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Research Article

Osmotic Fragility in Stored Non-Detergent Washed Human Erythrocytes

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ABSTRACT: Osmotic fragility (OF) of non-detergent washed erythrocytes was evaluated in Nigerian human male erythrocytes stored for 0h, 12h, 24h and 48h. Storage of these human erythrocytes for up to 24h did not significantly alter their membrane characteristics. Mean corpuscular fragility (MCF) diminished and correlated negatively ($r = -0.9668$) with storage time. Only the 48h storage-time MCF exhibited significant variance compared to 0h, 12h and 24h ($p < 0.001$). These results support OF-test data as reliable indices of alteration of membrane characteristics of non-detergent washed human erythrocytes stored for up to 24h. Non-detergent washing enhances erythrocytes OF test viability and hence the time window for meaningful OF test especially in situations of large sample volume.

Keywords: Osmotic fragility; Stored washed human erythrocytes; Mean corpuscular fragility.

INTRODUCTION

Osmotic Fragility (OF) test (OF test) is an empirical yet potent and relatively cheap test of membrane stability in both experimental investigations or in clinical evaluation of membrane based erythrocytopathies. Of recent, such tests have become valuable research tools especially in this era of "research grant-donor fatigue". When properly employed, OF test can also be of use in blood banking and clinical transfusion. The test exploits characteristic/ physiologic and structural changes which the erythrocyte membrane undergoes when the cell is subjected to osmotic stress. (Orcutt et al, 1995).

Variables such as techniques of blood 'sampling, handling and storage, changes in pH, temperature, blood gas level, age, humoral environment and other factors can influence OF test results. To illustrate the influence of some of these basic problems, a recent study investigated the OF of human male erythrocytes stored for up to forty-eight hours. The result of the study demonstrated that OF is a reliable index of changes in membrane characteristics of neat human erythrocytes stored for up to 12h. Beyond this storage time period, significant changes in membrane characteristics occur which may invalidate OF test data (Okwusidi, 2002).

Other current observations suggest that in addition to storage, the mode of collection and the preparative pre-assay processing of blood samples could influence assay test results (Farnett et al, 1999; Okwusidi, 2004). Specifically, in OF test assay, blood handling and preparative pre-OF assay processing of blood samples such as non-detergent washing of samples significantly impacts the erythrocytes OF-test (Okwusidi, 2004).

Taken together, these studies suggest a strong link between sample storage and preparative pre-assay blood sample processing. The effect of storage on the OF profile of non-detergent-washed human, erythrocytes stored for varying periods of time has hitherto not been described. This study was therefore conducted to evaluate the OF of non- detergent by

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washed human male erythrocytes stored for up to forty-eight hours.

MATERIALS AND METHODS

Blood Samples: Five *(5m1)* milliliters of blood samples were freshly collected from ten (10) apparently healthy consenting undergraduate medical students (*2 - 25* years), after an overnight fast. The samples were drawn into 10ml heparinized vacutainers. All blood samples were placed on ice upon collection. The samples were washed 3 times with 3 changes of ice cold phosphate buffered physiological saline (PBS, 0.85 NaC1) solution. The final erythrocyte pellets were then re-suspended in 5ml of washing buffer (0.85% PBS) solution to a final hematocrit of 43-*45%*, the approximate average hematocrit of the originally donated blood samples.

The blood samples were subsequently divided into 4 equal aliquots each stored for the designated period of time: 0h, 12h, 24h and 48h before the OF assay. Effect of temperature was kept minimal by maintaining all sample aliquots at 10°C until assayed (Okwusidi, 2004; Oyewale, 1993). Each aliquot was used once without a re-storage.

OF-Test: Osmotic fragility test was performed on the washed erythrocytes following standardized method essentially as previously described (Partpart, 1947; Oyewale, 1993; Okwusidi, 2002; 2004). Briefly, prior to assay; all samples to be assayed were allowed to equilibrate to the ambient (room) temperature for 5 minutes. The assays were conducted in a total final volume of 3ml using 1% phosphate buffered saline (1% PBS) solution, pH 7.7. The assay tubes were incubated for 30 minutes at room temperature; following which the samples were spun at 2500g for 10 minutes. Optical density of the resultant supernatants was determined spectrophotometrically at a wavelenth of *540nm* using distilled water as blank. The details of the content of each assay test tube are summarized in Table 1.

Statistical Analysis: All values are reported as mean ± standard error of the mean (SEM). The effect of storage was evaluated by comparing the mean. ± SEM of hemolysis of erythrocytes stored for 12h, 24h and 48h with the means (hemolysis) ± SEM of blood samples stored for Oh (Godfrey, 1985) using analysis of variance (ANOVA). A $p < 0.05$ was considered significant.

RESULTS

The hemolysis profile of non-detergent washed human erythrocytes stored for varying period of time are summarized in Table 2. For the control blood samples not previously stored (0h), evidence of hemolysis was noted at NaC1 concentration gradient of 0.45 — *5%*, concentration at which about 10% hemolysis had occurred. Hemolysis was essentially complete at NaC1 concentration of 0.2 — 0.1% where more than 90% erythrolysis had taken place. In blood samples stored for 12h and 24h hemolysis commenced at NaCl concentration gradient of 0.45 — *0.5%*, a salt gradient not significantly different from that of blood samples stored for 0h. In these latter blood samples, hemolysis was complete at NaC1 concentration of 0.1% (Table 2).

Hemolysis in the 48h-stored samples commenced at a salt concentration gradient of 0.4 — *0.3%*. At this salt gradient between 7-24% hemolysis was noted. More than 90% hemolysis occurred at a NaC1 concentration gradient of 0.1 - 0%. At 48h storage period, hemolysis of erythrocytes was significantly ($p < 0.001$) lesser than in the Hood samples stored for 0h, 12h or 24h at each hemolyzing NaCl concentration evaluated in this study (Table 2).

Table 1
Summary of Osmotic Fragility Assay Protocol

Tube	Blood vol. (ml)	1% PBS vol. (ml)	Distilled water vol. (ml)	Assay Final Total vol. (ml)	NaCl concentration
1.	0.02	0.00	4.98	5.00	0.00
2.	0.02	0.50	4.48	5.00	0.10
3.	0.02	1.00	3.98	5.00	0.20
4.	0.02	1.50	3.48	5.00	0.30
5.	0.02	2.00	2.98	5.00	0.40
6.	0.02	2.25	2.73	5.00	0.45
7.	0.02	2.50	2.48	5.00	0.50
8.	0.02	3.00	1.98	5.00	0.60
9.	0.02•	3.50	1.48	5.00	0.70
10.	0.02	4.00	0.98	5.00	0.80

Assay was conducted in a total final volume of 5m1. PBS, phosphate buffered saline

Table 2
Hemolysis (%) Profile of Non-detergent washed human Erythrocytes stored for varying Time Periods.

Storage Time (hr)	0.0	0.1	0.2	0.3	0.4	0.45	0.5	0.6	0.7	0.8
0h	100 ± 5	98 ± 9	93 ± 8	67 ± 10	32 ± 6	67 ± 10	7 ± 3	3 ± 2	2 ± 0.2	1 ± 0.1f
12h	100 ± 5	96 ± 8^{ns}	80 ± 8*	56 ± 5*	22 ± 3*	56 ± 5*	9 ± 2^{ns}	3 ± 0.5^{ns}	2 ±0.2^{ns}	1 ± 0.1^{ns}
24h	100 ± 5	93 ± 9^{ns}	77 ± 7*	52 ± 5*	25 ± 2*	52 ± 5*	9 ± 1^{ns}	03 ±0.5^{ns}	3 ±0.1^{ns}	2 ± 0.2^{ns}
48h	100 ± 6	82 ± 8**	53 ± 5**	24 ± 4**	7 ± 3**	24 ± 4**	2 ±0.5**	2 ±0.5**	1 ±0.5**	2 ± 0.2^{ns}

Hemolysis was measured spectrophotometrically at a wavelength of 540nm using distilled water as blank; ns = not significant vs 0h; *P<0.05; **P<0.05

Table 3
Summary of mean corpuscular fragility of non-detergent washed human erythrocytes stored for varying Periods of Time

Storage Time (h)	MCF x 10
0	35 ± 3*
12	32 ± 2^{ns}
24	29 ± 2^{ns}
48	19 ± 2*

*Mean corpuscular fragility (MCF) is [NaCl] for 50% hemolysis and was extrapolated from the plot of % Hemolysis vs [NaCl] (not shown); t, mean ± SEM; ns, not significant vs 0h; *p<005 vs (0h).*

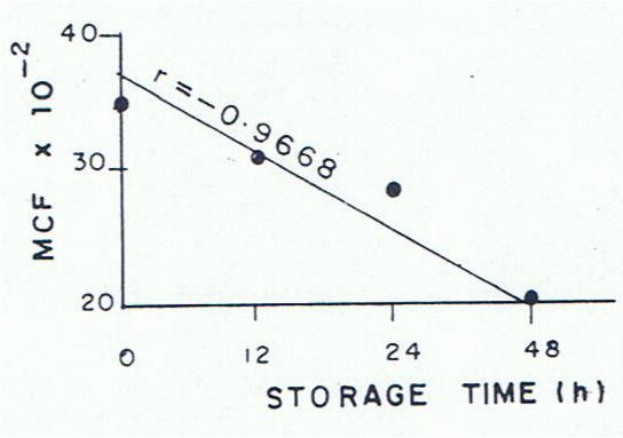

Figure 1
An illustrative non-linear correlation between mean corpuscular fragility (MCF) and storage time (h) of non-detergently washed human erythrocytes stored for: 0h; 12h; 24h and 48h; r=0.9668. The r-value does not necessarily reflect a steady r .te of OF with storage period. MCF is ENaC1] for *50%* hemolysis and was extrapolated from the plot of% Hemolysis Vs [NaC1] (not shown).

The mean corpuscular fragility (MCF) values of non-detergent washed erythrocytes are summarized in Table 3. MCF values were extrapolated from osmotic fragility curves. MCF represents the concentration of NaCI at which 50% hemolysis occurred (6). As depicted in Table 3, as these samples got older (longer storage up to 48h) they became more susceptible to lysis in hypotonic (lower NaCI gradient) medium. MCF decreased as storage time increased exhibiting a non-linear (or non steady rate of change in MCF) negative correlation coefficient. The r = -0.9668

Erythrocyte membrane stability was assessed by evaluating changes in MCF (Fig. 2). This was accomplished by normalizing the MCF data to the data of samples stored for Oh. The resulting values were then expressed as % of control. Obvious from fig. the greatest change was observed only in the samples stored for 48h when compared to 0h, 12h or 24h samples (p<0.05). There was therefore, no significant difference in variance of membrane stability of washed erythrocytes stored for. up to 24h.

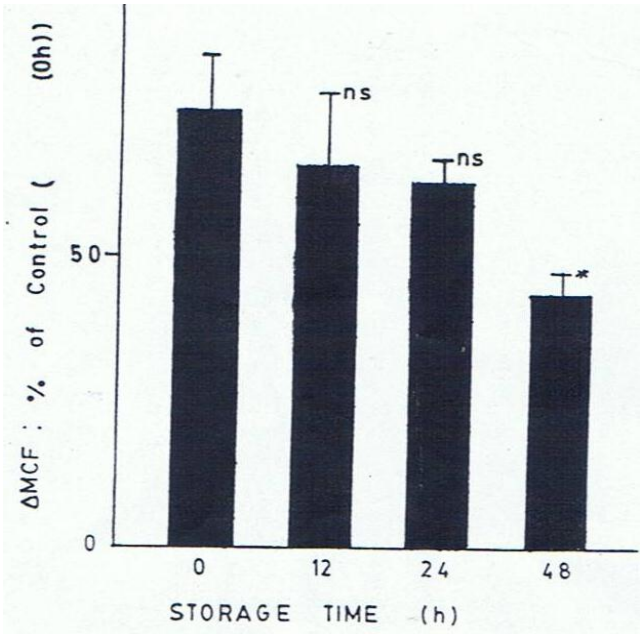

Figure 2
Changes in mean corpuscular fragility (AMCF) (% of control) as inoex if changes in membrane stability of non-detergent washed human erythrocytes stored fbr Oh, 12h, 24h and 48h. Vertical bars represent standard error of the mean (SEM); n, not significant Vs Oh; p<O.OS Vs Oh, 12hA24hfaiid '1-&l- MCF is {NaClj for *50%* hemolysis and was extrapolated from the plot of % Hemolysis Vs [NaCl] iot shown). 1Cf was obtained by normalizing the MCF data to the data of samples stored of 0h, Figure does not necessarily reflect a steady rate OF with storage period.

DISCUSSION

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 (Partpart, 1947; Murphy, 1967). Many problems frequently limit the utility and acceptability of the OF-test results and data. A host of factors (such as changes of pH, temperature, blood gas level, age. humoral environment and so on) influence OF and the values of OF-tests so obtained (Murphy, 1967, Mohandas et al, 1980). Consequently, various investigators tend to establish their own in-house normal OF- test values. Often these test values may not obtain in other laboratories. Another limitation to the viability and utility of OF-test results has to do delay that may come when analyzing large number of samples immediately upon collection (Oyewale, 1993). This study therefore investigated some aspects of this latter problem. Specifically, the effects of storage of non detergent washed human erythrocytes for varying periods of time (0h, I 2h, 24h and 48h) on the erythrocyte membrane osmotic fragility (OF) were evaluated in apparently healthy Nigerian male undergraduate medical students.

The results of this study demonstrated a storage-time (age) dependent modulation of the OF data of non-detergent washed erythrocytes stored over a period of time. Evidence from literature indicates a wide species specific variation in mammalian OF data. Whereas mammals such as goats and pigs with more fragile erythrocytes exhibit increases in fragility, erythrocyte osmotic fragility decreases with storage time in less fragile, more stable erythrocytes of cattle, mouse, rabbit and rat (Oyewale, 1993). Human erythrocyte on the other hand appears to exhibit a varied response to OF-test, depending on the ex-vivo age of the samples. Unwashed erythrocytes stay viable for OF-test assay for up to 12h (Okwusidi, 2002).

In the present study, storage of non-detergent washed human blood samples up to 24h did not significantly alter the erythrocyte membrane characteristics. This result is similar to the observations in the sheep erythrocytes data where storage for up to 24h had no significant effect on the OF-test. However, in this study, beyond 24h storage period, at 48h, decreases in OF were noted in human erythrocytes similar to the OF data obtained in rats, rabbits and cattle *(*Oyewale, 1993*).* After 24h storage and beyond, the washed human erythrocyte appears to become less fragile as storage time increases and OF decreases in these samples. Finally, changes in the OF data of these cells appear most pronounced at 48h storage where on the average a decrease in MCF occurred per rise in storage time.

In conclusion therefore, the results of the present study suggest that OF test values are viable indices of membrane characteristics in non detergent washed human erythrocytes stored for up to 24h. Non-detergent washing augments erythrocytes viability for OF test and hence extends by up to 12h, the time window for a meaningful OF assay in experimental investigations especially in studies where large samples are generated. Non detergent washing thus increases the patency of the test as an analytical test.

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REFERENCES

Orcutt, RH, Thurmond TS & Fersheu KE (1995); Mathematical modeling of the osmotic fragility of rabbit red blood cells, I. Pharmacol. Toxicol. Meth. 34(3): 169- 174

Okwusidi JI (2002); Forty-eight hour storage alters human erythrocyte osmotic fragility in Nigerian males. The Tropical Journal Health Sciences 9: 4 - 7.

Farnert, A. Arez Ap, Correia AT, Bjorkman A, Snounou G & do Rosario, V (1999): Sampling and storage of blood and the detection of malaria parasite by polymerase chain reaction. Transactions Royal Soc. Trop. Med. & Hyg. 93 (1): *50—53.*

Okwusidi JI (2004): Moderation of Osmotic fragility in non-detergent-washed human erythrocytes The Tropical Journal Health Science., 2004; 11:37 - 40.

Oyewale, J.0. (1993): Effect of storage of blood on the osmotic fragility of mammalian erythrocytes. *3.* Vet. Med. 1993: A40; 258 – 264

Partpart, AK (1947); The Osmotic resistance (Fragility) of Human Red *Cells,* J. Clin. Invest. 1947; 26 :636 cited in Dacie and Lewis: Practical Hematology 7ed. 1991 p. 196.

Godfrey, K. (1985): Statistics in practice. Comparing the means of several groups. N. Eng. J. Med.; 313: 1450—1456,

Murphy, JR (1967): The influence of pH and temperature on some physical properties of normal erythrocytes and erythrocytes from patients with hereditary spherocytosis. J. Lab, Clin. Med. 1967; 69: 758 - 775.

Mohandas, N., Clark MR., Jacobs, MS & Shohet SB (1980): Analysis of factors regulating erythrocyte deformability. *J.* Clin. Inv.; 66; 563 - 573.