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Progressive impairment of erythrocyte deformability as indicator of microangiopathy in type 2 diabetes mellitus

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Abstract. The erythrocyte deformability of blood samples, of diabetes mellitus (DM) patients with and without microangiopathic complications such as nephropathy and retinopathy, is determined and is compared with that of healthy control. The erythrocyte deformability is measured in terms of elongation index (EI) with microfluidic ektacytometer, which is very sensitive to detect changes in EI of erythrocytes due to hyperglycemic process. Each measurement of diffraction pattern of erythrocyte suspension in a highly viscous polyvinyl pyrroridone (PVP) solution in a disposable microchannel is carried out. The results show that EI is well correlated with the levels of glycated hemoglobin and creatinine, as determined from the blood samples of patients. A significant decrease in the EI in DM patients compared with that in normal control is observed. In patients with complications of chronic renal failure, end stage renal disease, retinopathy and with combination of retinopathy and nephropathy, the EI is significantly decreased in comparison with that of diabetes patients without these complications. Further reduction in EI is corresponded to the changes induced by the microangiopathy process despite the maintenance of blood glucose and glycated hemoglobin by drug therapy.

Keywords: Erythrocyte deformability, nephropathy, retinopathy, creatinine, glycated hemoglobin

1. Introduction

Erythrocyte deformability is the measurement of the ability of the cells to deform while flowing in large as well as small vessels in cardiovascular system. The major determinants of the deformability include cell shape, composition of the cell membrane and its cytoskeleton, and internal viscosity (mean cell hemoglobin concentration) [1–3]. The contribution of the erythrocyte membrane to the deformability is primarily regulated by the composition and arrangement of its structural constituents.

Diabetes mellitus (DM) affects the function of the erythrocytes through interaction with its membrane and intracellular constituents [4]. In diabetes the erythrocytes have increased malondialdehyde (MDA)

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[5], and decreased glutathione (GSH) and membrane – SH group [6], leading to formation of pathological proteins [7]. The ratio of cholesterol to phospholipids is significantly decreased [8]. The skeletal proteins beta-spectrin, ankyrin, and protein 4.2 are the most glycosylated while the spectrin is oxidatively damaged [9]. These cells further show the decrease of (Na^+/K^+)ATPase levels [10], diminished Ca^{2+}-ATPase activity [11] and increased intracellular uptake of Ca^{2+} [12]. These alterations produce impairment of normal functions of erythrocytes as measured in terms of erythrocyte deformability by rheoscopy [13], ektacytometry [14] and filtration techniques [15] and in the membrane bilayer in terms of its fluidity [16].

The impaired glucose tolerance is also associated with microvascular complications of diabetes**,** including retinopathy, nephropathy, and polyneuropathy. Vascular complications are contributed by inhibition of nitric oxide-mediated vasodilation, endothelial injury due to increased release of free fatty acids and adipocytokines from adipocytes, and direct metabolic injury of endothelial and end-organ cells [17]. On the other hand the progressive accumulation of glycated hemoglobin, which increases with the duration of disease [18], covalently bound to the membrane skeleton not only disrupts membrane organization but also threatens eventual phospholipids oxidation via a calcium-promoted quasi-lipoxygenase activity [19]. Due to appearance of phosphatidylserine in the external membrane layer, especially in nephropathy, this disrupts the phospholipids asymmetry in the membrane and subjects these cells to a risk of catastrophic breakdown [20].

The presence of micro-albuminuria is also a significant risk factor [21] which is associated with increased serum creatinine in diabetic nephropathy [22]. The increase of glycated hemoglobin is routinely used for long-term monitoring of glycemic status in diabetes patients [23]. This process is also associated with anemia, with 2–3 times greater prevalence and earlier onset than in patients with renal impairment from other causes [24].

The measurement of the various structural parameters thus provides information on the changes which are taking place in various constituents of erythrocytes but their influence could be measured through the change in erythrocyte deformability. The precise measurement of this erythrocyte deformability could provide information on the disease process, which is primarily contributing in its deterioration. Based on this parameter, the assessment of the possible changes in the microcirculation could also be made. The measurement of erythrocyte deformability in diabetes and its various diabetes complications forms the objective of the present work which has been carried out by a microfluidic ektacytometer, and to establish its correlations with creatinine and glycated hemoglobin, which are considered as the indicators of diabetes complications.

2. Materials and methods

2.1. Measurement technique

The erythrocyte deformability was measured by the microfluidic ektacytometer, Rheoscan-D (Sewon Meditech, Korea). The erythrocyte suspension, driven by differential pressure, flows through the disposable microchannel ($0.2 \times 4 \times 40$ mm, Sewon Meditech, Korea), and is collected in the waste chamber. During flow, a laser beam of wavelength 635 nm from a laser diode of power 1.5 mW passes through the diluted erythrocyte suspension. The diffraction pattern of flowing erythrocytes, at plural shear stresses is projected onto the screen, captured by a CCD-video camera, and is finally analyzed by an ellipse-fitting-program in a computer. The elongation index (EI) of erythrocytes is defined as $(L - W)/(L + W)$, where

L and W are the major and minor axes of the ellipse, respectively. After each measurement the microchannel was discarded. Further functional details of the microfluidic ektacytometry are given elsewhere [14].

2.2. Selection of DM and normal control subjects

The present study was conducted at the Kyungpook National University Hospital as a collaborative project of the Department of Laboratory Medicine and School of Mechanical Engineering of the University. Two hundred and twelve *type 2* diabetic patients participated in the present study: 65 DM patients without any apparent microvascular disease formed the Group-I. Group-II was comprised of 41 diabetic patients with chronic renal failure (CRF) and 42 diabetic patients with end-stage renal disease (ESRD) formed the Group-III. Similarly, Group-IV and Group-V were consisted of 23 diabetic patients with retinopathy (DMR) and 41 patients with retinopathy and nephropathy (DMR + DMN), respectively.

The control group was consisting 49 age-matched subjects without any history of DM and associated complications. Patients who had clinically evident cardiovascular diseases were excluded from the present study. Serum creatinine levels in Groups II, III and V were higher than 1.5 mg/dl. Control and patients selected for the present study were in the age group of 40–65 years and the duration of diabetes varied from 2–30 years. The levels of glucose, creatinine, hemoglobin, HbA_{1C}, LDL and HDL were determined by standard procedures in the Department of Laboratory Medicine at the Hospital. Prior to blood collection, the written consent from the patients and healthy subjects were obtained.

2.3. Sample preparation

Blood samples were obtained, from control and diabetes patients from the antecubital vein into an EDTA containing Vacutainers (BD, Franklin Lakes, NJ, USA). All analyses including EI measurement were completed within 6 hours after blood collection. For EI measurement the erythrocyte suspension was prepared by mixing, 5.0 μl of whole blood with 0.5 ml of high viscous PVP solution (31 mPa s) in phosphate buffered saline (0.14 mM), with a vortex stirrer. During the flow of the suspension in the microfluidic channel, the diffraction pattern was recorded and the EI was calculated.

The statistical analysis of the data was performed using an unpaired two-tailed student t-test. Linear regression was used to determine the correlation between the variables.

3. Results

The clinical parameters of blood of control and diabetic patients are given in Table 1. The glucose level is significantly higher in all diabetes groups compared to that of control. When compared to Group-I, the glucose level varies from significant ($p < 0.05$) to highly significant in Group-V ($p < 0.001$). Creatinine level increases in patients suffering from kidney disorder and is highly significant in nephropathy groups compared to Group-I, whereas, the creatinine level is not significant in retinopathy group. Hemoglobin concentration is decreased significantly in all the groups compared to Group-I, The concentration of HbA_{1C} in diabetes Group-II is highly significant compared to that of control (Group-I), whereas, in comparison with other groups, the concentration of HbA_{1C} is not highly significant, which could be attributed to the patients being on medication. The decrease in HDL concentration is not significant in

Table 1

The data on number of subjects and their age, and concentrations of glucose, creatinine, hemoglobin (Hb), glycated hemoglobin (HbA_{1C}), low density lipoproteins (LDL) and high density lipoproteins (HDL) of control and diabetes subjects of Group-I and with complications (Group-II to V). The data of diabetic are compared with control and diabetes with complications

	Control	Group-I	Group-II	Group-III	Group-IV	Group-V
	Healthy	Diabetic	Diabetic CRF	Diabetic ESRD	Diabetic Retinopathy	Diabetic CRF & Retinopathy
No. of samples	45	65	41	42	23	41
Duration	–	$12.8 \pm 6.1^{\#}$	17.0 ± 9.4	16.7 ± 5.4	16.6 ± 4.6	17.2 ± 7.1
			NS	*	*	*
Glucose (mg/dl)	105.2 ± 40.2	140.9 ± 38.4	179.1 ± 75.3	217.4 ± 45.5	211.5 ± 60.3	214.8 ± 53.2
	***		*	*	*	***
Serum creatinine	1.0 ± 0.08	0.81 ± 0.2	4.01 ± 1.54	9.02 ± 2.79	0.95 ± 0.34	5.05 ± 2.28
(mg/dl)	NS		****	****	NS	****
Hb (g/dl)	13.9 ± 1.1	12.8 ± 2.1	9.1 ± 1.5	9.6 ± 1.4	10.6 ± 1.9	10.0 ± 1.9
	**		****	****	***	****
HbA_{1c} (%)	5.5 ± 0.6	8.2 ± 2.0	7.4 ± 1.5	7.6 ± 1.4	8.1 ± 2.7	7.6 ± 2.0
	****		*	NS	NS	NS
LDL (mg/dl)	112.4 ± 20.4	113.8 ± 30.4	114.7 ± 31.2	112.2 ± 30.1	118.0 ± 11.7	116.3 ± 20.3
	NS		NS	NS	NS	NS
HDL (mg/dl)	54.3 ± 13.0	53.7 ± 14.0	43.2 ± 10.2	45.2 ± 9.7	47.8 ± 17.2	48.5 ± 20.1
	NS		**	*	NS	NS

$^{\#}$ Mean $\pm$ SD. P-value corresponding to symbols *, **, ***, and **** are $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. NS – not significant.

Table 2

Change in elongation index (EI) of erythrocytes of diabetes blood samples at increasing concentration of glucose, as measured by Rheoscan–D ektacytometer, This is based on the mean values of glucose concentration and the corresponding measured EI at shear stress 3.0 Pa

S. No.	Glucose (mg/dl)	Elongation index	Change in EI (%)
1	130	0.295	–
2	150	0.292	−1.09
3	170	0.284	−3.64
4	190	0.275	−6.75
5	210	0.271	−8.19

Group-I compared to that of control, Groups IV and V, whereas the decrease in Groups II and III is significant compared to that of Group-I. No significant change in LDL concentration among various groups is observed.

Prior to measuring the EI of erythrocytes of blood samples, the Rheoscan-D was tested with diabetes blood samples (Group-I) of different fasting plasma glucose concentration and its sensitivity analysis was carried out. The sensitivity parameter S was calculated by:

$$S = [\{(\mathrm{EI})_{\mathrm{X}} - (\mathrm{EI})_{130}\}/(\mathrm{EI})_{130}] \times 100,$$

where $(\mathrm{EI})_{130}$ and $(\mathrm{EI})_{\mathrm{X}}$ are the reference EI at 130 mg/dl and at higher concentration of glucose, respectively. The variation in the mean EI with increasing mean glucose concentration is given in Table 2. The

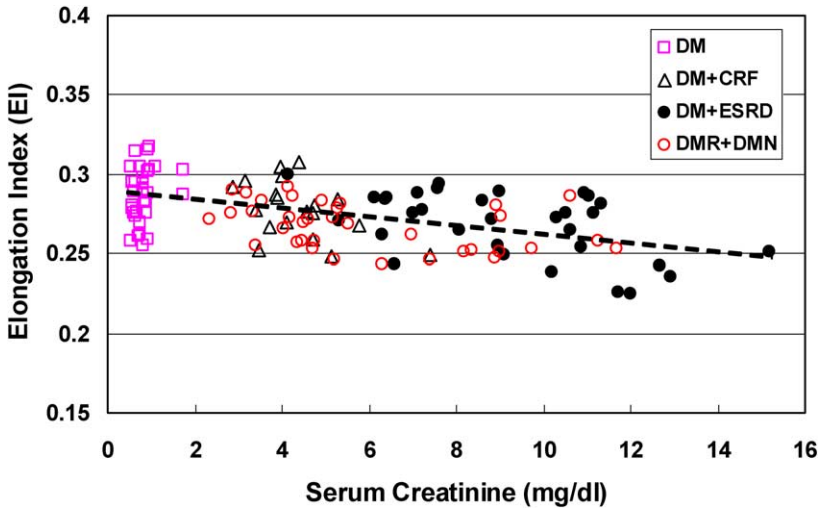

Fig. 1. Correlation between elongation index (EI) of erythrocytes and serum creatinine level at shear stress 3.0 Pa of healthy control and diabetes subjects of various groups ($n = 231$, $r = -0.508$, $p < 0.000001$).

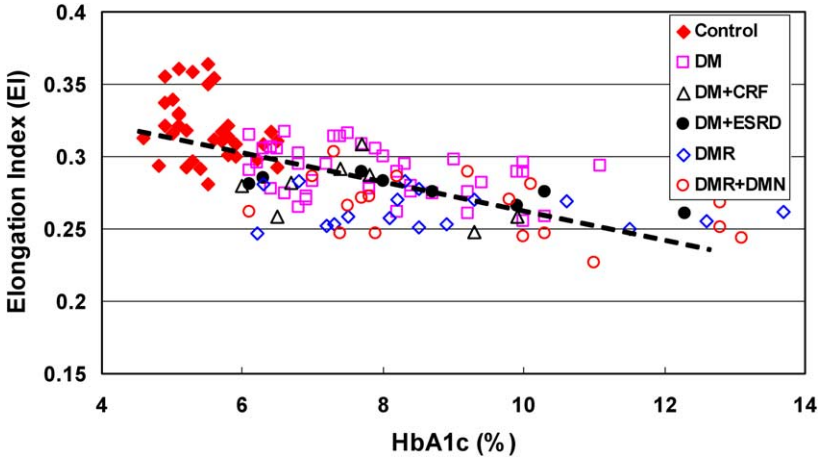

Fig. 2. Correlation between elongation index (EI) of erythrocytes and percentage of HbA_{1C} as measured at shear stress 3.0 Pa of healthy and diabetes subjects of various groups ($n = 231$, $r = -0.672$, $p < 0.00000001$).

EI with the increase of glucose concentration is reduced and the maximum change is observed at the concentration 210 mg/dl. This result shows that the present technique is sensitive to detect the changes in the EI with increasing glucose concentration but is associated with non-linearity, which could be attributed to the varying levels changes in cellular constituents.

The EI of various blood samples was measured and its variation with the concentrations of creatinine and glycated hemoglobin was analyzed. Figure 1 shows the variation of the EI with the increase of creatinine concentration. Good negative correlation coefficient between these parameters is observed ($r = -0.508$), indicating a decreasing trend of EI with the increase of creatinine level in the plasma. Figure 2 shows the variation of the EI at various concentrations of HbA_{1C}. A strong negative correlation ($r = -0.672$) between these parameters is observed. The EI decreases with the increase of HbA_{1C}.

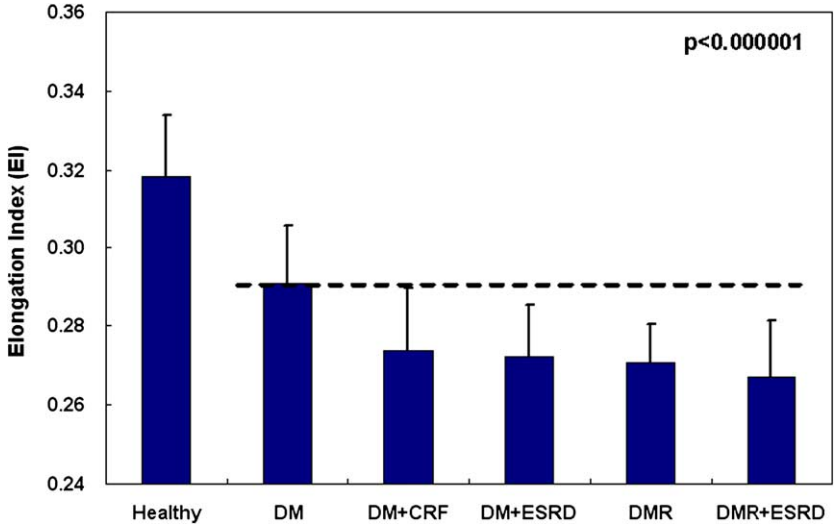

Fig. 3. Elongation index (EI) of erythrocytes as measured at 3.0 Pa for control and diabetes subjects with and without microangiopathic complications. Highly significant variation in the EI of erythrocytes of diabetes subjects compared to that of control ($p < 0.0001$) and of patients with diabetes complications is observed.

The variation of EI in diabetes groups and control subjects is shown in Fig. 3. The EI is the maximum for control and is decreased significantly ($p < 0.0001$) in the Group-I. A comparison of the EI erythrocytes of Group-II to V with that of Group-I further shows a significant reduction ($p < 0.0001$) in the EI. The EI is reduced to a minimum in erythrocytes of patients with dual complications due to retinopathy and nephropathy.

4. Discussion

Type 2 DM patients, due to improper utilization of glucose, cannot meet their energy requirements even at cellular level. Erythrocytes, after their release from bone marrow, undergo changes in their membrane bilayer, intracellular medium and cytoskeleton [5–12,18–20]. Increase of glycated hemoglobin leads to morphological changes in erythrocytes [15]. In combination with altered ratio of cholesterol to phospholipids in the core of the membrane [25], cross-linking of proteins [26] and enzyme dysfunction [27] due to oxidative stress, the erythrocytes become less deformable and are disrupted while flowing through microangiopathic blood vessels, thus leading to hemolytic anemia [25], resulting in decreased hemoglobin concentration as shown in Table 1. This severity is further increased in patients with renal failure [28]. Chronic hyperglycemia and oxidative stress in diabetes, especially in nephropathy, results in the formation and accumulation advanced glycation end products (AGEs) [29]. These changes also affect the erythrocyte deformability as shown by the decrease in elongation index. The recent morphological studies on erythrocytes from diabetes patients show that the erythrocyte shape with increase of glucose level in plasma deviate from its normal shape and the shape descriptor, form factor, shows an increasing trend similar to filtration time through the cellulose membrane [15]. These changes show that the alteration in cellular constituents is also associated with the morphological changes, leading to less deformable erythrocytes compared to that of healthy control.

Retinal circulatory alterations are thought to be early indicator of retinopathy. Involvement of two processes, development of neovascularization [19] and impairment of erythrocyte deformability [30, 31], lead to sluggish retinal microcirculation [32] associated with increase in blood viscosity [1,13]

The present study includes control and diabetic subjects with large variation of creatinine concentration, which increases in diabetic patients with nephropathy complications. Serum creatinine is frequently used for screening tests for nephropathy instead of glomerular filtration rate (GFR) [33]. There is strong and significant correlation between serum creatinine concentration and impaired erythrocyte deformability ($r = -0.508$, $p < 0.000001$) as shown in Fig. 1. Similar results are also reported in a recent study [34] that a significant correlation is observed between serum creatinine concentration and impaired erythrocyte deformability in diabetic nephropathy ($r = 0.43$, $P = 0.02$). However, there were wide standard deviation of EI and the distribution of EI overlaps somewhat at different creatinine levels in both studies. Further studies are required to establish the clinical significance of these findings.

In contrast to the serum creatinine, the increase in HbA_{1C} is controlled by medication but its level is still higher than that of control subjects. Despite the inclusion of medication effect, a good correlation coefficient between the EI and HbA_{1C} is obtained. The correlation coefficient between the EI and other parameters may further vary with the change in applied shear stress, as reported in the application of the laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Hoorn, The Netherlands) for malaria-infected erythrocytes [35], but for the present measurement an optimal value of the shear stress (3.0 Pa), similar to as in microcirculation, is preferred.

Previous studies with the St. George's filtrometer [36] and transparent microchannel capillary model and high-speed video camera system [37] have demonstrated the impaired deformability in type 2 diabetic patients. It is difficult to compare the results obtained by various methodologies, but erythrocyte deformability is strongly correlated with diabetes mellitus and complications. Increased serum creatinine was correlated with erythrocyte deformability in diabetic nephropathy using filtration method [34]. These results showed progressive impairment of deformability as the renal failure developed, which are in agreement with the present results.

In conclusion, the elongation index is a sensitive parameter in totality to describe the overall effect induced by the diabetic process in erythrocytes. Further significant reduction in the EI in various diabetes complications, as shown in present studies, could directly be taken as indicator of these complications. In contrast to the conventional levels of plasma and cellular constituents, the EI, in combination with flow related parameters could also provide information on the changes in blood flow through the affected region of the cardiovascular system.

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