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Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol

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Abstract

Granulocytes are currently transfused as soon as possible after collection because they rapidly deteriorate after being removed from the body. This short shelf life complicates the logistics of granulocyte collection, banking and safety testing. Cryopreservation has the potential to significantly increase shelf life; however, cryopreservation of granulocytes has proven to be difficult. In this study, we investigate the membrane permeability properties of human granulocytes, with the ultimate goal of using membrane transport modeling to facilitate development of improved cryopreservation methods. We first measured the equilibrium volume of human granulocytes in a range of hypo- and hypertonic solutions and fit the resulting data using a Boyle-van't Hoff model. This yielded an isotonic cell volume of $378 \mu\text{m}^3$ and an osmotically inactive volume of $165 \mu\text{m}^3$. To determine the permeability of the granulocyte membrane to water and cryoprotectant (CPA), cells were injected into well-mixed CPA solution while collecting volume measurements using a Coulter Counter. These experiments were performed at temperatures ranging from 4 to 37 °C for exposure to dimethyl sulfoxide, glycerol, ethylene glycol and propylene glycol. The best-fit water permeability was similar in the presence of all of the CPAs, with an average value at 21 °C of $0.18 \mu\text{m atm}^{-1} \text{min}^{-1}$. The activation energy for water transport ranged from 41 to 61 kJ/mol. The CPA permeability at 21 °C was 6.4, 1.0, 8.4 and 4.0 $\mu\text{m/min}$ for dimethyl sulfoxide, glycerol, ethylene glycol and propylene glycol, respectively, and the activation energy for CPA transport ranged between 59 and 68 kJ/mol.

Keywords

neutrophil; polymorphonuclear cell; cryoprotectant permeability; hydraulic conductivity; Coulter Counter; membrane transport

INTRODUCTION

Granulocyte transfusions have long been recognized as a potential therapeutic option for treating infections in individuals with neutropenia [6,38,39]. Nonetheless, the use of granulocyte transfusions has remained infrequent and only about 1000 granulocyte units are

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transfused per year in the US [1]. Recently, interest in granulocyte transfusions has increased for a variety of reasons, including the ability to collect granulocytes in higher numbers using granulocyte colony-stimulating factor (G-CSF), an increased incidence of therapy-related neutropenia and a rise in antibiotic resistance [20]. Moreover, recent research suggests that granulocyte transfusions could be used as a treatment for cancer [9,15,27]. US clinical trials are currently underway to investigate the use of granulocyte transfusions for treating infections in individuals with neutropenia [13] as well as for treatment of cancer [14].

However, the use of granulocytes for medical therapy is significantly hindered by their short shelf life. The current recommendation of the AABB is to use granulocytes for transfusions as soon as possible after collection, with a storage period at room temperature of no longer than 24 hr [11]. This short shelf life makes it impractical to bank granulocytes and the current clinical practice is to collect granulocytes from the donor on site and transfuse the cells shortly thereafter. The short time window between collection and transfusion makes it difficult to adequately ensure safety and at least one instance of disease transmission can be attributed to difficulties with completing disease testing [36].

The ability to cryopreserve granulocytes would greatly facilitate the logistics of granulocyte collection, banking, compatibility testing and infectious disease testing. However, granulocytes have been shown to be highly susceptible to damage during the cryopreservation process [3,5,41], and previous attempts at granulocyte cryopreservation have produced variable but largely unfavorable results [7]. At present, a clinically suitable method for granulocyte cryopreservation does not appear to be available [40].

The design of cryopreservation procedures can be facilitated by the use of mathematical modeling and optimization if the necessary cell-specific biophysical parameters are known [8,25,31,32]. In general, these mathematical modeling approaches rely on predictions of mass transfer across the cell membrane, which require knowledge of the cell membrane permeability to water and cryoprotectant (CPA). However, published CPA permeability data for human granulocytes is limited to a single study of the dimethyl sulfoxide permeability at temperatures between 20 °C and 30 °C [44] and two investigations of the glycerol permeability, neither of which considered the effects of temperature [17,23].

In this study, we systematically investigate the membrane permeability properties of human granulocytes and we report permeability measurements for several common CPAs (glycerol, dimethyl sulfoxide, ethylene glycol and propylene glycol) over a range of temperatures. These data will enable the use of membrane transport modeling to guide the design of granulocyte cryopreservation procedures.

MATERIALS AND METHODS

Granulocyte Isolation

Materials for granulocyte isolation were purchased from VWR (Radnor, PA) unless otherwise noted. Whole blood was collected from healthy human volunteers into 10 mL vacutainer tubes containing 18 mg of EDTA as an anticoagulant (Market Labs, Caledonia,

MI) using an IRB-approved protocol. The donors consisted of 4 males and 1 female with ages ranging between 22 and 33 years old. Granulocytes were isolated from whole blood using a procedure similar to that described in a previous study [30]. A volume of 5 ml of whole blood was carefully layered on top of 5 mL of Polymorphprep™ (Cosmo Bio USA, Carlsbad, CA) in a 15 mL centrifuge tube. Each donor gave 20 mL of blood, which corresponds to 4 different centrifuge tubes per donor. The tubes were centrifuged at 500g for 45 minutes while maintaining the temperature at 19 °C using a refrigerated centrifuge, which resulted in distinct bands for mononuclear leukocytes, granulocytes and red blood cells. The band corresponding to the granulocytes (i.e., the second band from the top) was carefully removed from each 15 mL centrifuge tube using a 1 mL micropipette and placed into a single 50 mL centrifuge tube. This resulted in a total volume of about 3 mL of granulocyte-rich cell suspension. The 50 mL tube was then filled with calcium and magnesium free Hank's Balanced Salt Solution (i.e., HBSS free) and centrifuged for 10 minutes at 400g and 19 °C. HBSS free has a pH of 7.1 and consists of 0.4 g/L potassium chloride, 0.048 g/L sodium phosphate dibasic, 0.06 g/L potassium phosphate monobasic, 8 g/L sodium chloride, 0.35 g/L sodium bicarbonate, 1 g/L glucose and 0.01 g/L phenol red. The resulting cell pellet was resuspended in 18 mL of pure water, allowed to equilibrate for 30-45 seconds, and then brought to isotonic conditions by addition of 2 mL of 10x Dulbecco's phosphate buffered saline lacking calcium and magnesium (DPBS). This brief exposure to a hypotonic solution was used to remove contaminating red blood cells that swell and lyse much more rapidly than granulocytes. Finally, the 50 mL centrifuge tube was filled with HBSS free, centrifuged for 10 minutes at 19 °C and 400g, and the resulting pellet was resuspended in 300 µL of HBSS containing 2 mM Ca^{2+} , 2 mM Mg^{2+} and 10 mg/mL bovine serum albumin. An appropriate volume of this suspension was added to 15 mL of isotonic (300 mOsm/kg) DPBS to create a final cell concentration of 200,000 cells/mL. After completing the isolation process, the granulocytes were stored at room temperature (~21 °C) until being used in experiments. The total time between blood collection and use of the cells in an experiment did not exceed 10 hours.

Determining the Purity of the Isolated Granulocytes

To confirm the purity of the cell suspension after granulocyte isolation, the cells were stained with Giemsa stain (VWR) using standard methods. Briefly, a small volume of cell suspension was smeared onto the surface of a microscope slide and allowed to dry. The slide was then submerged in methanol for 30 seconds. After allowing the slide to dry, it was submerged in a staining solution consisting of 0.08 g/mL Giemsa stain in a 50/50 mixture of glycerol and methanol. After staining for 30 min, the slide was removed, rinsed with water and carefully blotted dry using a paper towel. Granulocytes were identified by their distinctive multi-lobed nuclei.

Determining the Viability of the Isolated Granulocytes

Trypan blue staining was used to estimate the viability of the isolated granulocytes. The granulocyte suspension was diluted 1:10 in isotonic DPBS, and the resulting suspension was mixed 1:1 with 0.2% w/v trypan blue solution (VWR). This mixture was allowed to equilibrate for two minutes before counting the cells on a hemocytometer. Live cells were identified by their ability to exclude the trypan blue dye.

Experimental Solutions

Aqueous solutions for measuring cellular osmotic properties were either prepared using only nonpermeating solutes, or using both nonpermeating solutes and CPA. Throughout this paper, we use the term “nonpermeating” to describe solutes that are relatively impermeable to the cell membrane in comparison to water and CPA, such that transport of these molecules across the membrane can be considered negligible over the short (~1 min) experimental time frame. Both types of solutions were made using isotonic DPBS as the base solution. To prepare hypotonic solutions containing only nonpermeating solutes, the solutes present in isotonic DPBS were diluted by addition of purified water (these solutes can be considered nonpermeating). Hypertonic solutions were prepared by adding the nonpermeating solute sucrose to isotonic DPBS. CPA solutions were prepared by adding CPA to isotonic DPBS. Four different CPAs were used: dimethyl sulfoxide (Me₂SO), ethylene glycol, glycerol and propylene glycol (Sigma, St. Louis, MO). The osmolality of all solutions was confirmed to within 1% of the reported value using a freezing point depression osmometer (Advanced Micro Osmometer Model 3300, Advanced Instruments, Norwood, MA).

Measuring Cell Volumes

Granulocyte volumes were measured using a Coulter Counter Multisizer 3 (Beckman Coulter, Brea, CA) with a 100 µm diameter aperture tube. Several modifications to the standard operating procedure for the Coulter Counter were made to enable temperature control and rapid sample mixing. The modifications were similar to those described previously [28]. A custom glass jacketed beaker with an internal volume of about 15 mL was used instead of the standard Accuvette sample cups sold by Beckman Coulter. The jacketed beaker was fitted into a custom beaker holder on top of the Coulter Counter platform. A magnetic stirrer motor (Instech Laboratories, Plymouth Meeting, PA) was inserted into a cavity in the beaker holder which allowed the contents of the sample beaker to be rapidly mixed. Solution from a refrigerated water bath was circulated through the beaker jacket to control the sample temperature. In order to accommodate the tubes running between the sample beaker and the refrigerated bath, it was necessary to operate the Coulter Counter with the door open. Because the Coulter Counter software only allows operation of the instrument with the door closed, the software was tricked into registering that the door was closed by placing two small magnets on the right side of the door frame. To minimize electrical noise, the water in the refrigerated bath was made conductive by addition of 2% w/v sodium chloride and this solution was grounded using a copper wire inserted into the beaker jacket. In addition, the metal syringe needle used to inject the cell suspension into the sample beaker was grounded. To ensure that the cell suspension was rapidly mixed after injection, a short length of plastic tubing was attached to end of the needle and the tube outlet was situated near the magnetic stir bar at the base of the sample beaker.

Prior to the start of experiments, the Coulter Counter was drained and refilled with the appropriate experimental solution. The instrument was calibrated for each experimental solution and temperature by measuring the volume of 10 µm diameter latex beads (Beckman Coulter); these volume measurements were performed twice before beginning experiments with the cells and once after completing the experiments. For cell volume measurements, 0.5

mL of granulocyte cell suspension was injected into 15 mL of experimental solution in the sample beaker, resulting in a final concentration of about 6,000 cells/mL. This cell concentration was chosen to ensure that error due to coincidence was less than 5% [29].

Analysis of the Equilibrium Osmotic Response

To analyze the equilibrium osmotic response, the cell volume was measured after the granulocytes had equilibrated for 5 minutes in hypo- or hypertonic solutions containing nonpermeating solutes (but lacking CPA). The equilibrium cell volume V_e can be described using the Boyle-van't Hoff equation:

$$V_e = (V_0 - V_b) \frac{M_0}{M_n^e} + V_b \quad (1)$$

where V_0 is the cell volume under isotonic conditions, $M_0 = 300$ mOsm/kg is the isotonic osmolality, M_n^e is the osmolality of nonpermeating solutes at equilibrium and V_b is the osmotically inactive volume. This equation was used to estimate the osmotically inactive volume from experimental measurements of the equilibrium cell volume.

Analysis of the Transient Osmotic Response

Transient cell volume data was used to estimate the permeability of the granulocyte membrane to water and CPA. To acquire transient volume measurements, the Coulter Counter was setup to collect data over six consecutive 10-second intervals and data collection was initiated at the same time as the granulocyte suspension was injected into the sample beaker. The Multisizer 3 records the time at the start and end of each 10-second interval, as well as the order in which each volume measurement was acquired. However, it does not assign a unique timestamp to each data point. To estimate the times corresponding to each volume measurement, we assumed that the data points were equally spaced in time. On average, about 5,000 cell volume measurements were obtained in each 10-second interval, which corresponds to a volume measurement every 2 milliseconds. The assumption of equally spaced data points is equivalent to assuming that fluid with a constant cell concentration is drawn through the Coulter Counter aperture at a constant volumetric flow rate. Because the Coulter Counter imposes a constant volumetric flow rate, this assumption is expected to be accurate if the solution in the sample beaker is well mixed. Qualitative observations of mixing after injection of a dye-containing solution into the sample beaker suggest that mixing is complete in less than 1 second (data not shown). To quantitatively analyze this assumption, we injected calibration beads into the sample beaker and measured the number of beads counted in each of six consecutive 10-second intervals. The resulting data is shown in Fig. 1. The number of beads counted in each interval was approximately the same (to within 10%), supporting our assumption that average rate at which the beads are drawn through the aperture is constant over the entire data collection period. However, the number of beads counted in the first 10 second interval was slightly lower than the other counts, suggesting that there is a finite mixing time required for the beads to become evenly dispersed.

To determine the cell membrane permeability parameters, the transient volume data was fit with the two-parameter membrane transport model [33]:

$$\frac{dV_w}{dt} = L_p A R T \left(\frac{\rho_w M_0 V_{w0} + N_s}{V_w} - \rho_w M_n^e - \rho_w M_s^e \right) \quad (2)$$

$$\frac{dN_s}{dt} = P_s A \left(\rho_w M_s^e - \frac{N_s}{V_w} \right) \quad (3)$$

where V_w is the cell water volume, L_p is the hydraulic conductivity, A is the cell surface area, R is the ideal gas constant, T is the absolute temperature, ρ_w is the density of water (assumed equal to 1 g/mL), V_{w0} is the cell water volume under isotonic conditions, N_s is the moles of intracellular CPA, M_s^e is the extracellular osmolality of CPA and P_s is the CPA permeability. As is commonly done, we assumed that the cell surface area was constant and equal to the surface area of a sphere with the isotonic cell volume, i.e., $A = (36\pi)^{1/3} V_0^{2/3}$. This constant surface area assumption is based on the argument that changes in cell volume are accommodated by folding or unfolding of the cell membrane, rather than membrane stretching [35]. The differential equations were solved numerically using MATLAB's built-in differential equation solver *ode15s* to determine V_w and N_s as a function of time. The values of V_w and N_s were then used to determine the total cell volume using the following equation:

$$V = V_w + \nu_s N_s + V_b \quad (4)$$

where ν_s is the molar volume of CPA, which we assumed to have a value of $\nu_s = 73.3$ mL/mol for glycerol, 71.3 mL/mol for Me₂SO, 55.8 mL/mol for ethylene glycol and 73.6 mL/mol for propylene glycol [26]. The value of V_b was determined from the equilibrium cell volume measurements as described above. To determine best-fit values of L_p and P_s the sum of the error squared between the predicted and measured values of V was minimized using MATLAB's built-in function minimizer *fminsearch*. Only volume measurements obtained in the first 45 seconds that were in the range 150 μm^3 to 950 μm^3 were used (volume measurements outside this range were discarded).

To analyze the temperature dependence of the permeability parameters, we used an Arrhenius relationship. The Arrhenius models for water and CPA permeability, respectively, are

$$L_p = L_{p,ref} \exp \left[-\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right] \quad (5)$$

$$P_s = P_{s,ref} \exp \left[-\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right] \quad (6)$$

where E_a is the activation energy and $L_{p,ref}$ and $P_{s,ref}$ are permeability values at a reference temperature T_{ref} .

Statistical Analysis

All measurements were performed using granulocytes isolated from the blood of three different donors, and measurements for each donor were repeated 2-3 times. Results are reported as averages over the replicates for each donor and error bars show the standard error of the mean. Statistical analysis was performed using StatGraphics software. The data was analyzed using ANOVA, followed by Tukey HSD tests for pairwise comparisons. Differences were considered to be significant at a 95% confidence level (i.e., $p < 0.05$).

RESULTS

Purity and Viability of the Isolated Granulocytes

Fig. 2 shows a representative image of the isolated granulocytes. Granulocytes are easily identified because their nucleus is multi-lobed and stains dark purple. We analyzed random fields of view from 12 different isolations and estimated the granulocyte purity to be approximately $93\% \pm 1\%$, with the majority of the contaminating cells being lymphocytes. The viability of the isolated cells was assessed using a trypan blue exclusion assay, yielding an average viability of $89\% \pm 1\%$. The lowest viability after isolation was 80%.

Equilibrium Osmotic Response

Fig. 3A shows cell volume distributions for granulocyte suspensions after equilibration in solutions with various osmolalities. As expected, exposure to hypotonic conditions shifted the volume distribution to the right towards larger volumes, whereas exposure to hypertonic conditions shifted the volume distribution to the left toward smaller volumes. The mean of the volume data was determined for each solution composition and plotted in a Boyle-van't Hoff plot, as shown in Fig. 3B. The Boyle-van't Hoff equation (Eq. 1) was fit to the resulting data, yielding an osmotically inactive volume $V_b = 165 \pm 6 \mu\text{m}^3$ and an isotonic cell volume $V_0 = 378 \pm 6 \mu\text{m}^3$. Statistical analysis of the data revealed that the solution osmolality had a significant effect on the mean cell volume ($p < 0.0001$), as expected, but the effect of blood donor was not significant ($p = 0.40$).

Transient Osmotic Response

Fig. 4 shows representative volume measurements after exposure to ethylene glycol at 21 °C. Volume data was collected during six consecutive 10-second intervals, with short gaps between each interval during which no volume measurements were recorded. Each volume measurement is shown with a light gray symbol. Although the data is quite scattered, a typical shrink-swell trend is still apparent. To facilitate visualization of this shrink-swell trend, a running average was calculated over 60 consecutive volume measurements, as shown with the dark gray symbols. The volume trend is consistent with an initial period of shrinkage as water leaves the cell down its osmotic gradient, followed by a period of swelling as both water and ethylene glycol enter the cells. The volume data was fit using a two-parameter membrane transport model (Eqs. 2-3) to determine best-fit permeability parameters. As shown in Fig. 4, the best-fit model is in reasonable agreement with the cell volume measurements.

To examine the temperature dependence of the permeability parameters, Arrhenius plots were created, as shown in Fig. 5. Overall, these plots show that the water permeability values were similar in the presence of all four of the CPAs. However, the glycerol permeability was substantially lower than the permeability of Me₂SO, ethylene glycol and propylene glycol. The Arrhenius plots were fit with Eqs. 5-6 to obtain estimates for the activation energies and permeability values at a reference temperature of 21 °C. The resulting estimates are given in Table 1. Statistical analysis of the data by three-way ANOVA revealed that CPA type ($p < 0.0001$) and temperature ($p < 0.0001$) had significant effects on the CPA permeability, but blood donor did not ($p = 0.06$). In particular, the glycerol permeability was significantly lower than the permeability values for the other CPA types, and the propylene glycol permeability was significantly lower than that of Me₂SO and ethylene glycol. Analysis of the water permeability data yielded significant effects for temperature ($p < 0.0001$) and CPA type ($p = 0.009$), but not blood donor ($p = 0.86$). Pairwise comparisons revealed that the water permeability in the presence of propylene glycol was significantly lower than the water permeability in the presence of Me₂SO and ethylene glycol.

DISCUSSION

Although granulocyte cryopreservation was an active area of investigation in the two decades prior to 1980 [7], efforts to cryopreserve granulocytes appear to have been largely abandoned after the mid-1980s. Since that time, new strategies for mathematical optimization of cryopreservation procedures have been developed (e.g., [8,31,32]). Application of these mathematical optimization approaches to human granulocytes will require accurate knowledge of several cell-specific biophysical parameters, including the isotonic cell volume, cell membrane surface area, osmotically inactive volume and permeability properties.

Our value of the isotonic cell volume ($378 \mu\text{m}^3$) is within the range of isotonic volumes reported in the literature for human granulocytes: $392 \mu\text{m}^3$ [4], $345 \mu\text{m}^3$ [45], $448 \mu\text{m}^3$ [46], $299 \mu\text{m}^3$ [43]. To estimate the cell membrane surface area, we used this value of the isotonic cell volume and assumed a spherical geometry, yielding $A = 253 \mu\text{m}^2$. Although the assumption of a constant membrane surface area is common [16,17,23,44,45], many previous studies of the granulocyte permeability do not provide detailed information about how the membrane surface area was estimated. Toupin and colleagues [45] also assumed a spherical geometry, but estimated the cell membrane surface area from the maximum volume the cells achieved when induced to swell in hypotonic solution, yielding a value $A = 305 \mu\text{m}^2$. This value is about 20% higher than the cell membrane surface area used in this study.

We obtained an osmotically inactive volume of $165 \mu\text{m}^3$, which corresponds to an osmotically inactive volume fraction of 0.44. This value is slightly higher than osmotically inactive volume fractions reported in the literature, which include values of 0.36 [23], 0.43 [45], 0.36 [46] and 0.23 [43]. Truncation of the cell volume distribution has been shown to cause overestimation of the osmotically inactive volume fraction [28]. However, we do not expect that data truncation had a significant effect on our results because we used nearly all

of the cell volume distribution to calculate mean volumes, as shown in Fig. 3. Another factor that would affect estimates for the osmotically inactive volume fraction is the viability of the cell population, since non-viable cells would not be expected to respond osmotically. In the current study, the average viability of the granulocyte population was 89%, which means that 11% of the cells were non-viable. Assuming these non-viable cells remain at the isotonic volume and do not respond osmotically, our estimate for the osmotically inactive volume fraction would have to be adjusted to a value of 0.37, which falls within the range of published values.

The membrane water permeability of human granulocytes has been examined in several previous studies [16,44,45,46], yielding permeability values at room temperature (20-25 °C) ranging from 0.195 to 1.15 $\mu\text{m atm}^{-1} \text{min}^{-1}$. Most of these previous studies measured the water permeability in the absence of CPA, which makes direct comparisons to the present study difficult. Nonetheless, our values for the water permeability in the presence of CPA, which ranged from 0.13 to 0.21 $\mu\text{m atm}^{-1} \text{min}^{-1}$ at 21 °C, are approximately consistent with previously published values. Our values for the activation energy for water transport ranged from 41 to 61 kJ/mol, which is in line with the previously published activation energy for water transport in the absence of CPA (~48 kJ/mol [45]). However, we obtained an activation energy for water transport in the presence of Me₂SO of 56 kJ/mol, which is substantially larger than the value (26 kJ/mol) reported by Toupin et al [44]. A potential cause for this discrepancy is the fact that Toupin et al used a 3-parameter membrane transport model to fit their data, whereas we used a 2-parameter model.

To our knowledge, only Toupin et al [44] has measured the Me₂SO permeability of human granulocytes, yielding a permeability of 17.3 $\mu\text{m/min}$ at 20 °C. This value is nearly threefold higher than that obtained in the current study. In addition, Toupin et al [44] reported a relatively low value for the activation energy for Me₂SO transport (29 kJ/mol). In comparison, the value obtained in the current study was 60 kJ/mol, which is comparable to previously reported activation energies for CPA transport for other cell types [2,21,22,24,37]. It is important to note that Toupin et al used a 3-parameter model to fit their data, which may have contributed to the differences described above. In addition, Toupin et al performed experiments over a narrower temperature range (20-30 °C), and their model fits are not in very good agreement with the experimental data in several cases.

The majority of previous studies of granulocyte cryopreservation have involved the use of Me₂SO, typically at a concentration of about 10% [7,10]. The results of these studies have been variable, but most report a substantial decrease in the number of functional granulocytes recovered after cryopreservation [7,10]. Based on our permeability data, abrupt exposure to 10% Me₂SO is predicted to cause transient shrinkage to a relative volume of $V/V_0 = 0.76$ at 4 °C and $V/V_0 = 0.77$ at 37 °C. Abrupt removal of 10% Me₂SO is predicted to cause transient swelling to $V/V_0 = 1.48$ at 4 °C and $V/V_0 = 1.46$ at 37 °C. While volume changes of this magnitude have previously been reported to damage granulocytes to some extent, more extreme volume changes are required to cause extensive damage [4,18]. Therefore, it is unlikely that osmotic damage is the only cause of the cell losses reported in previous studies. Chemical toxicity may be an important factor [12,34], and it is also

possible that 10% Me₂SO does not sufficiently protect the granulocytes during freezing and thawing.

Glycerol is generally considered to be less toxic than Me₂SO and it has been used successfully for cryopreservation of other cell types, including red blood cells. Consequently, there have been several previous studies on the use of glycerol for cryopreservation of granulocytes [7,19,23]. To our knowledge, there are only two reports of the glycerol permeability of human granulocytes. Frim and Mazur [23] measured the glycerol permeability using an electronic cell counter, yielding a value of 0.4 $\mu\text{m}/\text{min}$ at 0 °C. This value is more than three-fold higher than that obtained using the best-fit Arrhenius model from the current study (see Table 1). On the other hand, Dooley [17] used radiolabelled glycerol to measure the permeability and obtained a value of 0.2 $\mu\text{m}/\text{min}$ at 26 °C, which is about 8-fold lower than the corresponding glycerol permeability from the current study. The reasons for the differences between our results and the previously published glycerol permeability values are unclear, but may be related to experimental limitations in the previous studies. For instance, the volume measurements reported by Frim and Mazur [23] were collected with low temporal resolution, and the model fit was obtained by eye.

Interestingly, the research groups that performed the glycerol permeability studies described above obtained conflicting results in subsequent studies of cell damage during glycerol addition and removal [3,19]. Armitage and Mazur [3] added and removed glycerol at 0 °C and used the permeability value from Frim and Mazur [23] to design multistep methods for maintaining the cell volume between $V/V_0 = 0.8$ and $V/V_0 = 1.7$. The resulting “slow” glycerol addition and removal procedures were found to produce substantial cell losses and the authors speculated that this damage may have been caused by glycerol toxicity. However, predictions using our glycerol permeability value indicate that osmotic damage is a more likely explanation for the cell losses. Addition and removal of 2 M glycerol according the method of Armitage and Mazur [3] is predicted to cause volume excursions of $V/V_0 = 0.77$ and $V/V_0 = 2.15$; such volume changes (particularly the cell swelling) would be expected to be highly damaging [4,18]. In contrast, Dooley and colleagues [19] were able to add and remove relatively high concentrations of glycerol at 26 °C with only modest cell losses. Dooley et al [19] used a cross-flow filtration system to continuously change the solution composition and selected the rate of concentration change based on cell volume predictions using the glycerol permeability value from Dooley [17]. Our glycerol permeability at 26 °C is about 8-fold higher and consequently the procedures used by Dooley et al [19] are expected to be very conservative for avoiding osmotic damage. Using our permeability value, the representative procedure presented in Dooley et al [19] for adding and removing 3500 mOsm/kg glycerol is only predicted to result in shrinkage to $V/V_0 = 0.94$ and swelling to $V/V_0 = 1.23$. Thus, our analysis of these previous studies [3,19] provides an explanation for the conflicting results and suggests that granulocytes can be equilibrated with high glycerol concentrations as long as osmotic damage is avoided.

While the cross-flow filtration method reported by Dooley et al [19] is promising for achieving high glycerol concentrations, the experimental approach is cumbersome. Therefore, to illustrate the value of membrane transport modeling, we have focused on the

design of a more convenient method for glycerol addition. Our group previously reported a method for mathematical optimization of CPA equilibration methods based on minimization of a toxicity cost function [8]. This optimization approach produced the surprising result that it is advantageous to induce swelling during CPA addition by using a hypotonic concentration of nonpermeating solutes in the CPA loading solution [8]. Fig. 6 illustrates a two-step procedure based on this concept for loading 30% w/v glycerol into human granulocytes at 37 °C. The first step consists of exposure to a 1020 mOsm/kg glycerol solution lacking nonpermeating solutes for about 1.4 min. This causes shrinkage initially, followed by swelling to a relative cell volume of $V/V_0 = 1.5$. In the second step, the cells can be exposed directly to the final glycerol solution without causing excessive cell volume excursions. In contrast, the conventional CPA loading approach would require a total of four steps over 12 minutes to achieve the same final intracellular glycerol content (Fig. 6, gray line). Thus, by inducing swelling in the first step of glycerol loading the number of steps can be reduced from four to two and the total loading time can be reduced from 12 min to 4.6 min. Although this method still needs to be evaluated experimentally, the predictions highlight the potential for conveniently loading human granulocytes with high glycerol concentrations.

Although ethylene glycol and propylene glycol have allowed the successful cryopreservation of other cell types, the use of these CPAs for cryopreservation of granulocytes has received little attention [42,47]. To our knowledge there are no previously reported measurements of the permeability of the human granulocyte membrane to propylene glycol or ethylene glycol. However, the results of Takahashi et al [42] suggest that ethylene glycol permeates more rapidly than Me₂SO and glycerol. Takahashi et al measured the viability and function of granulocytes after addition and removal of these CPAs and found the best results using ethylene glycol, suggesting that exposure to ethylene glycol causes the least osmotic damage. This result is consistent with our permeability measurements, which yielded a higher permeability for ethylene glycol than for the other CPAs.

In summary, we reported permeability values and activation energies for membrane transport of Me₂SO, glycerol, ethylene glycol and propylene glycol in human granulocytes. These biophysical parameters will allow the use of membrane transport modeling to re-evaluate and possibly improve previously reported procedures for granulocyte cryopreservation, which have primarily focused on the use of Me₂SO and glycerol [7]. Moreover, our results will facilitate the development of new cryopreservation procedures involving ethylene glycol or propylene glycol and those, in turn may lead to improved results.

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References

1. Report of the US Department of Health and Human Services. The 2009 national blood collection and utilization survey report. US Department of Health and Human Services, Office of the Assistant Secretary for Health; Washington, DC: 2011.
2. Agca Y, Gilmore J, Byers M, Woods EJ, Liu J, Critser JK. Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. *Biol Reprod.* 2002; 67:1493–1501. [PubMed: 12390881]
3. Armitage WJ, Mazur P. Toxic and osmotic effects of glycerol on human granulocytes. *Am J Physiol.* 1984; 247:C382–389. [PubMed: 6496725]
4. Armitage WJ, Mazur P. Osmotic tolerance of human granulocytes. *Am J Physiol.* 1984; 247:C373–381. [PubMed: 6496724]
5. Arnaud F, Yang H, McGann LE. Freezing injury of granulocytes during slow cooling: role of the granules. *Cryobiology.* 1996; 33:391–403. [PubMed: 8764847]
6. Atay D, Ozturk G, Akcay A, Yanasik M, Anak S, Devecioglu O. Effect and safety of granulocyte transfusions in pediatric patients with febrile neutropenia or defective granulocyte functions. *Journal of Pediatric Hematology/Oncology.* 2011; 33:e220–225. [PubMed: 21792027]
7. Bank H. Granulocyte preservation circa 1980. *Cryobiology.* 1980; 17:187–197. [PubMed: 6996919]
8. Benson JD, Kearsley AJ, Higgins AZ. Mathematical optimization of procedures for cryoprotectant equilibration using a toxicity cost function. 64. *Cryobiology.* 2012:144–151.
9. Blanks MJ, Stehle JR Jr, Du W, Adams JM, Willingham MC, Allen GO, Hu JJ, Lovato J, Molnar I, Cui Z. Novel innate cancer killing activity in humans. *Cancer Cell Int.* 2011; 11:26. [PubMed: 21813015]
10. Boonlayangoor P, Telischi M, Boonlayangoor S, Sinclair TF, Millhouse EW. Cryopreservation of Human-Granulocytes - Study of Granulocyte Function and Ultrastructure. *Blood.* 1980; 56:237–245. [PubMed: 6249429]
11. Brecher, ME., editor. Technical Manual. AABB; Bethesda, MD: 2005.
12. Cavins JA, Djerassi I, Roy AJ, Klein E. Preservation of viable human granulocytes at low temperatures in dimethyl sulfoxide. *Cryobiology.* 1965; 2:129–133. [PubMed: 5860597]
13. ClinicalTrials.gov. Safety and Effectiveness of Granulocyte Transfusions in Resolving Infection in People with Neutropenia (The Ring Study). National Library of Medicine; Bethesda, MD: 2008. Identifier: NCT00627393
14. ClinicalTrials.gov. A Study Using White Blood Cells from Healthy Donors to Treat Solid Cancers. National Library of Medicine; Bethesda, MD: 2009. Identifier: NCT00900497
15. Cui Z, Willingham MC, Hicks AM, Alexander-Miller MA, Howard TD, Hawkins GA, Miller MS, Weir HM, Du W, DeLong CJ. Spontaneous regression of advanced cancer: identification of a unique genetically determined, age-dependent trait in mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2003; 100:6682–6687. [PubMed: 12724523]
16. Diller KR, Bradley DA. Measurement of the water permeability of single human granulocytes on a microscopic stopped-flow mixing system. *Journal of Biomechanical Engineering.* 1984; 106:384–393. [PubMed: 6513536]
17. Dooley DC. Glycerol permeation of the human granulocyte. *Experimental Hematology.* 1982; 10:413–422. [PubMed: 7095016]
18. Dooley DC, Takahashi T. The effect of osmotic stress on the function of the human granulocyte. *Experimental Hematology.* 1981; 9:731–741. [PubMed: 7032958]
19. Dooley DC, Law P, Schork P, Meryman HT. Glycerolization of the human neutrophil for cryopreservation: osmotic response of the cell. *Experimental Hematology.* 1982; 10:423–434. [PubMed: 7095017]
20. Drewniak A, Kuijpers TW. Granulocyte transfusion therapy: randomization after all? *Haematologica-the Hematology Journal.* 2009; 94:1644–1648.
21. Ebertz SL, McGann LE. Cryoprotectant permeability parameters for cells used in a bioengineered human corneal equivalent and applications for cryopreservation. *Cryobiology.* 2004; 49:169–180. [PubMed: 15351688]

22. Fedorow C, McGann LE, Korbitt GS, Rayat GR, Rajotte RV, Lakey JRT. Osmotic and cryoprotectant permeation characteristics of islet cells isolated from the newborn pig pancreas. *Cell Transplantation*. 2001; 10:651–659. [PubMed: 11714201]
23. Frim J, Mazur P. Interactions of cooling rate, warming rate, glycerol concentration, and dilution procedure on the viability of frozen-thawed human granulocytes. *Cryobiology*. 1983; 20:657–676. [PubMed: 6661915]
24. Fry AK, Higgins AZ. Measurement of Cryoprotectant Permeability in Adherent Endothelial Cells and Applications to Cryopreservation. *Cellular and Molecular Bioengineering*. 2012; 5:287–298.
25. Gao DY, Liu J, Liu C, McGann LE, Watson PF, Kleinhans FW, Mazur P, Critser ES, Critser JK. Prevention of Osmotic Injury to Human Spermatozoa during Addition and Removal of Glycerol. *Human Reproduction*. 1995; 10:1109–1122. [PubMed: 7657750]
26. Hansen, CM. Solubility Parameters: A User's Handbook. CRC Press, Inc; Boca Raton, Florida: 1999.
27. Hicks AM, Riedlinger G, Willingham MC, Alexander-Miller MA, Von Kap-Herr C, Pettenati MJ, Sanders AM, Weir HM, Du W, Kim J, Simpson AJG, Old LJ, Cui Z. Transferable anticancer innate immunity in spontaneous regression/complete resistance mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:7753–7758. [PubMed: 16682640]
28. Higgins AZ, Karlsson JO. Curve fitting approach for measurement of cellular osmotic properties by the electrical sensing zone method. I. Osmotically inactive volume. *Cryobiology*. 2008; 57:223–233. [PubMed: 18805408]
29. Higgins AZ, Karlsson JO. Coincidence error during measurement of cellular osmotic properties by the electrical sensing zone method. *Cryo Letters*. 2008; 29:447–461. [PubMed: 19280049]
30. Itakura A, Verbout NG, Phillips KG, Insall RH, Gailani D, Tucker EI, Gruber A, McCarty OJ. Activated factor XI inhibits chemotaxis of polymorphonuclear leukocytes. *Journal of Leukocyte Biology*. 2011; 90:923–927. [PubMed: 21807745]
31. Karlsson JOM, Eroglu A, Toth TL, Cravalho EG, Toner M. Fertilization and development of mouse oocytes cryopreserved using a theoretically optimized protocol. *Human Reproduction*. 1996; 11:1296–1305. [PubMed: 8671443]
32. Karlsson JOM, Younis AI, Chan AWS, Gould KG, Eroglu A. Permeability of the Rhesus Monkey Oocyte Membrane to Water and Common Cryoprotectants. *Molecular Reproduction and Development*. 2009; 76:321–333. [PubMed: 18932214]
33. Kleinhans FW. Membrane permeability modeling: Kedem-Katchalsky vs a two-parameter formalism. *Cryobiology*. 1998; 37:271–289. [PubMed: 9917344]
34. Knight SC, O'Brien JA, Farrant J. Injury to human granulocytes at low temperatures. *Cryobiology*. 1980; 17:273–281. [PubMed: 7408518]
35. Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. *Cell Biophys*. 1990; 17:53–92. [PubMed: 1704816]
36. Meny GM, Santos-Zabala L, Szallasi A, Stramer SL. West Nile virus infection transmitted by granulocyte transfusion. *Blood*. 2011; 117:5778–5779. [PubMed: 21617013]
37. Mukherjee IN, Song YC, Sambanis A. Cryoprotectant delivery and removal from murine insulinomas at vitrification-relevant concentrations. *Cryobiology*. 2007; 55:10–18. [PubMed: 17533114]
38. Ofra Y, Avivi I, Oliven A, Oren I, Zuckerman T, Bonstein L, Rowe JM, Dann EJ. Granulocyte transfusions for neutropenic patients with life-threatening infections: a single centre experience in 47 patients, who received 348 granulocyte transfusions. *Vox Sanguinis*. 2007; 93:363–369. [PubMed: 18070282]
39. Quillen K, Wong E, Scheinberg P, Young NS, Walsh TJ, Wu CO, Leitman SF. Granulocyte transfusions in severe aplastic anemia: an eleven-year experience. *Haematologica*. 2009; 94:1661–1668. [PubMed: 19996117]
40. Sputek A, Kuhn P, Rowe AW. Cryopreservation of erythrocytes, thrombocytes, and lymphocytes. *Transfusion Medicine and Hemotherapy*. 2007; 34:262–267.
41. Takahashi T, Hammett MF, Cho MS. Multifaceted freezing injury in human polymorphonuclear cells at high subfreezing temperatures. *Cryobiology*. 1985; 22:215–236. [PubMed: 3996013]

42. Takahashi T, Bross JB, Shaber RE, Williams RJ. Effect of cryoprotectants on the viability and function of unfrozen human polymorphonuclear cells. *Cryobiology*. 1985; 22:336–350. [PubMed: 4028780]
43. Ting-Beall HP, Needham D, Hochmuth RM. Volume and osmotic properties of human neutrophils. *Blood*. 1993; 81:2774–2780. [PubMed: 8490184]
44. Toupin CJ, Le Maguer M, McGann LE. Permeability of human granulocytes to dimethyl sulfoxide. *Cryobiology*. 1989; 26:422–430. [PubMed: 2791611]
45. Toupin CJ, Lemaguer M, McGann LE. Permeability of Human-Granulocytes to Water - Rectification of Osmotic Flow. *Cryobiology*. 1989; 26:431–444. [PubMed: 2791612]
46. Yang H, Arnaud F, McGann LE. Cryoinjury in Human Granulocytes and Cytoplasts. *Cryobiology*. 1992; 29:500–510. [PubMed: 1395688]
47. Yang HY, Walterson M, McGann L. Responses of Human-Granulocytes to High-Concentrations of Propylene-Glycol and Rapid Cooling. *Cryobiology*. 1987; 24:588–588.

Fig. 1.

Evaluation of the assumption that particles are drawn through the Coulter Counter aperture at a constant rate. The number of 10 μm calibration beads counted in each of six consecutive 10-second intervals was normalized to the average count over all of the intervals. Each symbol represents the mean of 4 replicates and error bars show the standard error of the mean.

Fig. 2.
Representative image of isolated granulocytes stained using Giemsa stain. Scale bar is 20 μm .

Fig. 3. Equilibrium osmotic response of human granulocytes. (A) Cell volume distributions after equilibration in various hypo- and hypertonic solutions. The curves shown represent averages over 3 different donors, with 2-3 replicates per donor. (B) Boyle-van't Hoff plot. Mean cell volumes were determined by taking the average of measured volumes over the range $150 \mu\text{m}^3$ to $950 \mu\text{m}^3$ (300 mOsm/kg, 400 mOsm/kg and 600 mOsm/kg), or over the range $200 \mu\text{m}^3$ to $1150 \mu\text{m}^3$ (200 mOsm/kg). The different symbols represent data from different donors.

Fig. 4.

Response of cell volumes after exposure to a solution containing 1.7 Osm/kg ethylene glycol at 21 °C. Light gray symbols show the raw volume measurements and the dark gray symbols show a running average over 60 consecutive volume measurements. The line shows best-fit model predictions using Eqs. 2-3.

Fig. 5.

Arrhenius plots of the water permeability (L_p) and CPA permeability (P_s). Cells were exposed to either Me₂SO (1.7 Osm/kg), glycerol (0.7 Osm/kg), ethylene glycol (1.7 Osm/kg) or propylene glycol (0.7 Osm/kg) at temperatures of 4 °C, 21 °C or 37 °C. The different symbols represent data from different donors. Lines show the best-fit Arrhenius models (Eqs. 5-6).

Fig. 6.

Predictions of relative cell volume during addition of ~30% w/v glycerol using a new approach that takes advantage of cell swelling (black line), as well as the conventional approach (gray line). Cell volume excursions were constrained between $V/V_0 = 0.8$ and $V/V_0 = 1.5$. The inset table shows the solution concentrations and equilibration times in each step for both glycerol addition procedures.

Table 1

Permeability properties of human granulocytes at a reference temperature of 21 °C.

CPA	CPA Permeability		Water Permeability	
	$P_{s,ref}$ (μm/min)	E_a (kJ/mol)	$L_{p,ref}$ (μm atm ⁻¹ min ⁻¹)	E_a (kJ/mol)
Me ₂ SO	6.4	60 ± 4	0.21	56 ± 4
Glycerol	1.0	68 ± 4	0.18	41 ± 2
Ethylene Glycol	8.4	59 ± 2	0.21	61 ± 2
Propylene Glycol	4.0	60 ± 5	0.13	47 ± 4