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Prolong storage of blood in EDTA has an effect on the morphology and osmotic fragility of erythrocytes

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Abstract: Blood for various laboratory analyses are commonly kept in ethylenediamine tetra-acetic acid (EDTA). EDTA 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. The objective of this study was to investigate the storage effects of EDTA on erythrocytes morphology and osmotic fragility over a period of 4 days. A total of twenty- four (24) consenting, apparently healthy blood donors who passed the pre-donation screening were recruited for the study. Blood samples were collected into EDTA tubes and analysed for changes in erythrocyte morphology and osmotic fragility in 24 hour interval over the four day period. On Day 1(control), sample analysis were done within four hours after collection, they were then stored refrigerated (4 – 8^{o}C) and re-analysed from Day 2 to Day 4. Morphological changes observed in erythrocytes over time include echinocytosis, spherocytosis, sphero-echinocytosis and increase in rouleaux formation. Mean percentage haemolysis of erythrocytes increased from Day 1 to Day 4 ($p>0.05$). Again, the osmotic fragility curves of the RBCs exhibited a rightward shift suggestive of decrease in RBC membrane stabilization. Analysis of blood samples for haematological parameters should therefore be carried out as soon as possible, preferably within 4 hours after their collection to ensure clinically reliable results.

Keywords: EDTA, Morphological Changes, Erythrocytes, Haemolysis

1. Introduction

Laboratory testing is a very essential part of the clinical decision making process. The test results strongly influence medical diagnosis as well as the therapy applied [1]. In the clinical laboratory setting, the sequence of every test begins with the preparation of patient for the process, continues with collection, processing and analysis of samples and finally ends with reporting of the results [2]. Haematological 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 analysed [3]. Delayed sample analysis could result in haematological changes in the measured parameter, which could complicate the interpretation of the resulting data [4].

Blood testing is generally done on whole blood, plasma, or serum [5]. Whole blood is usually treated with anticoagulants to prevent them from clotting [6]. Ethylenediamine tetracetic acid (EDTA) salt (sodium or potassium) is regularly used as the anticoagulant in blood samples meant for routine laboratory analysis [7]. The EDTA anticoagulant action is based on inhibiting platelet aggregation and various reaction of the haemostatic cascade by chelating free calcium (Ca^{2+}) ions [8]. In fact, EDTA is the preferred choice for automated blood cell counts due to its general availability, ease of preparation, wide spread use and relatively low cost [9].

However, according to Rodak (1995), EDTA causes structural, biochemical and functional damage to blood platelets and other cells and the alterations induced are considered irreversible. EDTA effects on erythrocytes may include red cell crenation, spiculation and formation of echinocytes or Burr cells. There is often unacceptable

artefact of the blood cells from smears made from EDTA tubes that is kept at room temperature for more than five hours [10]. These artefacts are likely to be caused by a lysolecithin formation or fall in Adenosine Trisphosphate (ATP) as the blood is kept for a long time [11].

Keeping blood samples in EDTA tubes longer than normal before analysing 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 [12]. Identifying storage related changes is therefore important so that artefactual changes are not misinterpreted as pathologic findings.

2. Materials and Methods

2.1. Sample Collection and Processing

Samples from randomly selected replacement and voluntary blood donors were used for the study. During donation, 3 ml of whole blood was drawn from the satellite blood bag into a labelled di-potassium ethylenediamine tetra-acetic acid (K_2EDTA) tube. The blood collected was kept in a vaccine carrier with ice packs to maintain a temperature of 4 - 8°C before being transported to the laboratory for analysis. The samples were analysed within four hours of collection for osmotic fragility test and thin blood film to obtain the baseline values (Day 1). Subsequent daily analysis was done in 24 hours interval till the fourth day (Day 2 – Day 4). Day 2 was therefore equivalent to 24 hrs, Day 3 -48 hrs and Day 4 – 72hrs.

Leishman staining technique was used to examine the films for changes in erythrocyte morphology. Films were examined microscopically and comments made on each sample from Day 1 to Day 4 using the grading criteria of Cora et al (2012)**:** presence of altered RBC only in random fields**;** 1 to 5 altered RBC present in each field was scored as (1+)**,** an average of 6 to 15 altered RBC in each field (2+)**,** 16 to 25 altered RBC in each field (3+) and more than 25 altered RBC present in each field (4+).

For the Osmotic Fragility Test (OFT), a set of eleven (11) tubes containing 5 ml of NaCl solutions ranging from 0.0% to 0.90% were serially arranged in a test tube rack. 20ul of well mixed EDTA-anticoagulated blood was pipetted into each tube. The test tubes were allowed to stand at room temperature (25^0C) for 30 minutes followed by centrifugation at 3000 rpm for 5 minutes. The supernatant of each test tube was transferred into a plastic cuvette and the concentration of haemoglobin was measured at a wavelength of 540 nm using a Spectrophotometer (Aurora UV visible spectrophotometer, Aurora Instruments Ltd. Vancouver, Canada). The percentage haemolysis was calculated for each supernatant using the equation;

$$\%\ \text{haemolysis} = \frac{\text{absorbance of test}}{\text{absorbance of standard}} \times 100$$

Absorbance of standard = absorbance of haemolysis in 0.0% NaCl concentration (Fraukner and King, 1970). A fragility curve of percentage haemolysis versus NaCl concentration (%) was plotted. The Mean Cell Fragility (MCF) - saline concentration at which 50% haemolysis occurred was extrapolated from the curve.

3. Results

The outcome of the Osmotic Fragility Test (OFT) test and erythrocyte morphologic changes from Day 1 to Day 4 is shown in Figures 1-5. The RBC percentage haemolysis decreased as the saline concentration increased (0.0% – 0.9%) as seen in fig. 1. For the 3 different representative samples, Day 1 to Day 4 generally showed no haemolysis in 0.9% saline but 100% haemolysis in the standard saline concentration of 0.0% (distilled water only). Gradual increase in haemolysis from Day 2 to Day 4 as against Day 1 is again seen in all three samples with the most deviating being Day 4. Evidently, the osmotic membrane fragility curves exhibited a rightward shift (fig. 1 – Sample A -C).

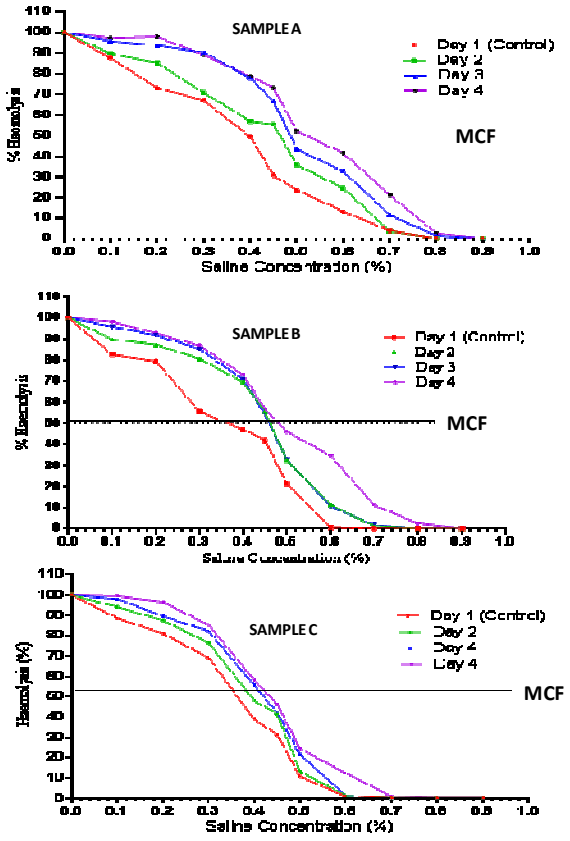

Fig 1. *Osmotic fragility curves of sample A–C stored from Day 2 to Day 4 as compared to Day 1(control).*

Mean cell fragility (MCF) values, representing the saline concentration in which 50% haemolysis occurred, were extrapolated from the osmotic fragility curves in fig.1. It was observed that increase in MCF occurred as storage days increased (fig. 2).

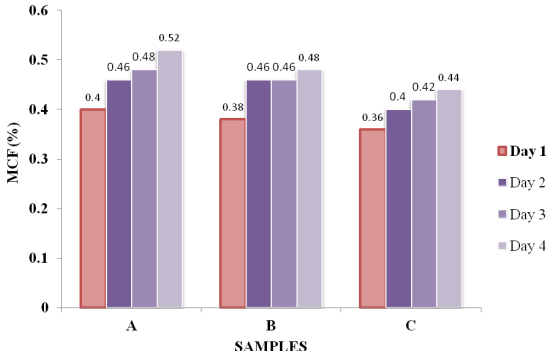

***Fig 2**. A graph of Mean Cell Fragility (MCF) of RBCs over the period showing increasing trends from Day 1 to Day 4.*

Corresponding thin blood films for sample A-C from Day 1 to Day 4 is shown in Fig. 3-4. The general morphology of the RBCs on Day 1 was normocytic normochromic. Morphological changes were however observed from Day 2 to Day 4. These included echinocytes (black arrow), spherocytes (blue arrow), and sphero-echinocytes (red arrow) increase in rouleaux formation. There was an average score of (1+) to (2+) for echinocytes and spherocytes whilst sphero-echinocytes score was (1+) to (2.5+) (fig. 3-5).

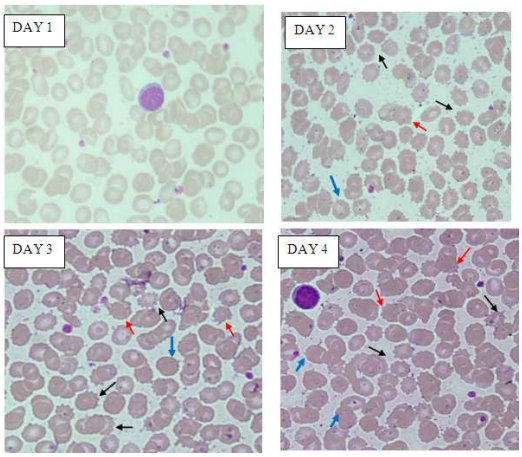

***Fig 3.** Leishman-stained thin films of sample A showing morphological changes from Day 1 to Day 4.*

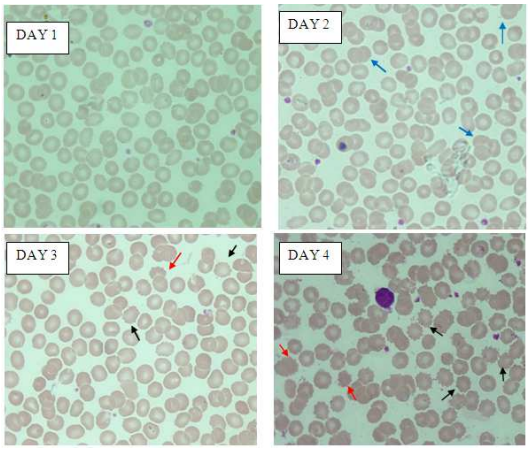

***Fig 4.** Leishman-stained thin films of Sample B showing morphological changes from Day 1 to Day 4.*

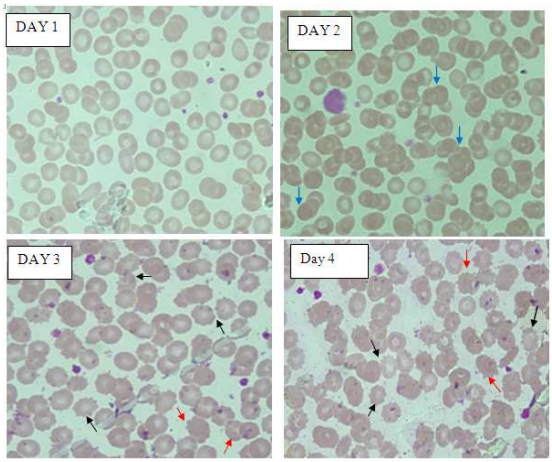

***Fig 5.** Leishman-stained thin films from Sample C showing morphological changes from Day 1 to Day 4.*

4. Discussion

Anticoagulated bloods for haematological analysis must be processed within the recommended time (4 hours) to avoid getting results that may not be reliable for patient treatment. This study confirmed that, storage of EDTA anticoagulated blood samples at 4 – 8^{o} C over a four day period (72hrs) causes some remarkable changes in erythrocytes morphology as well as their osmotic fragility. From the morphological findings, thin films prepared and stained within four hours of sample collection (Day1) generally appeared normocytic normochromic. This confirms the claims of Bain in reference [13] that immediate analysis of samples reduces artefactual changes in the samples which may be misinterpreted as pathological findings.

Increase in red cell size with time, due to degenerative changes that permits ingress of water into the cells was also observed. This observation was consistent with the findings in reference [2] as well as reference [12]. The latter also observed in their study that there was EDTA-induced erythrocyte swelling in stored fish blood. The findings of Cora et al. (2012) in their study on stored erythrocytes of rats also noticed this. They further indicated that the artefactual increase in RBC size should always be noted since it could mask microcytosis and lead to erroneous diagnoses of macrocytosis. Other morphological changes such as spherocytosis, echinocytosis and sphero-echinocytosis were observed from Day 2 onwards [11].

The osmotic fragility curves (fig. 1) showed an evident shift to the right as storage time increased. This is suggestive of storage time dependent membrane instability. Furthermore, increase in the RBC membrane instability was more pronounced by Day 4 where on average, more RBCs were haemolysed per unit rise in storage time. Also observed was increasing values of MCF for all the samples. It is known that as saline concentration increases, physiologically, fewer 'normal' RBCs should be haemolysing unless the RBC membrane has become compromised. In which way, even in higher saline

concentration, more RBCs will still be haemolysing. But for Sample A, in saline concentration as high as 0.52% as much as 50% of the RBCs haemolysed in Day 4 as against MCF of 0.40% in Day 1. Sample B and C also showed increasing MCF trend from Day 1 to Day 4. All these indicate that increasing storage days affected the membrane stability of the erythrocytes leading to increasing haemolysis.

5. Conclusion

The findings of the study showed that EDTA anticoagulated blood samples stored over time can lead to marked changes in erythrocytes morphology as well as their osmotic fragility. This is likely to result in wrong reporting and subsequently wrong diagnosis. Analysis of EDTA anticoagulated samples for haematological parameters should therefore be carried out within four hours after sample collection. It is recommended that in situations where the laboratory has decided to carry out the test on a sample that has been delayed, comments indicating the age of the specimen and its possible effects should be indicated clearly on the results. This way results misinterpretation will be avoided.

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