Investigating the Potential for Cryopreservation of Human Granulocytes with Concentrated Glycerol

by
Alden Moss

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
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Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

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Abstract approved:_____

Adam Higgins

Granulocytes are a class of white blood cells essential to immune function. Granulocyte transfusions hold promise to treat many conditions, but the short-shelf life of granulocytes makes transfusions infeasible without a cell preservation method. The purpose of this study was to investigate the potential for cryopreservation of granulocytes using 30% glycerol. Recently reported permeability data was used to design two different methods for addition and removal of glycerol: a fast method that is predicted to keep cell volumes between 80% and 150% of the isotonic volume and a slow method that is predicted to keep cell volumes between 80% and 115% of the isotonic volume. The fast method was designed to minimize glycerol toxicity, while the slow method was designed to minimize osmotic damage. The fast method resulted in cell recoveries of 31% ± 9% and 11% ± 3% before and after freezing, respectively, whereas the slow method resulted in even lower cell recoveries of 5% ± 2% and 4% ± 2%. The reduced cell recovery for the slow method is consistent with an increase in damage as a result of glycerol toxicity rather than osmotic damage. These results suggest that cryopreservation of granulocytes in concentrated glycerol is not feasible.

Key Words: Cryopreservation, Cryoprotectant, Granulocyte, Osmotic Damage

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Alden Moss, Author

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Introduction

Granulocytes, also known as polymorphonuclear leukocytes, are a class of white blood cells that includes neutrophils, eosinophils and basophils. What distinguishes granulocytes from other white blood cells are their multi-lobed nuclei and cytoplasmic granules that contain hydrolytic digestive enzymes. Granulocytes play an essential role in immunity by breaking down foreign invaders, including pathogenic microorganisms. Low granulocyte count can have serious implications. Neutropenia is a life-threatening condition defined as a low neutrophil count that results in difficulty fighting infections. The majority of cancer patients receiving chemotherapy experience neutropenia, making it a common condition [1, 2].

Granulocyte transfusions have been shown to benefit neutropenic patients. This has led to increased interest in granulocyte transfusions, and an increased demand for granulocytes [3]. Unfortunately, granulocytes have a very short shelf life of less than 24 h. To increase the availability of granulocytes for transfusion, cryopreservation or another method of long-term storage is necessary [4].

Cryopreservation is defined as the use of very low temperatures to preserve cells and tissues [5]. One major challenge in cryopreservation is the formation of ice crystals during freezing. Intracellular ice crystal formation damages cellular organelles, and extracellular ice crystal formation causes an increase extracellular solute concentration, which can cause osmotic damage. Cryoprotective agents (CPAs) are chemical additives that mitigate the detrimental effects of freeze/thaw cycles by inhibiting ice crystal formation. Common CPAs include dimethyl sulfoxide (DMSO),

glycerol, and ethylene glycol. Some CPAs, such as DMSO, are highly permeable across the cell membrane. In contrast, glycerol is known to have a particularly low permeability, which results in slower equilibration between the cytosol and extracellular solution. This introduces the possibility for osmotic damage during glycerol loading and removal. Success in cryopreservation entails employing CPAs at the correct concentrations, maintaining a slow cooling rate during freezing, and designing protocols to load and remove CPAs from cells that minimize osmotic damage and CPA toxicity [5].

While cryopreservation has been successful with many other cell types, granulocyte cryopreservation has long eluded scientists. Dimethyl sulfoxide has been the CPA of choice for granulocytes in lab experiments. While success has been demonstrated in some cases, the results are very inconsistent, and there is no consensus method [6,7]. Dimethyl sulfoxide also has negative side effects when administered clinically. Glycerol is a logical choice as a CPA since it has been used in high concentrations to successfully cryopreserve red blood cells. Cryopreservation of granulocytes with glycerol has been attempted before and failed consistently. Some have suspected osmotic damage as the culprit [8,9]. Others suspect toxicity as the leading obstacle to successful cryopreservation with glycerol [3,10]. Much of the trouble with designing an accurate experiment lies in predicting cell volume changes. Until recently, glycerol permeability data was limited to two studies which reported vastly different permeability values [10, 11].

Materials and Methods

The objective of this study was to design a convenient method for loading concentrated glycerol into granulocytes while limiting osmotic damage. Glycerol permeability values were based upon the most recent research, using modern equipment [4]. CPA loading and removal protocols were designed to keep cell volumes between 80% and 150% of the isotonic volume since cell volumes outside of this range have been known to cause osmotic damage. Cell volume predictions were made based on CPA concentrations and the time between glycerol loading or removal steps with a two-parameter model of cell membrane transport outlined in Vian and Higgins [4]. The model equations were solved numerically in MATLAB with the ode45 solver.

We examined two different approaches for equilibrating granulocytes with glycerol, a fast method (Fig. 1) and a slow method (Fig. 2). The starting point for the fast method was the plot in Vian and Higgins [4] for a two-step addition process, in which the cells are first exposed to a ~1 molal glycerol loading solution containing a hypotonic concentration of nonpermeating solutes. The cells are predicted to shrink to about 80% of their isotonic volume in this first step, followed by swelling to about 150% of the isotonic volume (Fig. 1). In the second step the cells are exposed to 30% w/v glycerol, which causes shrinkage to about 80% of the isotonic volume. A three-step removal process was used to prevent excessive swelling; this procedure is predicted to keep the cell volumes below 150% of the isotonic volume (Fig. 1).

The slow glycerol equilibration method consisted of 4 addition steps and 4 removal steps, and is predicted to maintain cell volumes between 80% and 115% of the isotonic volume (Fig. 2). All solutions contained an isotonic concentration of salts. Compared with the fast method, this approach is expected to result in less extreme volume changes, but requires more steps and is more time consuming.

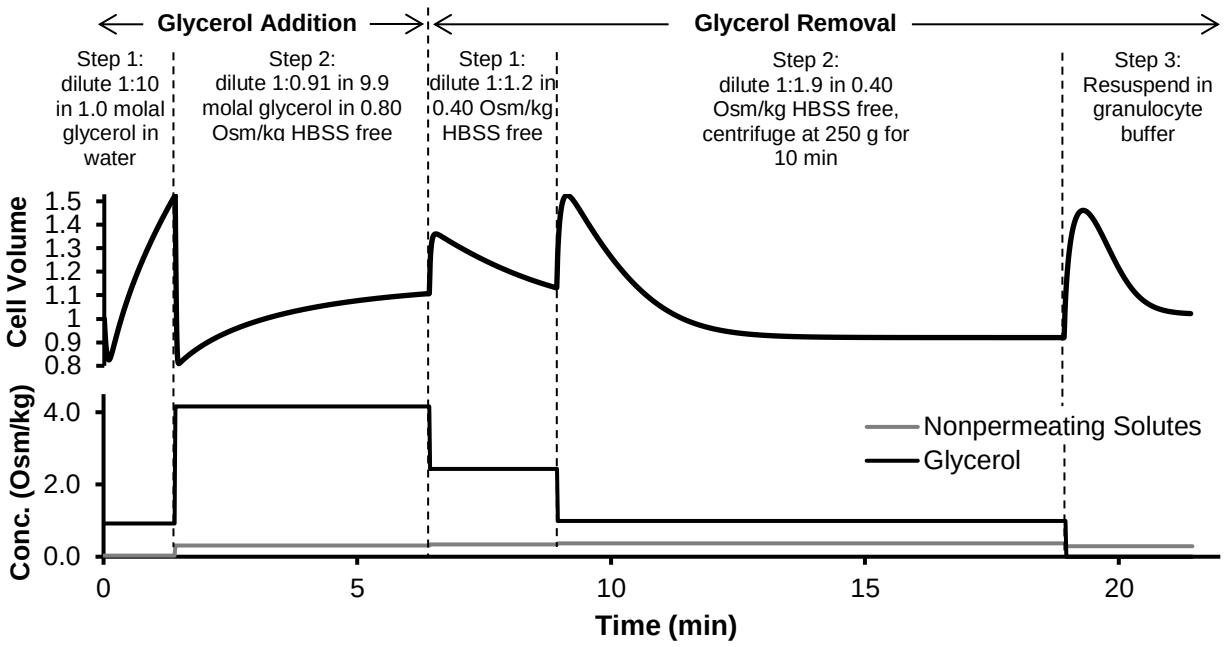

Figure 1: Mathematically modeled cell volume predictions of the fast method for equilibration of granulocytes in 30% w/v glycerol solution. All diluent solutions were pre-equilibrated in a 37°C water bath.

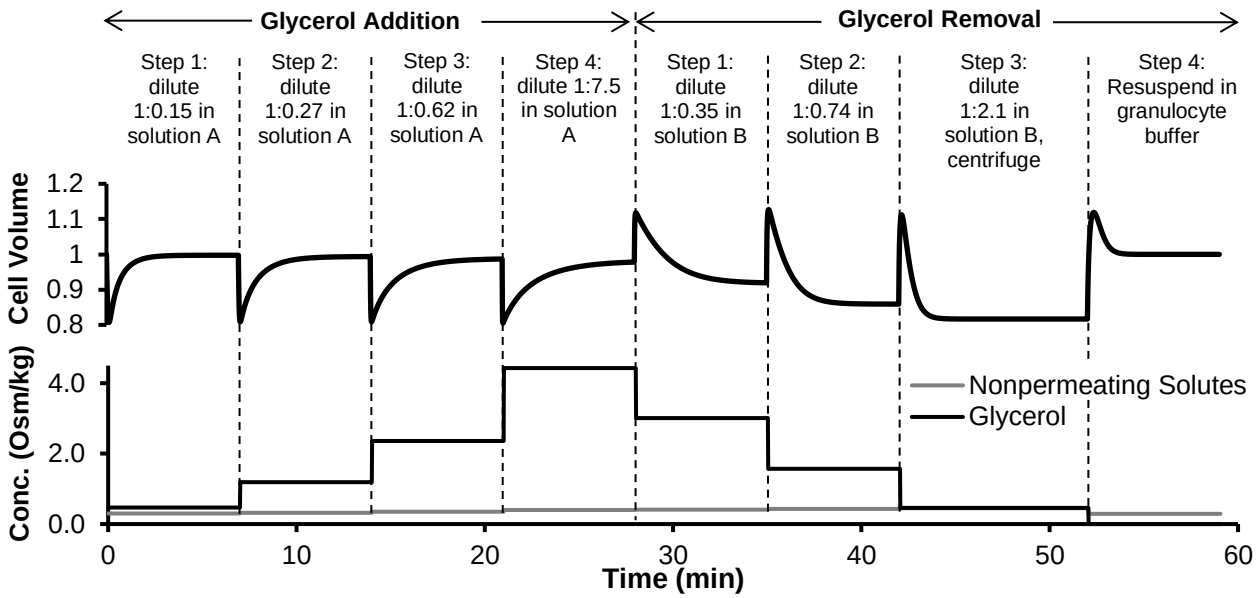

Figure 2: Mathematically modeled cell volume predictions of the slow method for equilibration of granulocytes in 30% w/v glycerol. All diluent solutions were pre-equilibrated in a 37°C water bath. Solution A consists of 4.75 Osm/kg glycerol in hypertonic (0.45 Osm/kg) HBSS free. Solution B consists of hypertonic (0.45 Osm/kg) HBSS free. Centrifugation was carried out at 250g for 10 min.

Granulocytes were isolated from whole blood following a procedure similar to our previous study [4]. Whole blood was collected from volunteer donors using an IRB approved protocol, layered on top of an equal volume of Polymorphprep (Cosmo Bio USA, Carlsbad, CA, USA), and then centrifuged to separate the cells into distinct layers. The granulocyte layer was transferred into a 50 mL centrifuge tube, washed with Hanks Balanced Salt Solution free of calcium and magnesium salts (HBSS free) and pelleted. The pellet was resuspended in 5 mL of ACK Red Blood Cell Lysis Buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) to lyse any contaminating red blood cells [12]. After sitting 5 min, the tube was again filled with HBSS free and centrifuged. The lysis process was repeated once more, letting the cells sit 3 min in

ACK. One final fill with HBSS free and centrifugation resulted in a pellet of pure granulocytes. This pellet was resuspended in 300 ml of granulocyte buffer (isotonic HBSS free containing 1% w/v BSA and 0.1% w/v EDTA). Cells counts and viability assessments were taken with a hemocytometer. Over 95% of the cells in the resulting suspension had intact membranes, as assessed by trypan blue dye exclusion.

Following granulocyte isolation and assessment of membrane integrity, aliquots of 100 ml of concentrated cell suspension were prepared for glycerol addition, freezing, thawing and glycerol removal. Freezing was carried out in 2 mL cryovials using a Mr. Frosty® freezing container, which produces a cooling rate of about 1 ℃/min. After storage for 2 h in a -80 ℃ freezer, samples were placed in the vapor phase of liquid nitrogen for 30 min prior to thawing in a 37 ℃ water bath. Samples were kept in the water bath until the last remaining ice crystal dissolved.

Results and Discussion

While the fast glycerol addition and removal procedure is predicted to maintain cell volumes within the osmotic tolerance limits of granulocytes (Fig. 1), it didn't yield a particularly high recovery of membrane-intact cells, as shown in Fig. 3. Glycerol addition and removal resulted in an average intact cell recovery of 31% ± 9% compared to the number of membrane-intact cells in the original cell suspension. When cells subjected to the fast glycerol equilibration method were frozen and thawed, the intact cell recovery was only 11% ± 3% compared to the original cell suspension. We also examined the effects of fast glycerol equilibration on membrane integrity by comparing the number of intact cells after treatment to the total number of cells present in the sample. Of those cells present after fast glycerol equilibration, just over half had intact membranes (57% ± 9% without freezing and thawing and 56% ± 4% with freezing and thawing). These results are summarized in Table 1.

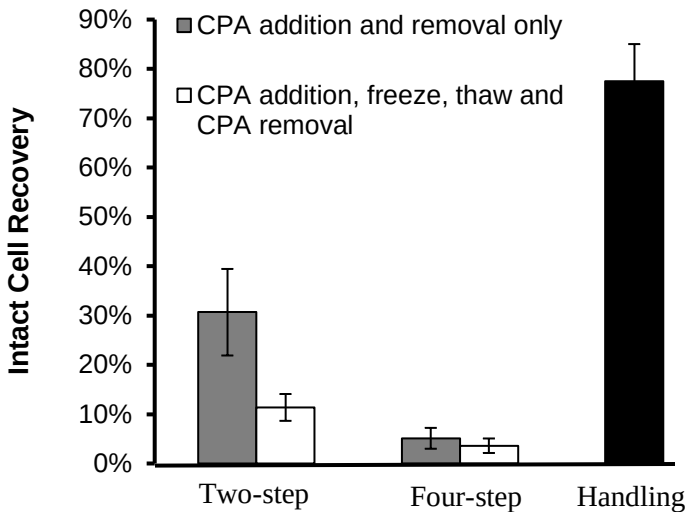

Figure 3: Effects of glycerol addition/removal and freezing/thawing on recovery of granulocytes with intact membranes. Cells were equilibrated with 30% w/v glycerol using either a fast method (Fig. 1) or a slow method (Fig. 2). For comparison, the cells were also subjected to the same handling as in the slow glycerol method, but using isotonic buffer only. Bars denoted with distinct letters represent significantly different recoveries of membrane-intact cells ($p < 0.05$, Fisher's Least Significant Difference tests, n = 4-6). Error bars represent the standard error of the mean.

Table 1: Effects of glycerol addition/removal and freezing/thawing on membrane integrity.

Experimental Treatment	Recovery of membrane intact cells compared to original cell suspension [a]	Percentage of cells in treated sample with an intact cell membrane [b]
Fast glycerol addition/removal	31% ± 9%	57% ± 9%
Fast glycerol addition/removal + freeze/thaw	11% ± 3%	56% ± 4%
Slow glycerol addition/removal	5% ± 2%	20% ± 4%
Slow glycerol addition/removal + freeze/thaw	4% ± 2%	35% ± 7%
Handling control	78% ± 8%	98.8% ± 0.3%

[a] $100\% \times \left(\frac{\text{membrane intact cells after treatment}}{\text{membrane intact cells before treatment}} \right)$

[b] $100\% \times \left(\frac{\text{membrane intact cells after treatment}}{\text{total cells after treatment}} \right)$

We hypothesized that these cell losses may have been caused by the nonconventional glycerol loading process, in which the cells were exposed to glycerol in a hypotonic carrier solution that has the potential to induce excessive swelling. Therefore, we also examined a more conventional, slow procedure involving exposure to glycerol in isotonic carrier solution and additional steps to reduce the extent of cell swelling (see Fig. 2). However, the slow glycerol approach resulted in even lower recovery of membrane-intact cells (5% ± 2% without freezing and thawing and 4% ± 2% with freezing and thawing), and the majority of the cells present after slow glycerol equilibration had compromised membranes (see Table 1). To rule out the potential for damage caused by sample handling, we also performed a control experiment in which the sample was subjected to the same handling as the slow glycerol method (including centrifugation), but using isotonic saline as the diluent. The handling control yielded an average intact cell recovery of 78% ± 8% compared to the original cell suspension, and over 98% of the cells present in the sample after handling had intact membranes.

Taken together, our results suggest that glycerol may be toxic to granulocytes. The slow glycerol equilibration method is gentler osmotically (keeping predicted volumes between 80% and 115% of the isotonic volume), but results in more prolonged exposure to concentrated glycerol solutions. This slow glycerol method resulted in significantly lower cell recovery than the fast method, pointing to toxicity as the mechanism of damage.

Interestingly, the fast glycerol equilibration method is similar to procedures designed to be minimally toxic based on minimization of a CPA toxicity cost function

[13,14]. These minimally toxic procedures also involve CPA loading using a hypotonic carrier solution that induces cell swelling. Swelling is potentially advantageous for reducing toxicity because it enables the use of a lower (and hence less toxic) CPA concentration to load a given amount of CPA into the cell. Our rationale for the fast glycerol method was to reduce the number of required steps by taking advantage of the full range of tolerable cell volumes, similar to the approach described by Meryman [15]. The resulting two-step addition procedure may have also had the added advantage of reducing CPA toxicity.

The literature provides conflicting evidence about the toxicity of glycerol to the human granulocyte. Dooley and colleagues used a cross flow filtration system for gradual addition and removal of glycerol and reported that granulocytes could tolerate exposure to up to 3.7 mol/L glycerol with little loss of chemotactic activity [8]. In contrast, substantial cell losses have been observed after exposure to glycerol concentrations as low as 0.5-1 mol/L using procedures that were predicted to be nondamaging osmotically [3,10], suggesting that glycerol may be toxic. Takahashi and colleagues provide the most thorough investigation of the effects of glycerol on human granulocytes [9]. While the authors were hesitant to conclude that glycerol was toxic, their results seem to indicate that this may be the case. A sharp decrease in cell recovery, phagocytosis and chemotactic activity occurred when granulocytes were exposed to 2 mol/L glycerol for more than an hour [9]. In addition, despite slow addition and removal to mitigate osmotic damage, the authors observed substantial loss of chemotactic activity after exposure to a glycerol concentration of 3 mol/L and complete loss of chemotactic activity for a glycerol concentration of 4 mol/L.

Nonetheless, it is difficult to conclusively rule out osmotic damage. Takahashi et al. obtained better results for slow addition and removal than rapid addition and removal and it is possible that their “slow” method was not slow enough to completely prevent osmotic damage.

Conclusions and Future Work

Through the use of the most recent granulocyte permeability values, our experiments provide new insight into the toxicity of glycerol. We compared slow and fast methods for glycerol equilibration, both of which were designed to avoid osmotic damage, and found that the fast method yielded better results than the slow method. It is unlikely that these results can be explained in terms of osmotic damage, as the slow method is predicted to result in less swelling than the fast method (see Figs. 1 and 2). Our results are, however, consistent with increased toxicity caused by prolonged exposure to high glycerol concentrations during the slow method. These results provide further evidence to the hypothesis that glycerol is toxic to human granulocytes, and suggest that cryopreservation of human granulocytes in concentrated glycerol is not feasible.

Future work would include repeating Takahashi's equilibration and glycerol exposure experiments while ensuring that the osmotic tolerance limits of the cells are not exceeded. This would involve designing protocols based on the permeability values employed in our study. Such a study would be specifically focused on identifying possible glycerol toxicity and allow for more glycerol concentrations and time points to be studied. A confirmation of the results in Takahashi et al. would further support the theory that glycerol is toxic to granulocytes.

While glycerol is likely not a feasible CPA for human granulocytes, other CPAs such as ethylene glycol could be explored. Other methods of preserving granulocytes for transfusions such as encapsulation in a gel matrix could also be

explored as preservation of stem cells in a gel matrix has been demonstrated [16]. Further effort to preserve granulocytes would be highly worthwhile due to the therapeutic potential of granulocyte transfusions.

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