

## ***Osmotic Tolerance Limits of Red Blood Cells from Umbilical Cord Blood***

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# Osmotic Tolerance Limits of Red Blood Cells from Umbilical Cord Blood

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## ABSTRACT

Effective methods for long-term preservation of cord red blood cells (RBCs) are needed to ensure a readily available supply of RBCs to treat fetal and neonatal anemia. Cryopreservation is a potential long-term storage strategy for maintaining the quality of cord RBCs for the use in intrauterine and neonatal transfusion. However, during cryopreservation, cells are subjected to

40 damaging osmotic stresses during cryoprotectant addition and removal and freezing and thawing that require knowledge of osmotic tolerance limits in order to optimize the preservation process. The objective of this study was to characterize the osmotic tolerance limits of cord RBCs in conditions relevant to cryopreservation, and compare the results to the osmotic tolerance limits of adult RBCs. Osmotic tolerance limits were determined by exposing RBCs to solutions of

45 different concentrations to induce a range of osmotic volume changes. Three treatment groups of adult and cord RBCs were tested: 1) isotonic saline, 2) 40% w/v glycerol, and 3) frozen-thawed RBCs in 40% w/v glycerol. We show that cord RBCs are more sensitive to shrinkage and swelling than adult RBCs, indicating that osmotic tolerance limits should be considered when adding and removing cryoprotectants. In addition, freezing and thawing resulted in both

50 cord and adult RBCs becoming more sensitive to post-thaw swelling requiring that glycerol removal procedures for both cell types ensure that cell volume excursions are maintained below 1.7 times the isotonic osmotically active volume to attain good post-wash cell recovery. Our results will help inform the development of optimized cryopreservation protocol for cord RBCs.

55 **Keywords:** hydraulic permeability, hemolysis, erythrocyte, osmotic fragility, cryopreservation, biobanking, cryoprotectant, transfusion medicine

**Abbreviations used:** RBC – red blood cell, CPA – cryoprotective agent, Hb – hemoglobin.

## INTRODUCTION

60 Fetal and neonatal anemias are serious complications that may happen during pregnancy and post-natal development. Fetal anemia may be caused by immune hemolytic disease [21], defects in the hemoglobin structure and synthesis, fetomaternal or twin-to-twin hemorrhages, and parvovirus B19 infections [6]. Neonatal anemia may carry-over from fetal anemia, or develop after birth from hemorrhaging due to obstetric incidents, frequent blood draws for laboratory  
65 testing, or impaired red blood cell (RBC) production by the bone marrow [6]. The most common treatment for fetal and neonatal anemia is intrauterine or intravenous transfusion of RBCs derived from adult donors [16; 21; 23; 31; 39; 44]. However, adult RBCs are different from the cells present in the blood of a fetus or neonate in terms of membrane composition [6; 28], biophysical properties [6; 28], hemoglobin structure [6; 18; 28; 32; 33; 34], as well as  
70 metabolism and enzymatic profiles [18; 34]. In particular, fetal hemoglobin is present in high concentrations in fetal and neonatal RBCs, but is almost completely absent in normal adult RBCs [24]. Fetal hemoglobin facilitates the fetus' uptake of oxygen from the placenta due to a higher affinity for binding oxygen when compared to hemoglobin present in adult cells [33]. These differences between fetal and adult RBCs may contribute to complications arising after  
75 transfusion of adult RBCs to treat fetal and neonatal anemias, including retrolental fibroplasia [9; 15; 26] and bronchopulmonary dysplasia [10; 11; 20].

Recently, there has been interest in the transfusion of RBCs derived from umbilical cord blood to treat fetal and neonatal anemia[2; 7; 8; 12; 17; 19; 42; 43]. RBCs in cord blood are commonly considered a waste product and are usually discarded after the isolation of stem cells  
80 [4; 37; 40]. However, cord RBCs contain high levels of fetal hemoglobin and consequently may offer a superior treatment alternative for fetal and neonatal anemia compared to adult RBCs. Various studies have demonstrated the efficiency of autologous transfusion of cord RBCs for treatment of neonatal anemia [1; 2; 7; 8; 12; 17; 43]; however, concerns have surfaced regarding the potential risk of bacterial contamination, low hypothermic storage stability, and the small  
85 harvestable volume of RBCs from umbilical cord blood [12; 41]. To overcome these concerns, an effective method for long-term preservation of cord RBCs is needed.

Adult RBCs are routinely stored at 1-6 °C in an anticoagulant/preservative solution for up to 42 days [38]. RBCs present in cord blood on the other hand, cannot be stored using the same hypothermic techniques as adult RBCs for more than 14 days without a significant decrease in

90 quality [12]. Cryopreservation is a potential long-term storage strategy for maintaining the quality of cord RBC for the future use in intrauterine and neonatal transfusion as well as blood banking. Adult RBCs are routinely cryopreserved in the presence of 40% w/v glycerol for clinical use, with cryopreservation protocols described in the literature [30; 46]. However, to our knowledge, no protocol for the cryopreservation of cord RBCs has been reported.

95 During cryopreservation, cells are subjected to potentially damaging osmotic stresses. For instance, addition of a permeable cryoprotective agent (CPA) like glycerol results in osmotically-driven water efflux and a concomitant decrease in cell volume, followed by swelling as both water and CPA enter the cell. The converse happens during CPA removal. Cryoprotectants, such as glycerol and dimethyl sulfoxide, must be removed prior to transfusion to avoid intravascular  
100 hemolysis of the RBCs and potentially serious adverse events associated with the cryoprotectants. Freezing and thawing also result in osmotic stresses. During freezing, growth of ice crystals removes water from the extracellular medium, concentrating the extracellular solution and creating an osmotic driving force for cell dehydration. Melting of ice crystals during thawing reverses this phenomenon. To design a successful cryopreservation procedure for cord  
105 RBCs, it will be necessary to avoid damaging osmotic stresses and the associated changes in cell volume. In particular, it will be necessary to maintain the cell volume within the osmotic tolerance limits, defined as the extent of volume excursions the cell can withstand before irreversible damage occurs [13].

This study investigates the osmotic tolerance limits of cord RBCs in conditions relevant  
110 to cryopreservation, and compares the results to the osmotic tolerance limits of adult RBCs. Our results will facilitate the development of a cryopreservation protocol specifically tailored for cord RBCs.

## MATERIALS & METHODS

### *Collection of RBCs from umbilical cord blood*

115 Cord RBCs were obtained from the Alberta Cord Blood Bank as a waste product after the isolation of stem cells from umbilical cord blood. The cord blood harvested from the placenta was stored at room temperature for up to 43 hours prior to the isolation of stem cells. A previous study showed that the storage period from harvest to the time of stem cell isolation does not cause a decrease in cord RBC quality [48]. The cord RBCs were then washed with isotonic

120 saline and centrifuged at 2200 g, 4 °C for 5 minutes. The washed cells were resuspended to a hematocrit of 60%, stored at 1-6 °C, and used in experiments within 24 hours. Ethics approval for the study was obtained from the University of Alberta Research Board (Biomedical Panel) and Canadian Blood Services Research Ethics Board.

### ***Collection of adult RBCs***

125 Adult RBCs were isolated from whole blood in citrate phosphate dextrose drawn from volunteer donors. Whole blood was centrifuged at 2200 g at room temperature for 6 minutes to separate the plasma and other components from the RBCs. The resulting packed cells were resuspended in isotonic saline, centrifuged at 2200 g at room temperature for 6 minutes, and the supernatant was removed. The RBCs were then resuspended in isotonic saline at a hematocrit of  
130 60%, stored at 1-6 °C, and used in experiments within 24 hours. Ethics approval for this study was obtained from the Oregon State University Institutional Review Board.

### ***RBC glycerolization, freezing and thawing***

#### ***Cord RBCs***

The supernatant from washed cord RBCs was removed to attain an approximate packed  
135 cell hematocrit of 90%. The packed cells were then weighed to achieve a starting mass of 4.1-4.2 g. This was done to ensure that the desired final concentration of glycerol, non-permeating solute, and cell density was achieved. Glycerolite 57 was then added at room temperature in two separate steps. In the first step, 1.5 mL of Glycerolyte 57 was added to the packed cells slowly over a three minute period accompanied by gentle agitation. The cells were then allowed to rest  
140 undisturbed for five minutes to ensure proper equilibration. In the second step, 5 mL of Glycerolyte 57 solution was added over a three minute period with gentle agitation to ensure proper mixing. After the second addition of Glycerolyte 57, the cord RBCs were either directly used for osmotic tolerance limit experiments or cryopreserved according to the following procedures. Glycerolized cord RBCs were transferred into cryovials (Thermo Fisher Scientific,  
145 Roskilde, Denmark) and frozen by placing them in a -80°C freezer to achieve a freezing rate of approximately 1°C per minute. Cord RBCs were left in a frozen state for at least 48 hours prior to being used in osmotic tolerance experiments. To thaw, the cryovials were placed in a 37°C water bath for one minute. Thawed cord RBCs were used in experiments immediately.

150 *Adult RBCs*

The adult RBCs were glycerolized, frozen and thawed in the same way as the cord RBCs except that, instead of using Glycerolyte 57, the adult RBCs were glycerolized using an aqueous glycerol solution with the following composition: 57.1 g glycerol, 0.03 g potassium chloride, 0.085 g magnesium chloride hexahydrate, 0.08 disodium phosphate, and 1.6 g sodium lactate in  
155 a total volume of 100 mL, adjusted to a pH of 6.8 [45]. The two glycerolization solutions are essentially identical except that Glycerolyte 57 lacks magnesium chloride and instead has a slightly higher concentration of phosphate buffer.

*Osmotic Tolerance Limit Experiments*

The general method of investigating the osmotic tolerance limits involves exposing the  
160 cells to solutions of different concentrations to induce a range of osmotic volume changes. Three treatment groups of adult and cord RBCs were tested: 1) RBCs in isotonic saline, 2) RBCs in 40% w/v glycerol, and 3) frozen-thawed RBCs in 40% w/v glycerol.

*Osmotic tolerance limits of RBCs in the presence of saline*

To induce shrinkage or swelling, a 1 mL volume of buffered anisotonic saline solution  
165 was added to 0.1 mL of washed RBCs in isotonic saline while the sample was gently agitated. After a 5 min equilibration period, 0.1 mL of the resulting mixture was removed from the vessel for hemolysis testing under anisotonic conditions. The remaining mixture was then brought back to isotonic conditions by adding 19 mL of either hypo- or hypertonic buffered saline solution, resulting in a final volume of 20 mL. Once the suspension was homogeneously mixed, a sample  
170 was obtained for hemolysis testing under isotonic conditions. All solutions used were buffered with 12.5 mM disodium phosphate and adjusted to a pH of 7. The exact anisotonic saline concentrations added to the RBCs are tabulated in Table 1.

The Boyle-van't Hoff relationship was used to estimate the change in osmotically active cell volume after equilibration in each of the anisotonic solutions described in Table 1. In  
175 particular, the following equation was used to calculate the ratio of the final volume of osmotically active cell water ( $V_w$ ) to the original, isotonic water volume ( $V_{w0}$ ):

$$(1) \quad \frac{V_w}{V_{w0}} = \frac{M_0}{M}$$

where  $M_0$  is the isotonic osmolality and  $M$  is the osmolality of the anisotonic solution. To estimate the solution osmolality, each mole of sodium chloride was assumed to contribute two osmoles, and the phosphate buffer was assumed to contribute an osmolality of 36 mOsm/kg (this is the measured osmolality of the buffer solution in the absence of saline). The expected volume changes calculated using Eq. 1 are also shown in Table 1.

*Osmotic tolerance limits of RBCs in the presence of 40% w/v glycerol*

The upper osmotic tolerance limit of RBCs in the presence of 40% w/v glycerol was measured – either immediately after glycerolization or after glycerolization, freezing and thawing – by inducing the cells to swell using a relatively hypotonic glycerol solution. The composition of the hypotonic glycerol solution was selected such that the equilibrium cell volume could be achieved by transport of water alone, avoiding the potentially confounding effects of glycerol transport (which occurs much more slowly than water transport). This was accomplished by preparing all solutions using a constant ratio of glycerol osmolality to non-permeating solute osmolality. The procedure for exposing the glycerolized RBCs to changes in solution composition was similar to that described above: a volume 0.1 mL of glycerolized RBCs was mixed with 1 mL anisotonic glycerol-saline solution, and the sample was gently agitated for 5 minutes to allow the cells to reach the expected volume change. Once the equilibration period was over, the cells were brought back to ‘isotonic’ conditions, the original starting concentration of the glycerolized RBCs, by adding the 18.9 mL of hypertonic glycerol-saline solution. After homogeneous mixing was achieved, a sample was obtained for hemolysis testing. All solutions were buffered with 12.5 mmolal disodium phosphate and adjusted to a pH of 7. The saline and glycerol concentrations added to the RBCs in these experiments are given in Table 1.

To estimate changes in the osmotically active volume, we assumed that the cells had reached chemical equilibrium. Because both water and glycerol are membrane permeable, this results in two equilibrium relationships that equate intra- and extracellular osmolalities:

$$(2) \quad \frac{N_n}{\rho_w V_w} + \frac{N_s}{\rho_w V_w} = M_n + M_s$$

$$(3) \quad \frac{N_s}{\rho_w V_w} = M_s$$



where  $N$  is the moles of intracellular solute,  $\rho_w = 1 \text{ g/mL}$  is the density of water and the subscripts  $n$  and  $s$  refer to non-permeating solute and glycerol, respectively. Given the subsidiary equations,  $N_n = \rho_w V_{w0} M_0$  and  $V_s = v_s N_s$  (where  $V_s$  is the intracellular volume of glycerol and  $v_s = 73 \text{ mL/mol}$  is the molar volume of glycerol), the following expression for the relative osmotically active volume can be obtained:

$$(4) \quad \frac{V_w + V_s}{V_{w0}} = \frac{M_0}{M_n} (1 + v_s M_s \rho_w)$$

To estimate the solution osmolalities for use in Eq. 4, each mole of sodium chloride was assumed to contribute two osmoles, each mole of glycerol was assumed to contribute 1 osmole and the phosphate buffer was assumed to contribute 36 mOsm/kg. The expected volume changes calculated using Eq. 4 are also shown in Table 2.

### ***Hemolysis Measurement***

Hemolysis was quantified by measuring the amount of free hemoglobin in the supernatant using Harboe's direct spectrophotometric method. The samples were first centrifuged at 2200 g for 6 minutes to separate the supernatant from the surviving cells. A portion of the supernatant was then diluted appropriately to fall within the linear absorbance measurement range at 415 nm. The concentration of the free hemoglobin (Hb) released by damaged cells into the supernatant was then measured using the following equation [14]:

$$(5) \quad Hb \text{ (g/L)} = 0.1672 A_{415} - 0.0836 A_{380} - 0.0836 A_{450}$$

where  $A_{415}$ ,  $A_{380}$ , and  $A_{450}$  are absorbance values measure at 415, 380, and 450 nm respectively and extinction coefficients are given in units of ( $\text{L g}^{-1} \text{ nm}^{-1}$ ). The percent hemolysis was calculated by dividing the mass of free hemoglobin in the supernatant by the mass of total hemoglobin from a sample that had been intentionally lysed by 100-fold dilution in distilled water followed by three consecutive freeze-thaw cycles. The hematocrit in the final sample was always less than 1%, so it was not necessary to account for hematocrit when calculating percent hemolysis.

### ***Statistical Analysis***

The results of the experiments are reported as averages of five replicates, with each replicate originating from a different batch of blood, except for the data for cord RBCs in the presence of saline which was reported as averages of seven replicates. Error bars show the

standard error of the mean. Statistical comparisons between adult and cord RBCs, as well as  
235 between different treatment groups, were performed using t-tests. Differences were considered  
to be significant at a 95% confidence level ( $p < 0.05$ ).

## RESULTS

### *Osmotic tolerance limits of untreated RBCs in the presence of saline*

The osmotic tolerance limits of untreated cord and adult RBCs were evaluated to  
240 assess the osmotic sensitivity of the cell membrane in its natural state. To provide insight to the  
mechanisms of osmotic damage, hemolysis was measured while the cells were in anisotonic  
conditions, and after the cells had been returned to isotonic conditions. The results of these  
experiments are shown in Figure 1. For both cord and adult RBCs, exposure to hypotonic  
245 conditions caused immediate damage, the extent of which increased as the solution hypotonicity  
increased. On the other hand, the majority of damage from exposure to hypertonic conditions  
did not manifest until the cells were returned to isotonic conditions. For example, exposure to  
the most hypertonic solution that we tested resulted in less than 20% hemolysis, but subsequent  
return of the cells to isotonic conditions caused the hemolysis to increase to nearly 100%.

A comparison of the hemolysis values between the two cell types reveals that cord RBCs  
250 are less resistant to osmotic swelling and shrinking when compared to adult RBCs. As shown in  
Figure 1B, there is a notable significant difference in hemolysis for swelling to 1.7 times the  
isotonic osmotically active volume ( $p = 0.0002$ ). Under these conditions, cord RBCs experienced  
a hemolysis of  $10.2\% \pm 2.3\%$ , which is significantly higher than the value of  $2.4\% \pm 0.6\%$  for  
adult RBCs. In addition, shrinkage to 10% of the isotonic osmotically active volume (and  
255 subsequent return to isotonic conditions) produced significantly higher hemolysis for cord RBCs  
( $60.1\% \pm 7.3\%$ ) than adult RBCs ( $33.2\% \pm 3.7\%$ ).

### *Osmotic tolerance limits of glycerolized RBCs*

To assess the effect of glycerol on osmotic sensitivity, both types of RBCs were  
glycerolized to a final concentration of 40% w/v and then exposed to relatively hypotonic  
260 glycerol solutions to induce a swelling to a range of final volumes. The results of these  
experiments are shown in Figure 2. To examine cell damage caused by the glycerolization  
process, the control hemolysis values in Figures 2 and 1B were compared. For cord RBCs, the  
control hemolysis (relative osmotically active volume of 1) significantly increased from  $1.6\% \pm$

0.2% for untreated cells to  $6.3\% \pm 1.8\%$  for glycerolized cells ( $p = 0.02$ ). For adult RBCs, the control hemolysis significantly increased from  $1.8\% \pm 0.5\%$  for untreated RBCs to  $6.9\% \pm 0.3\%$  for glycerolized cells ( $p < 0.0001$ ). The control hemolysis values for glycerolized cord and adult RBCs were not significantly different, indicating that both cell types incur a similar amount of damage as a result of the glycerolization process.

As shown in Figure 2, glycerolized cord RBCs appear to be more resistant to swelling when compared to glycerolized adult RBCs. In particular, there was a significant difference in hemolysis between glycerolized cord RBCs and glycerolized adult RBCs when induced to swell to 2 ( $p = 0.008$ ) and 2.5 ( $p = 0.014$ ) times the isotonic osmotically active volume, with the difference being particularly large at a relative osmotic volume of 2.

### *Osmotic tolerance limits after glycerolization, freezing and thawing.*

The upper osmotic tolerance limit after glycerolization, freezing and thawing was evaluated to assess the osmotic sensitivity of the cell membrane in a state that most closely resembles the condition of thawed clinically cryopreserved RBCs. The results of these experiments are shown in Figure 3. Comparison of the control data (far left) from Figures 2 and 3 show that both cord and adult RBCs survived the freezing and thawing process with no significant increase in hemolysis with respect to unfrozen glycerolized cells. A comparison of the hemolysis results from Figures 2 and 3 suggests that freezing and thawing sensitizes both cord and adult RBCs to hypotonic or swelling damage. In particular, frozen-thawed RBCs exhibited significantly higher hemolysis when induced to swell to 1.7 times the isotonic osmotically active volume than did glycerolized RBCs that had not been frozen and thawed.

The osmotic sensitivity of cord and adult RBCs after glycerolization, freezing, and thawing is compared in Figure 3. The osmotic damage incurred by frozen-thawed cord RBCs and adult RBCs was comparable when the cells were induced to swell to 1.7 times the isotonic osmotically active volume. However, cord RBCs exhibited significantly lower hemolysis than adult RBCs when induced to swell to 2 times the isotonic osmotically active volume ( $52.8\% \pm 3.8\%$  vs.  $74.9\% \pm 5.0\%$ ,  $p = 0.008$ ).

## **DISCUSSION**

To address the need for long-term storage of a cord RBC product, an obvious first step would be to use the 40% glycerol freezing method that was developed for adult RBCs.

295 However, the 40% glycerol method yields lower cell recovery for cord RBCs than adult RBCs [47]. Therefore it is important to understand the biophysical differences between cord and adult RBCs in the context of the cryopreservation process. To this end, we investigated the osmotic tolerance limits of cord and adult RBCs, both in the presence of saline as is commonly done in the literature, as well as in the presence of 40% glycerol.

300 There have been many previous studies of the sensitivity of cord RBCs to swelling in hypotonic saline solutions [3; 25; 27]. Typically, the results of such osmotic fragility experiments are quantified in terms of the hypotonic saline concentration required to induce 50% hemolysis. Reported values are in the range 130-160 mOsm/kg for both cord and adult RBCs [3; 5; 25; 27; 35], which corresponds with a relative osmotically active volume  $V/V_{w0}$  between 1.9 and 2.3. We observed about 50% hemolysis for both cord and adult RBCs when the cells were  
305 induced to swell to  $V/V_{w0} = 2.0$  (see Figure 1), which is in agreement with the previous literature. Whereas cord and adult RBCs do not appear to differ significantly in terms of the hypotonic concentration necessary to cause 50% hemolysis, previous studies do indicate that cord RBCs are more heterogeneous than adult RBCs and undergo hemolysis over a wider range of hypotonic concentrations [3; 25; 27]. Our results are consistent with this trend. As shown in Figure 1C,  
310 cord RBCs exhibited higher hemolysis than adult RBCs when induced to swell to  $V_w/V_{w0} = 1.7$ , which suggests that a portion of the cord RBCs are more sensitive to swelling damage than the adult RBCs. On the other hand, lower hemolysis was observed for cord RBCs at  $V/V_{w0} = 2.5$ , which suggests that a subpopulation of cord RBCs is more resistant to swelling than adult RBCs. The osmotic tolerance limit for cord RBCs should be defined in order to ensure the all cells in  
315 the population are not damaged. Because cord RBCs exhibit about 10% hemolysis when induced to swell to 1.7 times the isotonic osmotically active volume, the upper osmotic tolerance limit in the presence of saline should be set somewhere below this value.

The upper osmotic tolerance limit is primarily useful for designing procedures that avoid excessive swelling during removal of cryoprotectant after freezing and thawing. Therefore, we  
320 also investigated the osmotic tolerance of cord and adult RBCs after freezing and thawing in the presence of 40% glycerol. There do not appear to be any previous reports of the effects of glycerol, or of freezing and thawing, on the osmotic tolerance limits of cord RBCs. However, Meryman and Douglas [29] investigated the osmotic fragility of adult RBCs in the presence of glycerol, and their data was later analyzed by Pegg [36] in terms of the RBC volume. These

325 studies indicate that adult RBCs exhibit similar tolerance to swelling over a wide range of glycerol concentrations, with 50% hemolysis corresponding with a relative osmotically active volume of about  $(V_w + V_s)/V_{w0} = 2.2$ . We observed slightly higher hemolysis for unfrozen adult RBCs that had been glycerolized to 40% w/v: cells induced to swell to twice the isotonic osmotically active volume underwent about 70% hemolysis (see Figure 2).

330 Importantly, our results show that freezing and thawing makes both cord and adult RBCs more sensitive to swelling damage, which has implications for the design of post-thaw cell washing methods. The amount of damage experienced by both cell types was comparable when induced to swell to 1.7 times the isotonic osmotically active volume: hemolysis values were  $16.3\% \pm 2.4\%$  and  $20.3\% \pm 2.8\%$  for frozen-thawed glycerolized adult and cord RBCs, 335 respectively. Because these hemolysis values are relatively high, the design of glycerol removal procedures for both cell types should ensure that cell volume excursions are at least maintained below 1.7 times the isotonic osmotically active volume to attain good post-wash cell recovery. Linear extrapolation of the data between relative volumes of 1.7 and 2.0 allows estimation of safe swelling limits for both cell types, yielding  $(V_w + V_s)/V_{w0} = 1.5$  for frozen-thawed cord 340 RBCs and  $(V_w + V_s)/V_{w0} = 1.6$  for frozen-thawed adult RBCs.

Although the susceptibility of cord RBCs to damage in hypotonic solution has been fairly well studied, we are not aware of any previous reports of the sensitivity of cord RBCs to damage in hypertonic saline solutions. For both cord and adult RBCs, damage from exposure to hypertonic saline was not immediately apparent, and a significant increase in hemolysis was 345 observed when the cells were returned to isotonic conditions (see Figures 1A and 1B). This trend is consistent with the observations of Lovelock, who postulated that the RBC membrane becomes permeable to ions at high saline concentrations, resulting in uptake of sodium ions and subsequent dilution stresses upon return to isotonic conditions [22]. The fact that cord and adult RBCs both exhibited this trend suggests that hypertonic exposure damages both cell types by a 350 similar mechanism. However, cord RBCs appear to be more susceptible to hypertonic damage than adult RBCs, as evidenced by the higher hemolysis value for cord RBCs at  $V_w/V_{w0} = 0.1$  (see Figure 1). The data in Figure 1 can be used identify the safe limits for shrinkage of cord and adult RBCs; because both cell types exhibited less than 3% hemolysis when induced to shrink to 20% of the isotonic osmotically active volume, a relative osmotic volume of 0.2 would be a 355 conservative choice for the lower osmotic tolerance limit.

Our isotonic volume controls in Figures 2 and 3 provide useful information about the potential for cryopreservation of cord RBCs in the presence of 40% glycerol. While the glycerolization process significantly increased hemolysis from 1.6% to about 6%, subsequent freezing and thawing did not cause a further increase in hemolysis. These results indicate that 40% glycerol is adequate for protection of cord RBCs during freezing and thawing, and that glycerolization using the standard method (which was developed for adult RBCs) only causes modest damage to cord RBCs. These results, together with the previous observation that recovery of cord RBCs after cryopreservation in 40% glycerol is significantly less than recovery of adult RBCs [47], suggest that future efforts should primarily focus on refinement on the post-thaw washing process to maximize recovery of cord RBCs.

In this study we used cell lysis (hemolysis) as an endpoint measure of the stress that the RBCs could tolerate when exposed to anisotonic conditions. It is recognized that sub-hemolytic osmotic injury could occur to the RBCs, which may not be immediately detected. For this reason, future studies should consider looking at other measures of RBC injury (membrane remodeling, biochemical depletion, microparticle generation) when determining tolerable osmotic limits.

## CONCLUSIONS

This study investigated the osmotic tolerance limits of cord and adult RBCs under conditions relevant to cryopreservation using high glycerol concentrations. We show that cord RBCs are more sensitive to shrinkage and swelling than adult RBCs requiring that upper and lower osmotic tolerance limits are adjusted when considering protocols for the addition and removal of cryoprotectants. In addition, freezing and thawing resulted in both cord and adult RBCs becoming more sensitive to swelling damage requiring that glycerol removal procedures for both cell types ensure that cell volume excursions are at least maintained below 1.7 times the isotonic osmotically active volume to attain good post-wash cell recovery. Our results will help inform the development of optimized cryopreservation protocol for cord RBCs.

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**Table 1.** Saline concentrations (in molal units) used for investigation of the osmotic tolerance limits of washed RBCs in isotonic saline.

| Saline concentration in solution added to induce shrinkage or swelling (mmol/kg) | Saline concentration in solution added to return sample to isotonic conditions (mmol/kg) | Expected change in osmotically active cell volume ( $V_w/V_{w0}$ ) |
|--|--|--|
| 0 <sup>a</sup>   | 139  | 13   |
| 34   | 135  | 2.5  |
| 51   | 135  | 2.0  |
| 67   | 134  | 1.7  |
| 131  | 131  | 1.0  |
| 777  | 99 <sup>b</sup>  | 0.20   |
| 1580   | 59 <sup>c</sup>  | 0.10   |
| 2390   | 19 <sup>d</sup>  | 0.067  |

<sup>a</sup> Whereas all other solutions were buffered with 12.5 mmolal disodium phosphate, this solution did not contain any phosphate buffer.

395 <sup>b,c,d</sup> In addition to the indicated saline concentration, these solutions also contained glucose at concentrations of 3 mmolal, 6 mmolal and 10 mmolal (b, c and d, respectively).

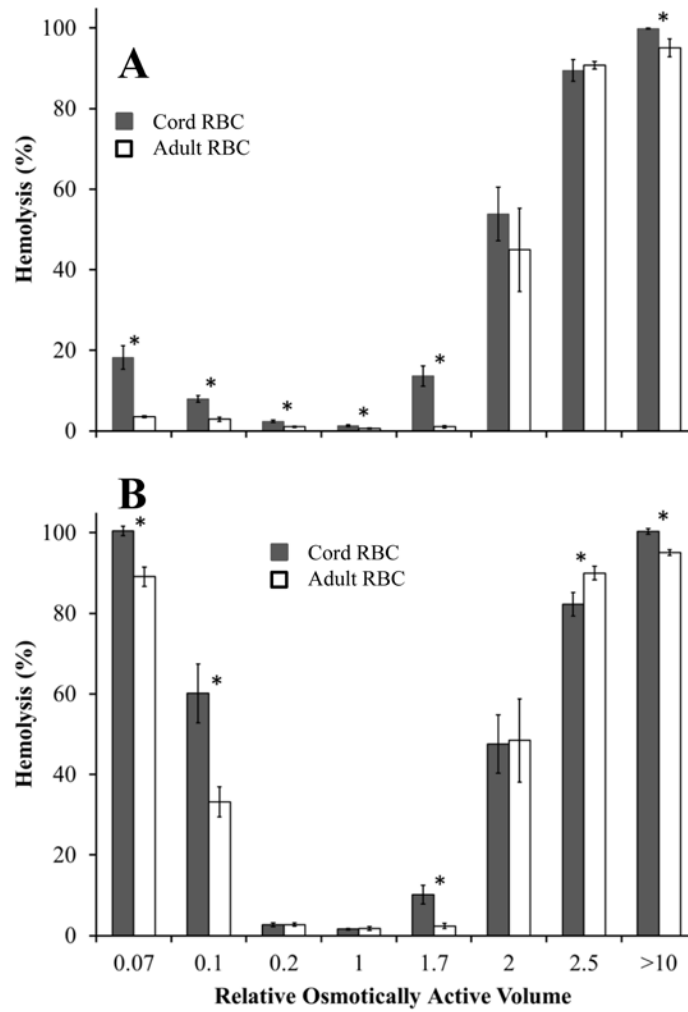


400 **Table 2.** Saline and glycerol concentrations (in molal units) used for investigation of the osmotic tolerance limits of glycerolized RBCs.

| Composition of solution added to induce swelling |                    | Composition of solution added to return sample to isotonic conditions |                    | Expected change in osmotically active cell volume $[(V_w+V_s)/V_{w0}]$ |
|--|--------------------|---|--------------------|--|
| Saline (mmol/kg)                                 | Glycerol (mmol/kg) | Saline (mmol/kg)  | Glycerol (mmol/kg) |  |
| 0*   | 0*                 | 216   | 7010               | 12   |
| 41   | 1670               | 213   | 6870               | 2.5  |
| 63   | 2290               | 212   | 6820               | 2.0  |
| 86   | 2900               | 211   | 6740               | 1.7  |
| 206  | 6480               | 206   | 6480               | 1.0  |

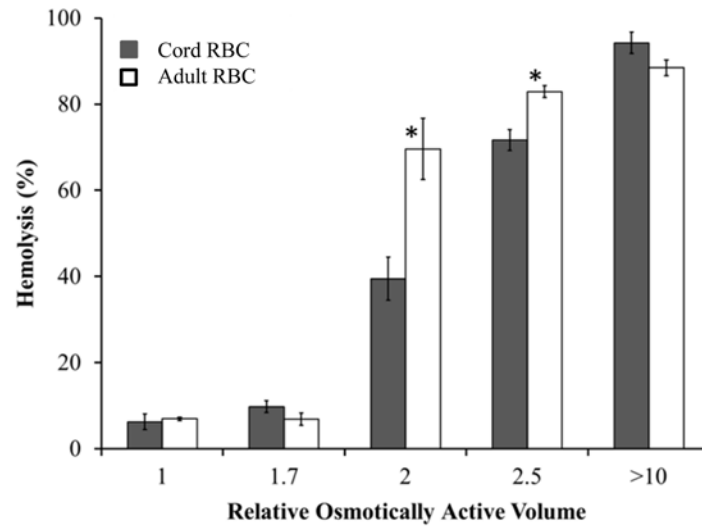
\* Whereas all other solutions were buffered with 12.5 mmolal disodium phosphate, this solution did not contain any phosphate buffer.

405 **FIGURES**

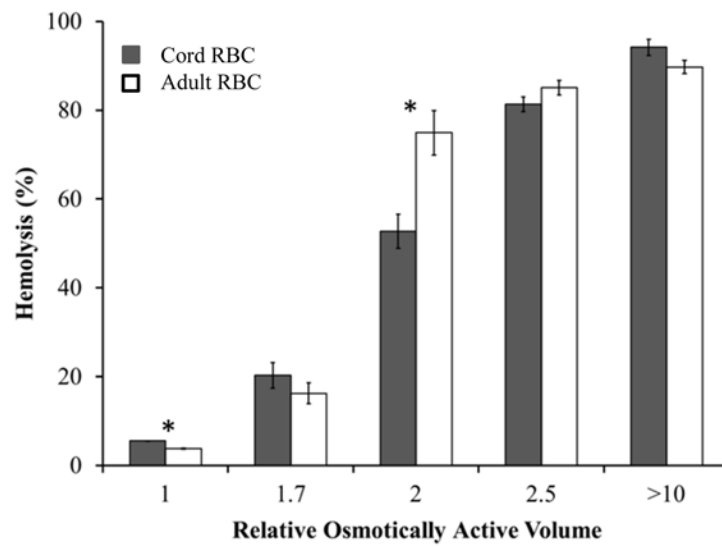


**Figure 1.** Osmotic sensitivity of untreated RBCs in the presence of saline. Hemolysis measured under anisotonic conditions (A) and after the cells had been returned to isotonic conditions (B). Asterisks denote significant differences ( $p < 0.05$ ).

410



**Figure 2.** Osmotic sensitivity of glycerolized RBCs. Asterisks denote significant differences ( $p < 0.05$ ).



415

**Figure 3.** Osmotic sensitivity of RBCs after glycerolization, freezing and thawing. Asterisks denote significant differences ( $p < 0.05$ ).

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