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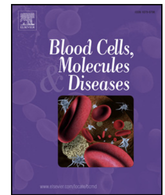

Association of abnormal erythrocyte morphology with oxidative stress and inflammation in metabolic syndrome

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ABSTRACT

In carrying out their role of free radical scavenging, erythrocytes become damaged due to oxidation of membrane lipids and proteins. Such damage may change the morphology of the erythrocytes. The present study aims to demonstrate change in erythrocyte morphology in MetS and associate the changes with increased oxidative stress and inflammation that were shown in our recent study. One hundred participants were recruited from a rural town of Australia. Whole blood viscosity, erythrocyte aggregation, erythrocyte deformability, lipid profile and blood sugar level, oxidative stress markers (erythrocyte reduced glutathione, superoxide dismutase, urinary isoprostanes) and inflammatory markers (high sensitivity C-reactive protein) were measured. Erythrocyte morphological study was performed by scanning electron microscopy. Recruited participants were classified into MetS and non-MetS following the National Cholesterol Education Program Adult Treatment Panel III definition. Data were analyzed by IBM SPSS 20 software. The mean percentages of biconcave cells were decreased whereas acanthocytes, stomatocytes and echinocytes were increased in MetS group compared to healthy controls. Morphologically abnormal erythrocytes were significantly correlated with oxidative stress and chronic inflammation markers. Free radicals generated in increased concentration in MetS seem to damage erythrocyte changing its morphology which possibly could affect other hemorheological parameters.

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Introduction

Metabolic syndrome (MetS) is the clustering of cardiovascular risk factors such as dyslipidemia, hypertension, obesity and insulin resistance [1]. The patients with the MetS are at twice the risk of developing cardiovascular diseases over the next five to ten years as individuals without the syndrome and the risk over a lifetime is undoubtedly higher [2]. Recently, we have shown increased oxidative stress and chronic inflammation in MetS [3]. Oxidative stress is increased in MetS [4,5] and erythrocytes play a significant role in scavenging the free radicals generated in MetS [6].

The flow properties of the erythrocyte (hemorheology) depend on the capacity of erythrocyte to deform in the blood vessels among others. Under physiologic conditions, deformability allows erythrocytes of 7 μm diameter to traverse through capillaries with diameters no more than 3–5 μm, thus facilitating tissue oxygenation. Alterations in deforming capacity due to oxidative damages and increase in the concentration of inflammatory molecules in the blood in MetS provide favorable environment for erythrocyte to aggregate and these increase the viscosity of blood. Recently, we have also shown decreased erythrocyte deformability, increased whole blood viscosity (WBV) and increased erythrocyte aggregation in MetS [7].

Thus, in carrying out their role of free radical scavenging, erythrocytes become damaged due to oxidation of membrane lipids and proteins and furthermore, the oxidation consumes endogenous reducing substances [6]. Such damage may change the shape of the erythrocytes [8]. Biconcave discoid shape is the physiological form of red blood cells and erythrocytes may respond by changing their morphology to any form of insult in their membrane or biochemical composition. In this context, the present study aims to demonstrate the change in erythrocyte morphology in MetS and associate the changes (if any) with increased oxidative stress and inflammation that were shown in our recent study [3].

Materials and methods

One hundred participants with and without MetS were recruited from a rural town of Australia from June–December, 2013. A modified National Cholesterol Education Program Adult Treatment Panel-III (NCEP ATP III) guideline was used to define MetS [9]. According to NCEP ATP III guidelines, the individual is said to be in the state of MetS

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if he/she fulfills three criteria from the following five criteria: waist circumference >102 cm in male and >88 cm in female; triglyceride (TG) ≥ 1.7 mmol/L or specific treatment; high density lipoprotein cholesterol (HDL-C) <1.0 mmol/L in male and <1.3 mmol/L in female or specific treatment; blood pressure ≥130/85 mm Hg or previously diagnosed hypertension; and fasting glucose ≥5.6 mmol/L or previously diagnosed diabetes mellitus. Pregnant women, non-ambulatory patients and children under 18 years of age were excluded from the study. Research participants were divided into three groups on the basis of number of MetS components present. Group I consists of the participants without any positive components of MetS (healthy controls); group II consists of the participants with one or two positive components; and group III consists of participants with three or more positive components. Written consent was taken from all the research participants and the proposal of the study was approved by the Human Research Ethics Committee, Charles Sturt University.

Inflammatory markers high sensitivity C-reactive protein (hsCRP) and thrombotic marker D-dimer were measured in the day of collection in a commercial clinical pathology laboratory. Erythrocyte reduced glutathione (GSH) was measured by the 5,5′-di-thiobis-(2-nitrobenzoic acid) method on the same day of blood collection from the erythrocyte lysate (from the washed cells) after protein precipitation by metaphosphoric acids [10]. Erythrocyte superoxide dismutase (SOD) was measured from hemolysate using a commercially available Cayman kit that uses xanthine oxidase and tetrazolium salts. Hemolysate was prepared from packed red blood cells. One mL of packed red blood cells was mixed with 4 mL of ice cold water and centrifuged at 1260 *g* for 9 min to obtain hemolysate. The hemolysate was stored in 1.5 mL Eppendorf tubes at −80 °C in an ultra low temperature freezer until tests were performed. 15-isoprostane F2t was measured in urine sample (NWLSS™) and was expressed as ng of isoprostanes per mmol of urinary creatinine (Cayman chemical). The urine sample was stored in 1.5 mL Eppendorf tubes at −80 °C in an ultra low temperature freezer until tests were performed.

Scanning electron microscopy (SEM) [11]

Three drops of whole blood were added directly from the syringe to 5 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, within seconds of being withdrawn. Fixation was allowed to proceed for at least 24 h before processing. This method of fixation has the least potential to artifactually change the erythrocyte shape. The low concentration of glutaraldehyde helps restore the normal discocyte shape of erythrocytes [12]. The cells were washed twice in cacodylate buffer, dehydrated with two washes in 70% ethanol, two washes in 95% ethanol, two washes in absolute ethanol and two washes in acetone. One drop of the cell suspension was applied to a coverslip and allowed to dry. Coverslips were mounted onto carbon tabs (eccentrically) which were already placed onto SEM mount pin type. Coverslips were stored in a desiccator until microscopy was performed. Since coverslips were placed eccentrically onto a carbon tab, small portion of coverslip was outside the carbon tab/SEM mount pin. The lower part of the portion of the coverslip, which was projected outside the mount pin, was coated with the Carbon/Graphite. Before microscopy, the specimen was coated with a thin layer of gold. This was done using a K550X sputter coater. The layer deposited was typically 10 to 20 nm thick, and nearly evenly coated the surface of the specimen, faithfully reflecting the surface morphology. A scanning electron microscope (JCM 5000, Benchtop SEM, Neoscope) was used to study the morphology of the erythrocytes. After the image was obtained, appropriate areas of the specimen were photographed using the instrument software. The erythrocytes in each photograph were examined, classified and counted.

Erythrocyte morphology classification given by Bessis [13] was followed to classify erythrocyte into different morphological subclass. Normal disc shaped cells were referred to as ‘discocyte’. Cells covered with crenations or spicules were referred to ‘echinocytes’. ‘Acanthocytes’ — though superficially resembling echinocytes were considered to be different cells with fewer spicules on their surface, which were irregularly arranged and were bent back at their tips. The cup shaped cells were referred to as ‘stomatocytes’ and spherical cells were referred to as ‘spherocytes’. The typical examples of morphological classifications are represented in Fig. 1.

The detailed methodology and the principles of the instruments used in the measurement of erythrocyte deformability, erythrocyte aggregation and WBV have been described in our recent study [3,7]. Erythrocyte deformability and aggregation measurements were carried out using a RheoScan-AnD 300 system (RheoMeditech Inc., Korea). Two indices were used to define erythrocyte aggregation: critical time and critical stress. The lesser the critical time, the faster the erythrocyte aggregation process and the higher the critical stress, the faster the erythrocyte aggregation [14]. Two measurement indices were used to define erythrocyte deformability: elongation index maximum (EI_{max}) and $SS_{1/2}$. EI_{max} is the maximum erythrocyte elongation index at infinite shear stress and $SS_{1/2}$ is the shear stress required for half of maximal deformation [15,16]. WBV measurement was carried out using a Brookfield DV-II+ programmable viscometer (MA, USA), using a CP40 spindle at 37 °C. All the rheological measurements were performed within 2 h of blood collection after adjusting EDTA anticoagulated whole blood to the hematocrit of 40%.

Statistical analysis

Data were analyzed by IBM SPSS statistics 20. ANOVA with post-hoc analysis was used to compare the percentage of different morphological cells in three different groups. Pearson's correlation test was used to correlate different morphological cells with oxidative stress and inflammatory markers. All the *p*-values were two-tailed, and those <0.05 were considered statistically significant.

Results

The present study is a part of a larger study that was undertaken to investigate the association of hemorheology with oxidative stress and systemic inflammation among participants with and without MetS. The demographic and baseline characteristics of the participants with and without MetS have been published in our recent studies [3,7]. Of the 100 participants, 36 participants fulfilled the criteria of MetS, 33 had one or two positive components and 33 were healthy controls.

The mean percentage of different erythrocyte morphologies in groups I, II and III is shown in Table 1. The mean percentages of biconcave cells were higher in group I when compared to groups II and III. Acanthocytes, echinocytes and stomatocytes were the abnormal cells that were found to be increased in group III.

Post-hoc analyses revealed that mean percentage of biconcave cells ($p < 0.0005$), acanthocytes ($p < 0.0005$), echinocytes ($p = 0.007$) and stomatocytes ($p = <0.0005$) significantly differed between groups I and III (Table 1). Similarly, the mean percentage of biconcave cells ($p = 0.001$) and acanthocytes ($p = 0.007$) significantly differed between groups I and II. In the same way, the mean percentage of biconcave cells ($p < 0.0005$), acanthocytes ($p < 0.0005$) and echinocytes ($p = 0.048$) significantly differed between groups II and III.

Morphologically abnormal erythrocytes were significantly correlated with oxidative stress and chronic inflammation markers (Table 2). The percentage of normal biconcave cells was negatively correlated with oxidative stress and chronic inflammation. Acanthocytes (%) showed a positive correlation with urinary isoprostanes and hsCRP level and a negative correlation with GSH level. Similarly, stomatocytes (%) was positively correlated with urinary isoprostane level.

The correlation of morphologically abnormal cells with other hemorheological parameters is shown in Table 3. Acanthocytes and echinocytes were positively correlated whereas biconcave cells were negatively correlated with erythrocyte aggregation. Acanthocytes

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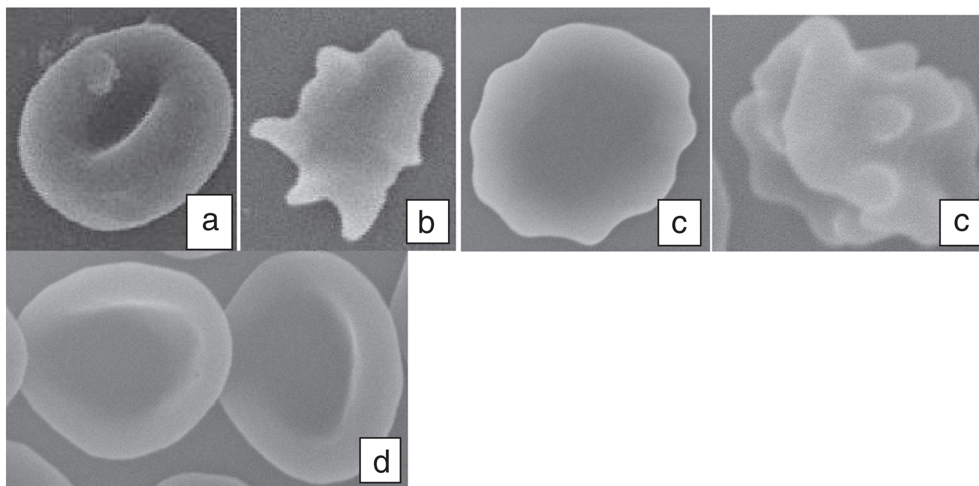

Fig. 1. Erythrocyte morphological variation identified from a scanning electron microscope: (a) biconcave cell (1500×); (b) acanthocyte (1500×); (c) echinocyte (5000×); (d) stomatocyte (5000×).

were also negatively correlated with maximum erythrocyte deformability whereas biconcave cells were positively correlated with maximum erythrocyte deformability.

Discussion

The present study investigated the morphological changes in erythrocytes in MetS and associated the morphological changes with oxidative stress and inflammation. This study showed an increase in abnormal erythrocyte morphology (acanthocytes, echinocytes and stomatocytes) and a decrease in biconcave shape erythrocytes in MetS participants when compared to healthy controls (Table 1). This is likely to be due to the continuous attack of reactive oxygen species, which consume endogenous reducing substances of erythrocytes. This may therefore damage erythrocytes by altering its lipid bilayer (peroxidation) and by oxidation of labile groups in the proteins of the cytoskeleton resulting in shape change. It has been suggested that treatment of physiological biconcave discoid erythrocytes with various agents can transform them into their two extreme opposite forms: stomatocytes and echinocytes [17]. This study is in agreement with a previous study that showed an increased number of acanthocytes in the participants with MetS in comparison to healthy controls [18]. Another similar study, reported diabetic participants to have higher 'acanthocytes' and 'stomatocytes' in comparison to non-diabetic participants which after treatment with antioxidant drug were restored to normal [19]. This restoration of morphologically abnormal cells to normal after antioxidant treatment highlights the effect of oxidant on blood cells.

In the present study, isoprostanes and hsCRP showed a negative correlation with biconcave cells and a positive correlation with acanthocytes and on the contrary, GSH showed positive correlation with biconcave cells and negative with acanthocytes (Table 2). This suggests that an important link may exist between erythrocyte morphology and oxidative stress and chronic inflammation. Erythrocytes may respond by changing their morphology to any form of insult in their membrane or biochemical composition. The findings in this study are consistent with past observations [20] that have demonstrated the conversion of normal erythrocyte population to echinocytes after incubating with hydrogen peroxide. The mechanism of echinocyte formation may be related to condensation of the inner monolayer lipids as a result of spectrin-hemoglobin complex formation due to oxidative damages as suggested by Snyder et al. [20]. Crenated forms of erythrocytes were observed in a dose dependent manner when blood samples from healthy donors were incubated with peroxynitrite [21]. The authors suggested that crenation of erythrocytes appears to be caused by both water and ion imbalance between the cells and surrounding medium and cytoskeletal structure changes as a result of oxidative stress [21]. These studies support the findings of the present study that the erythrocyte morphology is affected by oxidative stress and the increased count of abnormal erythrocytes seen in MetS group in the present study could be due to generation of oxidative stress in MetS.

The study of erythrocyte morphology is of great importance in hemorheology, since the deformability of circulating cells has a fundamental influence on rheological properties of the blood [22]. The present study showed a positive correlation of biconcave cells with erythrocyte deformability (Table 3). The principal accepted role for erythrocytes is transport of gases viz: oxygen, carbon dioxide and nitrous oxide. Erythrocytes are highly specialized for this task losing most of their organelles in development and containing mostly hemoglobin. Their biconcave shape permits flexibility, allowing them to squeeze through capillaries narrower than their own diameter. This is supported by the positive correlation of biconcave cells that was observed with erythrocyte deformability. More importantly, significant negative correlation of acanthocytes was seen with erythrocyte

Table 1
Erythrocyte morphology (means ± SD) for the percentage of morphological subclasses in three study groups.

Morphology	Group I (%)	Group II (%)	Group III (%)	*p*-value
Biconcave cells	89.60 ± 2.16	86.97 ± 3.11	84.04 ± 2.95	<0.0005
Acanthocytes	3.05 ± 1.01	4.48 ± 2.06	6.61 ± 2.18	<0.0005
Echinocytes	1.02 ± 0.46	1.13 ± 0.62	1.50 ± 0.75	0.006
Leptocytes	2.66 ± 0.98	3.07 ± 1.15	2.94 ± 1.18	0.335
Stomatocytes	2.00 ± 0.80	2.60 ± 1.32	3.20 ± 1.39	0.001

Table 2
The Pearson's correlation of abnormal erythrocyte morphology with oxidative stress and chronic inflammation parameters.

Morphology		Urinary isoprostanes (ng/mmol)	SOD (U/mL)	GSH (mg/dL)	hsCRP (mg/L)	D-dimer (μg/mL)
Acanthocytes	r	0.623	−0.006	−0.34	0.277	−0.057
	p	<0.005	0.956	0.001	0.005	0.572
Echinocytes	r	−0.039	−0.169	−0.172	0.183	−0.055
	p	0.703	0.094	0.087	0.069	0.592
Stomatocytes	r	0.341	−0.1	−0.153	0.164	0.081
	p	0.001	0.325	0.128	0.103	0.424
Leptocytes	r	0.144	0.018	0.02	−0.073	0.219
	p	0.152	0.86	0.841	0.472	0.029
Biconcave cells	r	−0.585	0.09	0.282	−0.292	−0.017
	p	<0.005	0.375	0.005	0.003	0.87

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Table 3
The Pearson's correlation of abnormal erythrocyte morphology with erythrocyte deformability and erythrocyte aggregation.

Morphology		Critical time	Critical stress	El_{max}	$SS_{1/2}$	WBV
Acanthocytes	r	−.296	.412	−.332	.167	.126
	p	.003	.000	.001	.096	.211
Echinocytes	r	−.149	.270	−.153	.039	−.151
	p	.139	.007	.130	.701	.134
Stomatocytes	r	−.182	.178	−.127	.168	.029
	p	.070	.076	.209	.095	.774
Leptocytes	r	−.077	.033	−.081	−.008	.019
	p	.445	.741	.421	.935	.848
Biconcave cells	r	.336	−.407	.355	−.201	−.115
	p	.001	.000	.000	.044	.254

deformability in the present study and this indicates that changes in morphology of cells due to oxidative stress and chronic inflammation lead to the generation of rigid cells, possibly compromising the rate of blood flow through capillaries and oxygen delivery in peripheral organs. Watanabe et al. [23], showed that erythrocytes from healthy donors incubated in hypoxanthine/xanthine oxidase system and hydrogen peroxide had decreased membrane fluidity and hence reduced deformability and this is supported by findings of the present study.

Conclusions

Abnormal erythrocyte morphology was found to be increased in MetS. Morphologically abnormal cells were correlated with oxidative stress and inflammation generated in MetS. Free radicals generated in increased concentration in MetS seem to damage erythrocyte changing its morphology which possibly could affect other hemorheological parameters. Alterations of erythrocyte morphology could have a negative impact in the circulation and gaseous exchange in the small blood vessels.

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