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# Osmotic parameters of red blood cells from umbilical cord blood ☆

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ABSTRACT

The transfusion of red blood cells from umbilical cord blood (cord RBCs) is gathering significant interest for the treatment of fetal and neonatal anemia, due to its high content of fetal hemoglobin as well as numerous other potential benefits to fetuses and neonates. However, in order to establish a stable supply of cord RBCs for clinical use, a cryopreservation method must be developed. This, in turn, requires knowledge of the osmotic parameters of cord RBCs. Thus, the objective of this study was to characterize the osmotic parameters of cord RBCs: osmotically inactive fraction ($b$), hydraulic conductivity ($L_p$), permeability to cryoprotectant glycerol ($P_{glycerol}$), and corresponding Arrhenius activation energies ($E_a$). For $L_p$ and $P_{glycerol}$ determination, RBCs were analyzed using a stopped-flow system to monitor osmotically-induced RBC volume changes via intrinsic RBC hemoglobin fluorescence. $L_p$ and $P_{glycerol}$ were characterized at 4 °C, 20 °C, and 35 °C using Jacobs and Stewart equations with the $E_a$ calculated from the Arrhenius plot. Results indicate that cord RBCs have a larger osmotically inactive fraction compared to adult RBCs. Hydraulic conductivity and osmotic permeability to glycerol of cord RBCs differed compared to those of adult RBCs with the differences dependent on experimental conditions, such as temperature and osmolality. Compared to adult RBCs, cord RBCs had a higher $E_a$ for $L_p$ and a lower $E_a$ for $P_{glycerol}$. This information regarding osmotic parameters will be used in future work to develop a protocol for cryopreserving cord RBCs.



## Introduction

The transfusion of RBCs derived from umbilical cord blood for the treatment of neonatal anemia has gained significant interest recently [4,5,12,13,19,25,28,30,62,63]. Fetal and neonatal anemias are among the most serious complications of pregnancy and postnatal development. The most commonly used treatment is transfusion of red blood cells (RBCs), either intrauterine or intravenous [24,36,64] to help replace the lost RBCs of the fetus or neonate. To date, RBCs used in intrauterine and neonatal (intravenous) transfusions are derived from adult donors [24,36,45,64]. Adult RBCs are different from those present in the blood of a fetus or neonate [11,27,39,48,50,51]. Perhaps not surprisingly, the practice of administering adult RBC transfusions to premature infants has been associated with a number of complications, such as retrolental fibroplasia [14,23,38] and bronchopulmonary dysplasia [15,17,32], usually caused by the delivery of unnecessarily high amounts of oxygen to tissues.

Neonatal RBCs obtained from umbilical cord blood (cord RBCs) may offer a superior alternative for intrauterine and neonatal transfusions [6,22]. Cord RBCs are usually discarded during the isolation of stem cells from cord blood [8,52,61]. Due to the high concentration of fetal hemoglobin (HbF), which is practically absent in adult RBCs, cord RBCs have a potential to deliver a physiologically suitable amount of oxygen to fetal and neonatal tissues upon transfusion [37]. A number of studies have demonstrated that transfusions of autologous cord RBCs are both safe and effective for the treatment of anemic neonates [4,12,13,19,25,63]. However, unlike RBCs from adult blood, cord RBCs deteriorate quickly during traditional storage at 1–6 °C [19,29] and thus would benefit from low temperature preservation. Despite several reports describing some of the effects of cryopreservation on cord RBCs

*Abbreviations:* RBC, red blood cell; HbF, fetal hemoglobin; b, osmotically inactive fraction; $L_p$, hydraulic conductivity; $P_s$, permeability to solutes; $P_{glycerol}$, permeability to glycerol; $E_a$, Arrhenius activation energy; CPD, citrate–phosphate–dextrose; SAGM, Saline–adenine–glucose–mannitol; NaCl, sodium chloride; PBS, phosphate-buffered saline; $V$, Volt; $V/V_0$, equilibrium relative RBC volume; $F/F_0$, equilibrium relative RBC fluorescence intensity; $\pi_0/\pi$, inverse relative osmolality; $P_f$, osmotic water permeability.

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[9,70], an effective cryopreservation method for cord RBCs still needs to be developed.

To design effective cryopreservation procedures for cord RBCs, it is critical to know the osmotic parameters of these cells [18,31,35,55]. A cell undergoes a number of osmotic changes during cryopreservation. Addition and removal of cryoprotectant, as well as freezing and thawing, cause changes in solute concentration inside and outside the cell and induce flux of water and permeating solutes across the cell membrane. Such changes in intra- and extra-cellular osmolality lead to corresponding changes in cell volume. A number of osmotic parameters define the movement of water and solutes across the cell membrane. Hydraulic conductivity ($L_p$) and osmotic permeability to solutes ($P_s$) describe the rate at which these substances cross the cell membrane and are typically determined by measuring the rate at which cell volume changes when placed in an anisotonic environment. The osmotically inactive fraction ($b$) is the fraction of the cell volume that does not participate in the osmotic response of the cell. The Arrhenius activation energy ($E_a$) describes the temperature dependence of the membrane's permeability to water and solutes [56]. These osmotic parameters can be used in mathematical models to predict an optimized cryopreservation protocol for the cells of interest [41,42,57].

It has been reported that, compared to adult RBCs, cord RBCs are less permeable to water [3,60], have a higher activation energy for osmotic water permeability [3], and are less permeable to some solutes, particularly the common cryoprotectant glycerol [46]. However, reports on cord RBC osmotic parameters are very limited at present. Moreover, with measurements made using different experimental techniques and under different experimental conditions, the absolute values of cord RBC osmotic parameters differ significantly between reports and, therefore, cannot be relied upon for use in mathematical modeling. The objective of this study is to measure and compare the osmotic parameters of adult and cord RBCs, such as osmotically inactive fraction, permeability to water and glycerol, and Arrhenius activation energies for these processes.

## Materials and methods

### *Source of red blood cells*

Two sources of RBCs were used in this study: RBCs from peripheral blood of adult donors (adult RBCs), and RBCs from umbilical cord blood (cord RBCs). The Canadian Blood Services Network Centre for Applied Development in Vancouver provided the adult RBCs. RBCs were leukocyte-reduced, stored in CPD (citrate–phosphate–dextrose) anticoagulant and SAGM (saline–adenine–glucose–mannitol) preservative at 1–6 °C, and were used in experiments within 15 days of collection. The hematocrit of adult RBCs was standardized to 60 ± 2%, if necessary, by the removal of supernatant or addition of saline. The Alberta Cord Blood Bank supplied cord RBCs, a waste product after stem cell isolation from umbilical cord blood. Cord blood collected from a placenta was stored at room temperature for up to 38 h prior to stem cell isolation (previously shown not to cause a decrease in RBC quality [68]). The leftover cord RBC product was washed 3 times with saline using centrifugation at 2200*g* at 4 °C for 5 min, to remove any residual pentastarch used in the stem cell isolation process [66]. The hematocrit of cord RBCs was then adjusted to 60 ± 2% by the addition of saline to the RBC pellet. Cord RBCs were stored at 1–6 °C and used in experiments within 24 h of isolation from cord blood. Ethics approval for the study was obtained from the University of Alberta Health Research Ethics Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

### *Experimental solutions*

Sodium chloride (NaCl) solutions were prepared by diluting 12% (w/v) NaCl stock solution (Baxter, Deerfield, IL, USA) with distilled water to yield final concentrations of 0.68%, 0.9%, 1.6%, and 3.5% (w/v). Phosphate-buffered saline (PBS) solutions were prepared by diluting 10× PBS solution (Calbiochem, Gibbstown, New Jersey) with distilled water to 0.5×, 1×, 3×, 5×, and 7× PBS. Lastly, a 5% (w/v) glycerol in 1× PBS solution was prepared by diluting 50 g glycerol (99.5+%, Sigma Aldrich, Inc., St. Louis, MO, USA) and 100 mL 10× PBS with distilled water to 1 L.

The osmolality of the experimental solutions was measured using a freezing-point depression osmometer Osmette (Precision Systems Inc., Natick, Massachusetts). Prior to each experimental run, the osmometer was verified through quality control checks using both a 290 mmol/kg Opti-Mole standard (Wescor, Inc., Logan, Utah) and a 1500 mOsm/kg standard (Precision Systems Inc., Natick, Massachusetts).

### *Hemolysis of RBCs in experimental solutions*

RBC hemolysis (membrane damage) was measured in experimental PBS and glycerol solutions. 50 μL of RBCs were pipetted into 1 mL of the experimental solution and allowed to equilibrate at room temperature for approximately 5 min. RBC hemolysis was determined by spectrophotometric measurement of total and supernatant cyanmethemoglobin according to Drabkin's method [1,71]. The hematocrit of the RBC sample was required for the calculation and, therefore, was determined, using a microhematocrit centrifuge (Hettich, Tuttlingen, Germany), as the ratio of the volume occupied by packed RBCs to the volume of a whole RBC sample. Controls for total hemoglobin were prepared from Stanbio Tri-Level Hemoglobin controls (Stanbio Laboratory, Boerne, TX, USA).

### *Measurement of RBC volume kinetics on stopped-flow*

Changes of RBC volume with exposure to solutions of different osmolalities were determined indirectly by monitoring changes in intrinsic hemoglobin fluorescence intensity, as described previously [69]. Using a SX20 stopped-flow reaction analyser (Applied Photophysics, Ltd., Leatherhead, UK), the RBC suspension was rapidly mixed with an equal volume of anisotonic experimental solution to induce osmotically-driven changes of RBC volume. RBC fluorescence intensity, which is directly related to RBC volume, was then recorded as a function of time after mixing.

The RBC suspension for stopped-flow experiments was prepared by adding 20 μL of RBCs to 1 mL of 1× PBS (final osmolality of 287 mOsm/kg). To determine hydraulic conductivity, RBCs were exposed to 0.75×, 2×, 3×, and 4× PBS solution (final osmolalities of 214, 562, 832, and 1112 mOsm/kg, respectively) – achieved by mixing the RBC suspension (in 1× PBS) rapidly in a 1:1 ratio with 0.5×, 3×, 5×, and 7× PBS, respectively. One thousand data points were collected during the 10 s period immediately following mixing. As a control, RBCs exposed to 1× PBS (osmotic equilibrium conditions) were assessed.

To determine glycerol permeability, RBCs were exposed to 2.5% (w/v) glycerol (final osmolality of 578 mOsm/kg) – achieved by rapid 1:1 mixing with 5% (w/v) glycerol in 1× PBS solution. Two thousand data points were collected during the 120 s period immediately following mixing: the time required for the complete equilibration of glycerol across the RBC membrane. As a control, RBCs were mixed at a 1:1 ratio with 1× PBS (establishing osmotic equilibrium conditions) and fluorescence was measured as a function of time. The background fluorescence of buffer solutions (without RBCs) was also measured. All samples were measured