EXPLORING THE POTENTIAL OF SUCRALOSE AS A CRYOPRESERVATIVE FOR RED BLOOD CELLS

By

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Abstract

Cryopreservation is a technique that involves freezing living cells to preserve them in long-term storage. Freezing and preserving living cells would not be possible without the assistance of cryopreservatives. Many cryopreservatives like DMSO and glycerol are currently used to protect living cells as they freeze. However, because of potential toxicity and the need for washing steps in blood transfusion medicine, there is need for alternative cryopreservatives that are both effective and safe for living cells. In this paper, sucralose, an artificial sweetener, is tested as a cryopreservative at varying concentrations. In addition, sucralose was paired with a traditional cryopreservative, DMSO, to observe if its efficacy was altered. The study showed that sucralose does offer cryoprotective ability, and its protection increases as the concentration increases. Pairing cryopreservatives was shown to be less effective. Presented data could be useful in further study of cryopreservation and transfusion medicine.

Introduction

Cryopreservation is a means to preserve structurally intact living cells and tissues at very low temperatures. Freezing intact living cells cannot be accomplished without the help of chemicals called cryoprotective agents (CPAs). Being that liquid water is a requirement for cells to survive, it is normal to believe that the freezing and solidification of water in live cells can cause cell death (Mazur, 1984). Cell death can be the result of ice crystal formation that pierces and destroys the integrity of cellular membranes (Leibo, 1978). Therefore, there is a need for CPAs that help the cell survive during freezing by limiting the formation of ice crystals. It has been shown that chemicals such as dimethyl sulfoxide (DMSO) and glycerol are effective as cryopreservatives (von Bomhard, 2016). However, CPAs like glycerol and DMSO can sometimes have toxic effects on cells (Gregory, 2010). For instance, it has been shown that glycerol causes shape changes and membrane destabilization of erythrocytes (Bakaltcheva, 1996). In addition, cryopreservatives like glycerol that are used in transfusion medicine must be washed out before transfusions, since they are toxic, plus the process is time-consuming and expensive (Lusianti, 2013). Because of the potential toxicity of current CPAs and the need for more effective protocols in red blood cell cryopreservation, there exists a need to find alternative cryopreservatives that are nontoxic and effective in red blood cell cryopreservation.

Sucralose, an artificial sweetener, is a cheap, nontoxic potential alternative to traditional cryopreservatives. It has been found that sucralose has the potential to displace water from cellular membranes, and therefore may be useful as a cryopreservative because it could inhibit ice crystal formation (Barker, 2017). Sucralose would classify as a non-penetrating cryopreservative because its chemical structure inhibits its ability to insert itself into cellular membranes. Other non-penetrating cryopreservatives, like hydroxyethyl starch (HES), have been

found effective in preserving red blood cells (Pogozhykh, 2017). The advantage of using non-penetrating CPAs is the elimination of the washing step before transfusion (Pogozhkyh, 2017). Pogozhkyh also found potential in using a combination of CPAs during freezing and thawing. The aim of this research is to test the ability of sucralose as a cryopreservative, and to find if its ability to protect is altered if combined with a traditional CPA like DMSO.

Materials and Experimental Design

Defibrillated sheep blood (Hemostat labs, Dixon, CA) was used to supply red blood cells. 1.5 mL of blood was dispensed into two 15 mL conical tubes and centrifuged at 500 g's for 5 minutes at 20 degrees Celsius. The supernatant was removed. The blood was then washed multiple times in 150 mM NaCl until the supernatant was completely clear or had a light yellow tint. The washes are needed to remove the serum from the blood. Once washed, 1 mL of blood from each tube was combined in a new 15 mL conical tube. Then, 1 mL of blood was taken and dispensed into a 50 mL conical tube and diluted 50-fold with phosphate buffer saline (PBS) by taking the total volume to 50 mL. At this stage a preliminary red blood cell count was performed to ensure there was enough red blood cells in the diluted blood solution.

The diluted blood was used in the experiment as the final blood solution. 200 µL of diluted blood was added to the desired number of wells in a 96-well plate. Each experiment was performed in triplicate so typically 18-24 wells were used. Then, aliquots of sucralose solution, PBS, and DMSO if needed, were added to the wells to attain the desired % (w/v). Aliquots were divided into multiple additions to ensure that osmotic collapse of the red blood cells did not occur. The total volume of each well was 250 µL. Once the well-plate was prepared, it was left at room temperature for one hour, then transferred to a -20 °C freezer for one hour, and finally the

plate was moved to a -80 °C freezer until ready for data collection. Shown below are the aliquots used to prepared the well-plates:

Aliquots for RBC Counting Experiment and Hemolysis without DMSO			
Well	**% (w/v) Sucralose**	**Sucralose added (µL)**	**PBS added (µL)**
1	0	0	4(12.5)
2	0.156	1.56	25+12.5+6.25+3.125+1.56
3	0.313	3.125	25+12.5+6.25+3.125
4	0.625	2(3.125)	25+3(6.25)
5	1.25	2(6.25)	3(12.5)
6	2.5	2(12.5)	2(12.5)
7	5	4(12.5)	0
Total Volume in each well = 250 µL			

Aliquots for Hemolysis Experiment with DMSO				
Well	**% (w/v) Sucralose**	**Sucralose Added (µL)**	**PBS Added (µL)**	**DMSO Added (µL)**
1	0	0	3(12.5)	12.5
2	0.156	1.56	2(12.5)+6.25+3.125+1.56	12.5
3	0.313	3.125	2(12.5)+6.25+3.125	12.5
4	0.625	6.25	2(12.5)+6.25	12.5
5	1.25	2(6.25)	2(12.5)	12.5
6	2.5	2(12.5)	12.5	12.5
Total Volume in each well = 250 µL				

When ready for analysis, the well-plate was transferred to a 37 °C incubator until thawed. The thawing process usually takes 40-60 minutes. If the cells were to be counted, 100 µL of each well was mixed with 100 µL of trypan blue solution to dye the cells and then observed under a microscope using a hemocytometer. Trypan blue is used because it dyes only dead cells. In these experiments, only live cells were counted.

To measure hemolysis, a micro-plate reader was utilized to measure the absorbance of each well. First, the well-plate was centrifuged until a clear supernatant was observed, and the supernatant was pipetted into different wells that corresponded with the original wells. The plate was then loaded into the plate reader, and the absorbance was measured at 450 and 492 nm. The data from the 450 nm reading was used in the results because hemoglobin has a peak absorbance at 420 nm.

Results

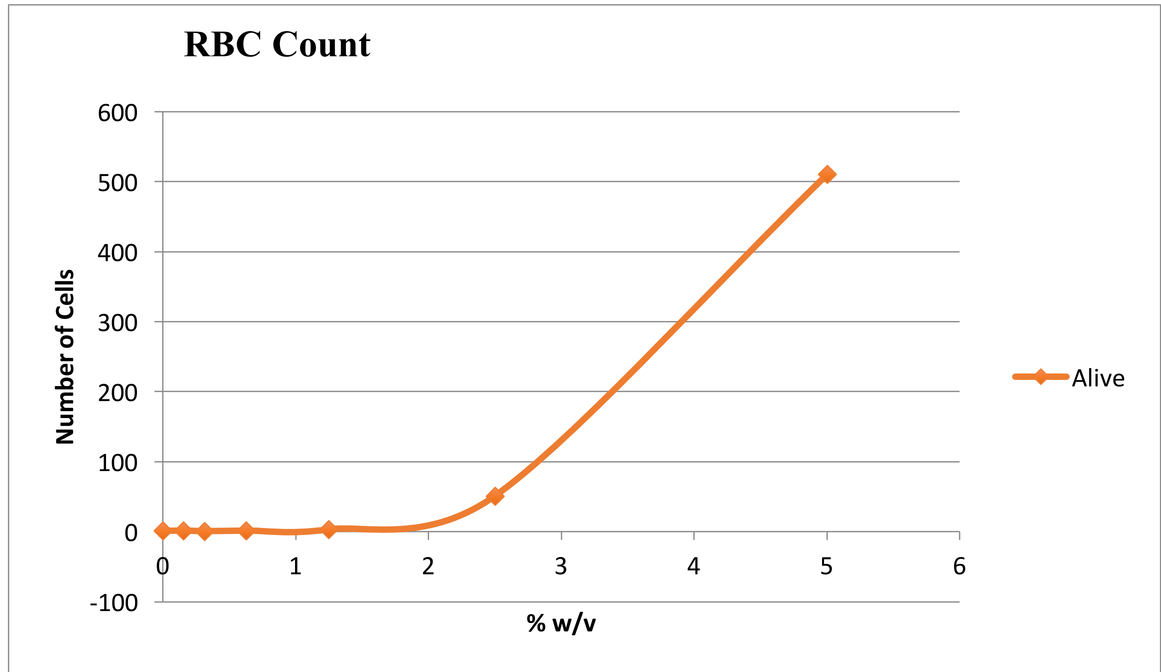

Figure 1: The results in this graph were compiled after following the protocol explained earlier. This experiment was executed to test how the concentration of sucralose affected the survival of red blood cells after during freezing and thawing. As shown, the number of surviving cells increases as the concentration of sucralose increases.

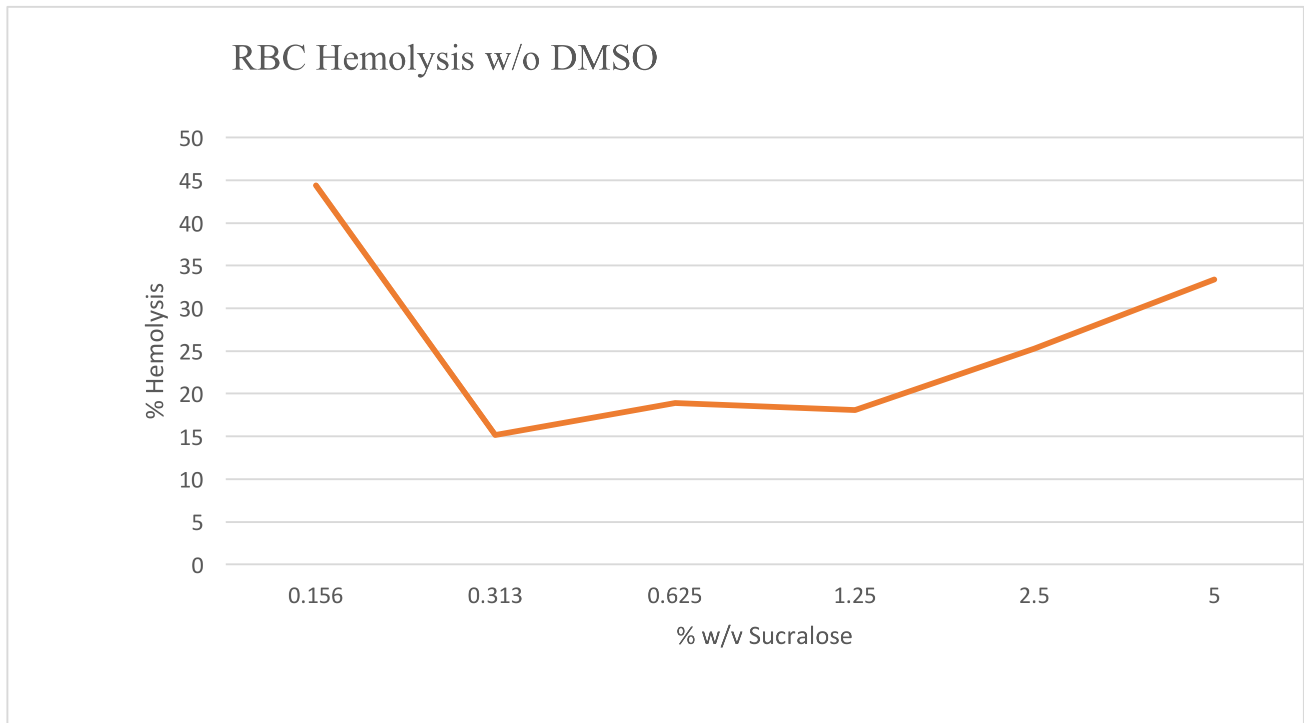

Figure 2: Shown above are average hemolysis results after following the protocol. As the concentration of sucralose increases, the % hemolysis decreases, meaning more cellular membranes remained intact. However, at higher concentrations of sucralose hemolysis begins to increase once more. The 0% concentration of sucralose results are not displayed because the % hemolysis in each of the trials was greater than 50%.

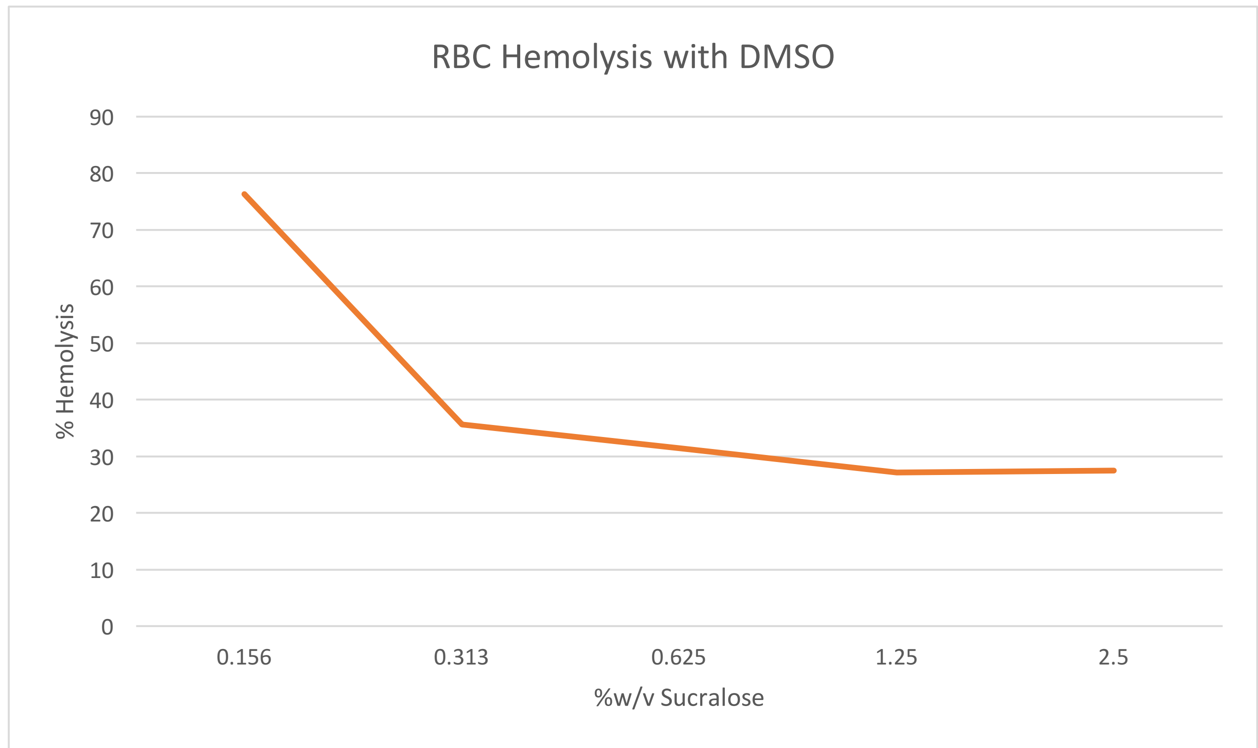

Figure 3: Shown above are hemolysis results following protocol, along with the addition of 5% (w/v) DMSO in each of the experimental wells. The average results are displayed. Again, as the concentration of sucralose increases, the % hemolysis decreases. There is no 5% (w/v) sucralose trial in this case, because the addition of 5% (w/v) DMSO would have made the total volume greater than 250 µL.

% (w/v) Sucralose	% Hemolysis Without DMSO	% Hemolysis With DMSO
0.156	44.38	76.31
0.313	15.19	35.68
0.625	18.87	31.57
1.25	18.10	27.15
2.5	25.28	27.49

Table 1: Shown above are results compiled to compare the differences in hemolysis percentages between the experiments performed with and without the addition of 5% (w/v) DMSO. As shown, there was better protection without DMSO present.

Discussion

Sucralose does provide some protection during the freezing and thawing process. In most of the results, as the concentration of sucralose increases, so does its cryoprotective ability. There is to be noted one discrepancy. In the red blood cell counting results, the 5% sucralose concentration yielded the most living cells after freezing and thawing. However, in the hemolysis results without DMSO addition, the 5% sucralose concentration yielded a higher hemolysis rate than lower concentrations of sucralose. Therefore, it could be that higher concentrations of sucralose can become toxic to cells, or there could be some error in the experiment to account for the discrepancy.

The combination of sucralose and DMSO was less effective than sucralose alone, although the same trend mentioned above did follow, which further supports that sucralose offers

protection during the freezing and thawing process. The best hemolysis rate in the experiments came at 0.313 % (w/v) of sucralose and conferred a 15.19 % hemolysis rate.

Conclusion and Future Experimentation

There is evidence to conclude that sucralose does offer protection for red blood cells during the freezing and thawing process. Its protection is likely to be best when not paired with penetrating CPAs like DMSO. However, there are some changes in protocol that could be made to increase the efficacy of sucralose as a cryopreservative. For instance, there is room to fine tune the optimal sucralose concentration by testing intermediate concentrations between those that were used in these experiments. Other freezing protocols like droplet freezing and rate freezing may prove more effective than the freezing protocol used here. There also is room for expansion in pairing sucralose with other cryopreservatives, although using penetrative CPAs would likely show the same results as the ones presented here. Finally, while these results show cell survival and hemolysis rates, they do not show that red blood cells are viable after freezing and thawing. Therefore, experimentation analyzing the viability of red blood cells after freezing by utilizing an enzyme assay is needed to show that the red blood cells both survive and function properly.

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