Changes in erythrocyte aggregation and deformability in diabetes mellitus: A brief review

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Diabetes mellitus (DM) is a metabolic disorder characterized by varying or persistent hyperglycemia either due to insufficient production of insulin by pancreas or improper utilization of the glucose. Erythrocytes remain in hyperglycemic environment throughout their life span and thus are subjected to series of compositional changes, which in turn affect their flow properties through alteration of deformation at individual level and aggregation at collective level. This brief review summarizes the changes in biochemical parameters primarily contributing to the erythrocyte deformability and aggregation as measured by various techniques, of blood samples obtained from diabetic subjects. The significant changes in erythrocyte aggregation and deformability, in comparison with that of control subjects show the relevance of these measurements. These changes are further supported by in vivo observations of blood flow through microvessels. Finally the relevance of these in combination with other clinical parameters is suggested.

Keywords: Deformability, Diabetes mellitus, Erythrocyte aggregation, Techniques

Blood, a carrier of metabolic products from and to the various regions of the cardiovascular system, is affected by the clinical status of the tissue environment. Due to arrival of altered levels of biochemical and tissue products in the blood and their interactions with blood constituents the functional properties of erythrocytes are changed, which in turn affect their hemotrological, i.e., blood flow properties under in vivo and in vitro conditions. At cellular levels the parameters which are affected include the aggregation and deformability of erythrocytes.

The shape transformation of erythrocytes while flowing through micro-vessels makes major contribution to flow resistance and is directly related to the erythrocyte deformability under in vitro and in vivo conditions. In combination with plasma proteins fibrinogen and globulins, the aggregation of erythrocytes, a reversible phenomenon related to formation of three-dimensional chain-like structure, takes place. As the aggregation process requires a specific shape of participating erythrocytes, each cell has to deform with intra-cellular layer of fibrinogen/globulin to form their chain-like structures. Thus the deformability individually and in combination for aggregation of erythrocytes is of vital importance to blood flow in cardiovascular system and under in vitro conditions.

Glucose is an essential nutrient for proper functioning of the body cells. In response to increase in glucose level in blood, insulin is released by the pancreas, eventually lowering the blood glucose and its transport to cells. Insulin therefore acts as a regulator of glucose metabolism in the body. Dysfunction of this auto-regulatory system results in lack of insulin and high blood glucose and thus leading to diabetic conditions. According to new classification while Type 1 diabetes mellitus (T1DM) is characterized by beta-cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency, Type 2 diabetes mellitus (T2DM) is associated with increasing blood glucose which can impair many body systems. Gestational diabetes mellitus appears in about 2-5 % of all pregnancies. It is temporary and fully treatable.

The increased glucose concentration may affect the basic functions of erythrocytes, related to exchange of metabolic products at the systemic and pulmonary capillaries. The biochemical alterations in plasma...
and erythrocytes in diabetes mellitus (DM) have
direct influence on the hemorheological properties of
cells. A critical assessment of the various parameters
and their effect on erythrocyte deformability and
aggregation in DM, as measured by various
techniques, is presented here.

Alterations in erythrocyte structure

Erythrocyte membrane lipids — Erythrocyte
membrane consists of two domains, the lipid bilayer
and cytoskeleton. The phospholipids are
asymmetrically dispersed in the bilayer. Cholesterol is
distributed evenly throughout the lipid domain, which
allows flexibility and provides stability to the
membrane. The cell membrane also contains
proteins and glycoproteins embedded in or attached to
the lipid bilayer. Proteins in the lipid domain are
asymmetrically organized forming integral proteins.
Structural changes in erythrocytes membrane are
found in the lipid bilayer at 0.6-0.8 nm below the
membrane surface. No essential immobilization of the
acyl-chains of phospholipids is found in deeper layers
as compared with the control group. As a result of these, the erythrocyte membrane becomes
rigid and non-deformable. The abnormal cells become
disrupted as they circulate through the
microangiopathic blood vessels. Diabetic subjects
showed a different erythrocyte age distribution, with
an almost double proportion of young red cells and
only one quarter of senescent ones, compared to those
of controls.

Erythrocyte membrane proteins — The
cytoskeleton of the erythrocyte membrane is
composed of several proteins including spectrin,
ankyrin, actin, and protein 4.1, forming a quasi-two-
dimensional meshwork under the lipid bilayer. Spectrin and actin are the two main structural
proteins, forming a sub-membraneous cytoskeletal
meshwork that is responsible for the viscoelastic
properties of the erythrocyte membrane. Labeling
studies of erythrocyte membranes with
[3H]borohydride, which labels glucose residues
bound to proteins, revealed that several proteins, in
particular beta-spectrin, ankyrin, and protein 4.1, in
diabetic membrane were heavily glycosylated
compared with nondiabetic erythrocyte membranes.

Enzymes and ionic balance — Serum and intra-
erthrocyte sodium and serum potassium levels are
increased significantly in diabetic patients as
compared to control subjects. The Na⁺/K⁺-ATPase
levels are significantly decreased which may cause
disturbance of intracellular ion balance and thereby
acceleration of cellular ageing. This further leads to
an increase in cell size and osmotic fragility, which
contribute to the disturbances in micro-vascular
circulation observed in DM.

The Na⁺/K⁺-ATPase and Mg²⁺-ATPase activities
of erythrocyte membranes of Type 1 diabetic patients
are significantly reduced compared to matched
diabetic controls, while the affinity of the pump for ATP is
lower in the membranes of Type 2 DM. This state is
further associated with excess uptake of calcium, and
diminished Ca²⁺-ATPase activity in cells in
comparison to healthy individuals.

Alteration in plasma components — Fibrinogen, an
acute-phase protein, and glucagon, a stress hormone,
are often elevated in many conditions of physical and
metabolic stress, including uncontrolled diabetes.
Thus, glucagon may be involved in the increase of fibrinogen concentration and fibrinogen fractional
secretion rate observed under stressed or pathologic
conditions. With the increase of these molecules
associated with accompanying thrombogenic tendency,
these cells are at much greater risk of a catastrophic
breakdown. In combination with platelets, the
diabetes of short duration was found to be associated
with a greater than normal number and size of
platelet-fibrin thrombi in the human retinal
capillaries. Another plasma protein albumin in
 diabetes is significantly reduced compared with
healthy subjects.

Oxidative stress — In erythrocytes from diabetic
patients, increased membrane lipid peroxidation might
lead to abnormalities in composition and function.
Diabetes erythrocytes have higher malondialdehyde
(MDA) (an indicator of lipid peroxidation) and
decreased glutathione (GSH) and membrane -SH
groups compared to normal erythrocytes. As quality
control system of the cell becomes overwhelmed,
conformational changes occur which lead to structural
transition from physiological protein to pathological
protein. Oxidative stress may be amplified by a
continuing cycle of metabolic stress, tissue damage, and
cell death leading to increased free radical production
and compromised free radical inhibitory and scavenger systems, which further exacerbate this stress. Morphological changes in erythrocytes — Analysis of blood flow under in vivo conditions revealed that erythrocytes are rigid compared to normal erythrocytes. These cells produce contortions and dilatation in capillaries as analyzed by finger-nail capillariscopy in children. The in vitro observation by a micropipette of 4 μm show the diabetic erythrocytes return to their discoidal shape less rapidly compared to control erythrocytes indicating an elevation of either intra-erythrocyte or membrane viscosity. This process is further associated with morphological alterations, resulting in significant decrease in the number of bowls and increase in discocytes.

Measurement of hemorheological parameters

There are various procedures for measurement of erythrocyte deformability and aggregation. Some of the recent techniques are as follows:

1. Measurement of erythrocyte aggregation — Erythrocyte aggregation as a reversible dynamic phenomenon can be observed both in vitro and in vivo conditions and is found to be responsible for much of the increase in viscosity at low shear rates. The aggregation combined with yield stress is expected to reduce blood flow compared to that of non-aggregating system. The plasma proteins, fibrinogen and globulins contribute significantly to the aggregation process. These aggregates disperse at high shear rates (or flow rates) and form again with the reduction of the shear rate to nearly zero. Primarily this is the rearrangement which is used as index of aggregation of erythrocytes. Some of the prominent techniques for measurement are given below:

Optical rheoscopy by light transmission and backscattering: Light transmission through a blood suspension varies depending on applied shear rate. In absence of formed aggregate the change in light intensity is due to cell orientation and deformation, and the level of hemoglobin oxygenation. With shear rate assumed to be constant in the couette flow, various techniques are Erythroaggregometer (EA; Regulest, France), Laser-assisted Optical Rotational Cell Analyzer (LORCA; Mechatronics, Netherlands) and Fully Automatic Erythrocyte Aggregometer (FAEA; Myrenne, GmbH, Germany). These instruments show significant correlation between the measured parameters.

Ultrasound scattering techniques: This procedure does not involve the shearing of the blood sample or erythrocyte suspension. This is carried out by backscattering of ultrasonic from blood sample placed in a chamber. The backscattered amplitude (BSA) is continuously monitored to determine the aggregation parameters of blood samples.

Microscopic image analysis: This is carried out in a rheoscope containing transparent cone and plate assembly. At a fixed shear rate the images of the formed aggregates are obtained. By processing these images a parameter, K-index, $K = 4\pi S/P^2$, is calculated, where $S$ and $P$ are the projected surface area and perimeter of the aggregates. By processing a sequence of images the velocity of aggregation and their growth process under various conditions are determined.

The aggregation/adhesiveness could further be analyzed by freshly prepared erythrocyte glass slide placed at an angle 30°. From the images of the formed aggregates the aggregation parameters are determined.

Optical hemorheometer: This is based on forward scattering of laser light passing through erythrocyte suspension of hematocrit 5% in plasma. During sedimentation of aggregates under gravitational field the transmitted intensity, associated with fluctuations, is increased. By analysis of this signal the aggregation process in terms of various parameters under dynamic conditions, is carried out.

2. Measurement of erythrocyte deformability — The shape transformation of erythrocytes is clearly observed during blood flow through in vivo capillaries and in vitro micro-channels of diameter lesser than that of erythrocytes. Under constant shear rate conditions the magnitude of deformation is used as a parameter related to erythrocyte deformability which is measured by various procedures. Some of the prominent techniques for this measurement are given below:

Change in optical diffraction pattern (ektacytometry) of erythrocytes: During stationary flow conditions in a rheoscope or microchannel the diffraction pattern of erythrocytes changes from circular to elliptic form. At constant shear stress the elongation index (EI), which is directly related to the deformability of erythrocytes, is determined.

Erythrocyte filtration through membrane: This is based on the measurement of passage time of erythrocyte suspension through micropore membrane, which is reciprocal of the erythrocyte...
deformability. For better correlation of this measurement the applied pressure should be comparable to that as in microcirculation, below 10 Pa. As erythrocytes flowing under low pressure may block the membrane pores, a low hematocrit (less than 10%) is preferable. The initial flow method, which minimizes the influence of gravitational field by operating within the specified range of applied pressure, has been used to measure erythrocyte deformability under varied conditions. The deformability is also measured from the change in erythrocyte count before and after filtration through a membrane under gravitational field.

Morphological analysis of erythrocytes: The morphological changes in erythrocytes during the disease process reflect the effect of the altered environment which is measured from microscopic images by variation in shape descriptors and application of wavelet transforms. The deformability of erythrocytes is measured by their shape alteration during constant flow conditions in the microvessels/microchannels. Similar measurement has also been determined by capturing the erythrocytes with optical tweezers and dragging these through a viscous fluid. The cellular parameters, as obtained by these procedures for individual erythrocytes, are comparable to that as obtained by micro-pipette technique.

Measurement of fluidity of erythrocyte membrane: The membrane, consisting of proteins with variability in cholesterol-rich phospholipids bilayer, is deformable with mechanical properties as elasticity, plasticity and viscosity. The fluidity is measured by paramagnetic labeling and fluorescence (photobleaching) methods. The photobleaching is consisting of a short and very intense excitation, which induces a photochemical destruction of a probe in the observation area of the order of a few square microns. The transport coefficients are determined from the kinetics of motion of the probe molecules from non-irradiated area to the observed area.

Alterations in erythrocyte aggregation and deformability

a. Erythrocyte aggregation — The erythrocyte aggregation depends on composition of the erythrocyte membrane and plasma proteins fibrinogen and globulin. The aggregation is increased with increased fibrinogen and decreased albumin. This is positively correlated with plasma concentrations of fibrinogen and alpha 2-macroglobulin, but negatively with plasma albumin concentration. The membrane anionic charge in diabetes is decreased which leads to increased tendency of erythrocytes aggregation. Fibrinogen production and plasma concentration increase in insulin-resistant type 2 diabetes. Enhanced fibrinogen production by insulin is likely to be a key alteration contributing to hyperfibrinogenemia and therefore cardiovascular risk in type 2 diabetes through aggregation of erythrocytes.

The erythrocyte aggregate morphology in diabetic patients, as analyzed by direct microscopic observation and numerical processing of recorded digitized images, show an increase in aggregate shape parameter, indicating significant increase in aggregate size, compared to that of controls. Analysis under dynamic conditions show that aggregation parameters such as aggregation size index, aggregate sedimentation duration and time of completion of sedimentation process are significantly decreased, indicating enhanced aggregation in diabetes subjects compared to that of controls. By cross-incubation studies it is observed that control erythrocytes are hyperaggregated when suspended in diabetes plasma but this was never found when cells of diabetic patients suspended in control plasma, indicating that plasma factors make significant contribution to aggregation process.

In diabetic patients without micro- and macroangiopathy the hemorheological disturbances are associated with erythrocyte hyperaggregation. These disturbances are not the consequences of chronic hypoxia and/or severe ischaemia but are likely among factors promoting the maldistribution of blood flow in nutritive capillaries. These disturbances may further lead to local acidosis and an increase in platelet aggregation contributing to endothelial cell damage.

b. Erythrocyte deformability — This parameter depends on the composition of membrane, cytoplasmic contents and age of erythrocytes. Excessive availability of glucose within the cell leads to formation of HbA1c at a higher rate. The ektacytometry measurement shows that the erythrocyte deformability is significantly decreased. Similar decrease in deformability by filtration techniques has been observed. In measurement with transparent microchannels similar decrease in erythrocyte deformability in diabetes patients is reported. Under in vitro conditions by incubation of normal erythrocytes in high
concentration of glucose (50mmol/l) a reduction in deformability has been observed. In another study by Petit et al it has been shown that the presence of more rigid cells is responsible for decrease in deformability in diabetes, as detected by nickel filters compared to that of polycarbonate filters. The initial red cell filtration ratio relative to buffer (mean±SD) of diabetic patients (type 1 and type 2) was significantly impaired. Thus the majority of the techniques indicate that there is a part of the cell population which is affected by the diabetes process and these erythrocytes are contributing to their decreased deformability.

The impairment of erythrocyte deformability is attributed to the specific changes in the membrane structure. The oxidative stress due to high glucose concentrations causes damage to the erythrocyte membrane proteins, even in a relatively short exposure time. In diabetes the ratio of cholesterol to phospholipids in the core of the membrane is altered; leading to reduced deformability of erythrocytes. The significant increase in the index of rigidity (IR) in diabetes compared to that of controls is attributed to significant glycosylation of membrane proteins, which is primarily responsible for significant decrease in erythrocyte deformability. Alterations in membrane lipid-protein interactions together with the increased glycosylation-derived internal viscosity may consequently imply altered viscoelastic properties of erythrocyte membranes and underlying the impaired deformability of red blood cells in the diabetic state. An alteration in the deformability of erythrocytes with changes in lipid composition of the membrane, suggests a relation to the altered enzyme activity in red blood cells and the development of diabetic complications. This impaired deformability can increase the blood viscosity leading to increase in shear stress on the endothelial wall.

c. Change in erythrocyte membrane fluidity of lipid region — Changes in the fluidity and composition of human blood plasma and erythrocyte membranes, in Type 1 diabetes are investigated. The increased microviscosity of erythrocyte membranes provides unambiguous proof of the structural deterioration in diabetes. The erythrocyte membrane fluidity in vascular atherosclerotic disease associated with type 2 diabetes is less than that of normal cells. In vitro studies, as carried out by incubation of erythrocytes with glucose (2g/l for 2, 4 and 8 hr) further show a progressive decrease in membrane fluidity. From these studies it is evident that glucose enhancement in plasma produces specific alterations in the erythrocyte membrane.

d. Flow and morphological changes — In vivo changes: In the nailfold capillaries in both types of diabetes the reduction in capillary perfusion is due to decrease in deformability of erythrocytes. In addition to stiffened red blood cells, diabetics also have increased plasma concentration of fibrinogen and capillary leakage leading to loss of albumin and water. There is an increased tendency for diabetic platelets to aggregate. The end result is increased hemorheological parameters and sluggish microcirculation. These parameters could lead to decreased TcPO2 in patients with chronic leg hypoxia or severe leg ischaemia.

In vitro changes: When erythrocytes are ejected from a micropipette of 4µm diameter they return quickly to a discoid shape using stored elastic energy. Slow motion photography revealed that diabetic erythrocytes restore their shape less rapidly than nondiabetic erythrocytes, indicating that their reduced deformability is due to an elevation of either intra-erythrocyte or membrane viscosity. The erythrocyte morphology index (EMI), as determined by the ratio of number of bowls (most deformable cells) to discocytes (stiffer form), correspond to the decrease in the deformability of erythrocytes indicating the increase in discocytes.

The morphological changes have been directly represented in terms of shape descriptors which are calculated from the contours obtained by processing of erythrocytes images. These descriptors are projected area and perimeter of erythrocyte. Based on these the form factor (FF), given as the ratio of projected square perimeter to area of erythrocyte or membrane viscosity, is calculated. The increase in the FF is correlated with the increase in filtration time of the erythrocytes, indicating the deviation from circular shape and decrease in deformability.

Conclusion and future perspectives

Diabetes mellitus has been on the increase internationally. Out of different types, type 2 diabetes is increasing more than others. Impairment of glucose utilization is the source of minor and major changes in the cardiovascular system. Erythrocytes are continuously subjected to various changes due to compositional changes in plasma associated with
some variations in type 1 and type 2 diabetes mellitus\(^9\). Presently it has been established that there is a direct correlation between alterations in cellular parameters and erythrocyte aggregation and deformability. The latter parameters even may help in predicting the occurrence of severity of changes in cardiovascular system. Progressive impairment of erythrocyte deformability is found to be as indicator of microangiopathy\(^9,10\). The existing sophisticated technologies along with newer development\(^10\) have contributed in determining the changes in these parameters, which are induced by the disease process. The variation in these parameters indicates the occurrence of the changes associated in blood flow, which could even be informative to show the changes in healthy subjects of various age groups\(^10\).

In vivo and in vitro studies show that the aggregation and deformability occur in the cardiovascular system\(^12\) and glass microchannels\(^52,103\). In the diabetes all the erythrocytes are not affected. The severity of change in erythrocyte shape depends on plasma glucose level\(^65\). The morphological changes in DM could also be related to levels of biochemicals in plasma, and erythrocyte deformability and aggregation through neural network procedure, similar to one reported for malaria, for prediction of the disease process\(^48\).

From this development it is evident that the microhemorheological parameters are of vital importance as this could predict, to some extent, the changes in blood flow in cardiovascular system. In fact, technology and its precision, over the last two decades have improved significantly. The size of the sample collection has also been reduced. Still there is a need for speedier process to measure these parameters through computerized procedures. This may require series of experiments dealing with blood samples of healthy and diabetic subjects and glucose enrichment process of erythrocytes under in vitro conditions. The present technique of glucose enrichment by incubating erythrocytes in glucose solution in phosphate buffered saline is not sufficient as this is not an ideal representation of autologus plasma. A combination of several constituents may help in simulating the plasma for enrichment of glucose in erythrocytes.

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