The Cryopreservation of Monolayered Neuroblastoma Cells (Neuro-2a)

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Introduction

- Prior studies in our laboratory have shown trehalose to increase the membrane integrity of monolayer human hepatoma cells (HepG2) during freezing [1]
  - Trehalose is a naturally occurring reducer of cell stress, which protects organisms from extremes in heat shock and osmotic stress [2]
- Trehalose is moved from the extracellular to intracellular compartment via endocytosis and intracellular accumulation depends on extracellular concentration [3]
- Proline concentrations were elevated in bacteria subjected to osmotic stress during growth [4]
- Human brain-specific high affinity L-proline transporter [5]
- **We hypothesized that compatible osmolytes and protective sugars, such as trehalose and proline, would be beneficial in the cryopreservation of neuroblastoma cell monolayers**

Materials and Methods

- Mouse neuroblastoma cells (Neuro-2a) were plated on collagen coated 24-well microtiter plates at 0.5 million cells per well in 500 µL of culture medium (Opti-MEM I supplemented with 5.5% fetal bovine serum and antibiotics)
- After 2 h the culture medium was removed and replaced with culture medium containing either:
  a. Trehalose (0 - 200 mM)
  b. Proline (0 - 200 mM)
  c. Trehalose/Proline [1:1] (0 - 200 mM)
- Following incubation the medium was removed and replaced with freezing buffer composed of either:
  i. Opti-MEM I, 5.5% FBS and 10% DMSO (a,b,c)
  ii. Opti-MEM I, 5.5% FBS, 10% DMSO, and 100 mM trehalose (a)
  iii. Opti-MEM I, 5.5% FBS, 10% DMSO, and 100 mM proline (b)
  iv. Opti-MEM I, 5.5% FBS, 10% DMSO, and matched mM solution (c)
- Cells were transiently exposed to the respective buffer for 10 minutes, and placed into a passive freezing device (-1 ºC/min) (Cool Cell MP, BioCision Lake Spur, CA), to -80 ºC for 24 h
- Cells were thawed by addition of pre-warmed (37 ºC) culture medium and incubated for 24 h
- Cell membrane integrity and growth was assessed via trypan blue exclusion assay
- Statistics accomplished with one-way ANOVA test with HSD post-hoc analysis

Results

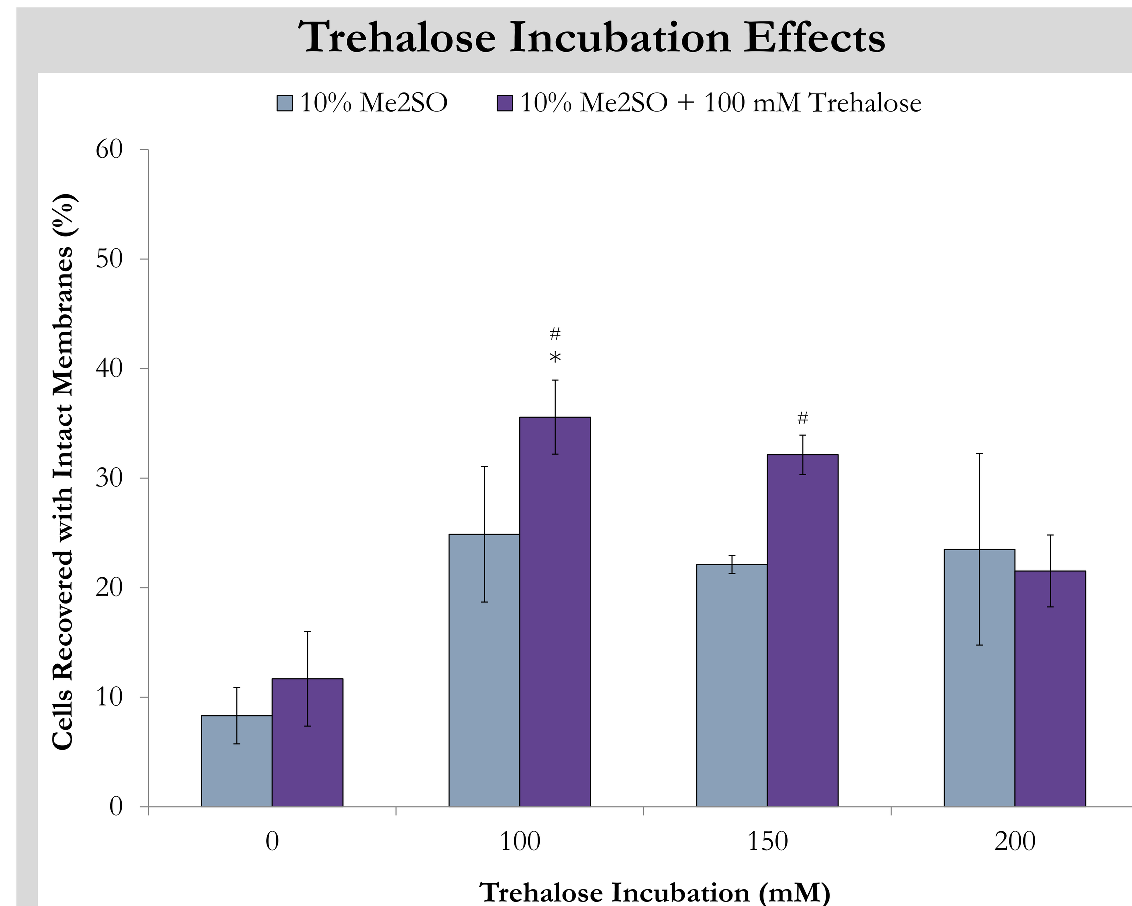

Figure 1. Membrane integrity of Neuro-2a cells after freezing and thawing was improved by addition of 100 mM trehalose (n=3 with 2 nested replicates, ±SEM; (# P < 0.05 compared to 0 mM trehalose with 10% Me2SO; * P < 0.05 compared to 0 mM trehalose with 10% Me2SO + 100 mM trehalose).

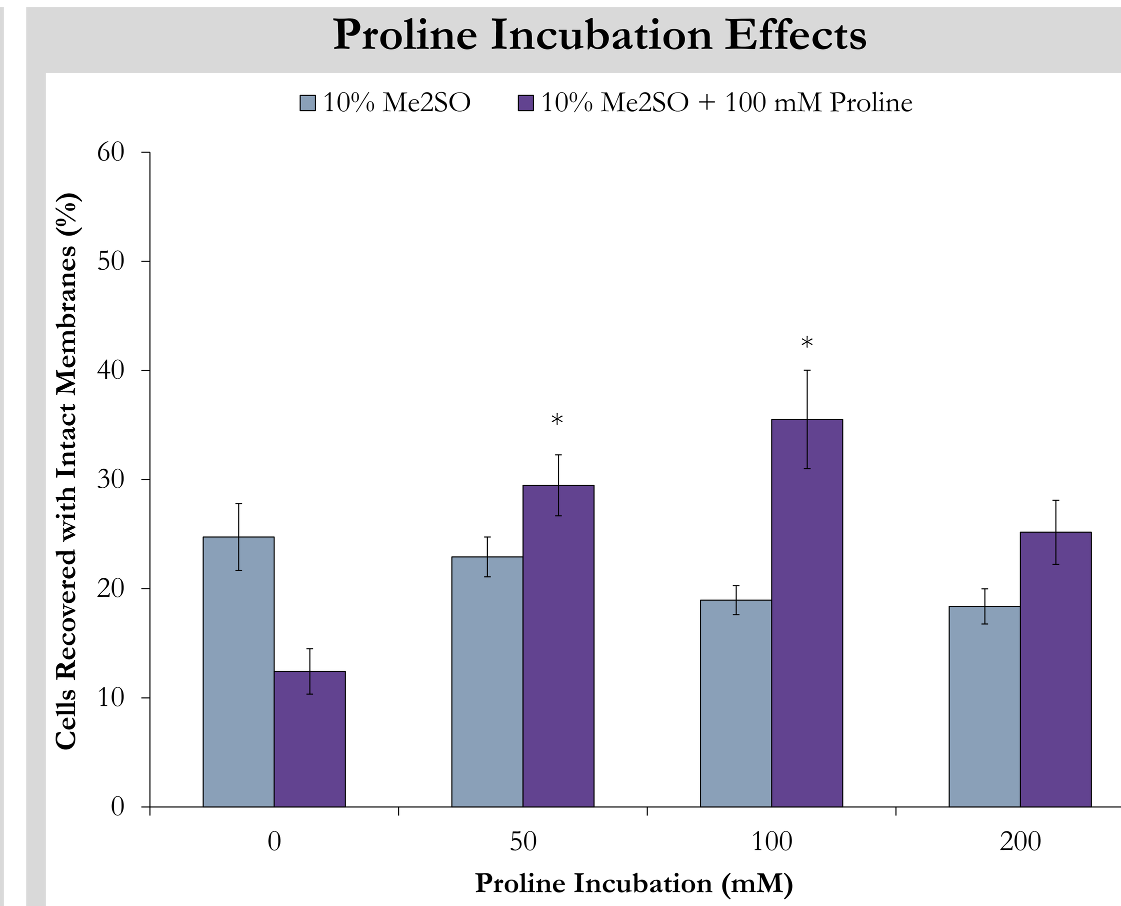

Figure 2. Membrane integrity of Neuro-2a cells after freezing and thawing was improved by addition of 100 mM proline (n=3 with 2 nested replicates, ±SEM; * P < 0.05 compared to 0mM with 10% Me2SO + 100 mM proline exposure).

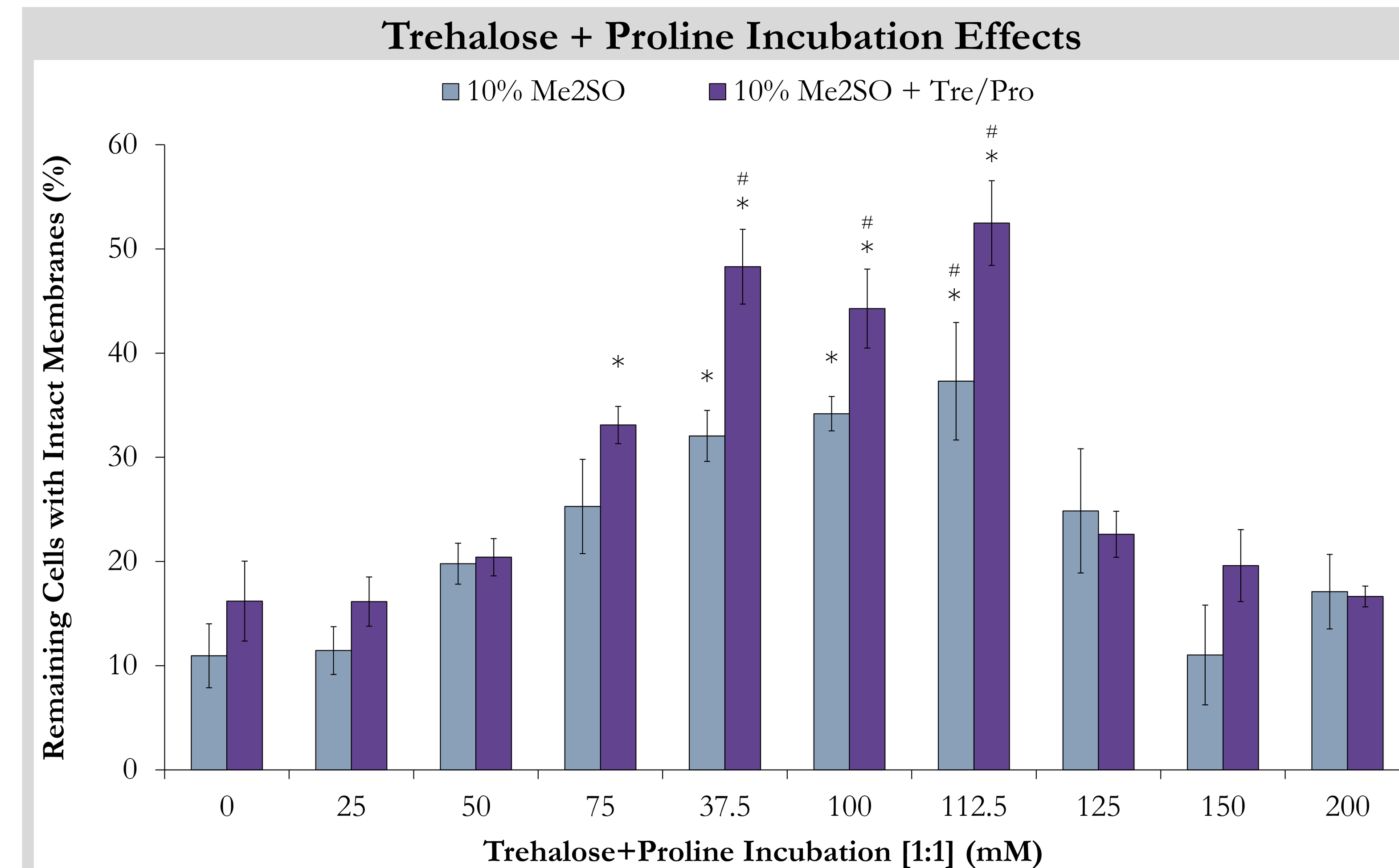

Figure 3. Membrane integrity of Neuro-2a cells after freezing and thawing was improved by addition of 112.5 mM solution of trehalose + proline (n=3 with 2 nested replicates, ±SEM; *P < 0.05 compared to 0 mM with Me2SO; # P < 0.05 compared to 0 mM with Me2SO + 25 mM trehalose+proline).

- We found significant differences in viability among trehalose concentrations (Fig. 1; P < .001) with 100 mM trehalose inclusion in the incubation and freezing buffer performing the best (36.7 ±4.5%)
- We found significant differences in viability among proline concentrations (Fig. 2; P < .001) with 100 mM proline inclusion in the incubation and freezing buffer performing the best (35.5 ±4.5%)
- We found significant differences in viability among trehalose + proline concentrations (Fig. 3; P < .001) with 112.5 mM of trehalose + proline inclusion in the incubation and freezing buffer performing the best (52.4 ±4%)

- We found a significant reduction in growth for cells incubated in solutions (Table 1; P < .001) with 100 mM trehalose+proline growth greatly reduced compared to control (55.9 ±2.0 vs. 4.8 ±1.8)
- We found growth rates returned to a higher fold increase when switched back to Opti-MEM on day 3 (Table 1; P < .001) with all conditions significantly higher than control

Trehalose + Proline Growth Rates

Table 1. Average fold change in growth per day in presence of proline and trehalose. 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 < 0.05 significant from control; *,$ P < 0.05 significant among treatments).

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
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 #$*
Control-Recovery	-	-	-	2.7 ±0.2	7.5 ±2.3	12.0 ±3.5
100 mM Trehalose-Recovery	-	-	-	4.0 ±1.2 *#	12.1 ±3.5 *#	19.7 ±5.7 *#
100 mM Proline-Recovery	-	-	-	2.2 ±0.7 *	7.5 ±2.4 *	16.2 ±4.7 *#
100 mM Tre+Pro-Recovery	-	-	-	2.1 ±0.6 *	5.8 ±1.7 *	18.7 ±5.5 #

Discussion

- **Trehalose:** we found that a 24 h incubation period with 100 mM trehalose provided the best cryoprotection for monolayers stored at -80 °C
- Trehalose is thought to act by altering or replacing the water shell that surrounds lipid and proteins [6,7]

Figure 3. Trehalose interacting with the bilayer [7].

- Desiccated *D. melanogaster* larvae could enter anhydrobiosis and revive upon rehydration and this strongly indicated the synthesis and accumulation of trehalose [8]
- **Proline:** we found that a 24 h incubation period with 100 mM proline provided the best cryoprotection for monolayer cells
  - When proline was incorporated into the tissues of *D. melanogaster* was able to survive when 50% of its body water was frozen [9]
  - High affinity L-proline uptake could provide an intracellular pool of L-proline, which serves a distinct metabolic or osmotic role [5]
- **Proline + Trehalose:** we found that a [1:1] 100 mM solution of trehalose + proline afforded the greatest cryoprotection for neuronal cells in a monolayer format
- **Growth:** incubation with [1:1] 100 mM trehalose + proline solution significantly reduced the growth rate
- **Our results suggest that a combination of solutes may be required to both stabilize the cells during the freezing process as well as manage signaling pathways to prevent apoptosis and down-regulate metabolic activity**

Literature Cited

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Additional Information

For additional information please contact the author at tlbailey@eiu.edu or visit http://ux1.eiu.edu/~mmenze/index.html