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Investigating the potential for cryopreservation of human granulocytes with concentrated glycerol

Alden C. Moss, Adam Z. Higgins*

School of Chemical, Biological and Environmental Engineering, Oregon State University, Corvallis, OR 97331-2702, USA

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ABSTRACT

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 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. Our results suggest that cryopreservation of granulocytes in concentrated glycerol is not feasible.

Increasing interest in granulocyte transfusions for neutropenic patients, antibiotic resistant infections, and patients with weakened immune systems due to chemotherapy have led to an increased demand for granulocytes. 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. Granulocyte cryopreservation has long eluded scientists despite successes with many other types of cells. Dimethyl sulfoxide has been the cryoprotective agent (CPA) of choice for granulocytes. While success has been demonstrated in some cases, the results are very inconsistent, and there is no consensus method [2,10]. 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 preserve red blood cells. Cryopreservation of granulocytes with glycerol has been attempted before and failed consistently. Some have suspected osmotic damage as the culprit [6,11]. Others suspect toxicity as the leading obstacle to successful cryopreservation with glycerol [1,7]. 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 [5,7].

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 [12]. 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 [12] 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). 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.

Granulocytes were isolated from whole blood following a procedure similar to our previous study [12]. Whole blood was

* Corresponding author.
E-mail address: adam.higgins@oregonstate.edu (A.Z. Higgins).

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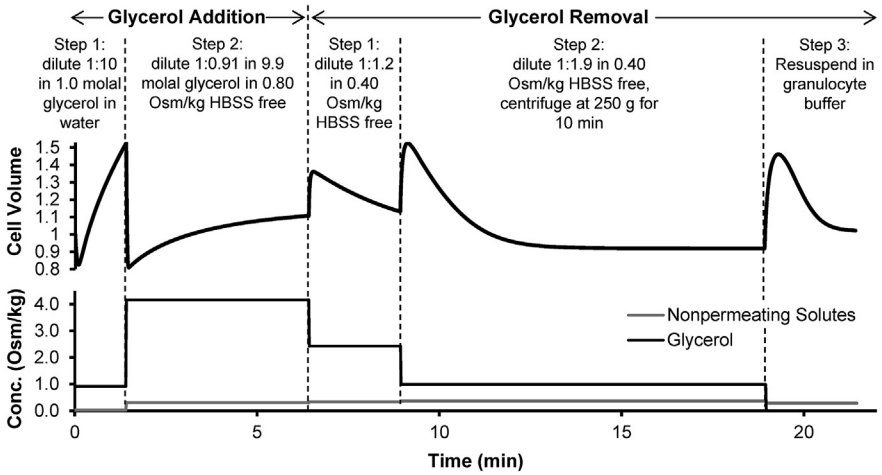

Fig. 1. Fast method for equilibration of granulocytes in 30% w/v glycerol. All diluent solutions were pre-equilibrated in a 37 °C water bath.

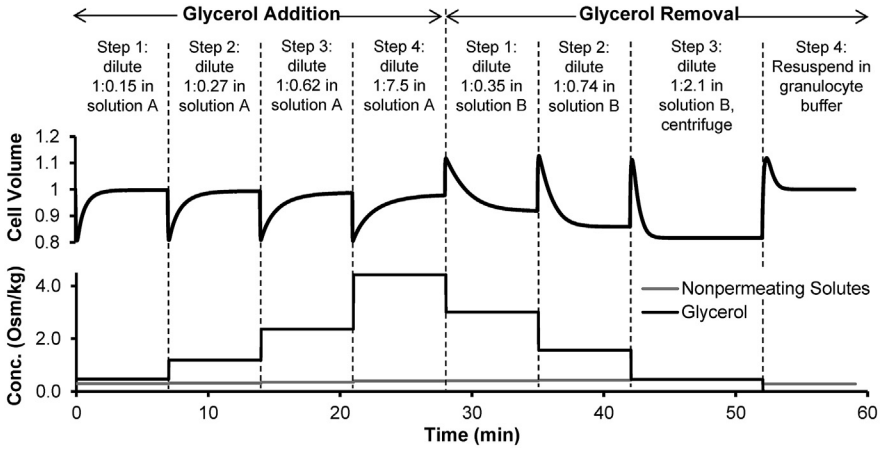

Fig. 2. Slow method for equilibration of granulocytes in 30% w/v glycerol. All diluent solutions were pre-equilibrated in a 37 °C water bath. Solution A consists of 4.75 Osm/kg glycerol in hypertonic (0.45 Osm/kg) HBSS free. Solution B consists of hypertonic (0.45 Osm/kg) HBSS free. Centrifugation was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

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 HBSS free and pelleted. The pellet was resuspended in 5 mL of ACK Red Blood Cell Lysis Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) to lyse any contaminating red blood cells [9]. 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 and centrifugation resulted in a pellet of pure granulocytes. This pellet was resuspended in 300 μl of granulocyte buffer (isotonic HBSS free containing 1% w/v BSA and 0.1% w/v EDTA). Over 95% of the cells in the resulting suspension had intact membranes, as assessed by trypan blue exclusion.

Following granulocyte isolation and assessment of membrane integrity, aliquots of 100 μl 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 °C/min. After storage for 2 h in a −80 °C freezer, samples were placed in the vapor phase of liquid nitrogen for 30 min prior to thawing in a 37 °C water bath.

While the fast glycerol addition and removal procedure is

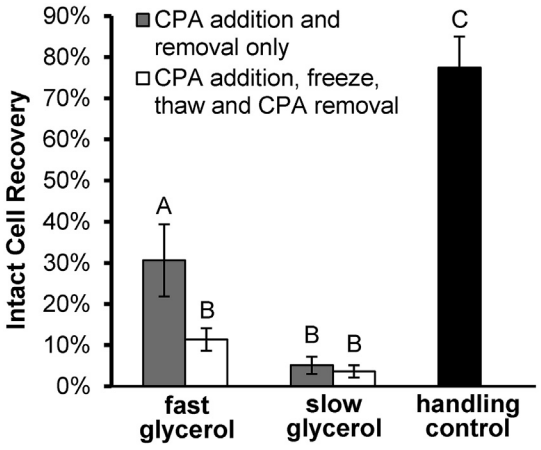

Fig. 3. Effects of glycerol addition/removal and freezing/thawing on recovery of granulocytes with intact membranes. Cells were equilibrated with 30% w/v glycerol using either a fast method (Fig. 1) or a slow method (Fig. 2). For comparison, the cells were also subjected to the same handling as in the 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.