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Lessons Learned from Nature: The Sweet Way to Preserve Cells

Shhyam Moorthy

Lessons Learned from Nature: The Sweet Way to Preserve Cells

by

Shhyam Moorthy

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Dr. Steven C. Hand

Department of Biological Sciences

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Steven C. Hund **Thesis Director** (*Required*)	Biol. Sci. **Department** (*Same Dept as student*)
E. William Wischusen **2nd Faculty within Dept** (*Required*)	Biological Sciences **Department** (*Same Dept as student*)
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Abstract

The extreme tolerance of the brine shrimp embryo (*Artemia franciscana*) to water stress is likely due in part to the presence of a non-reducing sugar trehalose. As an experimental model for water stress, we exposed human hepatoma cells (HepG2) to subfreezing temperatures. The impact of pre-treating cells with trehalose on viability after freezing was evaluated. Prior to freezing, HepG2 cells show a dose-dependent decrease in proliferation when exposed to trehalose concentrations between 0 mM and 150 mM for 24h. However, cells treated with 100 mM trehalose showed a pronounced increase in viability after freezing. The viability increased from 1.9 ± 0.2%, at 0 mM trehalose to 20.1 ± 0.8 %, at 100 mM trehalose (± SE, $n = 9$). To facilitate the uptake of trehalose into cells, HepG2 cells were transiently transfected with a gene encoding a trehalose transporter (TRET1) from the insect *Polypedium vanderplanki* (cf., Kikawada et al., 2007). Transfected cells were loaded with the sugar by exposure to 150 mM, 200 mM and 250 mM trehalose for only 90 min. Under these conditions the transfected cells in 250 mM trehalose maintained the highest viability of 50.8 ± 2.9 % post freezing (± SE, $n = 9$), compared to the values for non-transfected (control) cells after freezing of 6.5 ± 0.7 % (± SE, $n = 9$). In conclusion, the loading of trehalose into mammalian cells with TRET1 improves viability of cells measured after freezing.

Introduction

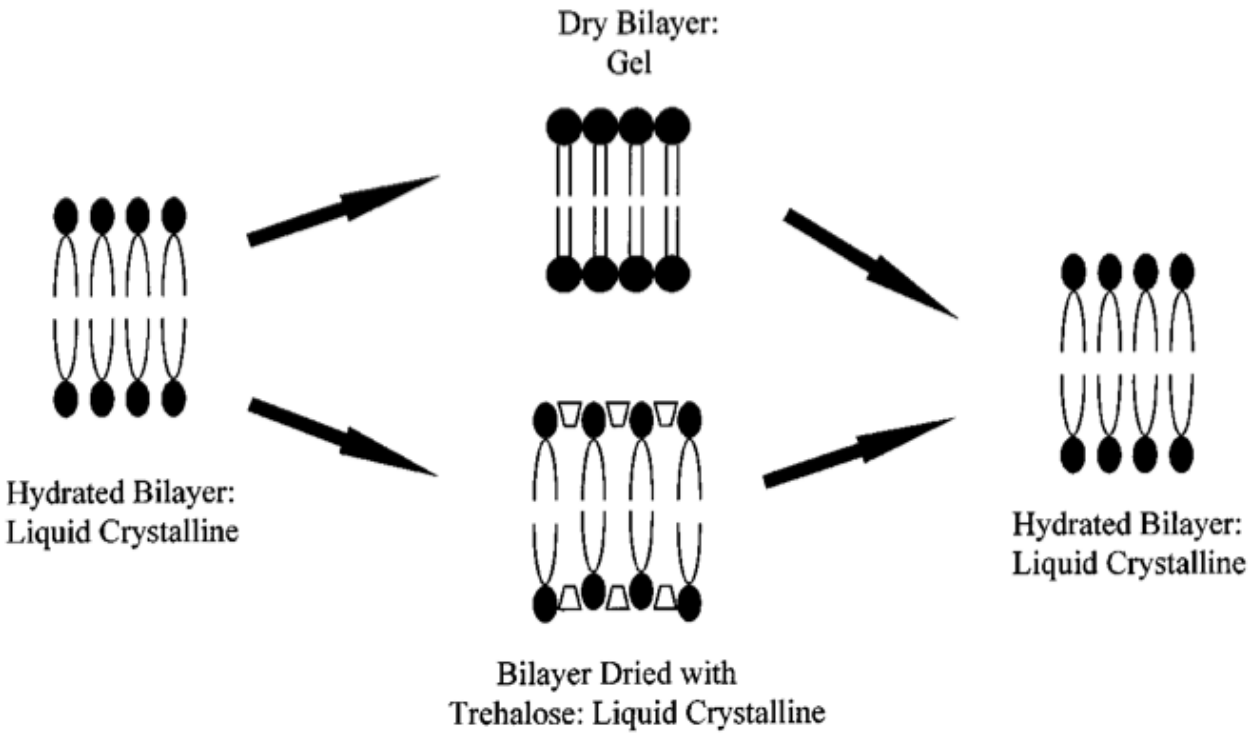
There has been an increasing interest in the long-term cryopreservation of mammalian cells as this could have many potential benefits particularly in the biomedical field. Cryopreservation of human hepatocytes reduces reliance on fresh liver tissue for research and biomedical applications. Most current cryopreservation techniques use dimethyl sulfoxide (DMSO), glycerol and liquid nitrogen for long term storage (Mazur, 1984). DMSO has been proved to be an efficient cryoprotectant, and viability of greater than 80% has been reported with human hepatocytes (Hengstler et al., 2000). However, one of the major disadvantages of using DMSO is its toxicity. After cryopreservation of mammalian cells, DMSO must be carefully removed to avoid any cytotoxic effects (Buchanan et al., 2005). Glycerol also has been shown to be toxic and damages cell membranes when used in high concentrations (Anchordoguy et al., 1987). Although the preservation of mammalian cells in liquid nitrogen has been widely used, replenishment of liquid nitrogen is periodically required. Frequent supply of liquid nitrogen is not always possible and may involve high costs and hazards (Mondal, 2009). Such limitations would be removed with successful freezing of mammalian cells in -80^{o} C freezers.

Certain species of invertebrates show great tolerance to various forms of water stress like desiccation and freezing (Hand and Hagedorn, 2008). These organisms include diapause embryos of the brine shrimp, *Artemia franciscana* (Clegg et al., 1997), several dry larvae and adults of nematodes (Madin et al., 1975), spores of some fungi (Sussman et al., 1959) and dry baker's yeast (Payen, 1949). These species can endure dehydrated states for long periods of time, and upon rehydration, are able to resume regular metabolic activities. Most mammalian cells die within a matter of a few minutes when exposed to water stressed environments such as desiccation (Hand and Hagedorn, 2008) and freezing (Mazur, 1984). The cell death mechanisms

associated with this phenomenon are not fully understood, but likely involves such issues as protein denaturation and phase transitions in lipid membranes.

Brine shrimp embryos are an exceptional example in that they can withstand long periods of extreme dehydration (Clegg, 1965; Clegg, 1974; Clegg, 1981; Clegg, 1984; Crowe and Clegg, 1973, 1978; Hand, 1992). During water stressed periods, the brine shrimp undergoes oviparous reproduction wherein the embryos released by the brine shrimp enter into a state of diapause. During this state, the embryo accumulates large amounts of a sugar called trehalose (Clegg. 1965). Trehalose is a non-reducing disaccharide composed of two glucose monomers bound in an α,α-1,1 linkage. This sugar is a key component in increasing water stress tolerance by preserving the structure and function of membrane proteins presumably by two mechanisms: water replacement (Crowe et al., 1984) and vitrification. (Crowe et al., 1998).

According to the water replacement hypothesis (Webb, 1965; Crowe et al., 1984), the unique orientation of hydroxyl groups on trehalose allows it to efficiently replace water molecules that are essential to maintain proper membrane and protein structure. The molecular architecture of trehalose allows it to interdigitate between phospholipid head groups and maintain its regular membrane structure. In hydrated conditions, water molecules spatially separate the polar head groups and when they are removed, the packing density of the head groups increases. As a result of such packing, there is an increase in Van der Walls interactions between the hyrocarbon chains. The temperature at which the hydrocarbon chains melt to form liquid crystalline phase (Tm) then increases. The result of such phase transitions is that the phospholipid bilayer becomes transiently leaky. This must be avoided to prevent the loss of critical cellular components. Trehalose interdigitates between phopholipid heads and prevents such phase transitions from occurring, thereby maintaining their proper spacing.



(Adapted from Crowe et al., 1998)

During the process of vitrification (Crowe et al., 1998) trehalose and similar sugars form amorphous sugar glasses at physiological temperatures. Thus the formation of crystalline sugars, which might prove to be lethal for the cell, is avoided. Understanding such principles that govern life without water might prove essential to increase our preservation capabilities of mammalian cells and tissues.

Trehalose is not permeable to plasma membranes; for efficient biostabilization of mammalian cells, the sugar must be present on both sides of the plasma membrane (Liu et al., 2005). Pinocytosis allows limited movement of trehalose across membranes. Due to the high extracellular concentrations of the sugar required, osmotic pressure could cause a reduction in cell proliferation. Trehalose can be more efficiently loaded into cells when its movement

facilitated by specific protein transporters**.** A number of methods such as lipid phase transition (Wolkers et al., 2001), hemolysin-H5 pores (Eroglu et al., 2000) and $P2X_7$ purinergic receptors (Elliott et al., 2006) have been employed to facilitate the uptake of trehalose in mammalian cells**.** However, with the recent discovery of a bidirectional trehalose transporter, TRET 1 (Kikawada et al., 2007), uptake of trehalose into mammalian cell lines has been improved. Kikawada et al. isolated the gene coding for TRET1 from the fat body of a chironomid fly larvae *Polypedium vanderplanki.* The isolated TRET1 was shown to facilitate trehalose uptake in three mammalian cell lines (Kikawada et al., 2007). The transfected cells showed a significantly higher uptake of trehalose compared to the non transfected cell lines.

As a mammalian model for water stress, we treated human hepatoma cells (HepG2) to sub freezing temperatures in the presence of trehalose. We report that HepG2 cells exposed to 100 mM extracellular trehalose concentration for 24 h and frozen at -80^0C had the highest viability of 20.1 ± 0.8 %, post freezing compared to all other concentrations. Prior to freezing however, treating HepG2 cells to varying external concentrations of trehalose at 37^0 C for 24 h showed a dose dependent decline in cell proliferation, presumably due to osmotic stress. This effect was not reversed even after the removal of the sugar and incubation in regular media. Further, HepG2 cells that were genetically altered to transiently express TRET 1 when exposed to 150mM, 200mM and 250mM trehalose for only 90 min and frozen at -80^0C maintained a viability of 17.3 ± 2.0%, 31.1 ± 2.4% and 50.8 ± 3.0% post freezing respectively, each of which was at least four fold greater than the non transected controls at each respective concentration. Thus, we conclude that the use of trehalose can have potential impacts in the field of cryopreservation, and with appropriate methods for the uptake of the sugar into cells, can be used for the biostabilization of mammalian cells during freezing.

Materials and Methods

Cell culture

HepG2 cells (ATCC, Manassas, VA), were grown in 75cm^2 cell culture flasks (Corning Incorporated, Corning, NY) containing OPTI-MEM media supplemented with 5 % fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen, Grand Islands, NY). Cells were maintained in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 ºC.

Trehalose treatment

Cells were detached from the flask using 0.05 % trypsin-EDTA solution (Invitrogen, Grand Islands, NY) and replated at 1 million cells per well in a two 6 well culture plates (Corning Incorporated, Corning, NY) containing 4 ml of trehalose media. Extracellular trehalose concentrations of 50 mM, 100 mM and 150 mM was used. Each trehalose solution contained trehalose (Mallinckrodt), 20% FBS and 20% distilled water. Trehalose solutions were sterile filtered using a 50mm, 250 mL vaccum filters (Nalgene, *Thermo Fischer Scientific Inc.*, NY) before adding them to cells. The cells were incubated in the same conditions as above for 24 h for uptake of the sugar by pinocytosis.

Freezing of cells at -80^{0}C

After completion of the 24 h incubation period for each treatment group, trehalose media was removed and cells were detached using 2 ml of 0.05 % trypsin-EDTA solution. 2 ml of OPTI-MEM media were then added and the whole solution was pipetted into a 15 ml tube. Each of the tubes was then centrifuged in a Centra CL Benchtop centrifuge (*Thermo Fischer Scientific Inc.,* NY) at 1000 rpm for 5 min. The supernatant was discarded and 1 ml of the respective trehalose media was added to each tube. 0.8mL of the cell solution was placed in 2 ml cryogenic vials (Nalgene, *Thermo Fisher Scientific Inc,* Rochester, NY), and 0.2 ml was used for cell counting with the trypan blue exclusion test to evaluate membrane integrity after trehalose

treatment. The vials where then placed in a Nalgene Cryo 1 °C Freezing container (controlled freezing module) (Nalgene, *Thermo Fisher Scientific Inc,* Rochester, NY). The controlled freezing module contains eighteen polyethylene 2 ml vial holders and is in contact with 100% isopropyl alcohol. The holder prevents direct contact between the vials and the alcohol and thus prevents contamination. The temperature within the controlled freezing module decreases at a rate of -1° C per min. The freezing module was frozen in a -80^{o} C freezer for 24 h.

Membrane integrity and viability post freezing

After the 24 h freezing period, the cryovials were thawed in a 37 ^{o}C water bath for 4 min. The cell suspension in the cryovials was then agitated using a vortex shaker for 30 sec. to suspend cells that might have settled in the bottom. 0.2 mL of the cell solution was used to determine membrane integrity by the trypan blue exclusion test and the remainder was plated in standard 6 well plates suspended with standard OPTI-Mem medium for 24 h. Viability was measured 24 h post plating by the trypan blue exclusion test.

Transient Transfection of cells with TRET-1

Cells were grown at a density of 500,000 cells/ml in 35mm cell culture plates containing regular OPTI-Mem media for 24 h. The regular cell culture media was replaced with 4 ml of fresh antibiotic free OPTI-MEM. 4.5 µl of the plasmid (2 µg plasmid total) was mixed with 95.5 µl OPTIMEM (no additions). 5 µl of FuGENE transfection reagent (Roche, Switzerland) was added to the above mixture and incubated for 15 min. The mixture was then added drop wise to the plate while continuously swirling the plate.

Alternative protocol for trehalose loading

In cells transfected with TRET1, a different method for trehalose treatment was used. After growth in cell culture plates for 24 h, transfected cells were detached using trypsin and

suspended in OPTI-Mem media (no additions) and centrifuged by the same method described earlier. The suparnatent was discarded and the cell pellet was suspended with the respective trehalose solutions. The tubes were then placed in a Forma Scientific Orbital Shaker (Thermo Fisher Scientific Inc, NY) and agitated at 450 rpm at 30^{o} C for 90 min. After 90 min., 0.8 mL of the cell solution from each tube was added to cryovials for freezing.

Statistical Analysis.

Results are reported as mean ± standard error. Statistical analyses were done using *Sigma Plot 9* (SYSTAT software). Statistical differences were evaluated using ANOVA and students t-test.

Results

Incubations with trehalose: passive uptake by pinocytosis

The effect of trehalose on cell proliferation is shown in Figure 1. HepG2 cells show a dose dependent decrease in cell proliferation when exposed to external trehalose concentrations between 0 mM and 150 mM at 37^{o} C for 24 h. In the presence of trehalose, cell proliferation presumably was inhibited due to the high osmotic pressure as a result of the elevated trehalose concentrations. The passive uptake of trehalose by pinocytosis is not a very efficient method for the influx of large quantities of trehalose into the cells. Trehalose concentrations of 150mM showed maximum inhibition in cell proliferation as manifested by the 57 % decline compared to the controls. The effect of osmotic stress was not reversed even after removal of trehalose (Fig. 2). After removal of trehalose, HepG2 cells were plated in standard culture medium, and cell counts were performed after 24 h. As can be seen in Figure 2, trehalose caused an inhibition in cell proliferation even after the removal of the sugar.

Trehalose loading improves freeze tolerance

HepG2 cells were treated with 50 mM, 100 mM and 150 mM trehalose for 24 h and frozen in freezing solutions containing100 mM trehalose. Viability of HepG2 cells was measured 24 h post-freezing after culturing in regular cell culture media. As seen in Figure 3, HepG2 cells treated with 100mM trehalose prior to freezing displayed the highest viability of 20.1 ± 0.8 %, while viabilities in the control (no trehalose), 50mM and 150mM treatments were 1.9 ± 0.2%, 3.3 ± 0.2% and 12.0 ± 0.7%, respectively. The result in Figure 3 is an average of nine replicates. This result indicates that the optimal concentration of trehalose for freezing and successful post recovery is 100 mM. Although the viability of HepG2 cells treated with 100mM trehalose was

significantly improved, a more efficient method for the uptake of trehalose was needed to increase cell viability.

Transfection of cells with a trehalose transporter (TRET1) from Polypedium vanderplanki *improves viability*

HepG2 cells were genetically altered to transiently express a gene that codes for the trehalose transporter, TRET1. Since TRET1 was previously proven to be effective in the uptake of trehalose in multiple mammalian cell lines (Kikawada et al., 2007), HepG2 cells were treated with 150 mM, 200 mM and 250 mM trehalose. Viability post-freezing was greatest for cells frozen with 250 mM trehalose compared to the non-transfected controls and all other trehalose concentrations (Fig. 4). The viability of non transfected control cells was similar in 150mM and 200mM trehalose, 4.4 ± 0.5% and 4.3 ± 0.5% respectively while a slight increase was seen in 250mM trehalose, 6.5 ± 0.7%. Viability of transfected cells at all concentrations showed at least a fourfold increase post-freezing compared to the respective non- transfected controls. The percentage viability of transfected cells post-freezing were 17.3 ± 2.0% for 150 mM trehalose, 31.1 ± 2.4% for 200 mM trehalose and 50.8 ± 3.0% for 250 mM trehalose.

Discussion and Future Directions

The approaches described here for the efficient loading of trehalose into cells can have potential impacts in the field of cryopreservation. When exposed to external concentrations of sugars, mammalian cells are subjected to osmotic stress since most sugars cannot easily penetrate the plasma membranes. However, the use of the trehalose transporter TRET 1 provides a more efficient procedure for trehalose uptake by cells.

Human hepatoma cells (HepG2) showed reduced cell proliferation at elevated trehalose concentrations (Fig. 1). Osmotic pressure of the 150 mM trehalose solution was about 450 mOsm compared to 300 mOsm for the control medium (with no trehalose). Osmotic stress is known to inhibit cell proliferation in a variety of cell lines. Silvotti et al. (1991) reported that SV.3T3 fibroblasts challenged with high concentrations of NaCl (400-500 mOsm) exhibited a dramatic decline of 75% in cell proliferation and a significant decline in protein synthesis and as well. In chicken fibroblasts exposed to 500-800 mOsm of NaCl for 30 min., protein synthesis declined by 75-95%. (Petronini et al., 1987). The molecular signals that trigger these changes are still unknown.

Many bacteria, plants and animals have been shown to adapt to an increase in external osmotic pressure by accumulating 'compatible solutes' (Yancey et al., 1982). These solutes include sugar alcohols (or polyols), neutral free amino acids, and methylamines plus urea. They are termed 'compatible' because their presence does not perturb the structure and function of intracellular cell macromolecules. Accumulation of such solutes occurs in response to osmotic stress either through an increase in internal synthesis of these compatible solutes or their uptake by cells (Burg et al., 2007; Burg and Ferraris, 2008; Garcia-Perez & Burg, 1991). Petronini et al. (1992) showed that addition of 10-25 mM of betaine to SV.3T3 fibroblasts subjected to 500 mOsm of NaCl prevented a 90% decline in cell proliferation. Even though trehalose can be

considered a compatible osmolyte, we did see a dose dependent decrease in cell proliferation. Presumably the effect was due to water loss from the cells, but this phenomenon needs further experimental study. The inhibition of cell proliferation was not reversed even after incubating cells for 24 h in regular media after the withdrawal of trehalose (Fig. 2).

HepG2 cells show a pronounced increase in cell viability after freezing in the presence of 100 mM trehalose loaded by pinocytosis without the TRET 1 transporter. There was a reduction in viability of cells treated with 150 mM probably because of the osmotic stress as a result of such high concentration. Similarly, a reduction in viability was also seen in cells treated with 50mM trehalose. The result could be attributed to this low trehalose concentration did not promote sufficient levels of trehalose inside the cells. Direct measurement of intracellular trehalose levels would be important for future studies. Katnez et al. (2007) conducted similar freezing experiments on primary human hepatocytes using a combination of DMSO and trehalose as cryoprotectants. In the presence of 1.4 M DMSO and varying trehalose concentrations, Katnez et al. found membrane integrity after freezing of HepG2 cells to be highest at 300 mM trehalose (54% relative to unfrozen control cells). The impact of 1.4 M DMSO and 300 mM trehalose mixtures on cell viability was not tested. DMSO is cytotoxic and care must be used to rapidly remove DMSO from cells during thawing (cf. Buchanan et al, 2005). Davis et al. (1990) showed that bone marrow grafts cryopreserved in 10% DMSO (i.e., 1.4 M) and then directly infused into patients had adverse effects on patients ranging from nausea, to transient hypertension which later required medication. Properties of DMSO that are considered to be particularly important to its therapeutic as well as toxic effects include: rapid cell penetration, enhanced penetration of other substances across plasma membranes, scavenging

free radicals, effects on coagulation, anti-cholinesterase activity, and induced histamine release by mast cells. (Brayton, 1986).

HepG2 cells genetically engineered to express a trehalose transporter (TRET1) showed a remarkable increase in viability post freezing compared to controls (Fig. 4). I predict this result was due to improved uptake of trehalose into cells, such that the sugar was present on both sides of the membrane at sufficiently high levels to induce effective biostabilization during freezing. Again, internal trehalose concentrations after loading were not measured in the present study; however, the data from Kikawada et al. (2007) suggests that when tranfected into mammalian cells TRET1 facilitates the uptake of large quantities of extracellular trehalose. Kikawada et al. isolated the gene that codes for the transporter from the chironomid fly larvae and transfected three mammalian cell lines with TRET1. These transfected cells were then treated to 100 mM extracellular trehalose concentration for 3 h. In all of the transfected cell lines, internal concentrations of trehalose were 4-14 folds higher than the non-transfected controls (measured by HPLC).

In our experiments, HEPG2 cells were transiently transfected with TRET1. At this point besides TRET1 serving as a facilitated transporter for trehalose, other consequences of expressing this foreign protein in human hepatoma cells are unknown. To prevent any potential negative effects that TRET1 may have on HEPG2 cells, we preferred transient transfection over stable transfection. First, TRET1 was needed only for a short time period for the uptake of the sugar into HEPG2 cells after which its function was not required. Second, after several cell passages, the TRET1 should be no longer present. Elimination of the foreign protein in this way could be of benefit, if in the future it became desirable to introduce a cryopreserved cell preparation into human patients.

With TRET1-transfected cells, we employed a shorter time period for trehalose incubation (90 min) compared to our earlier protocols for non-transfected cells. We also subjected transfected cells to vigorous shaking so that the cells did not settle during the loading period (perhaps increasing the effective surface area of cells exposed to the sugar). Although long-term (24 h) loading of trehalose without TRET1 did increase viability after freezing (Fig. 3), short-term (90 min) loading with shaking was even more effective in improving post-freezing viability (Fig. 4). The cells were attached to culture plates during long-term loading, again perhaps compromising the surface area available for sugar loading. Thus far our experiments were performed using freezing periods of 24 h only. Although 24 h freezing of hepatoma cells with trehalose has yielded encouraging results, the possibility that trehalose may serve as a beneficial cryoprotectant for long-term storage needs investigation after freezing periods of weeks to months. Such long-term experiments are planned for the future.

Trehalose has proved to be efficient protectant during periods of water stress. More recently it has been reported that Late Embryogenesis Abundant (LEA) proteins are expressed during life history stages of brine shrimp that tolerate severe desiccation and freezing (Hand et al., 2007, Menze et al., 2009). In addition to brine shrimp LEA proteins are also found in plants, bacteria, fungi, and certain insect larvae (e.g., Tunnacliffe and Wise, 2007). As reviewed by Tunnacliffe and Wise, LEA proteins have various physiological effects, but most important among them are a) stabilization of sugar glasses, b) protein stabilization via protein-protein interactions c) ion sequestration and d) formation of structural networks. All these factors have been hypothesized to decrease the stress a cell undergoes during desiccation. LEA proteins are not expressed in mammalian cells. Our next goal is to determine if there is a synergetic relationship between LEA proteins and trehalose in conferring freeze tolerance to mammalian

cells, as has been previously reported for drying of cells with trehalose and a small stress protein, p26 (Ma et al., 2006).

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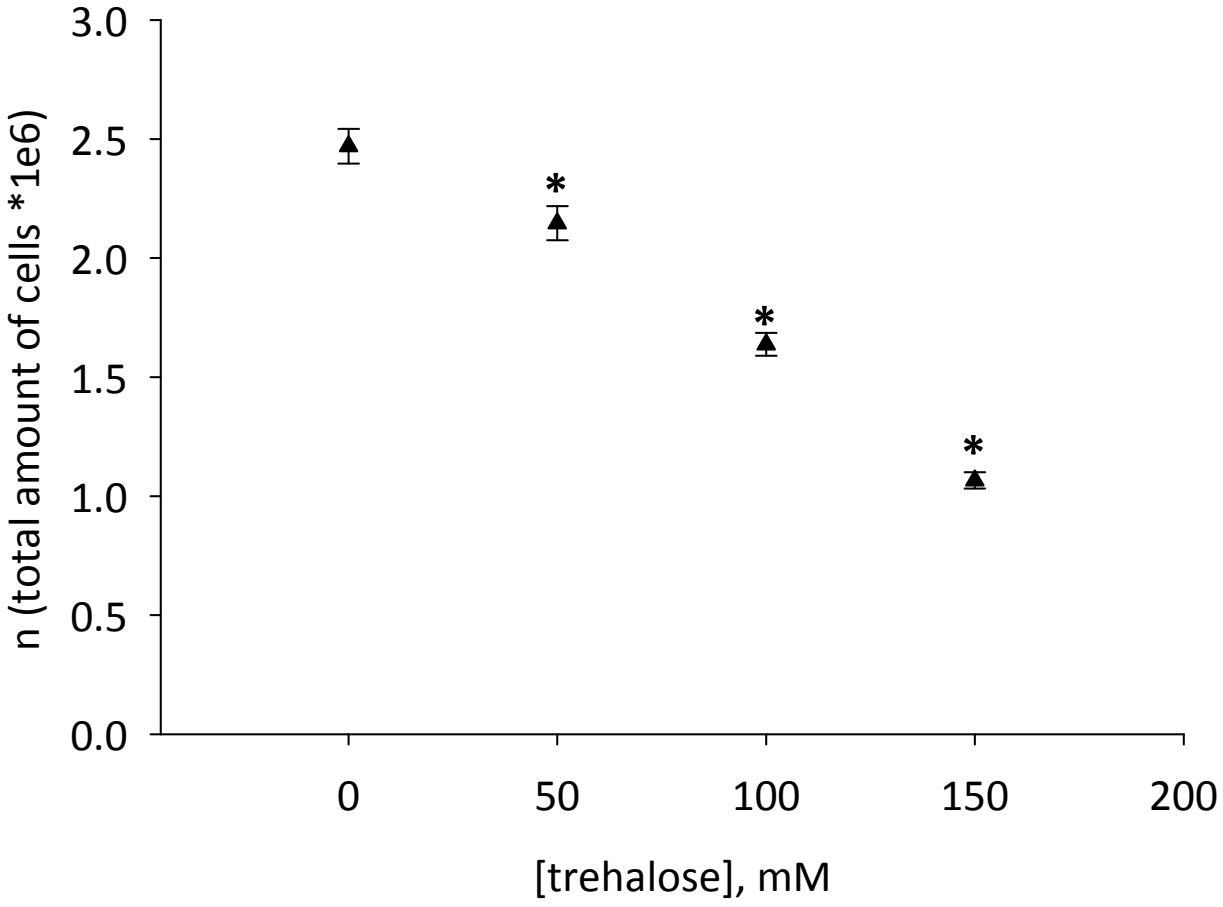

Figure 1. Impact of trehalose on cell proliferation. Human hepatoma cells (HepG2) were plated in standard culture medium at a density of 1 x 10^6 cells. Cell counts were performed after culturing the cells in presence of the indicated sugar concentrations for 24 h. Trehalose caused a significant inhibition in cell proliferation at all concentrations investigated (n = 9, ±SE). *statistically significant difference compared to 0 mM trehalose ($P < 0.05$).

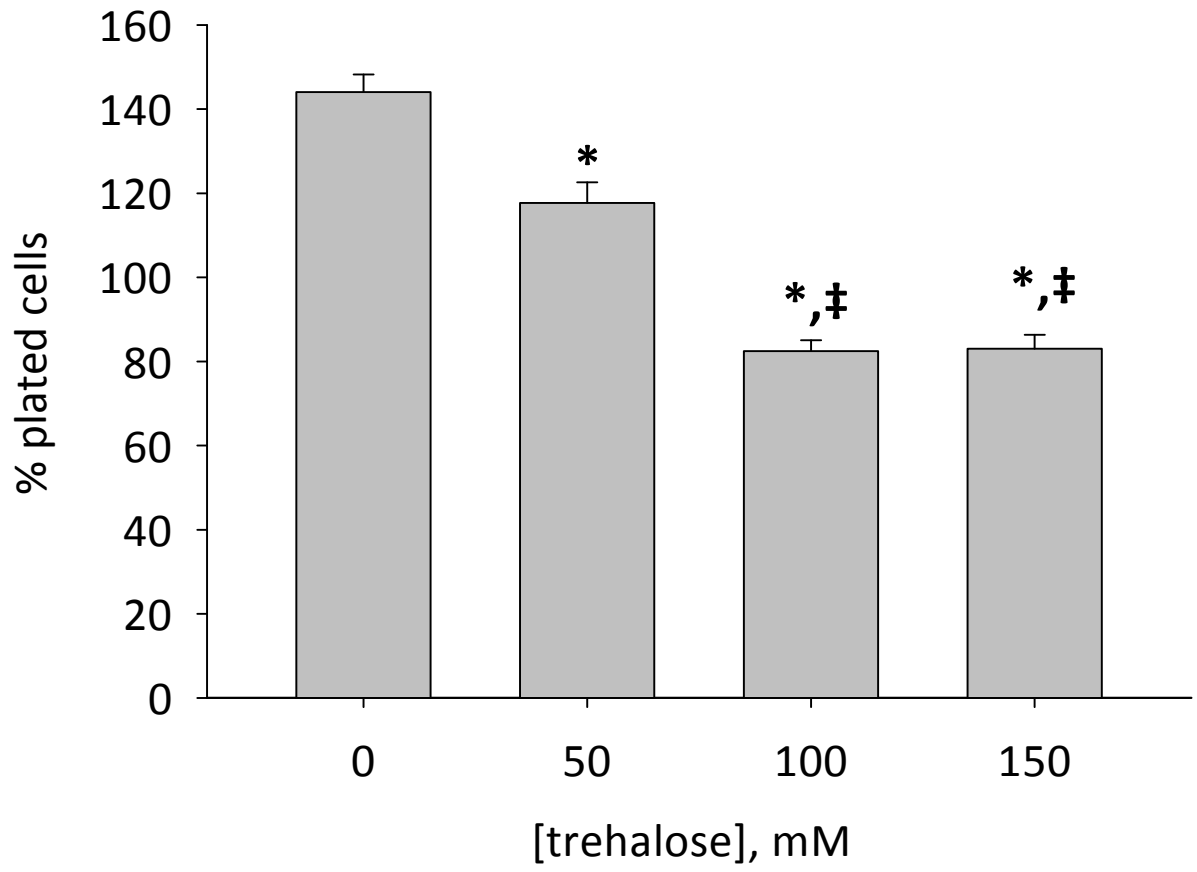

Figure 2. Recovery of HepG2 cells from exposure to trehalose. Human hepatoma cells (HepG2) were treated with trehalose for 24 h at concentrations between 0 mM and 150 mM. After these incubations, cells were detached, counted and subcultured in trehalose-free medium. 24h after sub culturing cells were counted and values expressed as percentage of plated cells. Inhibition of cell proliferation persisted after removal of the sugar (n = 9, ±SE). *Statistically different from 0 mM trehalose ($P < 0.05$). ‡ No significant difference between 100 mM and 150 mM trehalose (n = 9, ±SE).

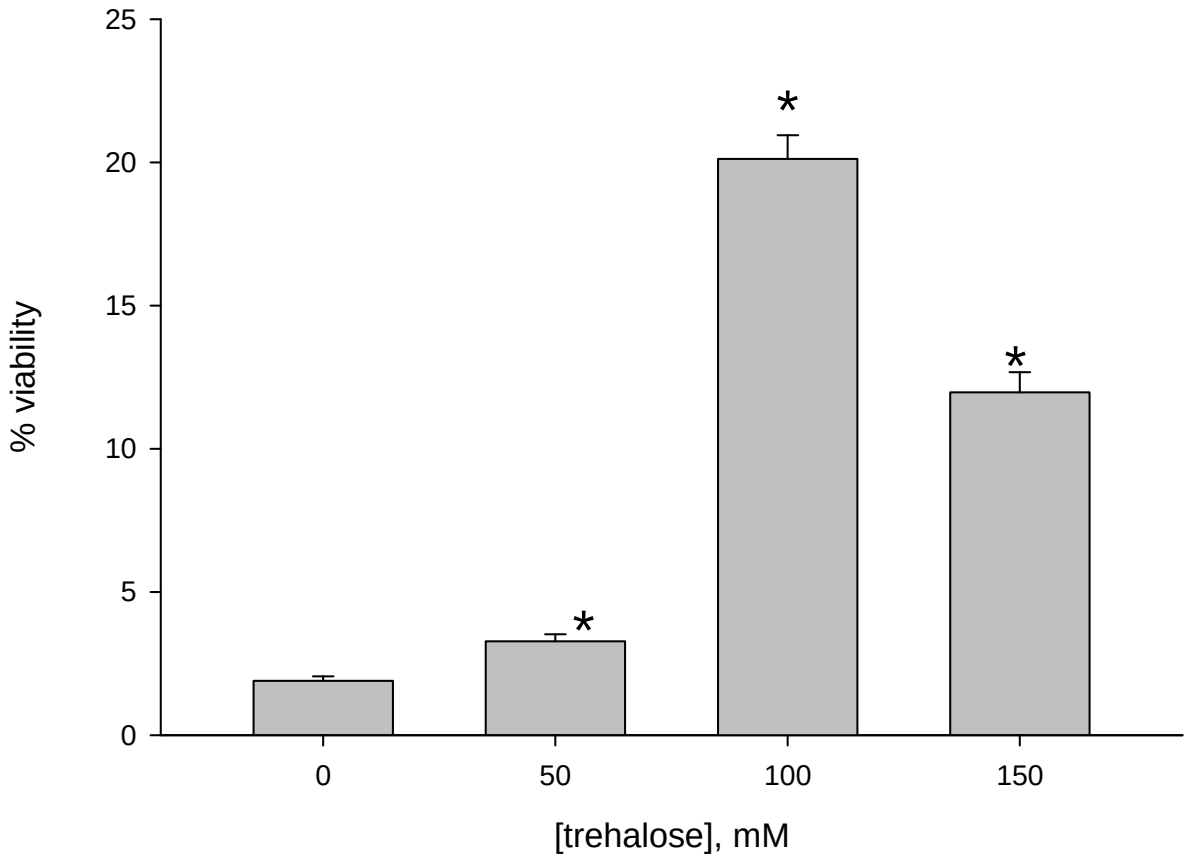

Figure 3. Viability after freezing. Human hepatoma cells (HepG2) were incubated with trehalose for 24 h at concentrations between 0 mM and 150 mM. After incubation cells were detached, counted and stored frozen at -80^{0}C for 24 h in freezing solution that contained 100 mM trehalose. Samples were thawed, cultured for 24 h and cell counts are expressed as percentage of cells before freezing. Incubation with100 mM trehalose for 24 h confers the highest amount of protection (n = 9, ±SE). *Statistically different from 0 mM trehalose ($P < 0.05$).

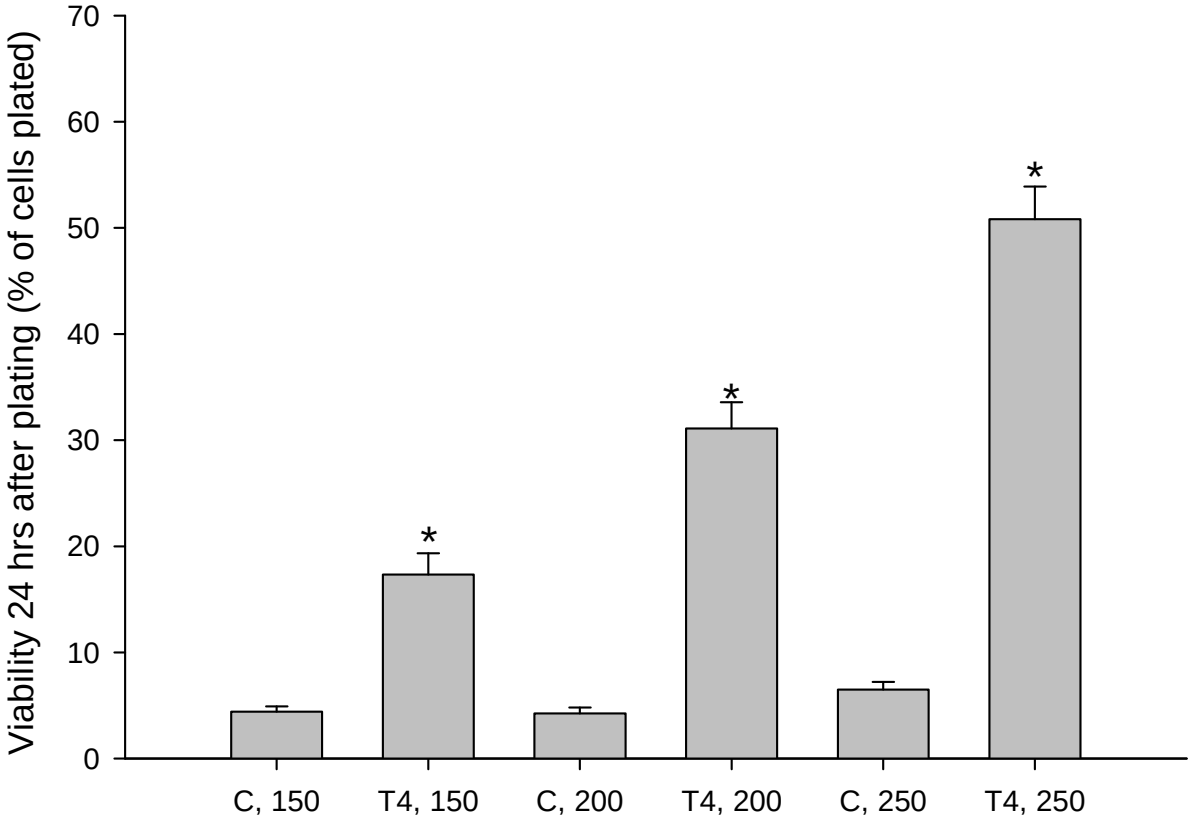

Figure 4. Viability of TRET1 transfected cells after freezing. Human hepatoma cells (HepG2) were transfected with TRET1 and suspended in trehalose solutions for 90 min at concentrations of 150 mM, 200mM and 250 mM with vigorous shaking at 30 ^{o}C in a Thermo Fischer Orbital shaker. After trehalose treatment, cells were stored frozen at -80^{0} C for 24 h in a controlled freezing module. Samples were thawed, cultured for 24 h and cell counts are expressed as percentage of cells before freezing. Incubation with 250 mM trehalose confers highest amount of protection (n = 9, ±SE). *Statistically significant differences compared to non transfected controls at each respective concentration ($P < 0.05$).