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Research Brief

Increased reticulocyte count from cord blood samples using hypotonic lysis

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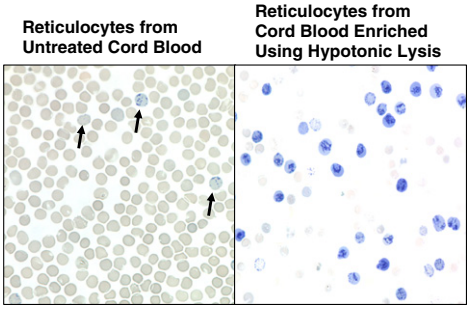
HIGHLIGHTS

- This is a simple and inexpensive method to increase the reticulocyte count of cord blood samples.
- By exposing cord blood to hypotonic saline for 5 min, normocytes are lysed.
- This method increases reticulocytemia by over 3-fold.
- The recovered blood has intact hemoglobin and is still able to support malaria parasites growth.

GRAPHICAL ABSTRACT



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ABSTRACT

Human reticulocytes are one of the fundamental components needed to study the *in vitro* invasion processes of the human malaria parasite *Plasmodium vivax*. Additionally examinations of reticulocytes and their binding proteins are difficult in areas of the world that do not have access to advanced equipment or stem cell lines. These issues are particularly relevant to malaria vaccine candidate studies that are directed against surface proteins that the parasites use to gain entry into erythrocytes. Described here is a simple and inexpensive method to increase the reticulocyte count of cord blood samples. Exposure of cord blood to hypotonic saline (0.2%) for 5 min selectively lyses the non-reticulocytes resulting in an average 3.6-fold increase in reticulocyte count. Our studies show that this enrichment process does not damage the hemoglobin of the remaining erythrocytes which are still capable of supporting *Plasmodium falciparum* invasion and growth. This economical and rapid method of enrichment could facilitate studies of *in vitro* laboratory culturing of other malaria parasite species which preferentially invade reticulocytes such as *P. vivax*.

1. Introduction

Most of the *Plasmodium* species of parasite causing malaria can use a number of pathways and receptors to bind to and invade erythrocytes. However, even though there is recent evidence that the malaria parasite species *Plasmodium vivax* (*P. vivax*) can use an alternative invasion pathway (Menard et al., 2010), this species is only known to use one receptor, referred to as Duffy, to facilitate host invasion. Duffy is a non-signaling chemokine receptor that functions to maintain chemokine homeostasis and is expressed on the surface of young reticulocytes (Woolley et al., 2000). However, the presence of reticulocytes dwindle when in *in vitro* cultures. Studies of *P. vivax* invasion of human erythrocytes are difficult because of the lower levels of reticulocytemia generally observed in adults (0.5–1.5%) (Bunn, 2008; Ferri, 2011). Additionally, the

Abbreviations: P. vivax, *Plasmodium vivax*; PBMC, peripheral blood mononuclear cells.

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expense and difficulty of obtaining reticulocytes for study through ultracentrifugation (Golenda et al., 1997) of blood samples or stimulation of hematopoietic stem cells (Giarratana et al., 2005; Panichakul et al., 2007), are barriers to research requiring reticulocytes from taking place in many malaria endemic countries. The goal of this study was to develop a simple, rapid, method that could be utilized, in laboratories studying reticulocytes and facilitate *P. vivax* invasion (Udomsangpetch et al., 2007) and those in the developing world, to further increase reticulocyte count of blood samples.

To overcome these hurdles and increase reticulocyte count, alternative methods for reticulocyte enrichment have been explored. Cord blood has a naturally higher reticulocytemia because of rapidly expanding blood volume up to 8% (Udomsangpetch et al., 2007) and is a readily available and often disposed of resource in most areas where *P. vivax* is endemic (Udomsangpetch et al., 2007) as well as at hospitals in non-endemic countries including the University Hospitals of Cleveland Case Medical Center. Unlike adult peripheral blood mononuclear cells (PBMCs), flow cytometry of cord blood mononuclear cells has been difficult because of the difficultly in removing contaminating reticulocytes. Even purpose made RBC lysis solutions from Becton–Dickinson, do not lyse all cord blood reticulocytes. This suggested that the osmotic pressure of cord blood reticulocytes is different enough from normocytes to allow for their selective enrichment. Exposing cord blood to a hypotonic saline solution greatly enriches the reticulocyte count of samples by an average of more than 3-fold. After steps to remove PMBCs and partially lysed cells, the remaining erythrocytes could support *Plasmodium falciparum* growth *in vitro*.

2. Methods

2.1. Blood samples

All human cord blood samples used in this study were collected in NaCitrate after obtaining consent from study participants under protocols approved by the Institutional Review Board of the University Hospitals of Cleveland Case Medical Center. All cord blood samples were obtained from non-malarious Caucasian patients.

2.2. Enrichment of reticulocytes

Cord blood samples from 10 separate donors were individually washed 3× at 200*g* for 5 min with RPMI 1640. The resulting pellet from each donor was suspended in 10 volumes 0.2% NaCl and incubated for 5 min at room temperature (21–27 °C). Time and temperature were crucial for successful outcomes (see Supplementary Fig. 1). A time course of cord blood exposure to the hypotonic saline indicated that 5 min was the optimal amount of time of exposure to the hypotonic saline solution. After this time point the selectivity of the hypotonic saline for reticulocyte rapidly drops off. At a temprature of 37 °C the method proceeds too quickly and all red blood cells are lysed after 5 min of exposure, and at 4 °C the lysis does not preferentially enrich for reticulocytes but lyses all cell types equally. Remaining reticulocytes were rescued with 10 volumes of 1.6% NaCl and spun as previously stated. The supernatant was removed and the pellet was washed 1× in RPMI 1640. To remove PBMCs and fractured/damaged cells the pellet was brought to 50% hematocrit with RPMI 1640 and gently layered over warm 37 °C Histopaque1077 (Sigma, St. Louis, MO. Layered cells were spun at 200*g* for 10 min. The resulting pellet was washed 2× with RPMI 1640. The final enriched pellet could be stored at 4 °C at 50% hematocrit. Reticulocyte count was determined using standard methods by staining blood samples with equal volumes of New Methylene Blue (Vajpayee et al., 2007) (Retic-Chex Stain, Streck, Omaha, NE, Fig. 1). It is important to note that this enrichment method does not work on normal adult blood and that the treated cells do not tolerate standard freezing protocols such as suspension in Glycerolyte 57 for later use.

2.3. Hemoglobin instability testing

To test for the presence of unstable hemoglobin as a result of the reticulocyte enrichment process, two common assays were performed (Old et al., 2004). In the first assay, the Isopropanol Test was used to weaken the hemoglobin hydrophobic bindings, which if already unstable, will precipitate out of solution. In brief, a sample of cord blood and enriched cord blood was completely lysed in pure distilled water. Resulting lysates were suspended in 10 volumes of tris-isopropanol buffer (Tris–HCl 0.1 M, pH 7.4 in 17% isopropanol). Solutions were heated at 37 °C for 30 min and were observed every 5 min for the presence of white flocculent, indicating the presence of unstable hemoglobin. Completely lysed samples from above were also exposed to a Heat Test for instability. Equal volumes of the water-treated lysates were suspended in 5 mL of Phosphate Buffered Saline pH 7.4 (1XPBS). Samples were mixed and centrifuge at 300*g* for 10 min. The supernatant was

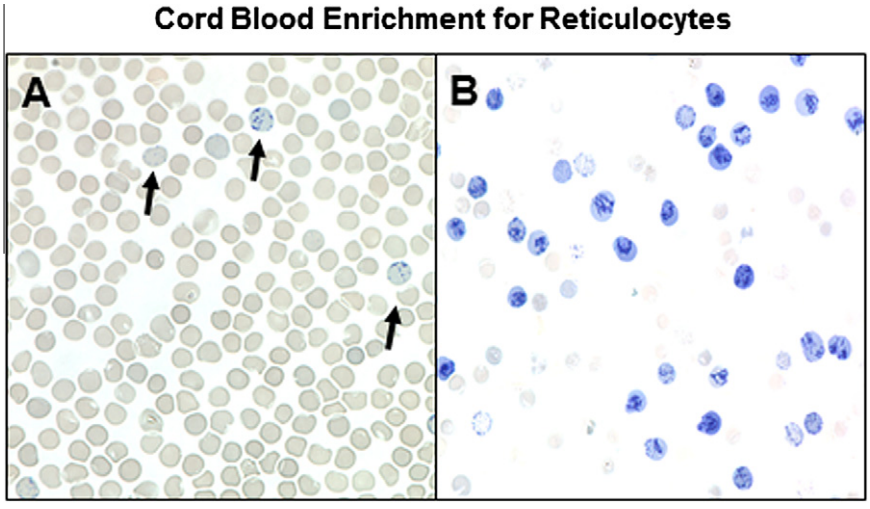

Fig. 1. Effect of hypotonic treatment of cord blood on reticulocyte count. The left panel (A) shows brightfield micrograph of whole cord blood with 6.6% reticulocyte count after staining with New Methylene Blue. Reticulocytes are indicated with black arrows. In the right panel (B), shows the sample after hypotonic treatment and removal of partially lysed cells and an increased reticulocyte count of 28%. All cells stained blue are reticulocytes.