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Alterations in Zeta Potential and Osmotic Fragility of Red Blood Cells in Hyperglycemic Conditions

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Abstract

The zeta potential is an electrokinetic property of red blood cells surface and in different disease conditions this property of the erythrocytes varies. The objective of the present study was to evaluate the alteration in zeta potential, lipid peroxidation and osmotic fragility of erythrocytes in hyperglycemic conditions. The zeta potential of the RBCs was measured using Zeta meter System 4.0. Lipid peroxidation, an indicator of tissue injury induced by reactive oxygen species was measured by the thiobarbituric acid assay (TBA). The percent haemolysis in terms of osmotic fragility was determined using UV-Vis spectrophotometer (JASCO). The mean erythrocytic ZP of the control group was found to be 22.13±0.2789 mV whereas, erythrocytic ZP for diabetes mellitus patients was found to be 8.559±0.4864 mV. Similarly, when erythrocytic ZP of control pregnant women was measured, and it was found to be 21.07±0.3393 mV which were slightly lower than a control group. Mean ZP of GDM patients was found to be 10.12±0.2294 mV which was significantly less than both control group and pregnant control group. Variations in zeta potential values were accompanied by increased osmotic fragility of RBCs. It was also observed from determination of lipid peroxidation of erythrocytes, that there was formation of higher concentration of malondialdehyde with the erythrocytes of hyperglycemic patients compared to control group. The findings suggest that the zeta potential value of erythrocytes can act as a key indicator for demonstration of increased oxidative stress.

1 Introduction

Diabetes is a chronic illness which needed continuous medical care with different risk-reduction strategies beyond glycaemic control[1]. Diabetes is an emerging public health problem and it is the major challenge to improve the long term health status of the diabetic patients[2]. In 2030, the prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4 %. With the rising prevalence of obesity & diabetes in younger population groups or family history of diabetes, women are at high risk of gestational Diabetes. As per American Diabetes Association gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. The prevalence depends on the population studied and the diagnostic tests employed may range from 1 to 14% of all pregnancies. As changes in membrane structure also contribute to the pathophysiology of the disease process, nowadays there is a growing interest in characterizing RBC membrane defects in several diseases.

Human RBCs contains about 95% of the glutathione which is responsible for scavenging reactive oxygen species[6]**.** Sialic acid is an important factor for maintenance of the surface electrical charge and stability of biological cellular system[7]. In hyperglycemic conditions, there is auto oxidation of glucose and non-enzymatic glycation of proteins, which are the main factors contributing to the production of reactive oxygen species (ROS) and there is excessive accumulation of oxidative stress within RBCs which reflects into cellular damage by generation of lipid hydro peroxides and detachment of sialic acid residues[6]. As a

result of alteration in the main contributor of the net negative surface charge i.e. carboxyl group of sialic acid in RBC extracellular membrane, there is an alteration in zeta potential value, indicating the extent of injury to its membrane[2].

Similarly, Osmotic Fragility index is a measure of the resistance of red blood cells to lysis by osmotic stress and resembles RBC deformity associated with the electro kinetic properties of the membrane[8]. Erythrocyte membranes are rich in polyunsaturated fatty acids; they are continuously exposed to high concentration of oxygen and powerful transition metal catalyst. In the form of phospholipids, polyunsaturated fatty acids are present in the erythrocyte membrane which appears to be particularly sensitive to oxidation damage, and this result in the formation of a lipid hydoperoxide and a new lipid radical[9]. Thus, by measuring the Lipid peroxidation of erythrocyte membrane, the stressed condition of the diseased RBCs can be evaluated.

In the present study, zeta potential (ζ) and osmotic fragility was evaluated which accounts for membrane deformity in hyperglycemic condition, and comparison were done between blood samples from normal human beings as control vs. diabetic patients and control pregnant women vs. gestational diabetic patients. Also, the study explored the effect of an oxidative stress condition on human erythrocytes by measuring lipid peroxidation of RBC membrane, which results in the formation of malondialdehyde.

2 Materials and Methods

2.1 Materials

Dextrose (Merck), Tris-buffer, EDTA, Thiobarbituric Acid, Trichloro acetic acid, HCl, Distilled water, Lancet, Rectified spirit, Zeta meter system 4.0

2.2 Clinical sample

Blood was collected from voluntary donors with history of Type 2 DM (n=26), Control pregnant women (n=60) and GDM patients (n=27), under treatment in Dalvi memorial hospital and research centre and Dr. Varma Pathology Laboratory, Nagpur. The control blood samples (n=84) were collected from healthy voluntary donors of the Department of Pharmaceutical Sciences, Nagpur University, Nagpur. None of the subjects (both control and patients) were addicted. Each volunteer provided written consent for the study of their blood sample.

2.3 Preparation of Isotonic Dextrose Solution

A 5 % w/v Dextrose solution was prepared by dissolving 5 g of anhydrous Dextrose (Merck) in 100 ml of distilled water.

2.4 Preparation of Blood suspension for Zeta potential measurement

About 0.04 ml of blood sample was transferred into 50 ml of freshly preparation 5% w/w dextrose to maintain osmotic pressure inside and outside the cell.

2.5 Estimation of zeta potential of prepared blood sample by Zeta meter System 4.0

The zeta potential of the RBCs was measured using Zeta meter System 4.0. Zeta potential is purely an electro kinetic property of the electrical double layer surrounding the system but the surface of the system itself. The value of zeta potential gives an indication about the stability of the system under study. This quantity is measured by determining the mobility/velocity of the particle under an applied electric field. The value of zeta potential can be obtained from the equation given by Helmholtz-Smoluchowski.

$$\zeta_d = (4\pi\eta/\epsilon)\, V$$

Where; ζ_d = electro kinetic potential/zeta potential, η = viscosity of dispersion medium, ϵ = dielectric constant of the dispersion medium, V = v/E (mobility of the particle), v = velocity of the particle in cm/sec, E = potential gradient in V/cm[10].

A special capillary cell called electrophoretic cell is used for the measurement of zeta potential. The capillary is embedded inside a chamber having electrodes at either of the two ends. Sample is placed from any one end of the electrophoretic cell and electrodes are connected to the cell and electric field at specific voltage is applied (200 V). Charged particles move towards oppositely charged electrode and their velocity is measured and expressed in terms of electro kinetic potential/zeta potential, which indicates the mobility of particle under applied electric field. Recently this method is widely used for determining the membrane potential of biological membranes.

In this experiment, fresh capillary blood samples were obtained from volunteer and blood suspension was prepared as described in above procedure. Prior to zeta potential measurement temperature of the RBC suspensions were measured and detection parameters for ZP measurements such as light intensity, focal plane and tracking duration were optimized for stable data collection. The RBC suspensions were then added to the previously cleaned and calibrated (using min-u-sil) zeta-meter cell placed under the zeta-meter stage and the mobility of individual RBCs was tracked by equipped Zeta meter-ZM4DAQ software using microscopically-acquired video images, and data were recorded 10 times for each sample and average zeta-potential in mv was determined using a standard Helmholtz–Smoluchowski formula.

2.6 Preparation of red blood cells and erythrocyte membranes for lipid peroxidation and osmotic fragility test[11,12]

The blood of healthy volunteers, Type -2 diabetic patients, Control pregnant women and GDM patients was drawn, and collected in vial containing EDTA solution (anticoagulant). Further, RBCs were separated from plasma by centrifugation at 3000 rpm for 10 minutes, followed by washing them thrice with

310 imOsM tris buffer pH -7.6, maintaining the temperature at 4 °c throughout the procedure. The washed cells were then suspended in the same buffer and used for testing.

2.7 Measurement of lipid peroxidation of erythrocyte membrane

Lipid peroxidation, an indicator of tissue injury induced by reactive oxygen species will be measured by the thiobarbituric acid assay (TBA). In this malondialdehyde (MDA) formed was measured based on the colour reaction of lipid peroxides with TBA, resulting in pink pigment with an absorption maximum at 532nm[13]. For this purpose, about 0.5 ml of prepared sample was reacted with 2 ml of TBA reagent containing 0.375% TBA, 15% trichloroacetic acid and 0.25N HCl in a glass centrifuge tube. Samples were then boiled in water bath for 15 minutes, cooled and centrifuged. Absorbance of the supernatants will be spectrophotometrically measured at 532 nm. Malondialdehyde formed was calculated using extinction coefficient of 1.56 $x10^5$ $M^{-1}cm^{-1}$.

2.8 Measurement of osmotic fragility of RBCs

In this test, different strengths of NaCl solution, from 0.2% to 0.9% concentration was prepared. To 5 ml of each solution of varying concentration, a fixed small volume of packed RBCs were added. Similarly, sample was prepared in double distilled water, which was considered as Standard. The amount of lysis in each mixture was compared with the standard mixture in double distilled water (100% lysis), at 415 nm using UV-Vis spectrophotometer (JASCO). The percent haemolysis in terms of osmotic fragility was determined.

2.9 Statistical analysis

The resulting experimental data are expressed as mean ± standard (SD). The statistical significance was evaluated by unpaired t-test with software PRISM 5. Differences between the groups were considered significant at $p<0.05$ which indicates that the control and other patient groups differ significantly from one another in all situations.

3 Results

3.1 Alteration in electro kinetic properties of erythrocytes in disease conditions

In this present study zeta potential of erythrocytes of four groups mainly control group, patients with diabetes mellitus, pregnant and gestational diabetes mellitus was studied. The results were obtained as shown in the Fig 1 & 2. The gestational diabetic erythrocyte membrane also showed a remarkable change in its characteristic ZP compared with control and pregnant volunteers. It was observed that erythrocytic ZP of a control group was 22.13±0.2789 mV whereas, erythrocytic ZP for diabetes mellitus patients was found to be 8.559 ±0.4864 mV. In this experiment, there is an alteration in the ZP value in diabetic patients this lowered value of ZP indicates that the charge between the RBC membranes has decreased, and they come together. Similarly when erythrocytic ZP of control pregnant patient was measured, it was found to be 21.07± 0.3393 mV which were slightly lower than control patients but ZP of GDM patients was found to be 10.12±0.2294 mV which is significantly less than both normal control and pregnant control group. This decrease in value of ZP of GDM vs. pregnant control group may be due to factors responsible for altering the membrane potential in pregnant diabetic patients or GDM Patient.

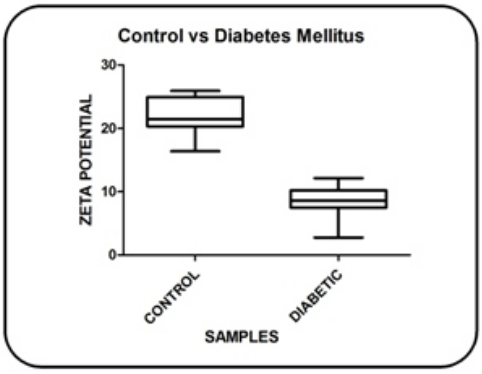

Fig 1: Comparison between the mean Zeta potential values of Control and Diabetic erythrocytes, obtained by using zeta meter system 4.0

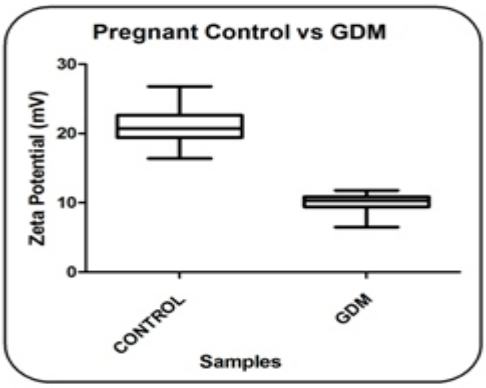

Fig 2: Comparison between the mean Zeta potential values of Pregnant Control and Gestational diabetes mellitus erythrocytes, obtained by using zeta meter system 4.0

3.2 Alteration in dynamic properties of erythrocytes in disease conditions

3.2.1 Osmotic fragility

Osmotic fragility is the measure of the resistance to lysis of erythrocyte membrane by osmotic stress. The result for osmotic fragility test reveals that (Fig 3 & 4) in Hyperglycemic condition RBCs becomes more fragile compared to erythrocytes of a control group. The represented graph depicts the comparison of osmotic fragility of erythrocytes in different condition.

3.2.2 Lipid peroxidation

As mentioned earlier, diabetes mellitus is a metabolic disorder which is characterized by hyperglycemia, causing auto oxidation

of glucose, which results in formation of lipid peroxides and increased free radicals. The levels of malondialdehyde as an index lipid peroxidation was measured, and it was found that there was formation of maximum malondialdehyde with diabetes mellitus patient and GDM, whereas low concentration of malondialdehyde was obtained with the blood sample of control (Table.1).

Table 1: Lipid peroxidation of erythrocytes resulting in formation of malondialdehyde in three different groups

Type of patients	Control group	Diabetes mellitus patients	Gestational diabetes mellitus patients
Malondialdehyde (MDA) produced in µmol/g of protein	0.0554±0.0093	0.0989±0.00861	0.135±0.0109

Results are expressed as mean ± standard error,; P value - P<0.0001; Are means signif. different? (P < 0.05);Yes

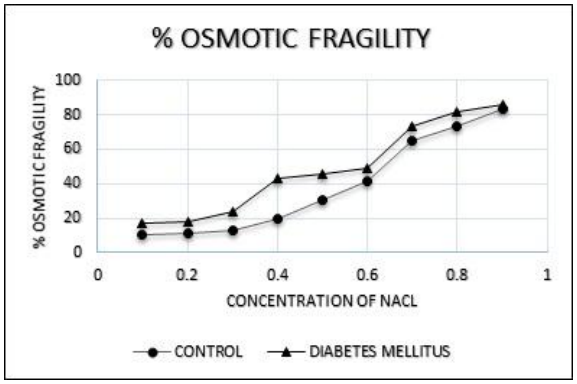

Fig 3: Comparison between the percent Osmotic fragility (haemolysis) of Control and Diabetes mellitus patients erythrocytes using different concentration (g %) of NaCl solution, at 415 nm using UV-Vis spectrophotometer (JASCO)

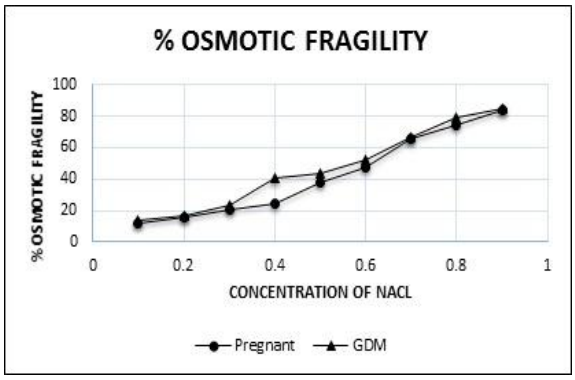

Fig 4: Comparison between the percent Osmotic fragility (haemolysis) of Pregnant Control and Gestational diabetes mellitus patients erythrocytes using different concentration (g %) of NaCl solution, at 415 nm using UV-Vis spectrophotometer (JASCO)

4 Discussions

Diabetes mellitus is a complex metabolic disorder which required monitoring of blood glucose level. The etiology is so heterogeneous that it requires sophisticated testing devices to diagnose it properly. In general, blood glucose level is the ordinary self-monitoring index that is used to measure hyperglycaemia. Also, Haemoglobin A1c (HbA1c), which is the irreversibly glycated haemoglobin, proved to be a reliable and valuable index to estimate how well diabetes is being managed over past few months. However, monitoring of hyperglycaemia was not sufficient for the prevention of many complications like developing cardiovascular disease and atherosclerosis in diabetic patients. Similarly, gestational diabetes mellitus (GDM) is a development of glucose intolerance during pregnancy, which leads to an increased incidence of maternal and fetal complications[14].

In this pilot study, the mechanisms that might underlie for alteration in osmotic fragility, zeta potential and lipid peroxidation in human RBCs during hyperglycaemic conditions was discussed.

The viscoelastic behaviour of red blood cell (RBC) membrane confers to the content of proteins and glycoproteins embedded in a fluid lipid bilayer. Also, a negatively charge surface which creates a repulsive electric zeta potential (ζ) between cells is due to the sialylated glycoproteins of the RBC membrane [15, 16]. These charges on the surface of RBCs help to prevent the interaction between them and the other cells and especially amongst themselves[17]. The fluidity of the system does not change, as long as the zeta potential of the system remains constant. But if the ZP of the system is lowered by the introduction of any cationic electrolytes or polyelectrolytes, then the system undergoes progressive changes in the stability resulting in simple agglomeration or fluid gel formation[2]. The present study, investigated the ZP of a control group vs diabetic and pregnant control vs GDM patients and found that ZP value decreases from 22.13 $\pm$ 0.2789 mV to 8.559 $\pm$ 0.4864 mV and from 21.07 $\pm$ 0.3393 mV to 10.12 $\pm$ 0.2294 mV in case of control group, diabetic, pregnant control and GDM respectively. The changes in ZP values of erythrocyte membrane may be attributed to decrease in membrane fluidity which indicates that blood begins to coagulate. As evident from the literature that the majority of cardiovascular diseases, even that originated from hyperglycemia, stem primarily from intravascular aggregation[18]. Studied literature reveals that zeta potential or surface charge of erythrocytes arises mainly from the dissociation of three functional groups, which bonds to the

different sites on cell membrane a) N-acetylneuramic acid (Sialic acid), b) α-carboxylic acid and c) weak amino base functional groups. The decrease of the surface charge and hence ZP may be mainly due to the increased friction among RBCs during blood circulation in hyperglycaemic state due to microvascular coagulation which would lead to a decrease of N-acetylneuramic acid and membrane-protein bound α-carboxylic acid. Another reason that might lead to reduction in ZP value is that diabetes causes the oxidative damage to the cell resulting from the decrease of the polyunsaturated fatty acid content of the membrane induced by the products of lipid peroxidation.

Similarly during pregnancy, the physiologic changes lead to red cell aggregation. The most dominant changes are Physiologic hemodilution, microvascular vasodilatation, and an increase in concentration of plasma proteins such as fibrinogen.

In a cross-sectional study, Ozanne et al demonstrated that during the course of Control pregnancy red cells aggregation increases [19]. Furthermore, Huisman et al reported in a longitudinal study that during normal pregnancy in spite of the physiologic hemodilution red cell aggregation considerably increases, mainly because of the increased fibrinogen concentrations[20]. Changes in ZP values of RBC membrane may be due to the above factors. Also GDM causes increase glucose intolerance which leads to decrease in viscoelasticity of erythrocyte membrane due to red cell aggregation.

Lipid peroxidation is an index of membrane damage, which promotes irreversible dysfunction of essential cellular components and ultimately triggers accidental cell death and necrosis[21]. It is evident from our work that higher concentration of malondialdehyde was observed with the erythrocytes of hyperglycaemic patients (diabetes mellitus and GDM patients) compared to control group. This may be due to the oxidative degradation of polyunsaturated fatty acids in the membrane, which are easily available due to dissociation of the three functional groups responsible for surface charge of erythrocytes. As mentioned earlier, that erythrocytes are susceptible to oxidative cellular damage when they are exposed to excessive oxidative stress. The higher values of lipid peroxidation reflect that diabetes leads to accumulation of excessive oxidative stress on RBCs.

Osmotic fragility experiment implicated that erythrocytes of hyperglycaemic patients are more fragile or susceptible to lysis compared to control volunteers. Study infers that, due to increased oxidative stress and alteration in N-acetylneuramic acid (Sialic acid), α-carboxylic acid and weak amino base functional groups in the membrane results in increased fragility of the membranes and reduced zeta potential of the membrane.

5 Conclusion

Thus, it can be concluded from the above discussion that alteration in osmotic fragility and zeta potential of erythrocytes can act as a key indicator for demonstration of increased oxidative stress and hyperglycaemic condition.

6 Acknowledgement

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7 Conflict of Interest

The authors declared no conflict of interest, financial or otherwise.

8 Author's contributions

SSG and MNK carried out literature review, participated in collection of data, statistical analysis of data and interpretation of results and conclusion. SSG drafted the manuscript. JGA supervised in the research study and drafting of final manuscript. All authors read and approved the final manuscript.

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