Effects of repleting organic phosphates in banked erythrocytes on plasma metabolites and vasoactive mediators after red cell exchange transfusion in sickle cell disease

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Background - Red blood cell (RBC) exchange (RCE) transfusion therapy is indicated for certain patients with sickle cell disease (SCD). Although beneficial, this therapy is costly and inconvenient to patients, who may require it monthly or more often. Identification of blood and plasma biomarkers that could improve or help individualise RCE therapy is of interest. Here we examined relevant blood and plasma metabolites and biomarkers of vasoactivity and RBC fragility in a pilot study of SCD patients undergoing RCE using either standard RBC units or RBC units treated with a US Food and Drug Administration (FDA)-approved additive solution containing phosphate, inosine, pyruvate, and adenine ("PIPA").

Materials and methods - In this prospective, single-blind, cross-over pilot clinical trial, patients were randomised to receive either standard RBC exchange or PIPA-treated RBC exchange transfusion with each RCE session over a 6-month treatment period. Pre- and post-transfusion blood samples were obtained and analysed for RBC O_2 affinity, ATP, purine metabolites, RBC microparticles, and cell free haemoglobin.

Results - Red blood cell O_2 affinity was maintained after PIPA-RCE in contrast to standard RCE, after which P_{50} fell (net O_2 affinity rose). Plasma ATP did not change significantly after RCE using either of the RBC unit types. Exchange transfusion with PIPA-treated RBC units led to modest increases in plasma inosine and hypoxanthine. Plasma cell free haemoglobin fell after either standard or PIPA-treated RBC exchange transfusion (novel findings), and to a similar extent. RBC-derived microparticles in the plasma fell significantly and similarly after both standard and PIPA-treated RCE transfusion.

Discussion - In summary, treatment of RBCs with PIPA prior to RCE elicited favourable or neutral changes in key metabolic and vascular biomarkers. Further study of its efficacy and safety is recommended and could ultimately serve to improve outcomes in chronically transfused SCD patients.

Keywords: *sickle cell disease, exchange transfusion.*

INTRODUCTION

Sickle cell disease (SCD), the most common inherited blood disorder in the United States, is characterised by abnormal sickle haemoglobin (HbS) production. As a result, the capacity of sickle red blood cells (RBCs) to transport and deliver oxygen is impaired. In low-oxygen conditions, HbS polymerises, altering RBC morphology and susceptibility to lysis and triggering recurrent vaso-occlusive episodes and pain crises[1]. Chronic periodic RBC exchange transfusion is indicated for patients with SCD for primary and secondary stroke prophylaxis and for patients with recurrent vaso-occlusive crises[2,3].

Red blood cell exchange (RCE) transfusion is a therapeutic intervention in SCD involving the removal of native sickle (SS) RBCs in exchange for the administration of multiple units of packed healthy RBCs. In RCE transfusion, RBCs containing normal adult haemoglobin (HbA) are given to largely replace the recipient's diseased HbS-containing RBCs and to reach a target clinically determined % HbA (e.g. >50%) level. The interval between RBC exchanges (typically every 3-6 weeks) is determined based in part on the projected rate of HbA clearance. This partial exchange of HbS for HbA reduces the number of SS RBCs present, lowering viscosity, and protecting against sickling and vaso-occlusion[2]. This can in turn reduce the rate of serious complications, including recurrent pain crisis, acute chest syndrome, stroke, and renal and other end organ damage. This intervention is critical in patients with SCD who require chronic transfusion for a number of indications, including secondary stroke prevention. However, the duration of benefit from transfusion is limited due, in part, to the clearance over time of transfused RBCs, which have already aged during storage[4]. Additionally, large-volume RBC transfusion is associated with other complications including iron overload, iron-mediated hypercoagulability, and progressive development of alloimmunisation, motivating the study of determinants of optimal RBC unit processing[5].

Transfused RBCs are also substantially altered by storage. Refrigerated storage is known to make RBCs more rigid, less tolerant of stress, and more likely to adhere to the vascular endothelium[4,6]. Additionally, they are depleted of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP), and have lower P_{50} (ie, higher O_2 affinity)[7]. As RBCs are cleared from circulation, they produce additional burdens of RBC breakdown byproducts, including cell free haemoglobin (CFH) and RBC microparticles (RMPs)[8-10]. RMPs are already elevated in SCD due to sickling and haemolysis and can impair vasodilation, decrease microcirculatory oxygenation, and damage vascular endothelium[11-13]. Thus, these burdens of cleared RBCs exacerbate problems already present in this high-risk patient population. Additionally, SCD patients who routinely undergo RCE face major health care burdens arising from the need for central vascular access, transfusion complications, work and school absences, and high costs[14].

RBC "rejuvenation" (incubation with "PIPA" additive solution, containing phosphate, inosine, pyruvate, and adenine) is a US Food and Drug Administration (FDA)-approved, standardised blood bank process that restores the organic phosphates 2,3-DPG and ATP, and raises the P_{50}, all of which are reduced during storage[15,16]. RBC transfusate incubation with PIPA increases RBC membrane stress tolerance, and microcirculatory oxygenation, while decreasing RMP generation and vascular endothelial adhesion[17-19].

In this pilot study, we studied a cohort of clinically stable, crisis-free, chronically RCE-transfused SCD patients and compared the effects of standard RBC exchange transfusion (STD-RCE) *vs* PIPA-treated RBC exchange transfusion (PIPA-RCE) on plasma vasoactive biomarkers/mediators. Specifically, we examined purine metabolites including and related to the vasoactive mediator ATP (which is augmented in PIPA-treated RBCs), and the RBC haemolysis biomarkers and vasoactive mediators plasma CFH and red cell-derived RMPs. Plasma ATP originating in RBCs or other cells may act as a vasodilator and antiadhesive agent[20,21]. ATP can also elicit pro-inflammatory effects[22]. CFH and RMPs reflect the balance between RBC fragility and related clearance mechanisms, and may also contribute to vascular pathophysiology in SCD or after RBC transfusion[10]. We hypothesise that changes in these

biomarkers might therefore provide information on the risk-benefit balance of metabolic modification of RBCs and ultimately be of benefit in frequently transfused SCD patients.

MATERIAL AND METHODS

Selection and description of research participants

This prospective, single-blind, cross-over pilot clinical trial allowed for randomisation of patients to receive either PIPA-treated or standard RBC exchange transfusion with each treatment session over a 6-month treatment period. Other results from this study have been published previously[23]. Eligible patients were ≥18 years of age, in stable clinical condition, therapy-compliant, chronically transfused SCD patients (HbSS) who were maintained with repeated RCE therapy and who had been crisis-free for a period covering at least the previous three consecutive sessions. No patient had tolerated chronic hydroxyurea therapy and none received it during the study period. Exclusion criteria included a baseline need for washed RBCs and pre-treatment SaO_2 <92%. After written, informed consent under a protocol approved by the Duke University Medical Center Institutional Review Board, blood samples were collected from these established SCD patients. Demographic data including age, sex, race, height and weight were obtained for each patient. Pre- and post-transfusion session vital signs were recorded and blood was collected for analysis just before and within 60 minutes after RCE.

Red blood cell units

Per institutional practice, only RBC units stored less than 21 days were used for RCE transfusions, and all RBC units selected for PIPA exposure were stored at least 7 days (after which decreases in DPG are substantial). Using standard erythocytapheresis, patients received crossmatch-compatible leukoreduced RBCs that were phenotypically matched for at least ABO, D(Rh), C, E, and Kell antigens. One patient had additional, low-frequency antigens (Lua and Wra) that were easily avoided. For standard RCE transfusion, no additional manipulations were performed. For sessions in which transfusate PIPA treatment ("rejuvenation") was performed, the final 4 units of blood were exposed to Rejuvesol® RBC Processing Solution (Zimmer Biomet Labs, LLC, Braintree, MA, USA), per standard protocol[24]. Briefly, RBC units are infused with PIPA solution for 1 hour at 37 °C, washed with saline in a Cobe 2991 Cell Washer (TerumoBCT, Lakewood, CO, USA), and suspended in 0.9% saline/0.2% dextrose for transfusion. The 4-unit treatment was based on practical considerations while projecting that this would equate to PIPA treatment of a high fraction of the patient's final circulating RBC volume.

P_{50} analysis

Whole-blood P_{50} was analysed before and after either standard or PIPA-treated RCE using a TCS Hemox analyser (TCS Scientific Corporation, New Hope, PA, USA), according to the manufacturer's instructions.

ATP, purine metabolites, and cell-free haemoglobin assays

Plasma from patient samples was analysed for ATP concentration, purine metabolites, and CFH. ATP was measured using a commercially available luciferase ATP assay (Sigma). Purine metabolites were measured using a previously described mass spectrometric method[25]. CFH was measured using multi-wavelength absorbance spectroscopy for haemoglobin, as we described previously[26].

Red blood cell microparticle analysis

Plasma from patient samples was incubated at room temperature with RBC-specific CD235a antibody (Invitrogen; 1:30 concentration, 50 µL samples) for 30 min as previously described to distinguish MPs derived from RBCs (RMPs) from other microparticles[27]. The number of RMPs in the plasma samples was determined by volumetric flow cytometry (Cytek, Aurora, CO, USA). A SCD blood sample was used as a positive control to set appropriate gates to identify RMPs based on size and CD235a staining; these gates were applied to all study plasma samples.

Statistical analysis

All values are reported as means±Standard Error of Mean (SE) unless otherwise noted. Outliers were defined as ±3 Standard Deviation (SD) from the mean in any sample set. No data points fit that criterion, and therefore no outliers were excluded.

A mixed error-component statistical model would be the ideal approach for the analysis of such data, but is not recommended when N <5[28]. Thus, ANOVA and t-test analyses were performed using GraphPad software version 8.2 (San Diego, CA, USA). p<0.05 was considered statistically significant. For each biomarker, at least one pair of values (pre and post) was available for each patient from one STD-RCE session and one PIPA-RCE session (n=4 throughout). In some cases, data from multiple STD-RCE or PIPA-RCE sessions was used for a given patient in the principal statistical analysis (n >4).

RESULTS

Demographics

The 4 patients were clinically stable, African-American males with HbSS disease, aged 19-32 years; none of the patients was currently receiving hydroxyurea. They were maintained free from vaso-occlusive crisis on a monthly RCE transfusion programme. Based on established %HbS targets and patient size, the RBC requirements at each RCE session were 7 units for 3 patients (post-RCE HbA target 75-80%) and 10 units for the remaining patient (post-RCE HbA target 80-85%). Patients underwent up to 3 standard and 3 PIPA-treated RCE sessions in varying sequence over the 6-month study period. The average storage age for blood units was 16±5 days for standard units and 22±13.5 days for the PIPA-treated units (p=0.002 vs STD units). Vital signs were stable throughout all RCEs, and no transfusion reactions were reported. All participants remained crisis-free with stable renal function during the study period (*data not shown*). No adverse effects (AEs) of any kind (including serious AEs and treatment-emergent AEs) occurred during the 4 weeks following the RCE transfusions of either standard or PIPA-exposed (*rejuvenated*) type.

Whole-blood P_{50}

We compared patients' whole-blood P_{50} before and after either a standard RCE transfusion or RCE transfusion using PIPA-treated RBCs (**Figure 1**). The P_{50} decreased after STD transfusion (pre, 28.31±1.6 vs post, 23.33±1.4) in contrast to no change after PIPA-treated RCE transfusion (pre, 28.57±0.4 vs post, 27.17±1.3). There was improved maintenance of the

P_{50} after PIPA-treated RCE transfusion (p=0.0024). The interaction between pre vs post P_{50} effects and STD vs PIPA effects was significant by two-way ANOVA (p=0.0064).
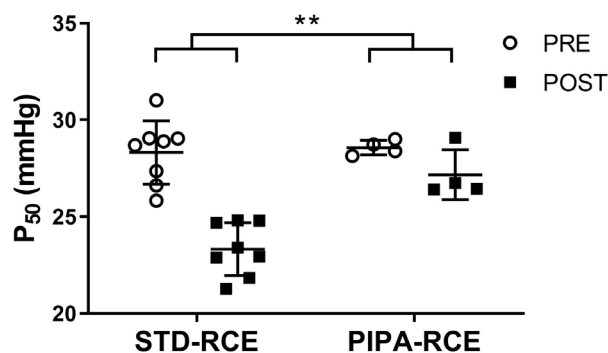

Figure 1 - P_{50} before and after standard or phosphate, inosine, pyruvate, and adenine (PIPA)-treated red blood cell exchange (RCE) transfusion showing improved maintenance of P_{50} with PIPA-treated RCE transfusion p=0.0024 for standard (STD)-RCE vs PIPA-RCE. p<0.0001 for Pre vs Post RCE. p=0.0064 for interaction of STD-RCE vs PIPA-RCE x Pre vs Post.

Plasma ATP

Plasma ATP levels were measured before and after either standard or PIPA-treated RCE transfusion. There was no greater preservation of ATP in the PIPA-RCE group post transfusion (**Figure 2**). There was also no overall difference in plasma ATP in pre- vs post-RCE transfusion when analysing both transfusion conditions (STD-RCE vs PIPA-RCE; p=0.23). There was no significant interaction between the pre vs post and the STD vs PIPA effects (two-way ANOVA).

Plasma purine metabolites

To further evaluate the impact of rejuvenation on RBC metabolism, we used a mass spectrometric method to measure ATP pathway metabolites in plasma from a subset of samples pre- and post-RCE with PIPA-treated RBCs. The primary ATP metabolic products, AMP and adenosine, were unchanged post transfusion (**Figure 3**). We observed significant increases in plasma inosine (p=0.003), a component of Rejuvesol, and in the inosine metabolite hypoxanthine (p=0.007) post-PIPA-RCE. However, further downstream metabolites including adenosine, xanthine and uric acid, were unchanged post-PIPA-RCE.
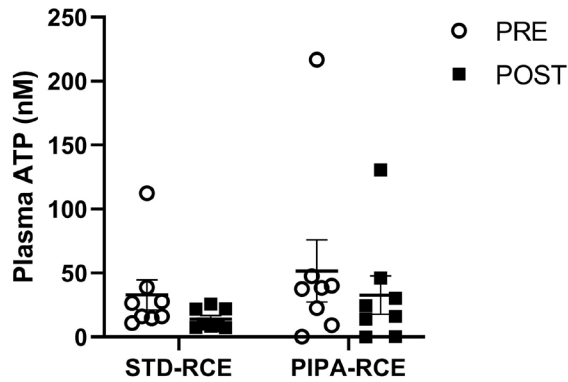
Figure 2 - Plasma ATP before and after standard (STD) or phosphate, inosine, pyruvate, and adenine (PIPA)-treated RCE

Two-way ANOVA was non-significant. In addition, there was no significant effect of PIPA and no overall post-red blood cell exchange (RCE) effect on plasma ATP.
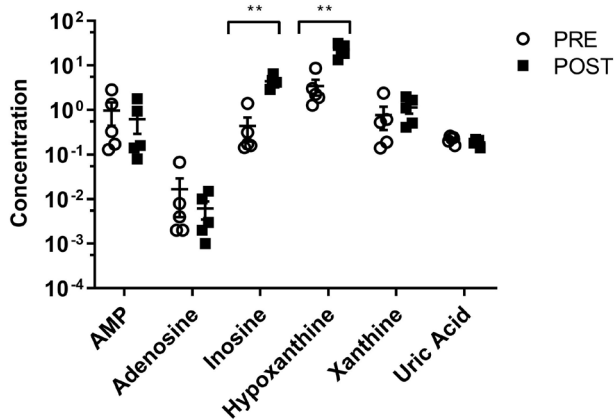

Figure 3 - Mass-spectrometric analysis of plasma obtained pre- and post-exchange transfusion of phosphate, inosine, pyruvate, and adenine (PIPA)-exposed red blood cells (RBCs)

Post-transfusion concentrations of inosine (p=0.003) and hypoxanthine (p=0.007) increased significantly without changes in other purine metabolites. Concentrations are in μM except for uric acid, which is in mM.

Plasma cell-free haemoglobin and red blood cell microparticles

Plasma haemoglobin levels were measured pre and post either standard or PIPA-treated RCE transfusion. **Figure 4** demonstrates the nanomolar CFH concentration. There is a statistically significant decrease in CFH after both STD or PIPA-treated RCE (p=0.047). There was no significant difference based on the RCE transfusion type (STD-RCE *vs* PIPA-RCE) in plasma RMPs quantified

pre and post either standard or PIPA-treated RCE transfusion (**Figure 5**).

There was a statistically significant decrease in RMP concentration after RCE transfusion of either type (pre *vs* post, two-way ANOVA with post-hoc Bonferroni, p=0.01), but no significant difference based on the RCE transfusion type (STD-RCE *vs* PIPA-RCE).
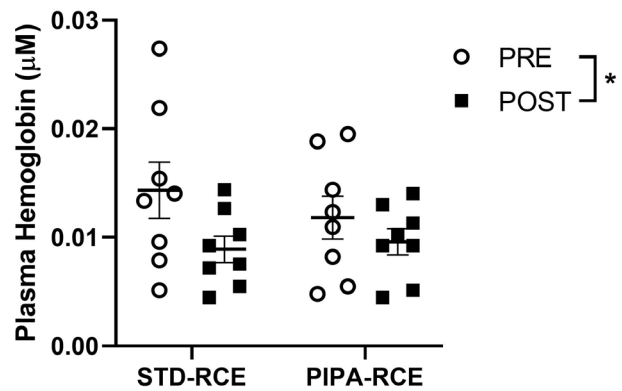

Figure 4 - Plasma cell-free haemoglobin (CFH) before and after standard (STD) or phosphate, inosine, pyruvate, and adenine (PIPA)-treated red blood cell exchange (RCE)

There is a significant decrease in CFH after both STD and PIPA-treated RCE (p=0.047).
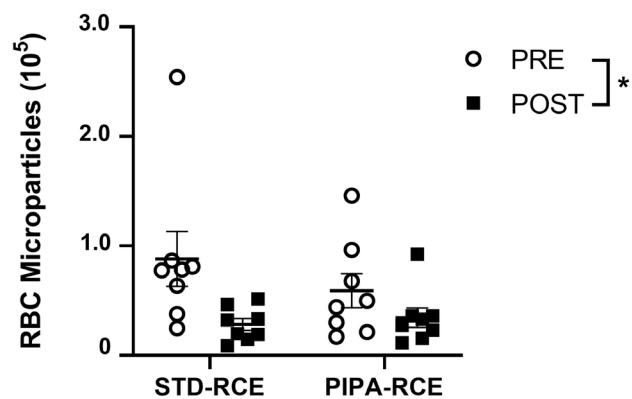

Figure 5 - Plasma red blood cell microparticles (RMPs) before and after standard (STD) or phosphate, inosine, pyruvate, and adenine (PIPA)-treated red blood cell exchange (RCE)

There is a significant decrease in RMPs after both STD and PIPA-treated RCE (p=0.01).

DISCUSSION

This pilot study examined the effects of PIPA treatment of RBC transfusates on changes in plasma metabolic and vasoactive biomarkers in patients with SCD requiring chronic RCE transfusion therapy. Previous studies have examined metabolic changes that develop secondary to RBC storage, but few have investigated the metabolic and functional changes in the plasma and RBCs of SCD patients transfused with metabolically modified RBC units. In theory, RBC transfusate modification such as PIPA treatment could extend the interval between RCE visits and thereby positively influence quality of life in SCD patients by reducing costs and the incidence of complications. We reasoned that a carefully controlled demonstration of favourable and safe changes in blood biomarkers of RBC metabolism and vasoactivity after PIPA-RCE in SCD was warranted before such an outcome study.

We found a statistically significant decrease in patient blood P_{50} (increase in RBC Hb O_2 affinity) after standard RCE transfusion. This finding was expected, given that RBCs transfused after storage have depressed 2,3-DPG, and thus elevated O_2 affinity (lower P_{50}) relative to fresh (or native HbA) RBCs. The P_{50}-lowering effect of RCE was essentially eliminated with PIPA-treated RCE transfusion. The relative preservation of patient blood P_{50} using stored-RBC exposed to PIPA is not novel, and is consistent with the well-established effect of PIPA in augmenting stored-RBC organic phosphates (ATP and 2,3-DPG)[29,30]. This corresponds to an increased O_2 off-loading capacity of the PIPA-treated (*vs* standard) transfused RBCs, which may be of clinical benefit in this patient population who have physiologically compensated for their anaemia by right-shifting their O_2 dissociation curve at baseline. Specifically, SCD patients compensate for their anaemia by increasing the production of 2,3-DPG in RBCs, in turn lowering RBC O_2 affinity (raising the P_{50}). Additionally, although RCE transfusion is generally beneficial, the overall decrease in P_{50} (leftward shift in the O_2-binding curve) resulting from transfusion of stored HbA RBCs could limit the potential O_2-delivery benefits of transfusion, especially in

SCD patients. In addition, it is possible that O_2 exchange may take place between the transfused HbA RBCs (with high O_2 affinity) and the native HbS RBCs (having low O_2 affinity). The net effect could be the movement of O_2 from low-affinity HbS RBCs to high affinity HbA RBCs and a net decrease in oxygen delivery from RBCs to the tissue. This could promote the sickling of SS RBCs and/or lead to local tissue hypoxia. Pre-transfusion PIPA exposure of HbA RBCs would tend to attenuate this exchange by restoring organic phosphates in the stored RBCs, thus moving P_{50} nearer to that of the native HbS RBCs.

Red blood cell exchange transfusion (both STD-RCE and PIPA-RCE) did not significantly alter plasma ATP concentration. The plasma ATP may reflect the net balance resulting from the steady-state rates of RBC export of ATP and intravascular haemolysis when SS RBCs are exchanged for AA RBCs, on one hand, and the hydrolysis of ATP on the other[20,21]. Thus, the RBC ATP-repleting effect of PIPA treatment of the transfused units may not result in increased ATP into the plasma[20,21]. PIPA treatment of stored RBCs does augment ATP export *in vitro*[29], but the net effect of transfusion of PIPA-treated RBCs on plasma ATP levels in SCD patients may also be driven by a slowing of intravascular haemolysis rates (tending to lead to lower plasma ATP). Notably, the effects of PIPA on post-RCE plasma ATP may have been blunted by the fact that the storage age of the PIPA-treated RBC units was (by chance) greater than that of the STD RBC units used in RCE.

In our purine analysis, we did not observe any changes in plasma adenosine monophosphate (AMP) or adenosine (ADO) post-RCE with PIPA-treated RBCs, suggesting that ATP metabolism was not altered by this process. Plasma ADO is of particular interest in terms of safety because it can promote pathophysiology in SCD when ADO promotes production of 2,3-DPG in erythrocytes, which shifts the O_2 affinity curve to the right (lower P_{50}). This lowered affinity for O_2 thus favours HbS deoxygenation, RBC sickling, and organ dysfunction[31]. The increased plasma inosine post transfusion is consistent with the presence of inosine

in PIPA, as was the increase in the inosine metabolite hypoxanthine, consistent with our prior findings[32]. Increased plasma hypoxanthine is also a biomarker of disease and can contribute to oxidative stress through its metabolism by xanthine oxidase[32]. The impact of RCE with PIPA-treated RBCs on oxidative stress will need to be determined in future studies.

In SCD, RMP generation is elevated at baseline as a result of accelerated RBC vulnerability and turnover[33]. We found that exchange transfusion (standard or PIPA-treated) decreased the overall steady-state RMP concentration in SCD patient plasma after transfusion, consistent with prior reports[34,35]. We report for the first time that RBC exchange transfusion also reduced plasma levels of CFH (resulting from intravascular haemolysis) regardless of RCE type. Because CFH and RMPs can promote intercellular adhesion, blood flow dysregulation, and coagulation, all contributing to vaso-occlusion and organ damage in SCD, their net reduction after RCE is expected to be favourable. However, transfused RBC units also contain (supernatant) CFH and RMPs, and the amount of clearance with RCE would depend in part on the RMP content of the transfused units, which may vary with factors including RBC storage time (which happened to be significantly longer in the PIPA group). Finally, the washing that necessarily follows PIPA treatment might, on the one hand, "clear" CFH and RMPs from the aging RBC unit, but can also contribute to generation of CFH and RMPs through the added mechanical shear stress of centrifugation and osmotic perturbations with washing. It is, therefore, reassuring that we observed a net decrease in plasma CFH and RMPs, and this favourable effect was quantitatively similar in both transfusion types (STD-RCE vs PIPA-RCE).

Collectively, these findings suggest some benefit of transfusing PIPA-treated RBCs with a smaller post transfusion drop in P_{50} after rejuvenated transfusion. Plasma ATP, exported from RBCs or other cells or sources, can act as a regional vasodilator and antiadhesive[20,21], and so the maintenance of ATP concentration after RCE (STD or PIPA) may also be reassuring in SCD. Additionally, PIPA-RCE demonstrates a similar favourable safety profile to STD-RCE with respect to decreased CFH and RMPs post transfusion. It also does not appear to contribute harmful purine metabolites in the plasma. This study is limited, however, in that it is a pilot study with a small patient sample. It would be logical in future studies to determine whether pre-transfusion rejuvenation of stored RBCs, or other strategies designed to preserve or replete organic phosphates, lead to longer post-transfusion circulating survival time after RCE, and to investigate patient-reported and clinical outcomes.

CONCLUSIONS

Chronic RBC transfusion including RCE is a required maintenance therapy for many patients with SCD and chronic complications. Metabolic optimisation of RBC units could extend the interval between RCE sessions, would reduce costs and risks, and could improve quality of life for SCD patients. As such, our findings support further investigation of the outcomes of measures to improve the metabolic and vasoactive sequelae of RBC transfusions in order to improve the risk-benefit balance in chronic transfusion therapy for SCD patients.

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DISCLOSURE OF CONFLICTS OF INTEREST

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