

## Time dependent changes in red blood cells during storage in the local blood banks of Khyber Pakhtunkhwa

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### Abstract

**Objective:** This project aimed at determining time-dependent ultrastructural and haematological changes taking place in blood stored in local blood banks of Khyber Pakhtunkhwa.

**Methods:** It was a longitudinal study with repeated measures design. Twenty healthy blood donors participated in this study. An amount of 250ml blood was collected from each donor and stored in Citrate Phosphate Dextrose Adenine-1 (CPDA-1)-containing blood bags. Within first four hours, baseline samples were taken while subsequent samples were obtained at 5 days interval till day 20th. Structural changes in RBCs were observed under light and scanning electron microscope (SEM) at different intervals. Furthermore, haematological parameters and osmotic fragility were also determined.

**Results:** Remarkable alterations were seen in RBCs morphology. From 5th day onwards, multiple visible spicules were observed on the RBC's outer membrane and more than 2/3rd cells were abnormal at day 20. There was a significant reduction in RBCs count and haemoglobin concentration while the remaining parameters remained unchanged. Osmotic fragility increased significantly over time, with <math>1\%</math> haemolysis noted in baseline samples as compared to 2.4% haemolysis on day 20th ( $p \leq 0.0001$ ).

**Conclusion:** Prolonged storage of blood results in distorted RBCs morphology and increased fragility. Transfusion of such cells would potentially result in rapid lysis in patients with hepatosplenomegaly and conditions requiring multiple blood transfusions.

**Keywords:** Red cells storage, scanning electron microscope, osmotic fragility, Transfusion, Blood storage, hemoglobin.

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### Introduction

The red blood cell is unique among human cell types in its structure and function. It carries oxygen from the lungs and distributes it to the various organs and tissues of the body.<sup>1</sup> The red cell contents are enveloped by a network of cytoskeletal and membrane proteins.<sup>2,3</sup> Lacking a nucleus, and cytoplasmic structures such as golgi bodies and endoplasmic reticulum, its membrane is involved in its diverse mechanical, structural, transport and antigenic properties.<sup>4</sup> The biconcavity of red cells results in relative abundance of membrane in comparison to cytoplasm. The unique discoid shape provides red cell with essential deformability needed for red cell survival through narrow capillaries during circulation.<sup>2</sup> Any changes of red blood cell membrane makes it vulnerable to disruption and can even obstruct the circulation.<sup>3</sup>

Red blood cell transfusion is one of the earliest human-human transplants. It is imperative for life threatening acute conditions such as severe blood loss following accidents and injuries, to equally life threatening but more chronic conditions such as thalassaemia and aplastic anaemia. Currently, millions of blood units are transfused in hospitals each year across the world. Blood is collected

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in blood bags containing anticoagulant CPDA-1 and stored in special storage refrigerators at 4°C.<sup>5,6</sup> With increasing duration of storage, the red blood cells begin to lose functional activity with decreasing 2, 3-diphosphoglycerate (DPG), ATP and pH values. This causes loss of cell membrane fragility and results in red cells dysfunction.<sup>4</sup>

The American Association of Blood Banks (AABB) recommends that stored blood can be used up to a maximum of 42-days of storage.<sup>7,8</sup> Studies performed on healthy volunteers have shown that transfusion of up to 5-weeks old blood is safe. It is clear that with increasing storage, red cells become rigid, less deformable<sup>2</sup> and undergo different morphological, structural and metabolic changes.<sup>5,6</sup> These morphological changes are called storage lesions, which give red blood cells echinocyte, stomatocyte and spherocytic appearances as compared to normal discoid shape. Transfusion of such a blood to a patient with no other comorbidities might not have adverse consequences. However, if transfused to patients with comorbidities such as hepatosplenomegaly, the aged red cells might result in rapid clearance from the body. There is a need for quantitative measurement of morphological and biomechanical changes in relation to the duration of storage. The timing, extent and the variety of changes occurring in red cells over long storage have not been looked into in a comprehensive way. This study sets

out to determine storage induced light and ultra-microscopic, haematological, and biomechanical changes in red blood cells stored in CPDA-1 containing blood bags. This is the first of its kind to investigate the ultra-structural changes along with changes in osmotic fragility.

## Methods

This was a longitudinal study with a repeated measures design. It was carried out over a period of six months after an approval from Advanced Study and Research Board (DIR/KMU-AS&RB/TD/000547) and ethical approval from the institutional ethical committee (DIR/KMU-EB/TD/000335). Sample size was calculated using the statistical software G\*Power<sup>7</sup>. The apriori effect size of 30 percent was expected. Power of the study was kept at 80% and alpha error at 0.05. With two groups (fresh blood and stored blood) and repeated measures at 5 time points (days 0, 5, 10, 15 and 20), the sample size was estimated to be 20. In addition, literature was searched for similar studies<sup>3,8-10</sup> and their samples sizes averaged. Simple random sampling was done and twenty healthy male participants between the ages of 17-40 years were included in the study. Aims of the study were explained and informed consent to collect blood was obtained. Total of 250 ml whole blood was collected from each donor in 250 ml paediatric blood bag containing CPDA-1 solution (JMS, Tokyo, Japan) and stored in a standard blood bank refrigerator at +2 to +6°C. Subsequently, 5 ml of blood was drawn from the blood bags at baseline (within 4 hours of collection) and at an interval of 5- days on the 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup> and 20<sup>th</sup> day of storage. The drawn blood was used to assess the haematological parameters using Complete Blood Counts (CBC), osmotic fragility, a thin film for examination under light and scanning electron microscope (SEM).

For haematological changes, the erythrocyte count, Haemoglobin levels, MCV, MCH and MCHC were determined using haematological analyzer BC 3000 (Mindray, Schenzhen China) at different time points from day 0 to day 20. Morphological changes in RBCs were examined via light microscopy using Giemsa staining.<sup>11</sup> For making peripheral blood smear, a commonly used push (Wedge) method was applied.<sup>12</sup> The dried smear was fixed with absolute ethyl alcohol and stained with a Giemsa stain for about 10 to 20 minutes to allow good fixation. The smear was diluted twice with buffered water and was kept for 5 to 10 minutes to allow stain absorption. Slides were then washed with running water and placed to air dry.<sup>10</sup> Multi head light microscope NIKON eclipse 50i was used for the examination of peripheral blood slide. Initially the slide was examined under 10 × and then 20 × power magnification to determine overall quality of slide and distribution of RBCs on head, tail and middle area of the

smear. At 40 × magnification, four random fields were selected and images of the selected field were taken via camera installed with multi head microscope. For quantification of RBCs, a 10 × 10 μm grid consisting of multiple squared boxes was placed on image using the imaging software installed in multi-head microscope. The images were imported into the Image J software for quantification.<sup>11</sup>

After placing the grid and flattening the image, a total of 200 red blood cells were marked in each field. The counting began from the box on the top left corner of image and continued from top to bottom and left to right manner. Only the RBC located closest to the lower right corner of each grid box was selected. This was continued till a total of 200 RBCs from each field were counted and observed for abnormal shape. A grading criteria of Cora et al, was used to determine the proportion of distorted RBC in random fields.<sup>13</sup> According to this criteria, 1 to 5 distorted RBCs in one field were graded as 1+, while for 6 to 15 RBCs, 2+, 16 to 25 3+ and for more than 25 RBCs, 4+ was used. In this way, samples from day 0,5,10,15 and 20 were assessed for deformed RBCs.

The changes from normal discoid to pointed or bubble like extensions on the membrane surface of RBCs are difficult to examine under light microscope, therefore scanning electron microscope (SEM) JSM5910, JEOL, Japan was used to examine these changes.<sup>13</sup> For SEM, blood smear from all time points was prepared on a glass cover slip. A blood drop was put on the cover slip and spread along base of the slide width using smooth end of spreader to make a proper tail on the slide. The coverslip was kept in the Petri dish on filter paper soaked with phosphate buffered saline for 10 minutes at 37°C. The samples were then washed for 20 minutes. The prepared blood slide was air dried at room temperature and then fixed in absolute ethyl alcohol. After that, cover slip was fixed with aluminum tap and for conduction purpose, all sides of the slide were covered with silver paste and finally coated with gold.<sup>13</sup>

The slips were observed for various magnifications under SEM and images were obtained. The osmotic fragility test was performed to assess the haemolysis of RBCs in isotonic as well as at various concentrations of hypotonic solutions. During this test, the solution changes its colour from red to pale and finally turns transparent, at various concentrations of sodium chloride (NaCl), which is then measured with absorbance of 540nm on spectrophotometer. Approximately 50 μl of blood was added to 2 ml serial saline dilutions (0.9, 0.8, 0.75, 0.6, 0.4, 0.3, 0.2 and 0.2% saline) and mixed instantly. The tubes were incubated for 30 minutes at room temperature. The tubes were mixed again and then centrifuged for five minutes at 1200 to

1500g. A supernatant from individual tubes were taken to determine absorbance at 450 nm in a spectrophotometer. The osmotic fragility was computed using the formula:<sup>14</sup>

Percent lysis of every tube calculated by. Hypotonic tube absorbance  $\times$  100 Absolute tube 12 absorbance.

Data were analyzed using the statistical software SPSS (IBM) version 22. The quantitative variables are presented with Mean  $\pm$  Standard Deviation. For osmotic fragility, the percentage lysis was computed using the formula mentioned above, while for microscopic field analysis, Cora et al grading system was used and percentage of abnormal cells was determined. Independent t-test was used to compare the findings of osmotic fragility test, mean reduction in RBC counts and HB levels on day 0 and 20. For all the remaining comparative analysis, repeated measure analysis of variance (ANOVA) with Greenhouse-Geisser correction was used for multiple time points. Furthermore, pairwise comparison based on estimated marginal means at all time points was performed with Bonferonni adjustment for multiple comparisons. A p- value of  $\leq$  0.05 with 95% confidence interval was considered as significant.

## Results

In this study, all volunteers were male between the ages of 17 to 40 years, while the mean age was  $23.75 \pm 6.77$  years. Out of twenty, eighteen blood donors were first time donors and the rest of the two had previous history of blood donation. The baseline RBC count varied from  $4.15 \times 10^{12}$  to  $6.68 \times 10^{12}$  with a mean value of  $4.97 \pm 0.67$  count/L, while the hemoglobin ranged from 13.2g/dl to 19.5g/dl with an average value of  $14.81 \pm 1.50$  g/dl. The mean corpuscular volume (MCV) had an average baseline value of  $84.0 \pm 7.06$  fl, ranging from 60.5 fl, to 90.8 fl. While for Mean Cell Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), the mean values of  $29.62 \pm 2.51$  pg and  $32.6 \pm .35$  g/dl were observed, respectively.

The mean RBCs count was  $4.97 \pm 0.67 \times 10^{12}$  /L at day zero, while it was reduced to  $4.40 \pm 0.37 \times 10^{12}$  /L at day 20. The mean reduction in RBC counts from day 0 to day 20 was

computed to be  $0.57 \pm 0.30 \times 10^{12}$  /L (p- value  $\leq$  0.001). When using an ANOVA with repeated measures with a Greenhouse-Geisser correction, mean scores for RBC count were statistically significantly different ( $F(2.971, 56.449) = 10.602$  p $\leq$  0.0005). Pairwise comparison showed that RBC count on day 0 differed significantly from that on day 10, 15 and 20 (p $\leq$  0.0005) while no significant difference was found in RBC count on day 10, 15 and 20.

Similarly, during storage the Hb concentration reduced slightly but significantly with time. The mean reduction in Hb from day 0 to day 20 was  $1.81 \pm 0.64$  g/dl (p  $\leq$  0.001), while with repeated measure ANOVA, a significant reduction in HB concentration was observed over period of time ( $F(2.002, 38.027) = 20.429$  p  $\leq$  0.0005) and based on pairwise comparison, HB concentration differed significantly at all time points (pairwise comparison p  $\leq$  0.0005)

Non-significant variations were seen in MCV, MCH values over period of time (MCV p=0.313, MCH p=0.761) while for MCHC, values differed significantly (repeated measure ANOVA p  $\leq$  0.05). On pairwise comparison, the values at day 0 differed significantly with those on day 15 and 20 (p $\leq$  0.0005) (table 1). Semi Quantitative analysis of red blood cells was performed using criteria mentioned by Cora et al.<sup>13</sup> The analysis suggested that almost all the day 0 samples scored +1, while the remaining samples of day 5, 10, 15 and 20 had a score of +4. On day 0, only a small proportion of cells were abnormally shaped ( $2.05\% \pm 0.5$ ). With increasing storage time, percentage of morphologically abnormal red cells rose significantly to  $68.10 \pm 7.92$  on day 20 ( $F(2.643, 50.212) = 157.853$ , p  $\leq$  0.001, repeated measure ANOVA) (Figure 1f).

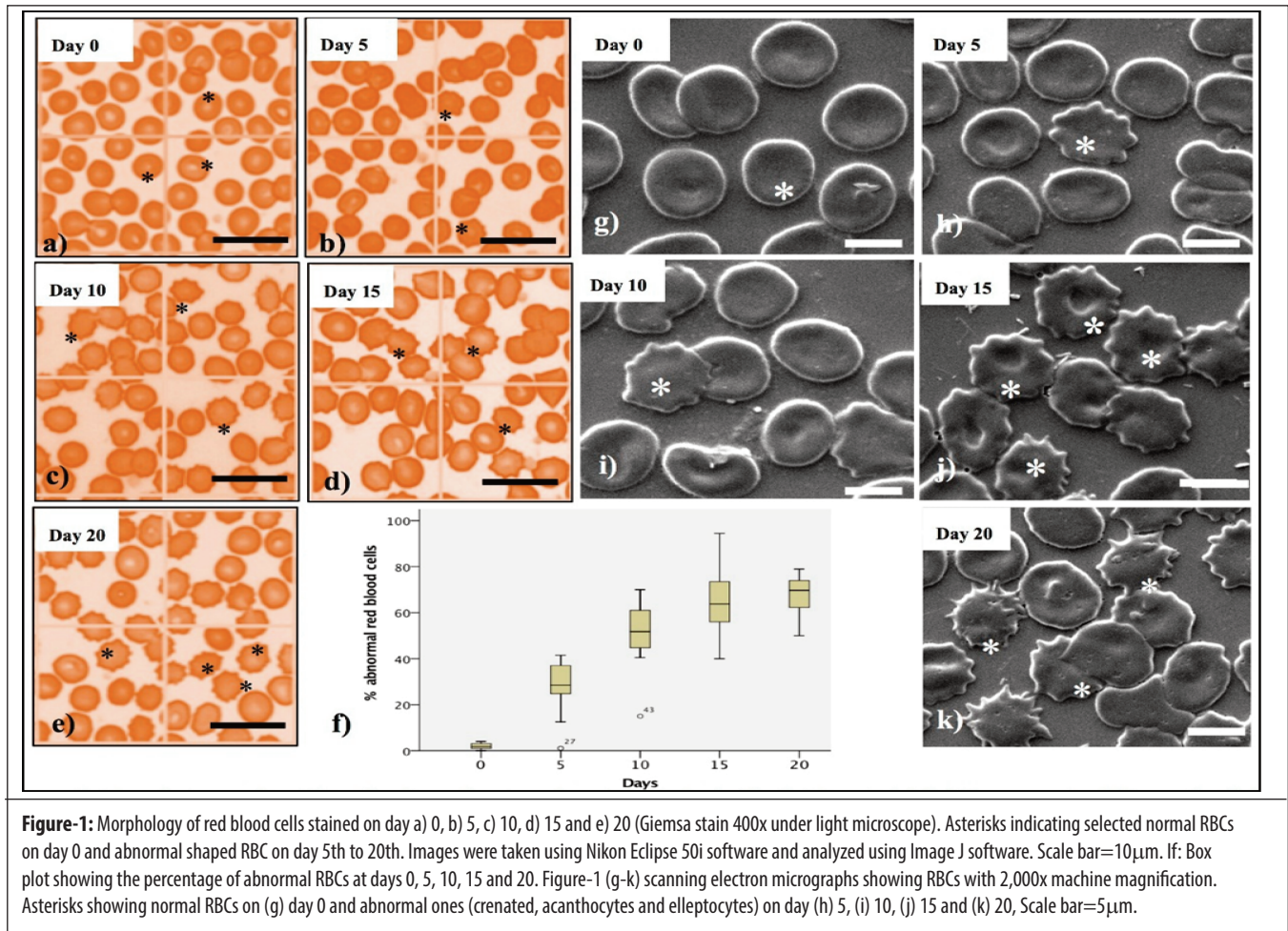
Furthermore, pairwise comparison with Bonferonni adjustment showed that proportion of abnormal cells differed significantly at all time points (Figure 1f).

On SEM, the number of abnormally shaped cells increased with each time point in all samples. On day 0, image of SEM showed normal biconcave discoid shapes of red blood cells, while on day 5 few RBCs appeared as acanthocytes, while on day 10, 15 and 20 the percentage of crenated cells (acanthocytes) and elliptocytes increased with visible multiple spikes on the outer membrane. On day 20, majority of cells appear to have lost the normal discoid shape and showed adhesion to each other. Notably, some cells showed doughnut shaped appearance with a central hole instead of central area of pallor (Figure 1g-k). There was a gradual increase in osmotic fragility of cells with increasing time points. The respective mean values of percent haemolysis for

**Table:** Showing mean (Mean  $\pm$  STD) of RBCs, HB, MCHC, MCH and MCV from day 0 to day 20 of stored blood

Parameters	Storage time (days)					p-values*
	0	5	10	15	20	
RBC (count/L)	$4.97 \pm 0.67$	$4.65 \pm 0.35$	$4.45 \pm 0.45$	$4.37 \pm 0.57$	$4.40 \pm 0.37$	<0.0005
HB (g/dl)	$14.81 \pm 1.51$	$13.65 \pm 0.80$	$13.12 \pm 1.05$	$12.92 \pm 0.74$	$13.01 \pm 0.87$	<0.0005
MCV (fl)	$84.01 \pm 7.06$	$84.27 \pm 6.99$	$84.18 \pm 6.88$	$83.88 \pm 6.46$	$82.97 \pm 7.49$	0.313
MCH (pg)	$29.62 \pm 2.52$	$29.61 \pm 2.44$	$29.39 \pm 2.52$	$28.88 \pm 2.62$	$28.83 \pm 2.65$	0.761
MCHC (g/dl)	$35.30 \pm 2.39$	$34.99 \pm 2.23$	$34.49 \pm 2.27$	$33.87 \pm 2.89$	$33.73 \pm 3.02$	<0.05

Calculated using repeated measure ANOVA, RBC: Red Blood Cells, HB: haemoglobin, MCV: Mean Corpuscular volume. MCH: Mean Cell Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration.

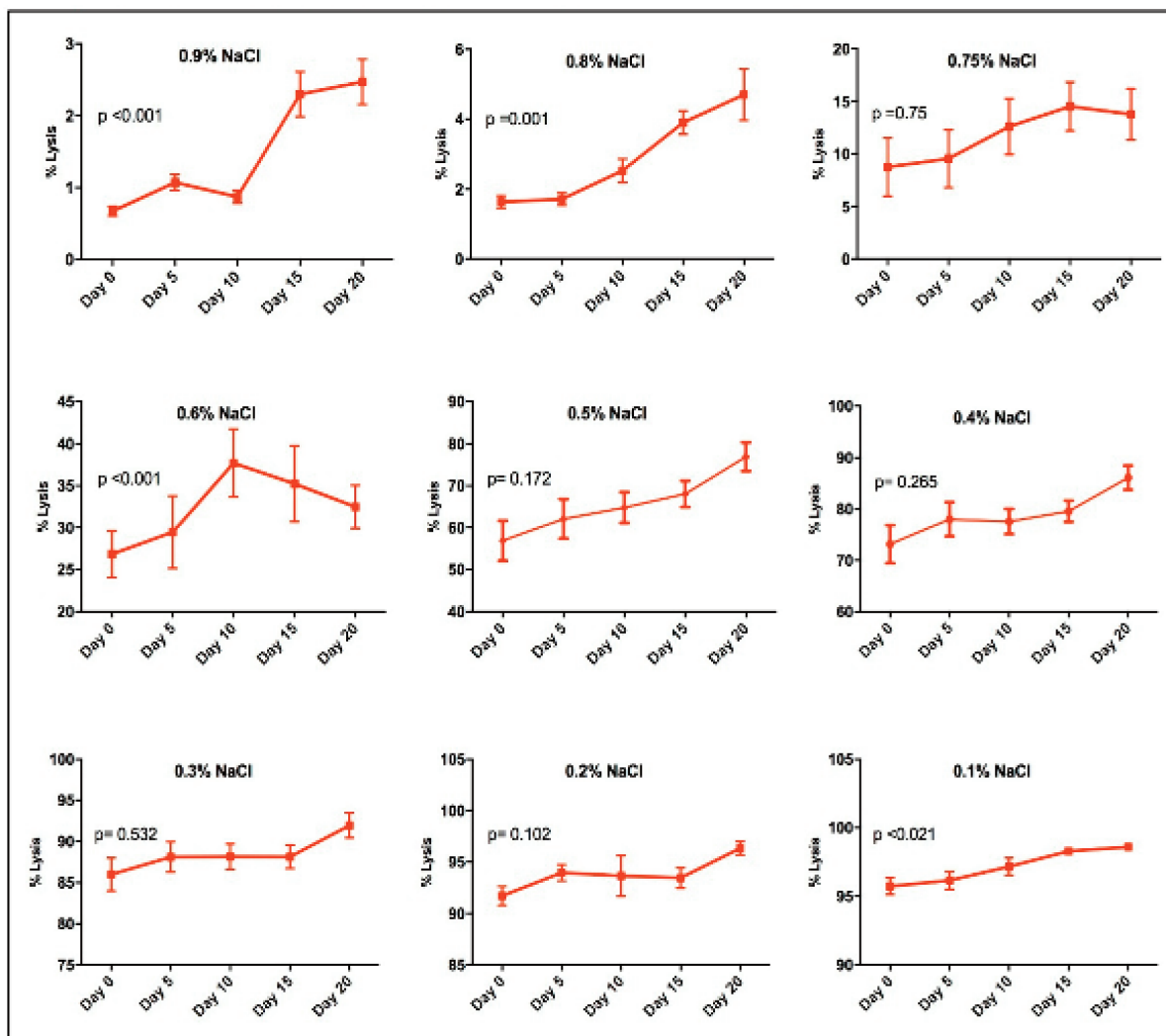


all time points showed that red cell lysis increased over period of time. Osmotic fragility under isotonic condition was noted on all time points compared to fresh sample. Less than 1% haemolysis was noted in freshly collected blood sample ( $0.67 \pm 0.09$ ), whereas 20-days old blood showed 2.4% haemolysis ( $2.47 \pm 1.4$ ,  $p < 0.0001$ ). With all the remaining saline concentrations i.e. 0.8%, 0.75%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2% and 0.1%, there was a gradual increase in the percent haemolysis over period of time and this difference was statistically significant for all concentrations except for 0.3%, 0.2% and 0.1% saline concentrations (Figure 2).

## Discussion

The aim of this longitudinal observational study was to record temporal changes in haematological, morphological and biomechanical properties of blood after storage in CPDA-1 blood bags. We, for the first time, showed morphological changes in stored blood using Scanning Electron Microscopy (SEM). We demonstrated that red cells become increasingly dysmorphic with loss of normal biconcave shape of red cells and appearance of crenocytes

and spiculated red cells.<sup>15</sup> In addition, haemoglobin concentration and red cell counts of stored blood showed significant reduction over a period of 20 days. Cells become increasingly prone to lysis in saline medium. With increasing duration of storage, RBCs begin to lose their normal biconcave shape as early as day 5 of storage and by day 20, most cells are acanthocytes, elliptocytes, spherocytocytes. These morphological abnormalities reflect disruption in the cells' cytoskeleton. These findings are consistent with previous reports with red cells stored in CPDA-1<sup>16</sup> and CPD-SAGM.<sup>17</sup> The latter observed morphological changes in RBCs through SEM with 14 days interval till 42nd day. Normal biconcave disc shaped RBCs were observed on day zero, while on day 14 significant morphological changes were seen. These changes could have happened as early as on 5th day as observed in our study, however, the different time points between the two studies might explain the discrepancy in the observed results. Also, since in our study whole blood instead of leukodepleted blood was used, this may also explain differences observed between the two studies. Similarly, irreversible morphological changes were observed under



**Figure-2:** Percent lysis at various concentrations (0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2% and 0.1%) of NaCl plotted against the time points (0, 5, 10, 15 and 20 days). Percent lysis was calculated by the formula: Hypotonic tube absorbance x 100/Absolute tube 12 absorbance. Data analyzed using one-way ANOVA.

SEM in 24% of the RBCs stored in SAGM blood on day 7.<sup>18</sup> These findings suggested that morphological changes in RBCs initiated earlier than the quantitative haematological changes. It has been proposed that ATP depletion in stored blood is directly related to red cell spherocytosis and membrane stiffness.<sup>9,13,18</sup> These abnormally shaped cells lose deformability and might be prone to rapid clearance in the reticuloendothelial system once transfused to the recipient. As to why not all the cells become equally dysmorphic, the most plausible explanation could be the differential age of red cells in the blood bag.<sup>19</sup>

A significant reduction in RBC counts and haemoglobin levels were observed with no changes in MCV, MCH and MCHC. The reduction in haemoglobin levels start as early as day 5. The plausible explanation to this observation is the haemolysis of the oldest cells in the stored blood. This

is also consistent with previous literature.<sup>15,19</sup> Free haemoglobin begins to appear in the plasma of CPDA-1 anticoagulate blood as soon as day 7 of storage, reflecting breakdown of red cells in blood bags during storage. There is also evidence to suggest that red cell senescence increases during storage. Tuo W, W et al,<sup>19</sup> demonstrated that the number of senescent red cells sharply increased after 14 days of storage. In another study, stored packed RBC were stored in adenine saline solution for 42 days and examined with 7 days interval.<sup>20</sup> They confirmed significant increase in RBCs haemolysis due to collapse of RBCs, started with 14 days of storage. In our study, we observed earlier haemolysis and consequent decrease in RBC counts. This relatively earlier haemolysis might reflect environmental factors such as higher room temperatures or issues with quality control of the blood bags. The insignificant changes

in MCV, MCH and MCHC results are consistent with previous reported literature.<sup>18-21</sup>

We also reported that red cell osmotic fragility increases significantly over time. Osmotic fragility reflects cells' ability to withstand lysis in hypotonic saline solution. The susceptibility to osmotic lysis is determined by surface-to-volume ratio and chloride substitution for 2-3 DPG.<sup>21</sup> Ageing red cells in blood bags lose biconcave shape and metabolic abnormalities. These cells when transfused to patients are rapidly cleared by the recipient's circulation. It has been previously shown that red cell recovery in patients transfused with old blood is significantly lower.<sup>21,22</sup>

This study was not void of constraints. Regarding impact limitation, the sample size was relatively small. However, since five further samples were acquired from each subject, the data has been based on a total of 100 samples obtained at different time points. The study was based on only two centers. The unavailability of osmium tetra oxide for electron microscopy might have affected the quality of electron micrographs, still clearly noticeable ultra structural changes were observed.

## Conclusion

Taken together, these findings suggested that the efficacy of transfusion may reduce due to increasing storage duration of blood, due to abnormalities in cell membrane resulting in increased fragility. Exact mechanisms of reduced cell stability including cell membrane protein-cytoskeletal abnormalities remain to be investigated. It is recommended that blood banks employ more efficient stock management systems in order to gain maximum benefit from each transfusion.

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**Conflict of interest:** None

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