

**Red Blood Cells Surface Morphology in Diabetic Ketoacidosis****<sup>1</sup>Naglaa Kholoussi, <sup>1</sup>Iman Helwa, <sup>2</sup>Fahmy Amara, <sup>3</sup>Oguz Baskurt, <sup>4</sup>Nazek Abd El Latif El-Gindy, <sup>2</sup>Magdy Megalaa, <sup>5</sup>Mohamed Korani, <sup>6</sup>Mohamed Amara, <sup>7</sup>Mohamed Mahmoud Abd El Aziz**<sup>1</sup>*Department of Immunogenetics, National Research Centre, Cairo, Egypt.*<sup>2</sup>*Unit of Diabetology, Alexandria University, Alexandria, Egypt.*<sup>3</sup>*Department of Physiology, Akdeniz University, Antalya, Turkey.*<sup>4</sup>*Department of Industrial Pharmacy, Alexandria University, Alexandria, Egypt.*<sup>5</sup>*Unit of Diabetology, Menoufia University, Egypt.*<sup>6</sup>*Medical Department, Maridive Company for Maritime Services, Alexandria, Egypt.*<sup>7</sup>*Diabetes Specialist, Alexandria, Egypt.*

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**ABSTRACT**

The aim of this work was to study the possible changes in red cells morphology certain morphology in patients suffering from diabetic ketosis or ketoacidosis before and after treatment in comparison to normal subjects. **Material and methods:** A total of 50 subjects; 32 males, 18 females were enrolled in the study from the outpatient diabetes clinic and ICU for critical diabetic patients in the Unit of Diabetes and Metabolism, Internal Medicine Department, Alexandria Main University Hospital. All studied individuals were subjected to full history taking, complete clinical examination and routine laboratory investigations. Determination of Fibrinogen level by functional assay, lipid profile and urine analysis were included in investigations in addition to measuring whole blood and plasma viscosities Red blood cell surface morphology changes for patients and controls in this study were detected using Scanning electron microscopy (SEM). **Conclusions:** Erythrocytes experience various changes due to changes in plasma composition that occurs in diabetes mellitus. These changes were reflected on cellular parameters directly in the form of erythrocyte aggregation and deformability. Morphological changes were revealed by SEM as spheroidal aggregates, many flat cells in addition to spherocytes with surface &/or marginal irregularities. Echinocytes were also occasionally encountered.

**Key words:** Diabetes mellitus, red blood cell morphology, rheology, scanning electron microscopy.**Introduction**

The function of blood is to feed all the tissues of the body with vital materials and to remove waste. To perform this function effectively, the blood must circulate above a limiting rate. This rate of circulation is determined by the driving pressure generated by the heart, by the geometrical resistance offered by the vasculature and by the flow properties of the blood. These flow properties are the concern of the hemorheologist and they are dependent on the composition of the blood and the properties of its constituents; hence, knowledge of them is vital to understanding hemorheology (Shi and Lian 1978).

Rheology is defined as the science which deals with the deformation and the flow of materials under the action of the stress (Shi, 1977- 1986). It was first put forward by the American scientist Bingham and was established in 1929.

Nevertheless, hemorheology, deals with behaviors and the courses of blood and the relation to vessel and heart, which is studied the most in the biorheological fields.

Photographs of normal erythrocytes in native plasma resemble in appearance the red cells seen in light microscope, but the three dimensional appearance is accentuated. However, the cell rims are more pronounced and the central depressions seem more like holes (Kayden and Bessis, 1970).

Diabetes mellitus is a clinical term denoting a group of metabolic impairments which affect glucose utilization and lead to hyperglycemia. Type 1 diabetes is characterized by the complete absence of insulin, while type 2 diabetes is characterized by hyperinsulinemia and insulin resistance which precedes the development of hyperglycemia. Hyperglycemia may influence hemorheological parameters through enhanced advanced glycation end product (AGE) formation; reducing sugars may react non-enzymatically with the amino groups in proteins or lipids, ultimately leading to the formation of stable covalent adducts. AGE can bind to biological membranes in a nonspecific manner. They also induce specific cellular responses, including the release of pro-fibrogenic and pro-inflammatory cytokines and by interacting with receptor for AGE (RAGE) with high affinity. The consequence of AGE-RAGE interaction is the generation of reactive oxygen species (ROS) as well as particular consequences in endothelial cells, namely; induction of vascular endothelial growth factor (VEGF)

expression, prostacyclin production inhibition and plasminogen activator inhibitor-1 (PAI-1) synthesis stimulation. Accordingly, AGE stimulate the growth of microvascular endothelial cells, leading to angiogenesis on one hand and to a prothrombotic state on the other hand (Le Devehat *et al*, 2004, - Mellinghoff *et al*, 1996- Vekasi *et al*, 2001- Yonekura *et al*, 2005).

Positive associations have been found between parameters of glycemic control (HbA1C, fructosamine), fibrinogen levels and red blood cell aggregation. Studies have reported that insulin improves hemorheological abnormalities in diabetes, and when studied in vitro, incubation of red cells obtained from diabetic patients with insulin results in improved cellular deformability as measured by micropore filtration.

Nevertheless, it seems that hyperglycemia affects red cell rheology via direct effects on the membrane, including alterations of the lipid membrane bilayer composition and microviscosity and changes in membrane Na<sup>+</sup>/K<sup>+</sup> ATP-ase function (Thomas *et al*, 2005).

## Materials and Methods

A total of 50 subjects; 32 males, 18 females were enrolled in the study from the outpatient diabetes clinic and ICU for critical diabetic patients in the unit of Diabetes and Metabolism, Internal Medicine Department, Alexandria Main University Hospital.

The DKA group ( $n = 20$ ) included 11 males and 9 females, with a median age of 43 years (range 15–66 years). The other 3 groups; good control, uncontrolled and control ( $n = 10$  each) included 7, 9, 5 males and 3, 9, 5 females respectively. There was no significant association between sex and group. The median age in the good control group was 26 years (range 3–47 years), while that in the uncontrolled group was 38 years (range 19–55 years), and that in the control group was 28 years (range 19–43 years).

All studied individuals were subjected to full history taking, complete clinical examination. Laboratory investigations for all subjects enrolled in this study included random blood sugar (Rampling *et al*, 2004), Hemoglobin A1c complete blood picture (Chien, 1988), blood urea, and serum creatinine (Somer and Meiselman, 1993).

Prior to SEM for red blood cells samples were prepared as follows: blood samples (5ml) were received from each patient using wide pore needles. Red blood cells were washed in 0.9% saline, then fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3) for 4 hours followed by post fixation in osmium tetroxide (OsO<sub>4</sub>) 1% for 2 hours. Samples were then rinsed three times in the same buffer, and then dehydrated through a graded ethanol series from 10% to 100%; ten minutes in each one except for 100% ethanol where we repeated this step three times. All samples which were dehydrated by the critical point method, mounted on copper stubs with double sided adhesive tape then coated with gold using S150A Sputter Coater, Edwards/England. Finally specimens were viewed in a scanning electron microscope JXA-840A Electron Probe Microanalyzer-JEOL/Japan.

## Results:

A total of 50 subjects; 32 males, 18 females were enrolled in the study. The DKA group ( $n = 20$ ) included 11 males and 9 females, with a median age of 43 years (range 15–66 years). The other 3 groups; good control, uncontrolled and control ( $n = 10$  each) included 7, 9, 5 males and 3, 9, 5 females respectively. There was no significant association between sex and group. The median age in the good control group was 26 years (range 3–47 years), while that in the uncontrolled group was 38 years (range 19–55 years), and that in the control group was 28 years (range 19–43 years). There was no significant difference in age between groups.

Results revealed that there was no significant difference between different groups regarding CBC parameters.

Random blood sugar levels were significantly different between groups; adjusted pair-wise comparisons showed a significant difference between the DKA and the diabetic in good control.

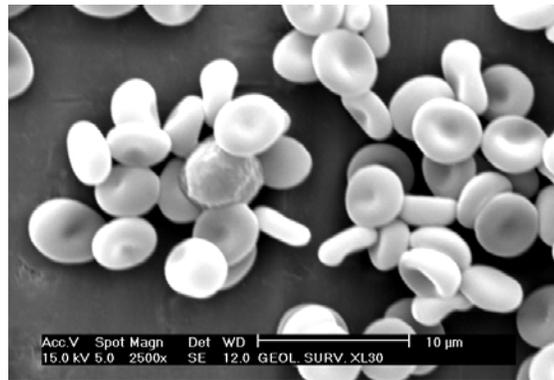
A significant difference was found between the DKA and the control group. The other pairs were not significantly different.

Moreover, HbA1c levels were significantly different between groups, where adjusted pair-wise comparisons showed a significant difference between control group and the uncontrolled diabetic group, while there was a weak significance between DKA and control group and between controlled and uncontrolled diabetic group. The other pairs were not significantly different.

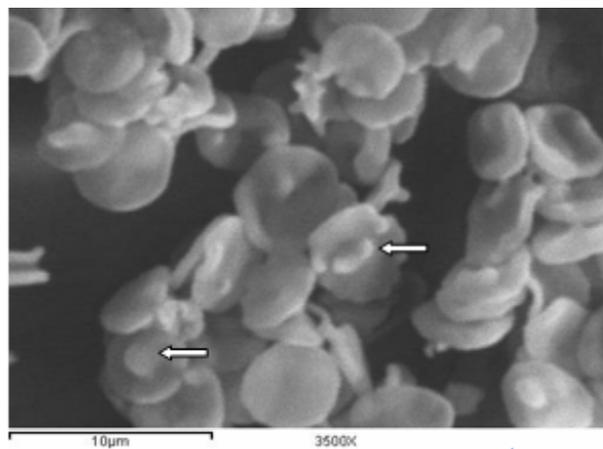
There was a significant difference in random blood sugar between the before and after measurements in the DKA group. Yet, there was no significant difference in Hemoglobin A1c.

Urea levels were significantly different between groups. Yet, there was no significant difference in the creatinine level between the different groups. Moreover, adjusted pair-wise comparisons showed a significant difference between the DKA and the control group while the other pairs were not significantly different regarding urea levels.

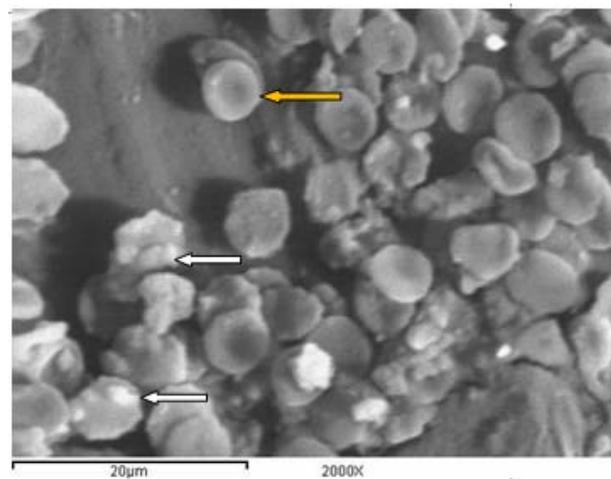
## Scanning Electron Microscopy results:



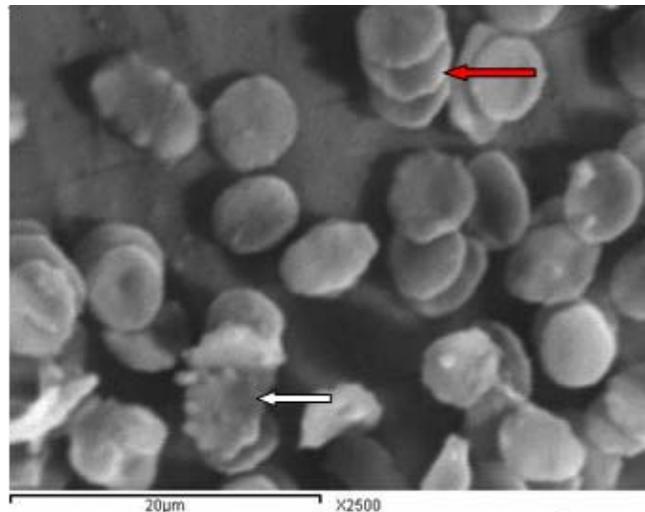
**Fig. 1:** Scanning electron micrograph for a collection of normal RBC's. They are normal in size) with mild variation in size & shape. Their biconcave shape is evident as a central depression. A leucocyte is seen among them (Mag.X2500).



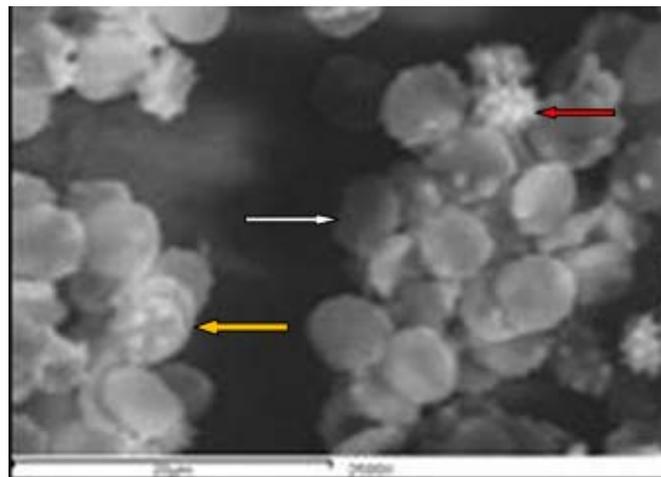
**Fig. 2:** Scanning electron micrograph (SEM) of red blood cells from a case of diabetic ketoacidosis before treatment showing spheroidal aggregates. Many flat cells and spherocytes are present; some of them show surface irregularity; in the form of surface protrusions (arrow) .Mag.X3500



**Fig. 3:** Scanning electron micrograph(SEM) of red blood cells from a case of diabetic ketoacidosis after treatment showing marked aggregation. Many flat cells and spherocytes are present (orange arrow); some of them show surface irregularity; in the form of surface protrusions (white arrow).Mag.X2000



**Fig. 4:** Scanning electron micrograph (SEM) of red blood cells from a case of diabetic ketoacidosis after treatment showing many flat cells and spherocytes; few of them show surface irregularity; margin irregularities are also encountered in few cells (white arrow). Occasional rouleaux can be seen (red arrow). Mag. X2500



**Fig. 5:** Scanning electron micrograph (SEM) of red blood cells from a case of diabetic ketoacidosis after treatment showing spheroidal aggregates (white arrow). Many flat cells and spherocytes are present; surface irregularity is frequently seen in the form of surface protrusions (orange arrow). Echinocytes (burr cells) are encountered (red arrow). Mag. X2500

#### Discussion:

Diabetes mellitus is a metabolic disorder characterized by varying or persistent hyperglycemia either due to insufficient or inefficient insulin action or improper utilization of glucose. Erythrocytes remain in hyperglycemic environment throughout their life span and thus are subjected to series of compositional changes, which in turn affects their flow properties through alteration of deformation and aggregation (Singh and Shim, 2009).

Devehat *et al*, 2001; reported that in diabetic patients without micro- and macroangiopathy there was an increase in erythrocyte aggregation associated with an increased fibrinogen level while albumin levels were decreased. Blood viscosity was reported to be significantly higher in patients with longstanding diabetes than in matched non diabetic controls and it was suggested that hyperviscosity and reduced erythrocyte deformability might be potentially important factors in the in the etiology or progression of microcirculatory disease in diabetes (Barnes *et al*, 1977).

In diabetic individuals, the elevated blood glucose stiffens the erythrocyte membrane, adversely altering the natural behavior of erythrocytes. One of the most important consequences of altered erythrocytes is the elevated

whole blood viscosity. This elevation is explained as the increased aggregation and reduced deformability of red cells. Furthermore, hematocrit was found to be elevated due to increased permeability of capillary vessel wall, which in turn increases the whole blood viscosity (Mooney, 2008).

In the present study there was no significant difference in sex or age between the four studied groups in accordance with McMillan in 1974 (Macmillan, 1974).

Regarding the glycosylated hemoglobin levels, there was a statistically significant difference between the four groups studied. This can be explained by the effect of long term hyperglycemia which is increased in uncontrolled individuals and much more increased in those with DKA together with an augmented effect due to ketone bodies.

Blood urea levels were significantly different between groups studied; while there were no significant difference in the serum creatinine levels. The difference in blood urea could be explained by the heavy diuresis and vomiting coding to dehydration in the uncontrolled and DKA groups. While serum creatinine was not different since in the short period of developing DKA there was not enough time for renal parenchymal damage.

It was reported that increased plasma fibrinogen and immunoglobulins have resulted in significant increase in plasma viscosity in patients with diabetic foot infection (McMillan DE *et al*, 1978).

It was reported that patients with type 2 diabetes mellitus show higher values of fibrinogen (Thomas *et al*, 2000).

It is known that there is a tendency towards hypercoagulation and disseminate intravascular coagulaopathy in patients with severe DKA and diabetic coma. This was found to be due to increase in the fibrinogen-fibrin degradation products (Alakhverdian and Koev, 1987).

In the present study, there was no significant difference in both total protein and total albumin between the different studied groups.

In the present study, SEM micrographs of blood samples of patients with diabetic ketoacidosis revealed several morphological changes compared to control samples. Red blood cells showed increased tendency to aggregation in the form of spheroidal aggregates in comparison to normal RBC aggregation which takes place in the form of rouleaux. Moreover, many flat cells and spherocytes were present; some of them show surface irregularity; in the form of surface protrusions while others showed marginal irregularities.

In addition to this, echinocytes (burr cells) were occasionally encountered.

These findings agree with (Cho *et al*, 2008) who found that diabetes markedly alters blood viscosity which is reflected as increased aggregation and reduced deformability of RBCs.

It was also stated that erythrocytes experience various changes due to changes in plasma composition which occurs in type 1 and type 2 diabetes mellitus (Blann *et al*, 1996). In accordance, Singh & Shin (Singh M & Shin S, 2009) stated that changes in cellular parameters directly correlate with erythrocyte aggregation and deformability.

Brown *et al* 2005 added that diabetic patients with renal insufficiency experience more impairment in red blood cell deformability.

Nevertheless, authors continued to explain that both hyperglycemia and impaired renal function could be remarkable factors that are important for developing red blood cell deformability and accumulation of AGEs.

It is worth mentioning that, growing evidence in the literature favors the association of haemorrhological abnormalities with diabetes mellitus (Rampling *et al*, 2004).

In 2012, Glaser *et al* stated that hyperglycemia is associated with impaired vascular endothelial function resulting in decreased vasodilation (Potenza *et al*, 2009), as well as with decreased erythrocyte deformability and alterations in blood flow properties.

In accordance, Petropoulos *et al* (Petropoulos *et al*, 2007) recorded several rheological disorders of the erythrocytes in diabetes mellitus as reduced deformability and increased aggregation and they also related these findings to the development of diabetic microangiopathy. Moreover; in 2000, Foresto *et al* , noted that the rouleaux pattern of RBC aggregate morphology characterizes normal aggregates while the formation of RBC clusters characterizes disease states and seems to be increased in the case of diabetic patients compared with normal controls; which come in agreement with our results.

They also reported remarkable difference in erythrocyte aggregation which increased in the case of diabetic patients compared with normal control subjects. Moreover, they added that aggregates of diabetic patients take a spheroidal shape compared to normal classical cylindrical shape aggregates known as rouleaux; both findings support our results.

#### Conclusions:

In diabetes mellitus, erythrocytes experience alterations in cellular parameters due to changes in plasma composition; namely, changes in erythrocyte aggregation and deformability.

By SEM, RBCs showed spheroidal aggregates, many flat cells in addition to spherocytes with surface &/or marginal irregularities. Echinocytes were also occasionally encountered.

There was no difference in morphological changes after treatment of diabetic ketoacidosis as revealed by SEM.

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