalose. Since sucrose has been shown to reduce cell death during cryopreservation of stem cells [84], we replaced either trehalose or proline with sucrose. We did not see improved cell viability with these solutions (Fig 4), suggesting the protective effects in Neuro-2a cells are specific to the combination of trehalose and proline and not solely a function of increased solute concentrations. Our results suggest that trehalose and proline are working in a partially additive manner to protect cells during freezing, implicating that each solute may be protecting the cell through different mechanism(s).

It has been shown that pretreatment with 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), a compound with the potential to mimic a number of the

metabolic features associated with metabolic down-regulation (e.g. diapause), promoted a significant increase in cell viability post-freezing and the improvement in cryopreservation outcome correlated positively with an increase in the observed inhibition of cell proliferation [62]. Similarly, we found that cells exposed to 100 mM trehalose plus 100 mM proline exhibited a proliferation rate of only 8% of the growth rate of control cells after 6 days of exposure to the osmolytes (Table 1), but recovered a significantly higher amount of cells post freezing and thawing than controls. Metabolic depression and cellular stasis are often prerequisites to survival for animals whose evolutionary history has provided natural adaptations to environmental insults such as freezing or drying [61]. Additionally, a requirement for survival after exposure to freezing in both *Caenorhabditis elegans* [82] and *Drosophila melanogaster* [60] was a reduction in metabolic activity. Our findings suggest that a reduction in cellular activity may precondition cells for cryogenic 'insults' and the combination of trehalose and proline may partly exhibit their protective properties through metabolic down-regulation prior to the cryogenic insult.

CONCLUSIONS

We have shown that the combination of 112.5 mM trehalose and proline has unique properties to protect cell monolayers during freezing and thawing, providing a post-freeze viability of 53%. Additionally, incubation with 100 mM trehalose plus 100 mM proline drastically reduced the growth rate of the cells to 8% of control cell proliferation. Our results suggest that a combination of solutes may be required to stabilize cells during the freezing process by engaging signaling pathways to prevent apoptosis and down-regulating metabolic activity. Future studies are needed to investigate the molecular and physiological basis of the protective effects in more detail along with testing additional cell types and tissues.

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