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Original Article

Effects of transfusion of stored blood in patients with transfusion-dependent thalassemia

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Abstract: Objectives: The aim of this study was to investigate the hematological and biochemical effects of stored blood transfusion on patients with transfusion-dependent thalassemia (TDT). Methods: In this quasi-experimental study, 20-patients with TDT were enrolled. Each participant received on first visit, freshly collected red cell concentrate (RCC) (<2-days storage) and 15-days later on second visit, 7-days stored blood. Blood samples were obtained immediately before and 24-hours after each transfusion. Differences in the Complete blood counts, bilirubin, LDH, C-Reactive protein, ferritin, and iron levels in the pre- and post-transfusion samples were compared between the first and second transfusion. Results: Fresh blood transfusion resulted in a higher (but non-significant) increase in hemoglobin and other red cell parameters. Notably, a significant increase in white cell counts (WCC) was seen in 7-days stored blood vs fresh blood (1.82×10^9/l vs 1.01×10^9/l, P=0.002). No statistically significant difference was found in LDH, direct and indirect bilirubin, creatinine, blood glucose, serum uric acid, serum ferritin, and serum Iron levels. There was a statistically significant rise in C-reactive protein levels in stored (6.43±7.46 mg/dl) versus fresh RCC (1.89±2.38 mg/dl), *p*-value =0.012. Conclusions: We show that in patients with chronic TDT, an increase in inflammation-associated markers (WCC and CRP) is observed. Further studies to assess the extent and duration of this increase are needed.

Keywords: Thalassemia, transfusion, erythrocytes, ferritin

Introduction

Red blood cell transfusion is one of the most common medical procedures performed in the world. Approximately 36,000 units of red blood cells are needed every day in the United States alone. The majority of blood transfusions are provided in hospital settings to patients undergoing surgery, or to those requiring multiple blood transfusions such as patients with thalassemia or aplastic anemia. To establish a continuous supply of safe blood, a comprehensive supply chain mechanism of blood collection, screening, storage and transfusion is required. Blood is collected from donors, screened for transfusion transmissible infections (TTIs), and separated into blood products such as red cell concentrate (RCC), platelets, and plasma. RCC units are stored in blood banks refrigerators for up to 42-days before transfusion. During storage, red cells undergo multiple physiologic changes. These changes, generally described as 'storage lesions', include hemoglobin oxidation and release of free hemoglobin, RBC membrane structural degradation, and increase cell fragility. In addition, metabolic abnormalities such as accumulation of lactate and 2-3DPG as well as depletion of ATP takes place over time. These cells when transfused, are at a risk of hemolysis, resulting in a release of free iron causing oxidative damage to the organs [1].

Recent evidence on morbidity and mortality associated with storage duration of transfused blood is inconclusive. A number of retrospective studies have investigated complications such as fever, hemolysis, hospital stay and death. However, these markers are relatively insensi-

tive and do not record short-term changes in blood parameters [2]. A meta-analysis comparing transfusion of fresh blood (1-10 days old) with older stored blood (2-3 weeks) in clinical trials, reported no significant differences in survival. However, in another meta-analysis based on 31 observational studies, increased risk of death was found to be associated with increasing duration of storage [3]. In a clinical trial, Hod *et al*. showed that autologous transfusion of stored blood after 1-7 weeks showed marked extravascular hemolysis, saturated serum transferrin, and circulating free transferrin after 6 weeks of storage [4]. Post-42 days of storage, transfusion of red cells produced extravascular hemolysis and circulating non-transferrin bound iron and was associated with proliferation of Escherichia coli [5]. Based on these findings, it has been suggested that the blood stored for up to 35 days appears to be safe for transfusion without significant morbidity and mortality. In another study, up to 21 days stored blood transfusion was not associated with multi-organ dysfunction in patients undergoing cardiac surgery [6]. However, the majority of these studies are either performed on healthy volunteers, or measured clinically insensitive outcomes such as morbidity or mortality. Based on this lack of evidence, the United States Food and Drugs Administration (US FDA) allows transfusion of up to 42 days old stored CPDA-1 containing blood [7]. Biochemical evidence stored blood transfusion shows that the transfusion of older red cells results in lysis and causes an inflammatory state [8]. This, in an otherwise healthy individual, might be of no major clinical consequence, however it may be harmful when transfused to patients with compromised hemodynamics or any other blood associated pathological conditions such as Thalassemia.

Thalassemia major is characterized by severe transfusion-dependent anemia, ineffective erythropoiesis, and extramedullary hematopoiesis. In addition, there is a splenomegaly with hypersplenism and increased red cell clearance within the sinusoids. It is plausible that storage of older red cells in patients with splenomegaly may result in lysis, release of hemoglobin and free iron, and causing a subclinical inflammatory state. Transfusion-dependent thalassemia patients receive multiple blood transfusions throughout their life. In developing countries blood donations are frequently through family/replacement donors rather than from voluntary donors. Therefore, there is scarce availability of blood products for the repeatedly transfused. Consequently, these patients rely on blood products left unused in blood banks. Therefore, there is higher likelihood of thalassemia patients receiving long-term preserved blood. There is a need to assess the effects of stored blood transfusion in such patients. Our study aimed at determining the biochemical changes in patients with transfusion-dependent thalassemia (TDT) following transfusion of 7-days stored RCC. Safety was determined clinically as well as with hematological and biochemical profiles by evaluating the short-term (24-hours) impact of transfusion of 7-days RCC on these profiles.

Materials and methods

Participant selection

This quasi-experimental study was conducted at Fatimid Foundation Thalassemia Care Centre, Peshawar, and the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar. Ethics approval was obtained from Khyber Medical university ethical committee (Ethical approval letter no. DIR/KMU-EB/CB/000472). Due to the potential risk of transfusion of old RCCs in sick children, ethical approval of transfusion of blood with storage duration longer than 7-days was not granted. Parents of children with TDT were approached. The aims and objectives of the study, procedure of testing, benefits, and risks were explained to patients and informed consent was taken by parents. Questionnaire data containing their age, sex, address, phone number, and transfusion demand per month were filled. A personal number was allocated to each patient. A total of 20 transfusion-dependent thalassemia patients with ages more than 5 years and less than 20 years were included in the study. Complete clinical history was obtained and physical examination was performed. Patients with end-organ failure or with acute infection or Transfusion Transmitted Infection (TTI) were excluded from the study.

Blood collection and storage

Blood donations were collected as per standard practice. Briefly, donor screening inter-

views were conducted and blood collection was performed in blood bags containing CPDA (BD 53877-001). Blood bags were screened for Hepatitis B and C, HIV, and Syphilis. Blood bags were then centrifuged in a high-speed blood bank centrifuge (PRP-benchtop) for plasma separation. Plasma was transferred in another blood bag and RCC was stored in a standard blood bank refrigerator. Leukoreduction was not performed due to a lack of a leukoreduction facility. RCC units were separated into 'fresh' and were transfused within 2-days of collection, or were labeled as 'stored' and kept in the refrigerator for 7-days before transfusion.

Transfusion of fresh and stored RCC

In the first visit, each patient received a transfusion of fresh RCC (2-days of storage). Before transfusion, a complete general physical examination was performed. Subsequently, blood samples were collected in EDTA-containing tubes (BD, cat no. 3K2356478) and z-serum clot activator containing serum separation tubes (BD, cat no. G00578897). Samples were transferred to the laboratory for analysis. After that, RCC was transfused to the patient as standard practice in the center. Twenty-four hours after the transfusion, patients were examined again and blood samples were collected in EDTA and gel tubes. Patients were then informed of their next transfusion schedule and asked to come on the scheduled date. Since the objective of the study was to investigate short-term changes in hematological and biochemical parameters. Current evidence suggests that these short-term changes normalize by 24-hours post-transfusion. Therefore, a wash-out period of 15-days was considered adequate.

Upon the next visit, the same examination, blood sampling, and transfusion procedure was adopted, except this time, 7-days old stored RCC was transfused. A 24-hour post-transfusion sample were obtained as before.

Laboratory analysis

In Laboratory, blood smear slides were prepared and stained with Giemsa stain (CAS number 51811) and analyzed. Complete blood count (CBC) of blood samples were obtained on Abbott's Cell-dyn 3200 hematology analyzer (Abbot Laboratories, Chicago, Il, USA). The machine contained pre-installed reagents for blood count which were supplied by the company. Serum ferritin levels of the samples were measured on Roche's Cobas e411 (Roche, Basel, Switzerland) analyzer. The blood sample was centrifuged at 4000 rpm for 3-5 minutes to obtain blood serum. A 10 µl serum was then aspirated in Roche's Cobas e411 analyzer to measure the serum ferritin level using a pre-installed ferritin kit. The machine was pre-calibrated using the company's supplied reagent before the test.

Alanine aminotransferase (ALT), bilirubin, uric acid, C-reactive protein (CRP), Lactate dehydrogenase, uric acid, and glucose levels of blood samples were measured on Roche's Cobas C111 analyzer.

Statistical analysis

Participant demographic and laboratory parameters were entered in Microsoft Excel 2016 (Microsoft Corporation, Redmond, Wa, USA) and exported to statistical software SPSS version 21 (IBM Corporation, Armonk, NY, USA) for analysis. Differences in each laboratory parameter were calculated by the following formula:

Difference = [post-transfusion (fresh RCC) - pre-transfusion (fresh RCC)] - [post-transfusion (old RCC) - pre-transfusion (fresh RCC)].

Mean difference and standard deviation were calculated. Paired sample t-test was performed to compare the differences in parameters between fresh RCC transfusion and stored RCC transfusion. *p*-value of less than 0.05 was considered significant.

Results

The study was conducted on 20 patients with TDT including 13 female (65%) and 7 male (35%) patients. The mean age of participants was 17.75±5.03 years. The demographic and clinical data collected from the patients i.e. age, sex, and other clinical and laboratory parameters are presented in **Table 1**. Transfusion of both 'fresh' and 'stored' RCC raised recipient blood Hb levels in 24-hour post-transfusion samples. This rise was higher with fresh RCC transfusion (1.55±0.64 mg/dl) compared to stored RCC (1.35±0.46 mg/dl). However, this difference was not statistically significant

Table 1. Demographics and baselines hematological and biochemical parameters of participants (n=20)

Variables	Observed Values	
Age (years)	Mean	Standard Deviation
	17.55	5.031
Gender	Ratio (Male:Female)	
	1:1.7	
Splenomegaly	Yes n (%)	No n (%)
	2 (10%)	18 (90%)
Hepatomegaly	4 (20%)	16 (80%)
Jaundice	9 (45%)	11 (55%)
	Mean ± SD	
Hemoglobin (g/dl)	7.58±1.59	
Red Blood Cells (10^{12}/L)	2.86±0.64	
White Blood Cells (10^9/L)	6.25±3.34	
Platelets (10^9/L)	199.9±147.2	
Packed Cell Volume (%)	22.6±5.06	
Mean Corpuscular Volume (fl)	79.5±4.85	
Mean Corpuscular Hemoglobin (pg)	26.81±2.36	
Mean Corpuscular Hemoglobin Concentration (g/dl)	33.70±1.75	
Bilirubin Direct (mg/dl)	0.53±0.30	
Bilirubin Indirect (mg/dl)	1.11±0.99	
Glucose (mg/dl)	125.3±76.77	
Uric Acid (mg/dl)	4.35±1.10	
Lactate Dehydrogenase (U/L)	345.15±173.36	
Creatinine (mg/dl)	0.31±0.10	
C-reactive protein (mg/dl)	17.30±39.57	
Ferritin (ng/ML)	2901.35±1855.63	
Iron (ug/dL)	547.19±235.86	

(paired-samples t-test, *P*=0.543) Similarly, transfusion of stored RCC resulted in smaller increases in other red cell parameters (RBC counts, MCV, MCH, MCHC) as compared to fresh blood but these results were also not statistically significant. No difference was seen in platelet counts between the two groups. Notably, an increase in white cell counts was seen in 24-hour post-transfusion samples in both the groups. The rise in white cells was significantly higher after transfusion of 7-days stored RCC (1.82×10^9/L±2.03) as compared to fresh RCC (1.01×10^9/L±1.1) (paired-samples t-test, *P*=0.002) (**Table 2**).

Following transfusion, red cells experience sheer pressure within the blood vessels and mechanical stress inside splenic sinusoids. The older transfused red cells may hemolyze resulting in increased LDH and bilirubin levels. To assess this, differences in LDH values, pre-and post-transfusion in both the groups were calculated and compared using paired samples *t*-test. Interestingly, an increase in LDH levels was seen in both fresh- and stored RCC groups. Although, this difference was significantly larger in the stored RCC group compared to the fresh RCC group (67.02±101.02 units/L vs 107.30±121.69 units/L), it was not statistically significant (*p*-value =0.13). No significant difference was observed in direct bilirubin (0.13± 0.14 mg/dL vs 0.12±0.15 mg/dL, *p*-value =0.76) and indirect bilirubin (0.42±0.55 mg/dL vs 0.32 mg/dL±0.32, *p*-value =0.45) levels. Similarly, no difference in Creatinine, glucose, and uric acid variations were noted (**Table 2**). Interestingly, levels of C-reaction protein (CRP) which is a marker of inflammation, showed a significantly higher rise when 7-days stored RCC was transfused in comparison with the fresh RCC (1.89±2.38 mg/L vs 6.43±7.46 mg/L, *p*-value =0.012).

Table 2. Difference in hematological parameters fresh blood vs 7-days stored RCC

Variables	Difference after fresh RCC transfusion (Mean ± SD) (Diff)	Difference after stored RCC transfusion (Mean ± SD) (Diff)	*p*-value (paired samples t-test)
Hb (g/dL)	1.55±0.64	1.35±0.46	0.543
RBC (10^{12}/L)	0.52±0.25	0.57±0.24	0.89
WBC (10^9/L)	1.01±1.1	1.82±2.03	0.002
Platelet (10^9/L)	19.9±18.17	22.85±26.55	0.65
PCV diff. (%)	4.67±2.51	3.57±1.91	0.91
MCV diff. (fl)	1.67±1.87	1.25±0.91	0.17
MCH diff. (pg)	0.93±0.64	0.83±0.77	0.46
MCHC diff. (g/dl)	1.33±1.06	0.87±0.84	0.85
LDH (U/L)	67.02±101.02	107.30±121.69	0.13
Bilirubin (Direct) (mg/dl)	0.13±0.14	0.12±0.15	0.76
Bilirubin (Indirect) (mg/dl)	0.42±0.55	0.32±0.32	0.45
Creatinine (mg/dl)	0.10±0.06	0.07±0.06	0.149
Glucose (mg/dl)	28.5±46.8	34.40±108.12	0.76
C-reactive protein (mg/dl)	1.89±2.38	6.43±7.46	0.012
Uric Acid (mg/dl)	0.50±0.511	0.42±0.34	0.52
Ferritin (ng/ML)	1072.70±1292.052	826.32±1088.94	0.55
Iron (ug/dL)	155.79±177.77	91.76±88.87	0.18

An increase in serum iron levels was noted for both the fresh RCC (155.79 mg/dl±177.77 mg/dL) and 7-days stored RCC (91.76 mg/dl±88.87 mg/dL). However, the differences in the serum iron raise were not statistically significant (paired samples t-test, *P*=0.18). Transfusion of both fresh and 7-days stored RCC resulted in increased ferritin levels in the post-transfusion sample. In fresh RCC transfusion, serum ferritin levels increased by 1072.70 µg/L±1292.052 µg/L (mean ± SD), and in 7-days stored RCC transfusion, serum ferritin levels increased by 826.32 ug/L±1088.94 (mean ± SD). These differences were statistically not significant (paired samples t-test, *P*=0.55) (**Table 2**).

Discussion

This pilot study aimed to investigate the differences in hematological and biochemical markers after transfusion of fresh RCC compared to 7-days stored RCC in patients with transfusion-dependent thalassemia (TDT). Generally, the one unit of RCC raises blood Hb levels by 1 gm/dL [9, 10]. In our study, transfusion of older RCC units resulted in a lower albeit non-significant rise in Hb and other red cell parameters compared to transfusion of fresh RCC. Similar findings have been reported by others [7, 11, 12]. We have previously reported a temporal decrease in RBC counts and Hb levels, and an increase in osmotic and mechanical fragility in banked RCC units [13, 14]. This, coupled with the fact that the majority of TDT patients have enlarged liver and/or spleen, increased red cell clearance is expected, and so is the smaller rise in Hb levels after transfusion. No significant differences in MCV, MCH, and MCHC were observed in both the groups. Although TDT patients' red cells have very low MCV, MCH, MCHC levels, our patients had already received multiple transfusions and therefore, their hematological picture may have been affected by previously transfused blood from healthy donors. Therefore, transfusion of normocytic red cells did not appear to increase MCV, MCH, or MCHC. These findings are in accord with previously published work [15].

We also observed a slight but significant post-transfusion increase in WCC. Although non-leuko-reduced RCC units contain leukocytes, the possibility of these raising counts in the blood recipients is low. The host immune system would be expected to immediately clear transfused leukocytes. An alternate explanation for the increase in WCC count may be the infusion of pro-inflammatory mediators in RCC units causing a mild inflammatory state. Mild leukocytosis is observed in patients who are trans-

fused with leuko-depleted blood [16]. An increase in post-transfusion WBCs count was also found by Izbicki *et al*. [17]. A proposed mechanism is the increased concentration of non-transferrin bound iron (NTBI) in the blood, leading to the production of pro-inflammatory cytokines including IL-6 and IL-8, which cause leukocytosis in the recipient's blood [4]. More recently, Straat *et al.* concluded that stored RBCs transfusion bags contain extracellular vesicles (EVs), which release TNF, IL-6, IL-8, and IL-10 resulting in a pro-inflammatory host response i.e. leukocytosis [18]. Consistent with this, we also observed a significant increase in serum C-Reactive Protein (CRP) following transfusion of stored RCC. This finding is in keeping with the results by Kapur *et al.* [19]. The authors have attributed this increase in CRP to EVs which are found in blood bank stored blood.

We also tested surrogate clinical markers of hemolysis i.e. bilirubin and Lactate Dehydrogenase (LDH). Although the values of these showed a slight increase in post-transfusion samples, this increase was not significant. Blood transfusion-related abnormalities in serum laboratory parameters such as bilirubin and LDH were demonstrated by Weisen *et al*. in transfusion-dependent patients [20]. A significant but transient increase in bilirubin and LDH levels was observed in patients receiving two units of RCC. This increase in bilirubin level may be attributed to the hemolysis of non-viable RBCs during the first couple of hours after transfusion and conversion of released hemoglobin into bilirubin by the liver. Although, this phenomenon is seen in almost all patients who receive blood, the bilirubin level returns to normal almost after 24 hrs [21]. Since we tested our post-transfusion samples 24 hours after transfusion, these values might have returned to normal.

A typical blood transfusion carries approximately 200-250 mg iron per transfusion. In repeatedly transfused patients, it elevates the ferritin and iron levels in the body [22, 23], and is therefore associated with increased morbidity and mortality [24]. Recent literature suggests an additional transient spike in circulating iron immediately following a transfusion. An interesting finding in our study was a non-significant increase in blood ferritin and free iron level in post-transfusion samples from both the groups. Rapido *et al*. [25] have reported that after transfusion of stored blood cells, there is a sharp increase in non-transferrin bound iron (NTBI), bilirubin, serum iron, and transferrin saturation. This spike peaks between 6-12 hours post-transfusion and drops at around 20 hours. In our study, the release of serum iron, ferritin, and bilirubin might have been metabolized 24-hours post-transfusion.

In summary, we report for the first time, the effects of transfusion of stored RCC in TDT patients with hepatosplenomegaly. We tested whether transfusion of 7-days stored blood in patients with transfusion-dependent thalassemia was associated with an increase in hematological and biochemical markers linked with RBC lysis and inflammation. We demonstrate that transfusion of 7-days stored RCC is associated with rise in markers of inflammation (WBC counts and C-reactive protein). Taken together, these data suggest that transfusion of stored blood in TDT with compromised hemodynamics may result in subtle increased red cell lysis, release of iron, and associated inflammation. These are in keeping with published literature [8]. However, this study is not without limitations. The sample size was small, and only one post-transfusion assessment was performed. Using multiple post-transfusion time points (earlier time points), in a larger cohort of samples, could better show slight and transient changes in these parameters. The hematological and biochemical markers we tested were relatively insensitive and may not have detected smaller changes. The RCC used in our study was not leuko-reduced. Residual donor leukocytes in the collected blood may increase EVs and contribute to changes seen in the recipients. Nevertheless, our data and other published literature points that the use of stored blood may be associated with at least a transient increase in red cell lysis. In transfusion-dependent thalassemia patients with hepatosplenomegaly, who already have organ damage due to iron overload, and may have compromised ability to neutralize iron metabolites, this may result in increased end-organ damage.

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Disclosure of conflict of interest

None.

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