Research Article

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Effect of Storage on Osmotic Fragility in CPDA-1 Stored Blood in Sokoto, Northwestern Nigeria

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Abstract

The aim of this study was to investigate the effects of storage on the in vitro osmotic fragility of erythrocytes of humans in a single unit of whole blood. Blood was collected by venepunture from a healthy adult male (70-75 kg) into CPDA-1(450ml) blood bag containing citrate phosphate dextrose as anticoagulant (63ml) and stored in a blood bank maintained at 4°c ± 2°c. The osmotic fragility of the erythrocytes was determined by measuring the release of haemoglobin from blood added to tubes containing serially diluted phosphate buffered saline (pH 7.4). The Blood samples were analyzed on day 1 to day 35 after collection (5 weeks). Increased erythrocyte osmotic fragility was observed at week 3 ($p=0.010$). The initial haemolysis (>5%) occurred between 0.50% and 0.55% PBS. The mean corpuscular fragility was between 0.35 and 0.45% PBS. Maximum haemolysis occurred in 0.35% PBS. Osmotic fragility was significantly affected by storage ($p<0.05$). In conclusion, this research showed that there is an increase in the osmotic fragility as donor blood is stored and that the effect is more pronounced from week 3. There is need to maintain the cold chain management of stored donor blood to ensure that the aim of red cell transfusion which is to manage anaemia and increase the oxygen carrying capacity is not compromised.

Keywords: Storage, Osmotic Fragility, CPDA-1 Stored Blood, Sokoto, Nigeria

Introduction

The first blood transfusions were made directly from donor to receiver before anticoagulation of blood was discovered. Later, it was discovered that by adding anticoagulant and refrigerating donor blood it was possible to store blood for some days, thus opening the way for the development of blood banks [1]. John Braxton Hicks was the first to experiment with chemical methods to prevent the coagulation of blood at St Mary's Hospital, London in the late 19th century, but his attempts, using phosphate of soda were unsuccessful [2]. The introduction by J.F. Loutit and Patrick L. Mollison of acid-citrate-dextrose (ACD) solution, which reduced the volume of anticoagulant, permitted transfusions of greater volumes of blood and allowed longer term storage [2]. Carl Walter and W.P. Murphy, Jr. introduced the plastic bag for blood collection in 1950 which replaced breakable glass bottles with durable plastic bags allowed for the evolution of a collection system capable of safe and easy preparation of multiple blood components from a single unit of whole blood [3]. To further extend the shelf life of stored blood, an anticoagulant preservative, CPDA-1 was introduced in 1979, which increased the blood supply and facilitated resource-sharing among blood banks [3].

Haematological therapy and laboratory results are often influenced by a number of pre-analytical variables. These include anticoagulants used, method of analysis, the storage temperature, and the time lapse between when sample was taken and when they were analyzed [4]. Storage of red blood cells in preservative medium is associated with harmful metabolic, biochemical, and molecular changes to erythrocytes that are collectively referred to as storage lesions [5]. Blood products such as red blood cells (RBC) stored with additive solutions in different temperatures contribute to storage lesions significantly [6]. The most probable sites of damage will be cytoskeletal proteins in RBC membrane [7]. These membrane changes will lead RBC to be fragile and increase osmotic fragility and changes in electrolyte imbalance. Great efforts have been done to provide a suitable and a safe supply of blood with more benefits than side effects. Currently, the storage procedures of blood bags in blood banks require some conditions to ensure the maximum storage time for a healthy and safe blood supply. However, pathological consequences can affect the stored blood, they are termed as storage lesions. Storage lesions are hypothesized to decrease the efficiency of stored blood and decrease their ability to play their required role after transfusion, but these hypotheses have no clear evidences yet [8]. Some reversible changes result from stored blood such as decreased Adenosine Triphosphate and 2,3 Diphosphoglycerate (ATP and 2,3-DPG). However, some other damage is irreversible and includes increased osmotic fragility, small echinocytic rigid red blood cells with reduced function, microvesiculation, and haemolysis. Leukoreduced packed red blood cells will have storage lesions due to lipid peroxidation of RBC membrane and that will result in morphological alterations in stored blood [9]. Blood for

various laboratory analyses are commonly kept in ethylenediamine tetra-acetic acid (EDTA). Keeping blood samples in EDTA tubes longer than normal before analyzing them is therefore likely to have an effect on the morphology of the blood cells particularly erythrocytes. Their osmotic fragility may be altered, which can also affect their viability and hence the results of analysis such as complete blood count, thin film comments among others Identifying storage related changes is therefore important so that artefactual changes are not misinterpreted as pathologic findings.

Investigating storage lesions and oxidative stress on RBCs is essential to assess the extent of damage to the cells, as this damage causes alterations and decreases the functionality and viability of the red blood cells after transfusion. The principle of osmotic fragility is to measure the resistance of RBCs to increasing osmotic stress. The osmotic fragility test is used to determine the susceptibility of red blood cells to osmotic stress. The shape of RBC mainly influences the resistance of RBC to haemolysis. In case of old red blood cells stored for 42 days, they lose the normal morphology of discoid shape and progress to spherocytes, with decreased surface area to volume ratio. Spherocytes can haemolyze in hypotonic environment earlier than normal shaped RBC. The main defect that occurs in old RBCs is in the proteins that connect the cytoskeleton to the membrane, which are spectrin, ankyrin, protein 4.2, and band 3 [10]. The increased production of reactive oxygen species with decreased antioxidants causes the oxidation and degradation of these proteins. This damage can result in losing parts from the unsupported membrane in the form of microvesicles, leading to decrease in surface area to volume ratio. In consequence, spherocytes will have high osmotic fragility with increased haemolysis. Biochemical changes such as reduced ATP concentration and increase in calcium can contribute to increase rigidity of the RBCs. In the body, these cells will have decreased viability because they are inflexible and unable to pass through the small blood vessels. It has been established that erythrocyte osmotic fragility (EOF) is a good indicator of stress in animals [11-13].

Some chemical compounds are used in blood transfusion bags to prevent blood from clotting. Various anticoagulant-preservative solutions have been formulated for better red cell preservation. Correct proportion of this anticoagulant to blood is crucial for effective anticoagulation and optimum preservation. Examples of commonly used anticoagulants in transfusions for whole blood include Acid Citrate Dextrose (ACD), Citrate phosphate Dextrose solution (CPD), Citrate phosphate dextrose adenine. (CPDA-1), while for frozen red cell includes high glycerol solution, low glycerol solution [14]. CPDA helps to maintain high ATP levels. Blood collected in CPDA is safe, well tolerated and has a shelf-life of 35 days when stored at 4°c ± 2°c [14]. Storage lesions in stored red blood cells are now considered well-known phenomena, yet there are structural and functional aspects of stored red blood cells to be explained. Blood for various therapeutic and laboratory analyses are commonly kept in storage medium. These storage medium can however cause morphological and fragility changes in blood cells particularly erythrocytes (RBCs) if the storage is prolonged. This can affect erythrocytes viability and hence their analytical results. The timing between blood sampling and analysis is therefore very important in achieving reliable results. In this study, we look primarily to see the progressive irreversible changes in RBC morphology to specially observed osmotic fragility changes during the course of storage time.

Whole blood and red cell concentrate must always be stored between 2°C and 6°C. A fall in temperature less than 2°C can cause freezing injury to the red cells leading to haemolysis. If haemolysed blood is transfused to a patient, it can lead to fatal consequences. Temperatures more than 6°C can lead to overgrowth of non- specific bacteria which may have entered the blood unit during collections or component preparation. As red cells consume glucose for their continued metabolism, storing a blood unit at 2-6°C will also decrease the rate of glycolysis [8].

Since the beginning of modern transfusion therapy, remarkable efforts have been done to provide a safe and adequate blood supply. Blood banks adopt storage procedures to ensure this blood supply and red blood cell (RBC) units are, nowadays, stored up to 42 days in most of developed nations. Length of storage of red blood cell units is based on the decision that they can be stored as long as the average haemolysis is below 0.8% and the proportion of red blood cells still alive 24 hours post-transfusion is higher than 75% [15].

Changes in RBC properties during storage have been known for decades, but the exact significance of these changes is still largely uncertain [16]. Researchers have carefully documented what storage does to red blood cells. It is well established that, when stored, red blood cells undergo several changes referred to as "storage lesion", the latter being potentially able to irreversibly alter RBC biological functions, including reduced deformability and increased osmotic fragility and a significant decline in adenosine triphosphate and 2,3- diphosphoglycerate [17].

These changes decrease oxygen transporting capacity and result in more rigid cells impairing the RBC microcirculation [15-19]. There has been increasing controversy about whether a given product unit's age is a factor in transfusion efficacy, specifically on whether "older" blood directly or indirectly increases risks of complications [20-21]. Studies have not been consistent on answering this question, with some showing that older blood is indeed less effective but with others showing no such difference; nevertheless, as storage time remains the only available way to estimate quality status or loss, a first-in-first-out inventory management approach is standard presently [22].

Osmotic fragility (OF) refers to the degree or proportion of haemolysis that occurs when a sample of red blood cells are subjected to osmotic stress by being placed in a hypotonic solution [23]. Osmotic fragility is affected by various factors, including membrane composition and integrity as well as the cells' sizes or surface-area-to-volume ratios [24]. Oxidative stress is a common feature of RBC storage lesions which increases with prolonged storage resulting in lipid peroxidation, protein oxidation and reduced integrity of the erythrocyte membrane, all contributing to the formation of exocytic microvesicles (microparticles) and it has been postulated that these oxidative events occurring in stored RBCs may be correlated with the risk of post-transfusion complication [25]. It is largely unknown how these storage lesions affect function and survival of RBCs in the circulation after transfusion, but most of these alterations occur in vivo, "allowing one to speculate that storage causes an acceleration of the normal RBC aging process [25]. It remains to be determined whether these changes translate into functional perturbations in the transfusion recipient [26].

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solution and it is used to determine the susceptibility of red blood cells to osmotic stress [27]. The shape of RBC mainly influences the resistance of RBC to haemolysis. In case of old red blood cells stored for 42 days, they lose the normal morphology of discoid shape and progress to spherocytes, with decreased surface area to volume ratio. Spherocytes can haemolyze in hypotonic environment earlier than normal shaped RBC. The main defect that occurs in old RBCs is in the proteins that connect the cytoskeleton to the membrane, which are spectrin, ankyrin, protein 4.2, and band 3 [27].

The findings of this study will provide further information about the effect of osmotic fragility of stored blood in the study area. The study will provide more on existing knowledge on the need to assess the extent of damage to the red blood cells, as this damage causes alterations and decreases the functionality and viability of the red blood cells after transfusion. CPDA-1 is the anticoagulants contained in blood donor bags in Nigeria. There is paucity of data on the effect of storage on osmotic fragility in CPDA-1 stored blood in Sokoto, Northwestern Nigeria. The aim of this study is to determine the effect of storage on osmotic fragility of erythrocyte.

Materials and Method

Study Area

This study was carried out in the Heamatology Department of Usmanu Danfodiyo University, Sokoto, Northwestern Nigeria. UDUS is a tertiary institution located within the Sokoto metropolis. The state is located in the extreme Northwest of Nigeria, and it lies between latitude 1 3◦ 3' 490N, longitude 5◦ 1 4' 890E and at an altitude of 272m above sea level. The State is in the dry Sahel, surrounded by sandy savannah and isolated hills, with an annual average temperature of 28.3°c (82.9°F). Sokoto is, on the whole, a very hot area. However, maximum daytime temperatures are for most of the year generally under 40°c (104.0°F). The warmest months are February to April when day time temperature can exceed 45°c (113◦c). The rainy season is from June to October during which shower on a daily occurrence. There are two major seasons, wet and dry which are distinct. Sokoto metropolis is estimated to have a population of 45,694.90764 people and by the virtue of its origin [28]. The indigenous inhabitant of the area are the Hausa and Fulani Other groups such as Gobirawa, Zabarmawa, Kabawa, Adarawa, Arawa, Nupe, Yoruba, Ibos and others. Occupation of the city inhabitants include farming, trading, and commerce, with a reasonable proportion of the population working in private and public sector.

Study Site

This study was conducted in the School of Medical Laboratory Science of Usmanu Danfodiyo University, Sokoto in collaboration with the Hematology Department of Usmanu Danfodiyo University. The site of the study and the collaborating hospitals have an enabling environment (both human and material endowment) for the study. The laboratory of Specialist Hospital Sokoto is capable of providing all the routine and special and specialized laboratory investigation for the study.

Study Subjects Selection

Inclusion Criteria

Consenting healthy male or female volunteer that is ≥18 years and free from transfusion transmissible infections.

Exclusion criteria

1. Male or Female donor under the age of 18 years.
2. Male or Female donor above the age of 65 years.
3. Male or female donor with any of the common TTI's

Study Design

This case study involved collecting a unit of blood from a consenting healthy volunteer donor that is ≥18 years and free from transfusion transmissible infections. Samples for this research project was collected after obtaining informed consent from the study participant.

Ethical Consideration

Ethical approval for the study was obtained from the Specialist Hospital Ethical Committee Sokoto, Sokoto, State.

Informed Consent

Written informed consent was obtained from the eligible participant.

Methods of Analysis

Sample Collection

One unit of whole blood was obtained from the volunteer donor. Institutional review board (IRB) approval was obtained from Specialist Hospital for using the donor's blood for the purpose of research. Whole blood, 450 ml was collected from healthy volunteer donor by venipuncture in citrate phosphate dextrose adenine (CPDA-1) anticoagulated blood bag following strict aseptic technique, according to standard blood bank procedures. The samples were analyzed in the Haematology Department, School of Medical Laboratory Science Usmanu Danfodiyo University, Sokoto, Nigeria. RBC units was stored at 2–6°C for 35 days and samples were withdrawn aseptically on day 1 to day 35 for the investigations [29].

Principle of test

The test determines the resistance of red cell to haemolysis in various concentration of hypotonic saline solution. The ability of erythrocyte to absorb sodium chloride solution without lysis depends on the ratio of volume to surface area of the cell. In normal red cells, the volume may increase up to 70% before lysis can occur. Spherocytes have reduced surface to volume ratio and therefore absorb less water.

Stock Solution

The stock solution of sodium chloride osmotically equivalent to 10 % was prepared as follows
Sodium chloride 90 g
Disodium hydrogen phosphate 13.65g
Sodium dihydrogen phosphate 2.34 g
Distilled water 1 liter

Working Solution

The stock solution of sodium chloride prepared above was diluted 1:10 with distilled water to obtain a 1 % solution.

Calculations

Percentage Haemolysis (%) =Absorbance of the test / Absorbance of 100% lysis ×100

Procedure

1. The stock solution of sodium chloride was diluted 1:10 with distilled water to obtain 1% solution.
2. 12 test tubes were arranged and dilution was prepared as follows
3. 0.05 ml of blood was added to each of the tubes and mixed

immediately by gently inverting several times.

4. The tubes were allowed to Stand at room temperature for (18-20◦c) for 30 minutes.
5. The mixture was then Remixed and centrifuged at 3000rpm for 5 minutes.
6. The amount of lysis in each tube was determined colorimetrically using a green filter, at 540nm. The first tube in the series serve as a blank (0% haemolysis) as it contains 0.9 % isotonic saline. The 12th tube containing the 0.1% saline serves as 100% lysis since this gives complete lysis.

Table 1: Dilution of the stock solution to obtain various concentration of Sodium Chloride Solution

Table number	1% Nacl (ml)	Distilled water	Final concentration of Nacl (%)
1	4.5	0.5	0.9
2	3.75	1.25	0.75
3	3.25	1.75	0.65
4	3	2.0	0.6
5	2.75	2.25	0.55
6	2.5	2.5	0.5
7	2.25	2.75	0.45
8	2.0	3.0	0.4
9	1.75	3.25	0.35
10	1.5	3.5	0.3
11	1	4	0.2
12	0.5	4.5	0.1

Sampling Method

Consecutive method was applied in recruiting the eligible subject for this research work.

The laboratory investigation was carried out on the sample collected in plain container from the volunteer.

Statistical Analysis

Data obtained was entered into a statistical package for the social science (SPSS) version 22) on a computer to define the nature of the distribution of data for each group. Statistical differences of data were analyzed using mean, standard deviation, and ANOVA. Probability ($p \leq 0.05$) was used to determine the level of significant for all statistical analysis.

Results

The evaluation of the effect of storage on osmotic fragility was carried out using citrate phosphate dextrose adenine (CPDA-1). Anticoagulated blood was drawn from a single healthy donor and placed on the quarantine shelf of the blood bank refrigerator. The blood was kept for 35 days and the sample was evaluated on day 1 to day 35. Table 2 shows the Osmotic Fragility of Red cells for 5 weeks (35 days). There was a statistically significant difference in osmotic fragility at week 3 (day 14-21) ($p=0.010$). Figure 1 shows the mean corpuscular lysis (obtained by plotting the graph of percentage lysis against concentration of Nacl) against the number of weeks (5 weeks), which shows an increase at week three.

Table 2: Osmotic Fragility of Red cells for the 5 weeks storage period

	N	Mean	p-value
Week one	7	0.4057±0.03690	0.060**
Week two	7	0.3986±0.06122	0.100**
Week three	7	0.4729±0.02059	0.010*
Week four	7	0.4371±0.03861	0.110**
Week five	7	0.4557±0.04117	0.200**
Total	35		

* p-value< 0.05 (statistically significant value)
**p-value > 0.05 (statistically non-significant value)

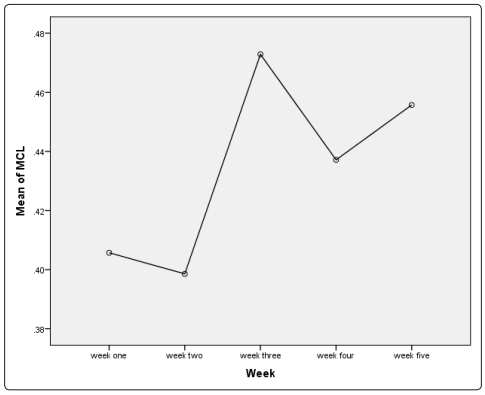

Figure 1: The mean corpuscular lysis (MCL) against the number of weeks showing an increase in osmotic fragility at week 3

Discussion, Conclusion and Recommendations

Discussion

This study investigated the effect of storage on osmotic fragility of CPDA-1 stored donor for 35 days. We observed by comparing the mean values of the osmotic fragility based on length of storage and observed that there was a statistically significant increased osmotic fragility at week 3 ($p=0.010$). This study has showed that storage of blood for a long period of time affect the osmotic fragility of human erythrocyte. Red cells that have been stored for prolonged period of time manifest a markedly increased osmotic fragility. This abnormality may not due to membrane loss, but rather to the presence of osmotically active substance within the red blood cell that render the interior hyperosmolar with respect to isotonic saline. The chief substance responsible for this hyperosmolar state is probably lactate. Other substance that are transported slowly from red cell such as phosphate and glucose may also play a role in establishing the reversible hyperosmolar state that exists in stored erythrocyte. Osmotic fragility test has the potential of serving as a potent and relatively cheap evaluative test of membrane stability in both experimental investigations or in clinical evaluations of membrane-based erythrocyte pathology. The test exploits characteristic structural changes which the RBC membrane undergoes when the cell is subjected to stress [30-31]. A number of factors frequently limit the utility and acceptability of the osmotic fragility test results (changes of pH, temperature, blood gas level and age). Humoral environment influences osmotic fragility and the values of osmotic fragility tests so obtained [32,33]. This study therefore investigated some aspects of these factors.

In this study, we specifically investigated the effects of storage of human erythrocytes for varying periods of time (week 1 to week 5) on the erythrocyte membrane osmotic fragility using a single unit of blood obtained from a healthy adult ≥18 years and observed that there was a statistically significant difference in the osmotic fragility the longer the blood is stored (p=0.010). Human erythrocyte on the other hand appears to exhibit a varied response to OF-test, depending on the ex-vivo age of the samples. In the present study, storage of human blood samples for up to 24h did not significantly alter the erythrocyte membrane characteristics. However, in this study, beyond 24h storage period, at 48h, a decrease in osmotic fragility was noted in human erythrocytes. The blood bags used for storage are made up of polyvinyl chloride (PVC) with plasticizer, di-(2-ethylhexyl) phthalate (DEHP). These bags are easily permeable to CO2. Our finding re-enforces the need to put in place measures to minimize RBC storage lesion. Current regulatory measures in place to minimize RBC storage lesion include; First-in, first-out (FIFO) primary inventory-management approach to minimize product expiration. There are however some deviations from this policy both in current practice as well as under research. For example, exchange transfusion of RBC in neonates calls for use of blood product that is five days old or less to ensure optimal cell function [34]. Also, some hospital blood banks will attempt to accommodate physicians' requests to provide low-aged RBC product for certain kinds of patients (cardiac surgery) [35]. There is also the need to implement restrictive protocol. Many physicians have adopted this method whereby transfusion is held to a minimum-due in part to the noted uncertainties surrounding storage lesion, in addition to the very high direct and indirect costs of transfusions along with the increasing view that many transfusions are inappropriate or use too many RBC units [34,36]. Maximum shelf life (currently 42 days), a maximum auto-haemolysis threshold (currently 1% in the US), and a minimum level of post-transfusion RBC survival in vivo (currently 75% after 24 hours) [37]. However, all of these criteria are applied in a universal manner that does not account for differences among units of product; for example, testing for the post-transfusion RBC survival in vivo is done on a sample of healthy volunteers, and then compliance is presumed for all RBC units based on universal (GMP) processing standards. RBC survival does not guarantee efficacy, but it is a necessary prerequisite for cell function, and hence serves as a regulatory proxy [34]. Opinions vary as to the best way to determine transfusion efficacy in a patient in vivo [36]. In general, there are not yet any in vitro tests to assess quality deterioration or preservation for specific units of RBC blood product prior to their transfusion, though there is exploration of potentially relevant tests based on RBC membrane properties such as erythrocyte deformability and erythrocyte fragility [37]. More recently, novel approaches are being explored to complement or replace FIFO. One is to balance the desire to reduce average product age (at transfusion) with the need to maintain sufficient availability of non-outdated product, leading to a strategic blend of FIFO with last in, first out (LIFO) [35].

Conclusion

In this research we found that, there is an increase in osmotic fragility in blood donor sample that has been stored for 5 weeks (35 days) more especially on week three. On storage the red cell viability is reduced which may be influenced by several factors such as change of pH, and temperature. Also, in this study we observed that red blood cell become less deformable and more fragile as they undergo long storage time.

Recommendations

1. There is need for further research to be carried out on different storage time and temperature.
2. It's also recommended that the level of osmotic fragility of stored blood should monitored during storage by maintaining appropriate storage temperature.

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