Fall 9-29-2020

Sonoporation-mediated loading of trehalose in cells for cryopreservation.

Charles W. Shaffer IV
University of Louisville

David F. Grimm
University of Louisville

Michael A. Menze
University of Louisville

Jonathan A. Kopechek
University of Louisville

Sonoporation-mediated loading of trehalose in cells for cryopreservation

Charles W. Shaffer[1], David F. Grimm[1], Jonathan A. Kopechek[2], and Michael A. Menze[1]

[1]Department of Biology and Department of Bioengineering[2], University of Louisville, Louisville, KY

Background

Trehalose, a non-reducing disaccharide, is present in many microorganisms and metazoans. In these organisms, trehalose acts as a stress protectant and helps preserve lipid membranes of cells during states of desiccation and freezing[1]. Trehalose is required on both sides of the cell membrane to achieve a significant cryoprotective effect. Specific loading methods for trehalose are required since this sugar is impermeant to mammalian cells[3]. Trehalose loading in mammalian cells has been achieved by fluid-phase endocytosis and genetic modification for the expression of trehalose transporters, however cryoprotective outcomes are unable to compete with established methods of cryopreservation for mammalian cells[4]. Sonoporation was achieved using a microfluidics device modified with an ultrasound emitter in the presence of microbubbles. Ultrasound frequencies emitted by the transducer result in a process called cavitation, which is the rapid expansion and collapse of lipid-coated gas-filled bubbles present in the solution. Cavitation of microbubbles creates small jets of liquid that can create membrane pores that are 150-300 nm in size and quickly reseal through budding and exocytosis allowing for uptake of impermeant compounds, such as trehalose[5].

Materials and Methods

Human hepatocellular carcinoma (HepG2) cells were used as a model to compare the effectiveness of trehalose loading using sonoporation or fluid-phase endocytosis before cryopreservation. Cells that were incubated in culture medium containing 100 mM trehalose were frozen in DMEM + 20% FBS + 100 mM trehalose to -80° C for 24 h at -1° C/min while cells exposed to trehalose during sonoporation were frozen in either DMEM + 20% FBS + 100 mM or 200 mM trehalose. To assess membrane integrity after freezing cells were rapidly thawed to 37° C for 2 minutes and intact cells were identified using a trypan blue exclusion assay. Cells loaded with trehalose via sonoporation require the presence of lipid microbubbles to cause cavitation. Cells were exposed to the microfluidics device and sonoporation with concentrations of microbubbles ranging from 0-10% as v/v additions. Intact cells were enumerated after sonoporation using a trypan blue exclusion assay.

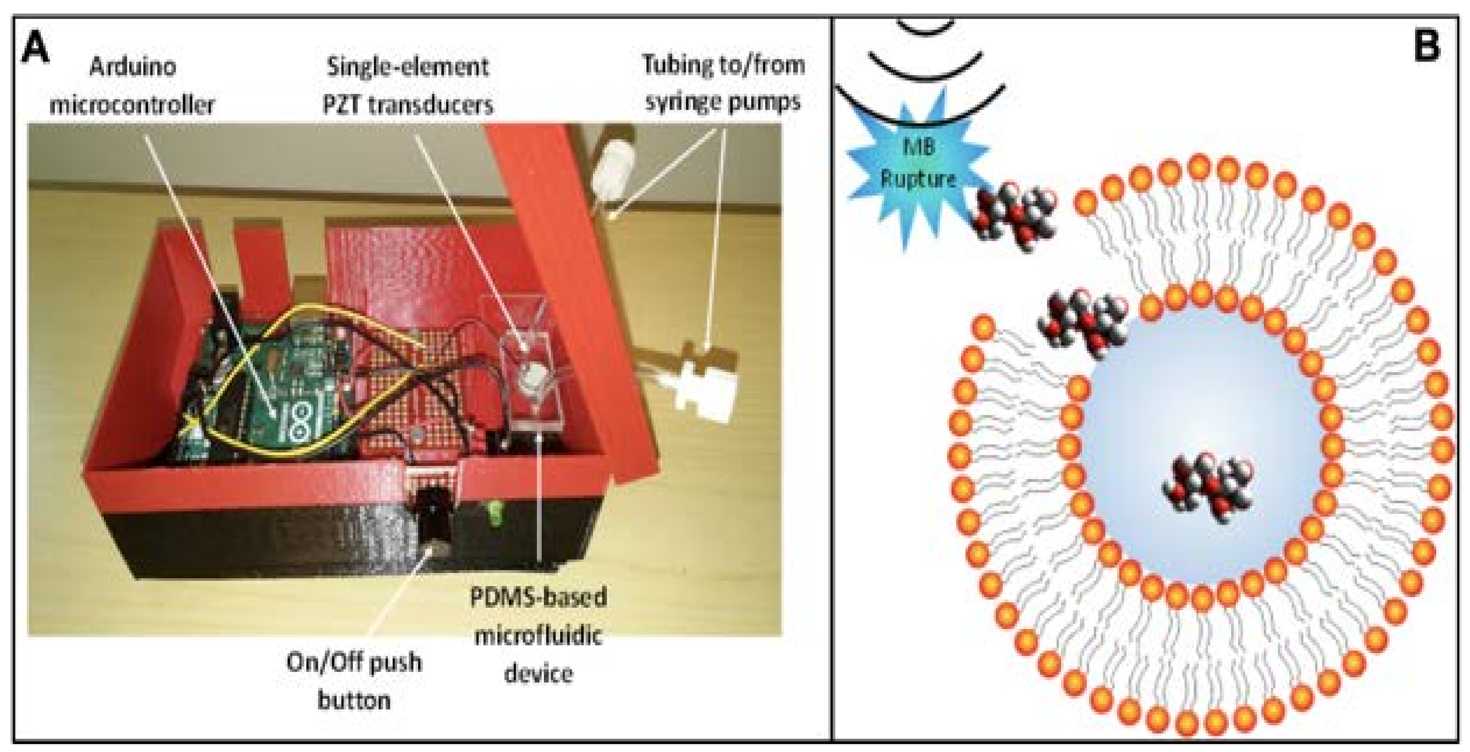

Fig. 1. A) Picture of the ultrasonic flow system. Ultrasound transducers are combined with PDMS-based fluidic devices inside a 3D-printed portable case to enable sequential and consistent loading of compounds across cellular membranes. **B)** Cartoon of microbubble (MB) rupture leading to active transport of compounds across cell membranes (not to scale)[2].

Results

Fig. 2

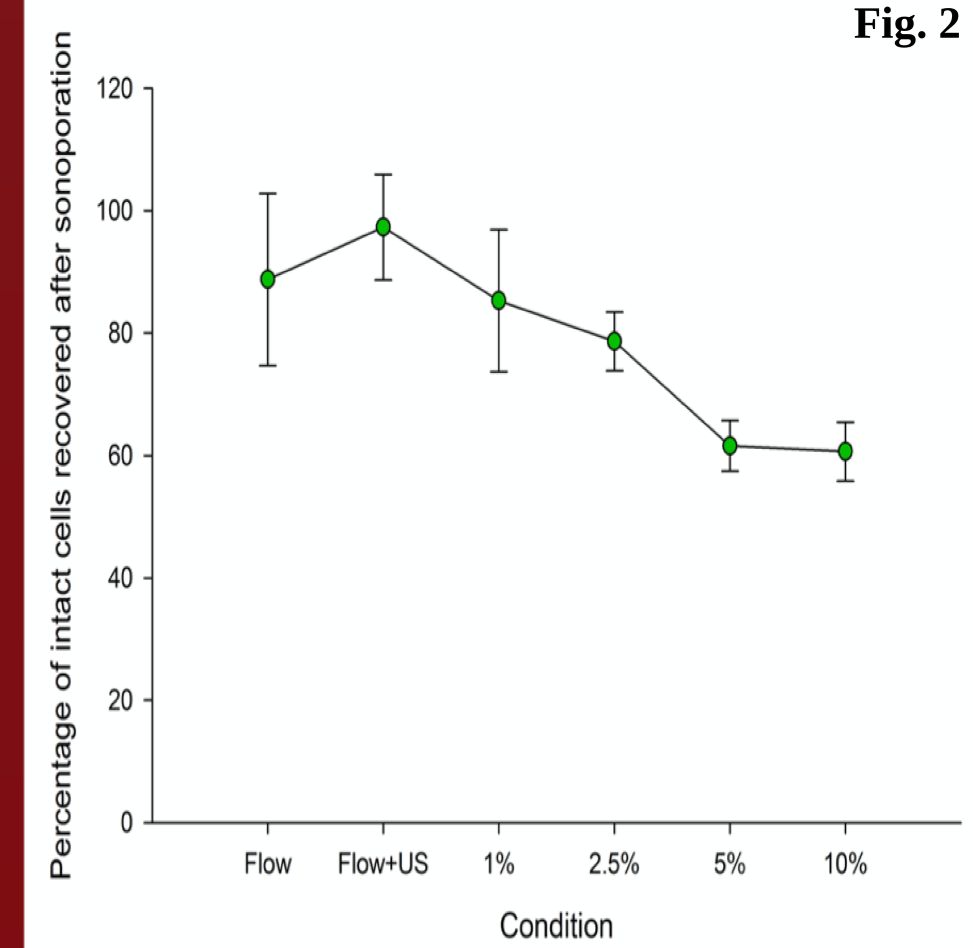

Fig. 3

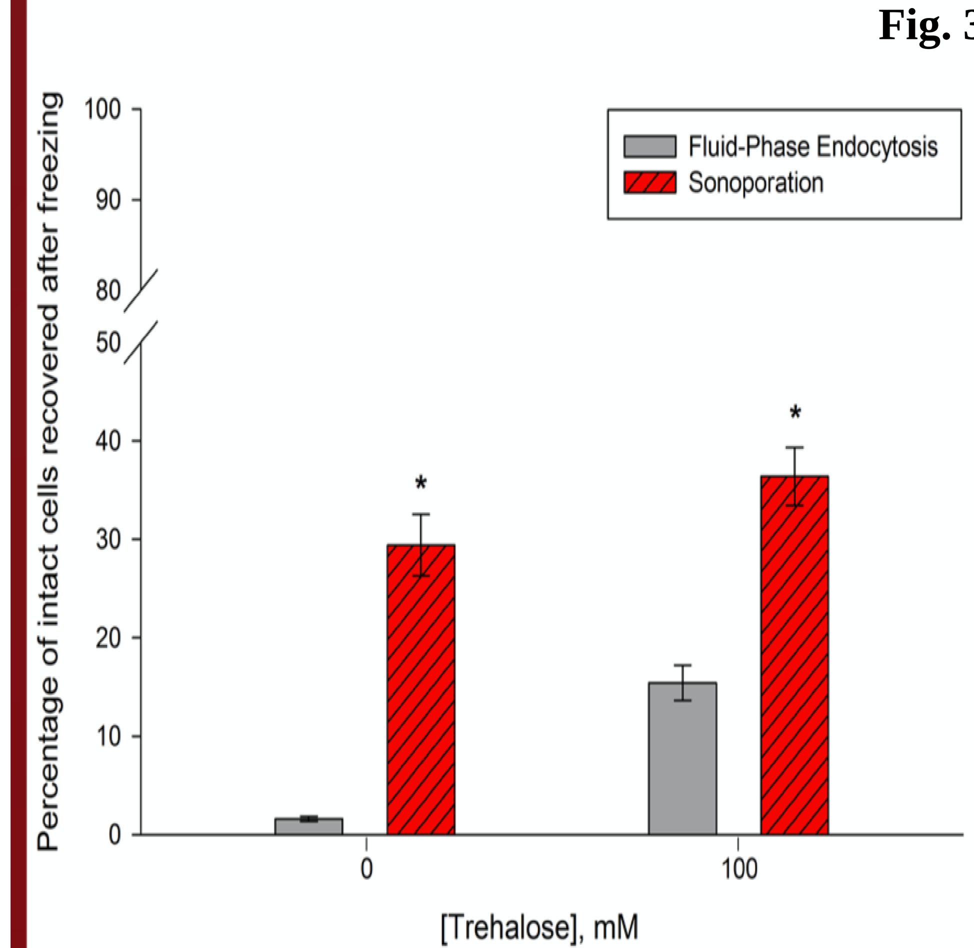

Fig. 4

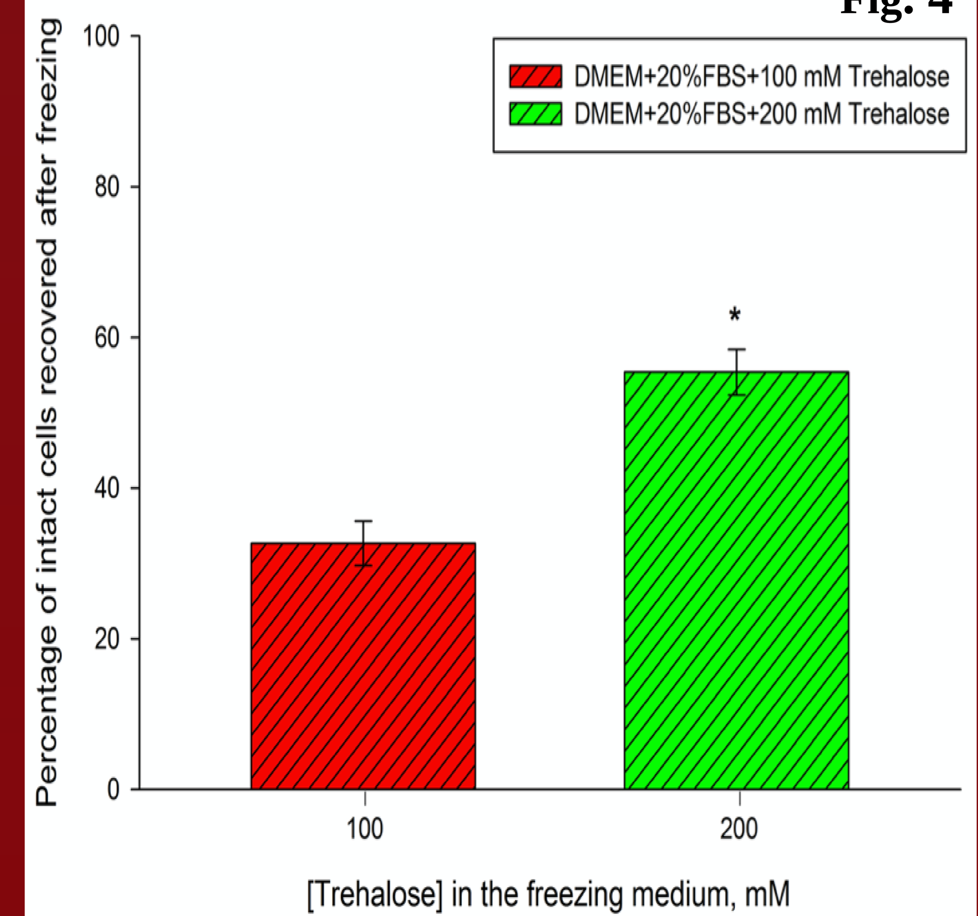

Fig. 5

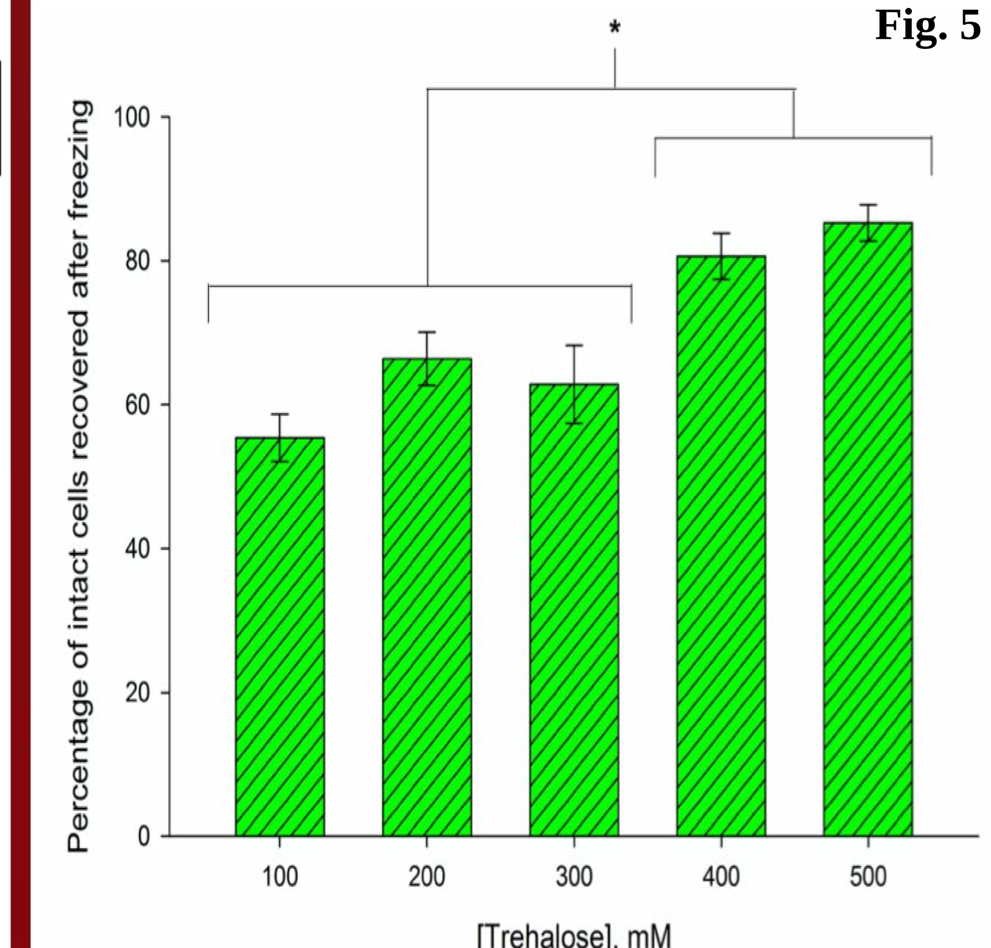

Fig. 2. Assessment of microbubble and process toxicity for HepG2 cells. The percentage of cells with intact plasma membranes was calculated after flowing through the device without exposure to microbubbles or ultrasound (Flow), without microbubbles in presence of ultrasound (Flow + US), or with ultrasound and a range of microbubble concentrations. Microbubble dosages were calculated as v/v additions (n = 3, ± SE).

Fig. 3. Percentage of intact cells recovered after freezing of suspended cells exposed to trehalose via fluid-phase endocytosis or sonoporation. HepG2 cells were either incubated with trehalose for 24 h (gray bars) or were exposed to trehalose during sonoporation (red bars) and all cells were frozen to -80 °C in culture medium supplemented with 100 mM trehalose and 20% FBS. An increase in recovery of intact HepG2 cells after freezing is observed for sonoporated cells (n = 3, ± SE). *Indicates statistically significant difference compared to fluid-phase endocytosis. Significance level was set at $p \leq 0.05$.

Fig. 4. Percentage of intact cells recovered after freezing of suspended cells exposed to 100 mM trehalose during sonoporation. HepG2 cells were frozen in culture medium supplemented with 100 mM trehalose (red bar) or 200 mM trehalose (green bar) and 20% FBS. Freezing of cells in medium containing 200 mM trehalose yielded the highest recovery of intact cells (n = 3, ± SE). *Indicates statistically significant difference compared to freezing medium containing 100 mM trehalose. Significance level was set at $p \leq 0.05$.

Fig. 5. Percentage of intact HepG2 cells recovered after freezing of cells loaded with trehalose via sonoporation. 400 and 500 mM trehalose yielded the highest intact cell recovery (n = 3, ± SE). *Indicates statistically significant differences between groups. Significance level was set at $p \leq 0.05$.

Discussion

Fluid-phase endocytosis is inefficient as a method of trehalose loading for cryopreservation. Exposing HepG2 cells to 100 mM trehalose during sonoporation improved the recovery of intact cells after freezing when compared to cells incubated in medium containing 100 mM trehalose. Using a freezing medium containing 200 mM trehalose increased the recovery of intact cells after freezing compared to medium supplemented with 100 mM trehalose. Further, increasing the trehalose concentration to 400 and 500 mM during sonoporation improved intact cell recovery to 80 and 85% respectively. A 2.5% v/v dose of microbubbles was determined to be optimal for the loading of trehalose without excessive toxicity to HepG2 cells. These results indicate that ultrasound-mediated sonoporation is an effective trehalose loading strategy for cryopreservation of HepG2 cells.

Conclusion

Sonoporation in a microfluidics device as a method of loading trehalose vastly increases the recovery of HepG2 cells with intact plasma membranes after freezing compared to results utilizing fluid-phase endocytosis. This strategy eliminates the need for long incubation periods where cells are exposed to hyperosmotic environments. Sonoporation of cells in medium that does not contain trehalose improved the recovery of intact cells after freezing, suggesting that compounds in the medium itself may confer cryoprotective effects. Further testing is required to assess the viability and function of HepG2 cells preserved with this method, however this approach has the ability to eliminate the need for cytotoxic cryoprotectant agents, such as DMSO, in clinical settings.

References

1. Behm, CA (1997). *Int. J. Parasitol,* 24(2), 215-226. **2.** Centner, C., et al (2020). *Biomicrofluidics*, *14*(2), 024114. **3.** Satpathy, GR, et al (2004). *Cryobiology*, 49(2), 123-36. **4.** Stokich, B., et al (2014). *Cryobiology*, 69(2), 281− 90. **5.** Zarnitsyn V., et al (2008). *Biophys J.*, 95(9), 4124–4138.

Acknowledgments

Support of this work by Mentored Undergraduate Research and Creative Activities Grant and the National Science Foundation (NSF-PFI-1827521) is gratefully acknowledged.