Increased Shear Rate Resistance And Fastest Kinetics Of Erythrocyte Aggregation In Diabetes Measured With Ultrasound

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**OBJECTIVE** – To measure with ultrasound the increased erythrocyte aggregation (EA) kinetics and adhesion energy between red blood cells in patients with type 2 diabetes mellitus and a poor metabolic control.

**RESEARCH DESIGN AND METHODS** – Blood samples were analyzed in a Couette rheometer at 32 MHz following shear rate reductions from 500 s\(^{-1}\) to residual shears of 0 (stasis), 1, 2, 10, 50, 100 and 200 s\(^{-1}\). The increase in EA was determined with the integrated backscatter coefficient (IBSC) as a function of time and shear rate.

**RESULTS** – The time required to form aggregates was shorter in diabetic patients at shear rates below 200 s\(^{-1}\) (\(p < 0.01\)). Erythrocytes formed larger aggregates in patients than in controls (\(p < 0.05\) at 2 to 100 s\(^{-1}\)).

**CONCLUSIONS** – Ultrasound can potentially demonstrate non-invasively, *in vivo* and *in situ* the impact of local abnormal EA on arteriovenous flow disorders in diabetes mellitus.
Flow disorders in diabetes often lead to severe outcomes in various organs and tissues; abnormal rheology of erythrocytes (RBCs) likely impairs macro- and microcirculatory blood flow, tissue oxygenation and vascular tone regulation in those patients (1-3). Diabetic retinopathy is attributed to microvascular flow disorders and enhanced RBC aggregation (4). Erythrocyte aggregation (EA) and plasma viscosity are also predictive of diabetic foot syndrome deterioration (5). EA is a reversible phenomenon responsible for increased blood viscosity at low shear rates. RBC hyper-aggregation can also promote flow stasis and thrombosis in the macrocirculation. This study proposes an ultrasound method that has the potential to non-invasively detect early rheological disorders in situ in blood vessels. The method is based on backscattering of ultrasound by blood; it measures the extent of EA and its shear rate dependency.

**RESEARCH DESIGN AND METHODS**

**Populations:** Recruited individuals were non-smoking males. They completed a questionnaire on current medications and medical history. Body mass index (BMI) and blood pressure were measured. Nine patients with type 2 diabetes mellitus and eight healthy controls gave their informed consent to the approved protocol. Patients were intentionally chosen with a poor metabolic control. Lipid profile and inflammatory proteins (fibrinogen - Fb, Von Clauss method; haptoglobin - Hp and immunoglobin G - IgG, immunonephelometric method; C-reactive protein - CRP, latex agglutination technique) were determined for each participant. Six patients were on oral antidiabetic and aspirin medications, 3 on insulin, 5 on a cholesterol lowering therapy and 4 were treated for hypertension. Those on insulin had a history of coronary artery disease and 2 of them had suffered from myocardial infarction. Distal angiopathy or cutaneous trophic disorders were not present. Mean age of patients was 58.3 ± 8.8 years (range 41-70). Healthy subjects were age matched (51.1 ± 8.7 years, range 41-64), did not take regular medications and had no history of cardiovascular disease. None had lipid disorders or hypertension.

**Couette experiments:** A Couette instrument made of two concentric cylinders and installed in an incubator at 37 °C generated homogeneous shear rates by rotating outer cylinder. Fifty mL of EDTA anticoagulated blood at 40% hematocrit was introduced between both cylinders. Stationary inner cylinder held ultrasound transducer perpendicular to flow. The 32 MHz PVDF ultrasound transducer (Visualsonics, Toronto, CANADA) had a -6 dB bandwidth of 15-45 MHz. It was pulsed with bipolar square waves (Avtech #AVB2-TA-C-CRIMA, Ottawa, CANADA). Received radio-frequency (RF) echoes (Miteq #AU-3A-0120, Hauppauge, NY) were amplified by 54 dB, filtered between 10-50 MHz (Panametrics #5900 PR, Waltham, MA) and digitized at 250 MHz (GageScope #8500 CS, Montreal, CANADA).

The protocol consisted in imposing a 500 s⁻¹ shear rate for 120 seconds to disrupt aggregates. Then, recording of aggregation kinetics for 380 seconds was performed at reduced shear rates of 0, 1, 2, 10, 50, 100 and 200 s⁻¹ randomly applied. One hundred RF echoes were acquired every 2 seconds. For each blood, the protocol was repeated three times for averaging. RF signals were
compensated for blood attenuation at each shear rate and integrated backscatter coefficient (IBSC) was determined, as in (6). It reflects number of RBCs per aggregate (7).

RESULTS
BMI, blood pressure, triglyceride, cholesterol, HDL and LDL cholesterols, Fb and CRP were not different between groups \( (p > 0.05) \), unpaired \( t \)-tests), whereas IgG \( (8.4 \pm 1.0 \) versus \( 11.3 \pm 1.8 \) g/L, \( p < 0.001 \) ), Hp \( (1.0 \pm 0.4 \) versus \( 1.6 \pm 0.5 \) g/L, \( p < 0.05 \) ) and HbA1c \( (5.5 \pm 0.8 \) versus \( 8.8 \pm 2.1\% , \( p < 0.01 \) ) were significantly higher in patients (mean ± SD). Couette flow protocol resulted in these observations: disaggregation of RBCs and minimum IBSC at 500 \( s^{-1} \); formation of aggregates and rapid increase in IBSC depending on reduced applied shear (e.g., see Fig. 2 of (7)); stable aggregate sizes and plateaus of IBSC after few seconds for most shears to minutes at 0 \( s^{-1} \).

Figure 1a summarizes mean raising slopes of IBSC between 2-8 s after shear rate reductions. In controls, maximum slope at 2 \( s^{-1} \) was not different from those at 1 and 10 \( s^{-1} \) \( (p > 0.89) \), whereas in patients, maximum occurring also at 2 \( s^{-1} \) was similar to that at 1 \( s^{-1} \) \( (p = 1.0) \). Except for 200 \( s^{-1} \) \( (p = 0.11) \), kinetic slopes were always faster in diabetic patients, which is indicative of higher rates of neighboring RBC clustering.

IBSC at plateaus averaged between 180-380 s after shear rate reductions are presented in Fig. 1b. In controls, maximum IBSC at 2 \( s^{-1} \) was similar to those at 0 and 1 \( s^{-1} \) \( (p > 0.95) \). Similarly, IBSC at 1 and 2 \( s^{-1} \) were similar in patients \( (p > 0.95) \). IBSC were statistically higher in patients between 2-100 \( s^{-1} \), which reflects stronger adhesions and bigger steady state aggregate sizes in diabetes.

CONCLUSIONS
Statistically significant differences in Figure 1 were noted for shears between 2-100 \( s^{-1} \), which correspond to normal flow at center streams and pathological flow stasis in recirculation zones of large systemic veins and arteries. Accordingly, EA in diabetes can be related to lower limb artery ischemic events, microangiopathy in foot extremities and retinopathy. Inflammation is involved in pathogenesis of type 2 diabetes mellitus and RBC aggregation, which agree with our results (legend of Fig. 1). Subacute inflammatory state promoting RBC aggregation is also associated with obesity (8) and metabolic syndrome (9). Thus, reducing inflammation (and indirectly aggregation) with statins, and HbA1c with antidiabetic medication and/or diet, are indicated as both have known benefits on cardiovascular consequences of diabetes. We reported measurements in a laboratory instrument but a short-term objective is sizing RBC aggregates in vivo with ultrasound (10). At 32 MHz, superficial 5-6 mm depth vessels can be scanned. The proposed non-invasive method should be investigated further as it may have potential benefit for diagnosis and follow-up of diabetic foot complications, and for monitoring therapy.

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REFERENCES
Figure 1:
(a) Raising slopes from 2 to 8 seconds of the integrated backscatter coefficient (IBSC) as a function of the shear rate applied on blood samples (means ± standard deviations).
(b) IBSC at the plateau of RBC aggregation as a function of the shear rate (means ± standard deviations). The power of 0 dB corresponds to that of a perfect flat stainless-steel reflector. Two-way analyses of variance (Tukey method for multiple comparisons) confirmed impact of shear rate ($p < 0.001$) and population ($p < 0.001$) on IBSC slopes and IBSC at plateaus. The $p$ values shown on the figure correspond to multiple comparisons between populations. IBSC slopes at 2 s$^{-1}$ were correlated with physiological variables (Pearson coefficient $r = 0.59$, $p = 0.02$ for HbA$\text{A}_1\text{C}$; $r = 0.53$, $p = 0.03$ for Fb; $r = 0.54$, $p = 0.02$ for IgG; and $r = 0.72$, $p = 0.001$ for Hp). Forward stepwise regressions explained IBSC kinetic slopes at 2 s$^{-1}$ by the following model ($r = 0.94$): IBSC kinetic = -1.00 ($p = 0.009$) + 0.67 Hp ($p < 0.001$) + 0.11 IgG ($p = 0.003$) + 5.60 HbA$_{1c}$ ($p = 0.048$). Only IgG was positively correlated with the plateau of IBSC at 2 s$^{-1}$ (Pearson coefficient $r = 0.49$, $p = 0.046$).