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ORIGINAL ARTICLE

Study of Hemolysis During Storage of Blood in the Blood Bank of a Tertiary Health Care Centre

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Abstract The aim of RBC storage system in a blood bank is to counteract damage to the metabolic machinery and the membrane, to improve post-transfusion viability. In recent years, the need for strict control over the quality of blood has been emphasised. Such quality indicator includes extend of hemolysis and morphological changes of RBC during storage. This study was design to see extend of hemolysis and level of plasma lactate dehydrogenase (LDH) and plasma potassium, during processing and storage at different intervals under blood bank condition. Forty-six donors were selected and blood units were collected and stored under blood bank conditions. Mean plasma haemoglobin of stored blood was estimated by tetra methyl benzidine method (TMB) and percentage hemolysis was calculated on day 0, 1, 7, 21, 28, 35 and 42 days. Similarly plasma LDH and plasma potassium level was also assessed during storage. It was noted that free haemoglobin level and percentage hemolysis progressively increased with storage along with the level of LDH and potassium. However, extend of hemolysis did not exceed the permissible limit of 0.8% up to 42 days of storage. 15 blood bags which showed visual hemolysis on day 28 did not exceeded the threshold of 0.8% hemolysis, when interpreted by TMB method. It was concluded that TMB method is better than visual method for determination of hemolysis. The reduced hemolysis at this centre may be accounted for the use of additive solution SAGM (Saline, Adenine, Glucose, Mannitol) and DEHP (di-2-ethyl hexyl phthalate) as plasticizer in blood bags for storage.

Keywords Hemolysis · Blood bag · TMB method · Storage

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Introduction

Red blood cell transfusions are given to raise the hematocrit level in patients with anemia or to replace the volume after an acute bleeding episode, but the main problems arising with storage of packed red cells in blood bank are hemolysis and morphological changes of RBC with time.

The quality of red cells stored for 5–6 weeks is dependent on the nature of anticoagulant, the initial handling, processing during component separation, composition of additive solution and nature of storage condition within transfusion service [1, 2]. When blood is mixed with an anticoagulant solution and stored at 4 °C, the red cells change shapes from discs to echinocytes and finally to spheres, become more rigid, shed lipid, exhibit various biochemical changes, particularly a fall in ATP and DPG content, and progressively lose the ability to survive in the circulation after transfusion. The cause behind these effects is paralysis of red cell membrane function including the membrane associated Na^+/K^+ pump [1–3].

The aim of storage system is to counteract damage to the metabolic machinery and the membrane, maintain a concentration of ATP and adenosine to improve post-transfusion viability. Other substances of potential benefit are

inorganic phosphate (both as substrate for the maintenance of organic phosphate compounds and for buffering), and citrate (for anticoagulation buffering and influencing intracellular pH) [4, 5]. Mannitol (and sorbitol) had been used for osmotic support and for counteracting spontaneous storage associated hemolysis. For these reason anticoagulant used are ACD (adenine, citrate and dextrose), CPD (citrate, phosphate and dextrose) and CPDA-1 (citrate, phosphate, dextrose and adenine) [4, 6]. Most common additive solution used for RBC storage is SAG-M (Saline, Adenine, Glucose and Mannitol) [2, 4].

SAG-M is an additive solution which not only increases the shelf life of packed red cells but mannitol also reduces haemolysis rate and vesiculation probably because it acts as free radical scavenger and membrane stabilizer [2, 4].

In recent years, the need for stricter control over the quality of blood and its products has been emphasized. One such quality indicator for stored red cell units is the extent of hemolysis. Detecting excess hemolysis due to component processing and storage has important implications for the transfused patient [7].

Lactic acid dehydrogenase (LDH) is abundant in RBCs and its level in plasma can function as another marker of red cell haemolysis in stored blood. Plasma potassium levels are also found to be raised in stored red blood cell units [1, 2].

The release of various chemicals and enzymes, especially proteases from the leukocytes contributes significantly to an increase in red cell hemolysis during storage. Therefore various leukocyte reduction filters are commercially available to decrease the rate of hemolysis in stored red cell units. In spite of the increasing use of additive solutions and all the preventive measures taken for storage of red cells, some amount of hemolysis is inevitable [7, 8]. The extent of hemolysis can be estimated by various techniques like visual assessment, spectrophotometric assays (TMB method), photometric method, and microplate technique [2, 9].

This is a prospective study conducted in blood bank to see the extend of hemolysis during processing and storage at different intervals of time under blood bank condition, along with the plasma level of LDH and potassium.

Materials and Methods

The present study was a prospective study on hemolysis during storage of blood in the blood bank of a tertiary health care centre. The study was approved by Institutional Ethics Committee. Forty-six donors were selected after elaborate medical history, necessary physical examination and haemoglobin estimation. Whole blood was collected in quadruple blood bag (450 ml) with CPD as standard anticoagulant in primary bag and SAGM as additive solution in the secondary bag, made of PVC with DEHP as plasticizer. After a holding period of at least 30 min, components are separated from the blood units collected. For preparation of packed red cells, the whole blood units are centrifuged with a "soft spin (1750 rpm for 9 min at 22 °C) in Heraeus cryofuge 6000i centrifuge. The platelet rich plasma (PRP) is separated and 78 ml of SAGM solution of pH 5.7 which is present in one of the satellite bag is added into the packed red cell bag. Subsequently all packed red cell units are stored under standard storage condition at 1–6 °C in blood bank refrigerators with continuous graphic temperature monitoring.

Sampling was performed from forty-six units of blood, before and after the component separation using sterile technique in a laminar airflow cabinet.

The prestorage whole blood samples taken from primary bags, immediately after phlebotomy, were considered as day 0 sample. 2 ml of this whole blood sample was taken in dipotassium ethylenediaminetetra-acetic acid EDTA (K2E) vacutainer and a 5 ml blood sample was collected in plain vacutainer. Hemoglobin (Hb) and hematocrit was measured by automated hematology analyser. Sample in plain vacutainer was centrifuged at 1000 rpm for 10 min and plasma was separated for biochemical parameters. The prestorage samples were considered as control sample for study and statistical analysis.

Similarly, after component separation, samples were collected on day 1 and weekly thereafter up to 42 days from packed RBC SAGM bags. The blood bag tubings were uniformly stripped and refilled to collect representative samples. The separated (plasma) samples were stored at −18 °C in deep freezer and later all the samples were thawed in 37 °C water bath for 30 min for assessment of biochemical parameters. Assessment of plasma haemoglobin was done by tetra methyl benzidine (TMB) method [6] Percentage hemolysis was calculated by (100-hematocrit) X Plasma Hb/Total Hb [3]. Assessment of plasma lactate dehydrogenase (LDH) level was done by two point calorimetric method using forward reaction of conversion of lactic acid to pyruvic acid. Plasma potassium level was assessed using automated electrolyte analyser [10].

Results

Mean age of the donors was 36 years ranging from 18 to 56 years, with a M:F ratio of 4.1:1. The most common blood group among donors was B (46%) followed by O (37%), rest being A (10%) and AB (7%).

Table 1 shows variation in mean plasma hemoglobin level during storage from day 0 to day 42. With storage,

Table 1 Mean plasma hemoglobin and percentage hemolysis during storage

Days of storage	Mean Hg ± SD (range) mg/dl	*P* value <0.05 (statistically significant)	Range of hemolysis (%)	Mean hemolysis (%)
Day 0	23.8 ± 7.6	<0.001	0.03–0.12	0.081
Day 1	48.5 ± 14.5		0.06–0.16	0.099
Day 7	86.9 ± 24.1	<0.001	0.12–0.28	0.177
Day 14	116.0 ± 36.5	<0.001	0.16–0.47	0.231
Day 21	135.0 ± 36.0	<0.001	0.19–0.53	0.269
Day 28	149.9 ± 37.1	<0.001	0.24–0.62	0.297
Day 35	161.8 ± 38.7	<0.001	0.27–0.68	0.327
Day 42	173.1 ± 39.8	<0.001	0.31–0.75	0.359

there was rise in plasma hemoglobin level from day 0 to day 42 (Table 1). The highest level of mean plasma haemoglobin observed in any sample was 247.4 mg/dl. Day 0 value was considered as control for statistical analysis because the plasma was separated prior to storage and processing. A statistically significant level was observed on day 1 and up to 42 days.

The total Hb content of all 46 red cell units ranged from 13.4 to 17.6 g/dl and mean ± SD was 15 ± 1.16 g/dl. The average hematocrits of RBC units ranged from 69.1% on day 1 of storage to 71.5% on day 42.

Table 1 also shows percentage hemolysis from day 0 to day 42 of storage. The percentage hemolysis on day 0 was 0.08% which rose to 0.1% after processing and component separation on day 1. Further increase in percentage hemolysis was seen with storage up to 42 days (Table 1).

Thus the least hemolysis was observed on the day of collection (mean = 0.08%) and the maximum hemolysis was observed on the 42nd day of storage (mean = 0.359%). The maximum percentage hemolysis shown by any sample was 0.75%. An overall mean hemolysis of 0.38% (range, 0.03–0.75%) was obtained, which was much below the permissible level of 0.8% hemolysis according to European Guideline and 1% limit according to FDA.

Fifteen red cell units (33%) showed visual evidence of hemolysis on day 28. Sterility of these 15 units was confirmed by the absence of fungal and bacterial growth. The plasma hemoglobin levels of the above 15 units ranged from 27.5 to 245.7 mg/dl but none of them exceeded the threshold of 0.8% hemolysis (Table 2).

Table 3 shows variation in mean plasma LDH levels and mean plasma potassium ion levels during storage. Plasma LDH showed increase in levels during storage. The rise in plasma K^+ level was statistically not significant when day 1 value was compared with day 0 values; however it was significant on day 7 and up to 42 days.

Table 2 Mean plasma hemoglobin and % hemolysis of 15 units showing visual hemolysis

Days after collection	Mean plasma hemoglobin (mg/dl)	% Hemolysis
Day 0	27.5	0.09
Day 1	53.4	0.11
Day 7	93.8	0.18
Day 14	123.6	0.23
Day 21	210.0	0.53
Day 28	245.7	0.62

Discussion

Hemolysis is considered to be an obvious marker of the failure of RBC storage system and also bacterial and fungal contamination. Hemolysis can be in the form of rupture or loss of micro vesicles from the surface of still intact cells.

This study showed that the plasma hemoglobin levels progressively increased from day 0 to day 42 of storage (Table 1). On day 0 the mean plasma haemoglobin levels of packed red cells in this study was 23.8 mg/dl (range, 10.7–52.1 mg/dl). This level was similar to that reported by Sawant et al. [2]. The plasma Hb levels were estimated weekly after processing, component separation and adding SAGM as additive solution from day 1 to day 42. This study reported a rise in plasma Hb level with storage very much similar to the other studies [2, 5, 6]. Mukherjee et al. [11] also studied the plasma Hb level in CPDA-1 packed RBCs without adding additive solution (SAGM) and reported a rise in level of plasma Hb from day 1 to day 21. The result of plasma Hb in another study in CPDA-1 PRBCs without adding additive solution on day 28 was 194 mg/dl [5]. It was observed that the plasma Hb levels in this study were lower than the other studies not using SAGM as an additive solution [5, 6, 11].

Table 3 Variation in mean plasma LDH and potassium level during storage

Days of storage	Mean ± SD of LDH (U/L)	*P* value <0.05 (statistically significant)	Mean ± SD of potassium (mEq/L)	*P* value <0.05 (statistically significant)
Day 0	164.3 ± 41.5	<0.001	3.59 ± 1.6	$P = 0.45$ (not significant)
Day 1	194 ± 39.7		4.33 ± 1.8	
Day 7	568.8 ± 110.1	<0.001	11.3 ± 4	<0.001
Day 14	898.4 ± 125.5	<0.001	23.2 ± 7.1	<0.001
Day 21	1291 ± 266.1	<0.001	33.6 ± 8.5	<0.001
Day 28	1698 ± 403	<0.001	40.3 ± 9.7	<0.001
Day 35	1845 + 306	<0.001	45.2 + 4.5	<0.001
Day 42	2190 + 209	<0.001	48.1 + 3.3	<0.001

The average hematocrits of RBC units increased from 69.1% on day 1 of storage to 71.5% on day 42. This increase can be explained by the morphological changes of the RBCs membrane. The red blood cells lose parts of their membrane as microvesicles due to storage lesions, resulting in the decrease in the surface area-volume ratio and ending up with microcytic spherocytes. Besides, much of the RBCs populations are echinocytes with many membrane projections protruding outward, which prevents the normal packing of the RBCs [8].

As shown in Table 1, the average % hemolysis in this study was 0.38% (range 0.03–0.75%). This was in accordance with the results of other workers [2, 11, 12]. Hemolysis rate in this study was much below the permissible threshold of 0.8% laid by European council and 1% limit recommended by FDA at the end of storage period for long term storage of packed red cells. Mannitol in additive solution SAGM reduces hemolysis rate and vesiculation probably because mannitol acts as free radical scavenger and membrane stabilizer [2, 4]. This might be the reason for lower levels of plasma Hemoglobin in present study as compared to the levels in CPDA-1 stored red cells without SAGM.

As shown in Table 2, all the 15 units showing evidence of hemolysis on visual examination in present study have plasma Hb levels ranging from 27.5 to 245.7 mg/dl. However the percentage hemolysis of all these units was within the 0.8% hemolysis threshold. These units would have been discarded had we not objectively evaluated the extent of hemolysis by the TMB method. One study have reported that a subjective visual inspection would have resulted in discard of approximately 50% of units collected in CPDA-1 and 10% of units stored in Adsol [13]. All these 15 units were issued from the blood bank subsequently. Rest of the blood bag units were issued on 42nd day of storage and none of them reported any type of transfusion reaction.

Various studies have figured out that the visual method is biased, inaccurate, misleading and results in overestimation of hemolysis in RBC units. Grossly visible pink discoloration of plasma or red cell suspending medium occurs with plasma Hb levels as low as 25 mg dl^{-1} ($\approx$0.09% hemolysis) [13]. Hence, spectrophotometric method such as the TMB method, have been traditional "gold standard" for measurement of free Hb as previously established [13, 14].

Red cell concentrates relatively void of plasma are more viscous and difficult to infuse in emergency situation. To overcome this problem red cell concentrates with hematocrit <80% are prepared. This allows adequate plasma to remain for red cell nourishment and also improves flow properties. The use of additive solutions allows recovery of maximum amount of plasma and preparation of RBC units with a final hematocrit of 60%. The mean hematocrit in present study was 69.1% on day 1 of storage and 71.5% on day 42. Additive solutions provide nutrients to red cell for improved viability. It also increases the shelf life of stored blood to 42 days, allows extraction of more plasma/platelet rich plasma for optimal production of platelets and other components [2, 4, 12].

In present study, the containers used for storage were made of polyvinyl chloride (PVC) with di-(2-ethylhexyl) phthalate (DEHP) as plasticizer. DEHP has been shown to decrease the rate of hemolysis and membrane loss by microvesiculation during storage. DEHP being an extremely lipid-soluble compound may intercollate into the red cell membrane and act as a membrane stabilizer. Polyvinyl chloride (PVC) plasticized with DEHP is the standard material used for RBC storage bags [2, 15, 16].

Lactate dehydrogenase is found in abundance in red cells and is usually raised in cases of intravascular hemolysis. In the present study plasma level of LDH shows progressive increase from day 0 to day 42 (Table 3). As evident from the present study, the rise in plasma LDH levels in stored blood run parallel to the rise in plasma haemoglobin levels indicating that plasma LDH can also be used as an indicator of progressive hemolysis and can be used for monitoring purpose. The results were compatible with the study conducted by other authors [2, 17]. Latham et al. [18] suggested that the progressive increase in LDH and plasma haemoglobin during storage of blood is due to continuous cell damage and transfusion of blood with such

characteristics ordinarily do not cause any problem to the recipient.

In this study significant rise in plasma K^+ was seen with storage (Table 3). The rise in plasma potassium levels on different days was in accordance with other studies [2, 11]. The volume of red blood cells is maintained by ATP dependent Na^+/K^+ pump present on RBC membrane. With storage, the ATP concentration in human red cells falls owing to deprivation of glucose. Whittam and Willey have stated that there is a parallelism between the ATP concentration and the potassium influx at any one time. Hence the fall in ATP concentration is associated with continuous net potassium efflux [19, 20]. Hyperkalemia may be of importance in neonates and massive blood transfusion associated with serious complication of cardiac arrest [20]. However, high potassium level in stored blood may be of little concern in transfusion of single unit of red cells due to in vivo restoration of electrolyte balance in recipient's body [21].

Conclusion

It was concluded from the study that Hemolysis is a very important parameter for assessing the quality of stored red cells. However, the extent of hemolysis did not exceed the permissible limit for hemolysis of 0.8% up to 42 days of storage. The reduced hemolysis in blood units at this center may be accounted for the use of additive solution SAGM and the use of DEHP as plasticizer in blood bags for storage. Although visual inspection of the blood unit is an easy and quick method to detect hemolysis, but at the same time it results in overestimation of hemolysis. Hence routine quantitative analysis for hemolysis in a blood must be done by TMB method to avoid inadvertent discard of precious RBC units. The Plasma LDH level and potassium level progressively increased with storage which can also be used as an indicator of progressive hemolysis and can be used for monitoring purpose.

Compliance with Ethical Standards

Conflict of interest The authors declare that the study and manuscript have no conflict of interest and is not supported/funded by any agency.

Ethical Standard The procedures followed are in accordance with the ethical standards of the responsible committee on human and with the Helsinki Declaration of 1975, as revised in 2000. The study was approved by the institutional ethics committee.

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