Chapter

Zeta Potential as a Diagnostic Tool to Determine the Angina Risk

Swati S. Gaikwad, Jasmine G. Avari and Mansi Liladhar Patil

Abstract

Red blood cells (RBCs) moving in circulation are continuously exposed to the reactive oxygen species (ROS) that are circulating within the vascular system of body. The objective of the study is to determine the erythrocyte zeta potential and its morphological changes caused by oxidative stress in hypertensive patients in relation to the risk of development of cardiovascular disease (myocardial infarction). In this retrospective study, we investigated 186 samples, which include hypertension patients (n = 64), myocardial infarction patients (n = 52), treated myocardial infarction patients (n = 20), and normal healthy volunteers (n = 50). Morphological and electrochemical characteristics of RBCs in patients were analyzed using scanning electron microscopy and zeta potential study. These parameters were also statistically analyzed applying one way ANNOVA (Tukey's comparison) using Graph Pad. Statistical analyses of data showed that Hypertensive (ZP-16.06 mV) and cardiac patients (ZP-9.94 mV) RBCs possessed significantly reduced zeta potential relative to that of RBCs from healthy individuals (ZP-23.39 mV) (P-value <0.0001). Microscopic imaging of Hypertensive RBCs revealed increased anisocytes and poikilocytes. These parameters were found exacerbated in patients suffering from Myocardial infarction. Hence, it can be concluded that ZP can be used as an effective diagnostic tool for detection of hypertension associated cardiovascular disorder risk.

Keywords: red blood cells (RBCs), zeta potential, erythrocyte fragility, lipid peroxidation, hypertension, myocardial infarction

1. Introduction

Hypertension is an emerging public health problem of this millennium and it is a major challenge to disclose the mechanism involved in the coexistence of hypertension and cardiovascular disease to improve the health of the Hypertensive patients. Patients with symptoms of a cardiovascular disease frequently present without striking evidence of cardiac specific enzymes in blood laboratory assessments or specific electrocardiogram findings. Recently, researchers have reported higher mortality risk associated with higher RDW in patient populations with cardiovascular disease (CVD) [1]. Nowadays, also there is a growing interest in characterizing RBC membrane defects in several diseases, as changes in membrane structure also contribute to the pathophysiology of the disease process.

Human erythrocyte contains about 95% of the glutathione which is responsible for scavenging reactive oxygen species [2]. Sialic acid is an important factor for

maintenance of the surface electrical charge and stability of biological cellular system [3]. Erythrocytes and Erythrocyte membrane are more vulnerable to peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acid, respectively [4]. The interaction of Reactive oxygen species (ROS) with particularly with fatty acids available in membrane can result in undesirable irreversible changes in cellular membrane. The membranes are therefore naturally protected by available anti-oxidative enzymes like superoxide dismutase, catalase, and glutathione peroxidase and vitamins E and A from oxidative damages [5]. By-products of peroxidation have been shown to cause profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids [6]. Reactive oxygen species are the responsible moiety that are involved in the generation and progression of atherosclerosis and that contribute to the development of plaque instability in acute MI [7].

Blood viscosity is strongly affected by the surface charge of RBCs and is responsible for the spacing between them. A higher surface charge causes repulsive forces to increases distant RBCs, preventing their close aggregation, lowers the viscosity, and results into very low peripheral resistance to flow. Therefore, it can be hypothesized that stress like condition develops in hypertension generate ROS which can induce membrane deformity and as a result affect the membrane surface charge. The occlusive arterial disease resulting from RBC aggregation may develop due to comparable membrane deformity [8]. Besides vascular and cardiac tissue integrity, blood and especially the RBC, is critical to performing this critical assignment of CV risk assessment considering it makes up over 90% of formed elements within blood. In cardiovascular diseases, impaired blood rheology has been observed. A direct relationship has been found between increased RBC deformability and increased risk for arterial hypertension [9]. Accurate risk stratification of patients with chronic heart failure is critically important to efficiently target the use of evidence-based therapies and identify high-risk patients who may benefit from advanced treatments [10].

We tested the hypothesis that variations in zeta potential and deformation of erythrocytes were associated with risk of adverse cardiovascular outcomes in a population with hypertension that were free of symptomatic heart failure [11]. In the present work we envisaged to study and evaluate morphological changes taking place in RBCs, erythrocyte fragility, lipid per oxidation and zeta potential which can act as invaluable aid in the diagnosis of a hypertension and risk of Cardiovascular Disorder in hypertension patients. Hence, the aim of this study was to test the hypothesis of association of variation in RBC morphology, erythrocyte fragility and zeta potential in hypertension and its relation with the risk of cardiovascular disorder in hypertension patients.

2. Morphological characterization

2.1 Preparation of blood Smear

Blood smear was prepared with the aid of wedge method [12]. In this method a drop of blood was placed on base closed to one cease of the slide at least 1 cm away from the edged of the slide. Another slide with the smooth end was used as spreader and smear was prepared by moving spreader inclined at 30–45° angle to the blood.

This Smear was air dried and fixed with Leishman stain and located and observed beneath a trinocular research microscope (RXT4, Radical).

2.2 Scanning electron microscopy

To observe the morphological variations in the erythrocyte membrane structure in Erythrocytes of patients suffering with hypertension and MI, erythrocytes were analyzed by scanning electron microscopy. With this motive blood sample was taken in Eppendorf tube containing 10 μl of Heparin (5000 UI/ml) in 900 μl of pH 7.4 phosphate buffer saline. The blood suspension was then centrifuged (1000 rpm for 10 min) and washed with buffer three times. The supernatant was removed and replaced by same volume of buffer. One drop of these separated erythrocytes were then exposed to 500 μl of 2.5% Glutaraldehyde in distilled water overnight at 4°C to fix. Again samples were washed thrice with distilled water and centrifuged. About 40 μl of each sample was placed on glass covered studs and air dried at room temperature. The Scanning electron microscopy (SEM) analysis of prepared samples was accomplished using Jeol, Japan (Model—JSM 5610LV).

2.3 Preparation of iso-osmotic dextrose solution

About 5 g of anhydrous Dextrose (Merck) was solubilized in 100 ml of distilled water to prepare a 5% w/v iso-osmotic Dextrose solution.

2.4 Preparation of blood sample for zeta potential measurement

About 0.1 ml of blood sample was transferred into 50 ml of freshly prepared 5% w/w dextrose solution which is isotonic with the red blood cells [12].

2.5 Zeta potential measurement by zeta meter system 4.0

Zeta meter System 4.0 instrument was used to measure the zeta potential of the Erythrocytes [12]. Zeta potential of the system is measured by applying the electric field to the samples using electrodes and determining the mobility/velocity of the particle under the applied field. Zeta potential was calculated according to the simplified Helmholtz-Smoluchowski equation as follows: Mean velocity of the 10 readings was used to calculate the zeta potential Eq. (1).

$$\text{Zeta Potential, } \zeta = \frac{113000 \times v \times EM}{D} \quad (1)$$

where ν = viscosity of sample in poise at temperature "t"; EM = electrophoretic mobility; D = dielectric constant.

The electrophoretic cell consists of capillary which is embedded inside a chamber having electrodes at both ends having cavity for sample connection with electrodes. From the cavity of any one end of the electrophoresis cell the Sample is introduced into the capillary to fill it completely. Electrodes are connected to the cell with the applied electric field at specific voltage of 200 V. Due to the applied electric field charged particles move towards oppositely charged electrode. Their velocity under the applied electric field is measured and expressed in terms of electro kinetic potential/zeta potential. Nowadays, this system is preferably used for determining the zeta potential of various types of biological membranes.

In this method, fresh blood samples were collected from volunteer and blood suspension was prepared as described in preparation of sample. Prior measurement of zeta potential temperature of and other parameters for ZP measurements were adjusted such as light intensity; focal plane and tracking duration were optimized for stable data collection with minimal error. The collected and prepared samples were then added to the previously cleaned zeta-meter cell, which were then connected to electrodes and placed under the lens over stage. The Erythrocytes were tracked by using remote by microscopically-acquired video images, values were recorded 10 times for each sample and average zeta-potential in mv was obtained standard deviation from software Zeta meter-ZM4DAQ software.

2.6 Statistical analysis

The experimental results for ZP are expressed as mean ± standard (SD). Investigated groups were compared by the statistical one way analysis of variance (ANOVA) and evaluated by Tukey's multiple comparison tests using Graph Pad Prism version 5.00 for Windows. Experimental Results were considered significant at $p < 0.05$ for the different groups which indicates that the control and other patient groups differ significantly from one another in all situations.

3. Results and discussion

Morphological study using the smear reveals that there is deformation in RBC shape (Anisocytosis) in majority of patients. In Hypertension and patients with MI showed RBC deformation from spherical biconcave shape to tear shape which further continues to Rouleaux formation (**Figure 1a–f**). Also, it was observed that there is increased aggregation of Erythrocytes in patients suffering from Hypertension and Myocardial Infarction compared to normal volunteers.

SEM studies revealed that there are number of deformed erythrocytes observed in myocardial infarction patients (**Figure 2c**) in aggregated form. It was observed in patients with hypertension, Erythrocytes appears with uneven surface of cell membrane which indicates the oxidative damage in the cell membrane (**Figure 2b**) as compared to normal human erythrocyte as shown in (**Figure 2a**).

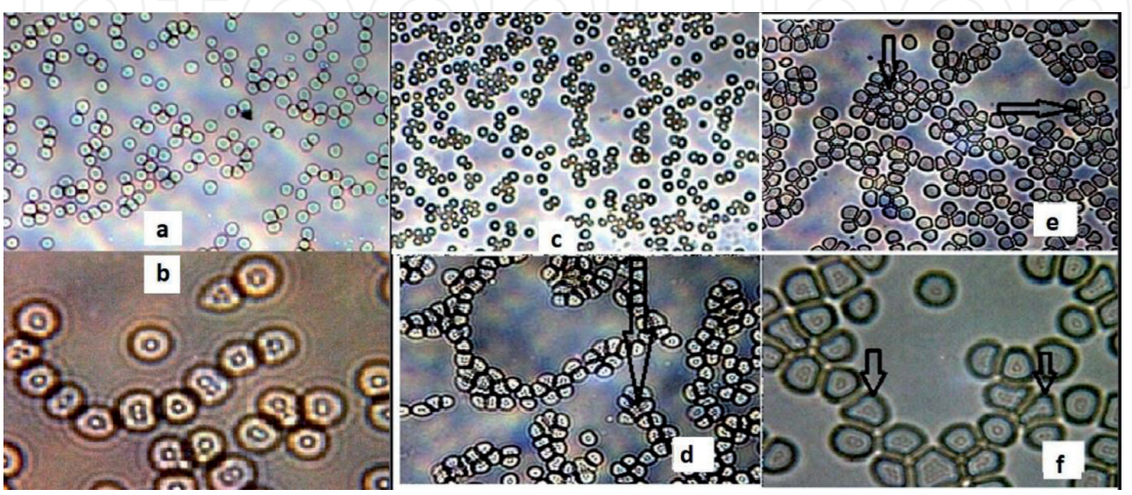

Figure 1.
(a, b) Erythrocytes of normal human erythrocytes. (c, d) Erythrocytes of patient suffering from Hypertension, blood smear showing Rouleaux formation. (e, f) Erythrocytes from patient suffering from myocardial infarction, blood smear showing anisocytes.

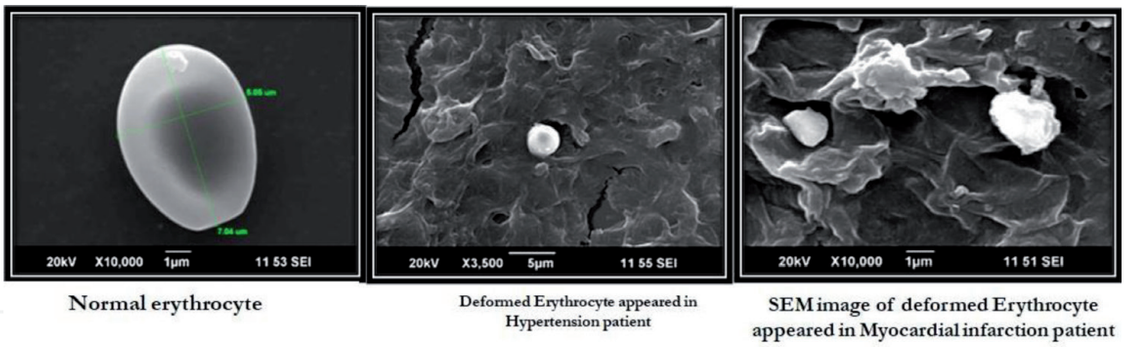

Figure 2.
Scanning electron microscopic images of normal human erythrocytes, patients suffering from hypertension and myocardial infarction showing deformed erythrocytes.

Similarly, the result for erythrocyte membrane osmotic fragility in test samples reveals that erythrocytes of patients with MI and hypertension become more fragile compared to erythrocytes of control group (**Figure 3**). The erythrocytes are very much susceptible to oxidative cellular damage on exposure to the excessive oxidative stress.

By using a Zeta meter system 4.0, the zeta potential of human Erythrocytes was obtained. The results obtained showed ZP values ranged from −20.13 mV to −26.46 (+1.87) mV for the healthy individuals with a mean value of −23.39 mV. But the ZP values obtained from the Patients with MI is much lower and ranges from −2.58 mV to −22.76 (+3.57) mV compared to patients suffering from only Hypertension values ranges from −12.13 mV to −19.61(+1.20) mV. Also it was observed that Patients with MI who underwent CABG and angioplasty and receiving medications the zeta potential of such patients was ranging from −21.53 mV to −32.59 mV(+2.95) indicating higher stability of erythrocytes in blood vessels (**Figure 4**).

It was specifically observed that the patients with Hypertension had a mean ZP value −16.06 mV and patients with MI mean ZP value was obtained as −9.938 mV ($p < 0.001$). Data obtained from the analysis of blood samples from patients with hypertension and healthy volunteers was subjected to one-way ANNOVA with the application of Tukey's multiple comparison tests, and results indicated that there is a significant lowering of zeta potential of Patients with Hypertension from that

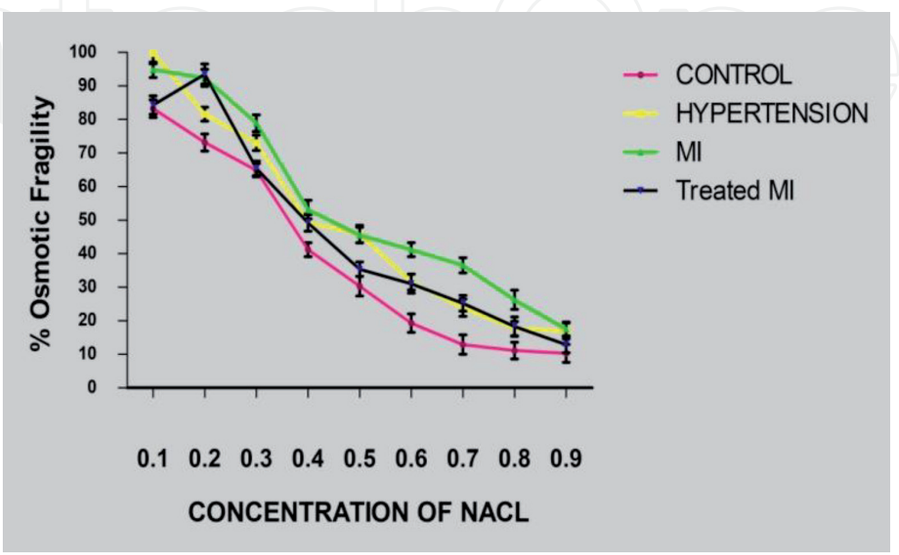

Figure 3.
Comparison between mean erythrocyte fragility of erythrocytes in normal volunteers (CONTROL), patients with hypertension and myocardial infarction and myocardial infarction patients on treatment.

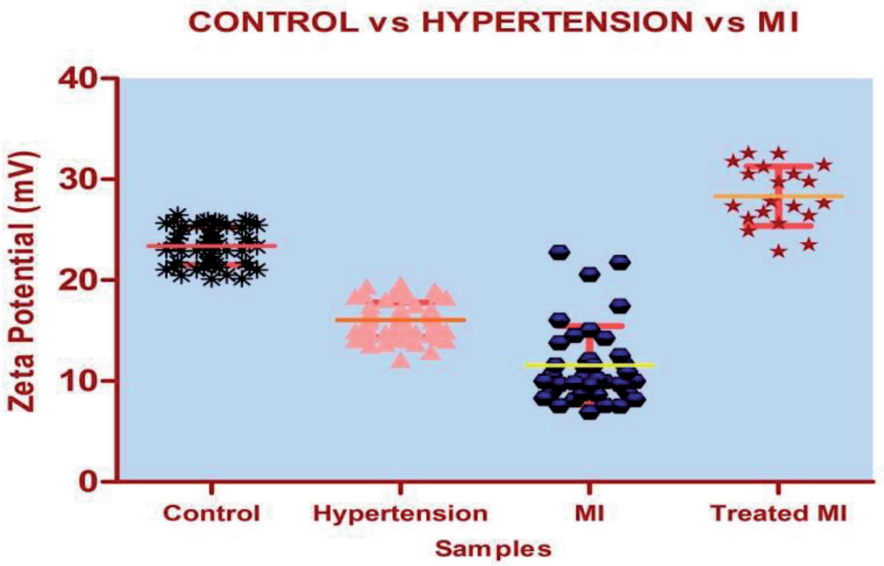

Figure 4.
Comparison between mean ZP values of normal volunteers, patients with hypertension and myocardial infarction patient and myocardial infarction patients on treatment.

of the healthy volunteers. Also, there is greater decrease in ZP values of patients with Hypertension suffering from MI indicate the exacerbation in RBC deformity in patients. This may be due to hypertension induced complications. Higher ZP in treated patients indicates the increased stability of erythrocytes due to reduced oxidative stress.

4. Conclusion

The exact mechanism of development of CVD is complex and is not yet fully understood. But from the literature survey it was clear that ROS plays an important role in the progression and development of CVD. Also, it was known that there is a strong relation between ROS and the pathophysiology of CVD. From the present research work it can be concluded that due ROS in Patients with Hypertension the erythrocytes are affected, their membrane gets oxidized resulting in various types of morphological deformity (Anisocytosis). Also, membrane potential (ZP) which is a characteristic property of RBC responsible for free flowing of RBC in the blood stream without aggregation, get affected. These conditions get exacerbated in erythrocytes of patients suffering from myocardial infarction. Development of membrane deformity directly reduces the membrane potential of RBC. Due to oxidation of RBC membrane by ROS, the membrane becomes fragile and therefore the fragility of erythrocytes increases in Patients with Hypertension and MI compared to healthy volunteers.

Results obtained in our lab suggest that variations in erythrocyte morphology, zeta potential, lipid peroxidation and erythrocyte Fragility can act as a key indicator to determine the risk factor of myocardial infarction in hypertensive patients. In the present study, we have developed a greater understanding of effect of ROS on morphology of RBCs, effect on its membrane potential (ZP) due to deformity in the membrane and its erythrocyte fragility. This could be a new way to realize a better treatment in hypertensive patients and a prevention of cardiovascular complications (i.e.: myocardial infarction, TIA, etc.). However, more works in the near future are necessary to improve the detection and treatment of the ROS mediated dysfunction.

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