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Effects of hyperglycaemia and sorbitol accumulation on erythrocyte deformability in diabetes mellitus

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SUMMARY Erythrocyte deformability was studied in a total of 83 poorly controlled diabetics (mean blood glucose 12·2 mmol/l) who were divided into three groups, each with matched healthy controls. There was no appreciable difference between diabetics and matched controls regarding the filtration of erythrocytes through 3 μm diameter straight channel pores (25 diabetics) or tortuous channel pores (28 diabetics), or for the measurement of erythrocyte elongation over a range of osmolalities in the Ektacytometer (30 diabetics). When erythrocytes from 17 additional diabetics and 17 healthy controls were incubated for two hours at 37°C in hyperglycaemic (50 mmol glucose/l) buffer, however, there was a considerable reduction in erythrocyte filterability for both diabetics and controls in parallel with an increase in erythrocyte sorbitol concentration. This loss of filterability was prevented by the addition of an aldose reductase inhibitor (Sorbinil). High glucose concentrations ($\simeq$50 mmol/l) impair the filterability of erythrocytes through 3 μm pores, and the intracellular accumulation of sorbitol in poorly controlled outpatients is therefore unlikely to have a major adverse effect on erythrocyte rheology in diabetes mellitus.

Erythrocytes from uncontrolled diabetics were originally found to have impaired deformability when suspended in autologous plasma and filtered through 5 μm diameter pores.[1] It was subsequently suggested that the impaired erythrocyte filterability could be corrected in vivo and in vitro by the addition of insulin.[2] Several factors extrinsic to the erythrocyte, however, may contribute to this impairment of filterability in uncontrolled diabetes. These include an acute phase increase in plasma fibrinogen concentration[3]; leucocytosis[4]; plasma hyperosmolality,[3 5] which can increase erythrocyte mean cell haemoglobin concentration; and a low blood pH[6] or low plasma sodium concentration,[7] which can increase erythrocyte mean cell volume.

The individual rheological effects of these extrinsic factors can be studied by resuspending leucocyte depleted washed erythrocytes in an appropriate buffer. In two earlier studies normal erythrocytes suspended in hyperglycaemic buffer showed impaired filtration through 5 μm diameter pores.[8 9] As water crosses the erythrocyte membrane in milliseconds[10] and glucose loss from the cell has a half life of <30 seconds,[11 12] hyperglycaemia of plasma or buffer is unlikely to impair erythrocyte rheology by a simple osmotic effect. Human erythrocytes can respond to a hyperglycaemic environment, however, by forming intracellular sorbitol,[13 14] and as sorbitol does not readily diffuse out of cells its concentration in the diabetic erythrocyte is raised.[15] Accumulation of sorbitol via the polyol pathway could thus affect the osmotic properties and consequently the deformability of erythrocytes:

$$\text{D-glucose} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{aldose}} \text{sorbitol} + \text{NADP}^+$$

$$\text{sorbitol} + \text{NAD}^+ \xrightarrow[\text{dehydrogenase}]{\text{sorbitol}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

where $NADP^+$ = nicotinamide adenine dinucleotide phosphate and NAD^+ = nicotinamide adenine dinucleotide.

Intracellular accumulation of sorbitol in diabetics has previously been implicated in the development of cataracts,[16] peripheral neuropathy,[17] and arterial wall lesions.[18] We therefore compared the rheology of erythrocytes from poorly controlled outpatient diabetics, with that of matched controls, and we also investigated the rheological effect of incubating diabetic and normal erythrocytes in high concen-

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trations of glucose in vitro with and without inhibition of the polyol pathway by the aldose reductase inhibitor Sorbinil.

Methods

PATIENT GROUPS

Rheological measurements were performed on poorly controlled outpatient diabetics, with a mean blood glucose concentration for the 83 patients of 12·2 mmol/l (range 2·4–28·1). Table 1 gives the clinical and biochemical data.

Erythrocyte filtration (3 μm polycarbonate membrane) Twenty five poorly controlled, insulin dependent diabetics (11 men; mean age 46·0 years, range 21–78) were matched for age and sex with 25 healthy controls (11 men; mean age 40·7 years, range 17–86).

Erythrocyte filtration (3 μm silver membrane) Twenty eight diabetics (14 insulin dependent; 17 men; mean age 49·4 years, range 17–70) were matched for age and sex with 28 healthy controls (17 men; mean age 48·4 years, range 21–68).

Laser viscodiffractometry Thirty diabetics (14 insulin dependent; 19 men; mean age 47·9 years, range 19–72) were matched for age and sex with 30 healthy controls (19 men; mean age 45·9 years, range 20–69).

Blood samples for rheological tests were taken from an antecubital vein into lithium heparin (15 IU/ml blood; Sterilin Ltd, Feltham, Middlesex) and analysed within four hours.[19]

A pure suspension of washed erythrocytes was prepared for filtration studies by passage of whole blood through Imugard IG500 cotton wool (Terumo Corporation, Tokyo, Japan) followed by two washes in phosphate buffered saline (75 mmol/l phosphate) of pH 7·4 and osmolality 290 mmol/kg.[20 21] Erythrocyte filtration was measured by the initial flow rate method using a Hémorhéomètre SPO2 (IMH, 95470 St Witz, France) with water jacket. Filtration membranes comprising disposable straight channel polycarbonate membranes (Nuclepore Corporation, Pleasanton, California, United States of America) of 3 μm (batch 62A2 B26) measured pore diameter,[22] and sintered silver Selas/Hytrex membranes with tortuous channels (Osmonics Inc, Minnetonka, Minnesota, United States of America) of nominal 3 μm diameter (batch 013) were used; the latter were cleaned by ultrasonication in aqueous sodium dodecyl sulphate[23] and reused. Filtration results were expressed as an index of filtration (IF) after correction for haematocrit,[24] as measured by a Coulter S counter (Coulter Electronics Ltd, Luton); IF values were also expressed as a percentage change in the in vitro study (Table 2). An increase in IF corresponds to a loss of filterability (reduction in deformability).

Laser viscodiffractometric measurements of erythrocyte elongation were made at ambient temperature using the Ektacytometer (Technicon International Division, Saint Denis, France), as previously described.[25] Whole blood was sampled by the Ektacytometer in the clinical study and an osmotic deformability profile, or Osmoscan plot,[26] was obtained by measuring erythrocyte elongation at a shear stress of 14 Pa in a continuously increasing osmotic gradient (80–500 mmol/kg) to give the following valves:

EI max = maximum value for erythrocyte elongation adjusted for mean cell volume[27]

O max = osmolality at EI max

O min = osmolality at which erythrocyte elongation reached its minimum in hypotonicity

O hyper = osmolality at which erythrocyte elongation decreased to half the EI max value in the hypertonic arm of the curve.

In the clinical studies erythrocyte mean cell volume was measured using a Coulter S Plus IV counter; blood glucose by a glucose oxidase method using blood anticoagulated with sodium fluoride; and haemoglobin A_1 (Hb A_1) by affinity chromatography (Glycogel B; Pierce UK Ltd, Cambridge).[28]

In vitro incubation of erythrocytes (from 17 poorly controlled, insulin dependent diabetics and 17 healthy controls) was performed under hyperglycaemic conditions using Imugard IG500 filtered and washed erythrocytes resuspended at 0·07 (7%) haematocrit in phosphate buffered saline. Incubation was performed with added D-glucose (Analar grade, BDH Chemi-

Table 1 *Mean (range) values for clinical and biochemical data in the three groups of diabetics in clinical study*

	Hémorhéomètre polycarbonate membranes (n = 25)	*Hémorhéomètre silver membranes (n = 28)*	*Ektacytometer (n = 30)*
Duration of diabetes (years)	9·3 (1–23)	6·8 (1–20)	7·8 (1–42)
Blood glucose (mmol/l)*	14·1 (6·4–28·1)	11·9 (2·4–25·3)	10·8 (3·1–27·6)
Erythrocyte HbA_1 (%)	14·7 (7·6–22·2)	11·8 (6·2–17·8)	11·3 (6·2–16·7)
Erythrocyte mean cell volume (fl)	88·6 (81–98)	87·4 (79–95)	86·8 (74–95)

*1 mmol/l glucose = 18 mg/100 ml.

Table 2 *Results of clinical study showing mean (SEM) values for Hémorhéomètre and Ektacytometer in diabetic patients and matched controls*

	Diabetic patients	*p value*	*Healthy controls*
Hémorhéomètre IF (No of matched pairs)			
3 μm polycarbonate membranes (25)	97·2 (3·3)	NS	104·0 (4·3)
3 μm silver membranes (28)	61·6 (2·2)	NS	61·8 (2·8)
Ektacytometer (No of matched pairs)			
EI max (30)	0·55 (0·01)	NS	0·55 (0·01)

cals Ltd, Poole) at concentrations of 5, 25, and 50 mmol/l giving final osmololities of 295, 315, and 340 mmol/kg, respectively. After incubation at 37°C for two hours erythrocyte filtration through polycarbonate membranes of 3 μm pore diameter was measured at 25°C using the Hémorhéomètre. Ektacytometric measurements were performed at ambient temperature on the same samples of washed erythrocytes.

A further in vitro incubation experiment was performed using Imugard IG500 prefiltered and washed erythrocytes from 17 healthy controls. Incubation was performed in phosphate buffered saline with added D-glucose at 5 and 50 mmol/l with and without 5 μg/ml Sorbinil (Pfizer Central Research, Sandwich). Erythrocytes from eight additional controls were similarly incubated in phosphate buffered saline with D-glucose (5 and 50 mmol/l) but with and without 200 μU/ml insulin (Actrapid MC; Novo Laboratories Ltd, Basingstoke).

Before and after the above incubations osmolality (Advanced Osmometer Model 3W, Advanced Instruments Inc, Needham Heights, Massachussets, United States of America); pH; erythrocyte morphology (interference microscopy of erythrocytes fixed in 1·25% w/v glutaraldehyde in phosphate buffered saline); and erythrocyte indices (calculated from Coulter S Plus IV erythrocyte count and haemoglobin concentration and centrifuged microhaematocrit) were determined for all erythrocyte suspensions. Sorbitol estimations were made on representative samples from diabetics and controls at the end of the incubation period using a modified enzymatic method,[15] in which sorbitol dehydrogenase was used to convert sorbitol to fructose with measurement of the stoicheiometrically produced NADH.

Significance (two tail) was determined by the Mann-Whitney U test for comparison between diabetics and healthy controls and by Wilcoxon's signed rank test for comparison of in vitro paired samples.

Results

Table 1 shows clinical and biochemical details of the three patient groups studied. In this clinical study the filtration of diabetic erythrocytes through 3 μm straight channel polycarbonate membranes (25 patients), or through 3 μm tortuous channel silver membranes (28 patients) was not significantly different from the filtration of erythrocytes from healthy matched controls (Table 2). When erythrocyte elongation (EI max) in the Ektacytometer was measured, there was also no significant difference between 30 diabetics and their matched controls. The Ektacytometer Osmoscan curve valves, O min, O max, and O hyper also showed no significant difference between diabetics and controls (data not shown). Erythrocyte mean cell volume did not differ significantly between patients and controls in any of the three clinical groups.

In vitro incubation of diabetic erythrocytes in a higher (50 mmol/l) glucose concentration for two hours resulted in pronounced accumulation of erythrocyte sorbitol, the concentration increasing from 1–5 μmol/l to >100 μmol/l erythrocytes. This increase was associated with a significant ($p < 0·02$) reduction in erythrocyte filterability through 3 μm diameter straight channel pores compared with filterability after incubation in 5 mmol/l glucose (Table 3). Incubation in 25 mmol/l glucose did not cause a significant loss of erythrocyte filterability. When normal erythrocytes were similarly incubated for two hours in 50 mmol/l glucose (Table 3), there was again a pronounced increase in sorbitol accumulation to >100 μmol/l erythrocytes, together with a significant ($p < 0·01$) reduction in their filterability compared with incubation in 5 mmol/l glucose (Table 3). Incubation in 25 mmol/l glucose again had no effect on erythrocyte filtration. At a glucose concentration of 50 mmol/l glucose, the erythrocytes from healthy controls showed a quantitatively greater loss of filterability ($p < 0·025$) than the erythrocytes from diabetics (Table 3). Values for EI max, measured by the Ektacytometer, were not significantly different between the three glucose concentrations (Table 3) and the Ektacytometer Osmocan curve variables (O min, O max, and O hyper) were also not significantly different (data not shown).

When erythrocytes from an additional 17 healthy controls were incubated for two hours in 50 mmol/l glucose there was an almost identical (12%–13%) loss of erythrocyte filterability through 3 μm diameter straight channel pores compared with the loss that occurred with incubation in 5 mmol/l glucose (Table 4). Addition of 5 μg/ml Sorbinil to the hyperglycaemic buffer prevented this loss of filterability (Table 4). In contrast, when 200 μU/ml insulin was

Table 3 *Results of in vitro study showing mean (SEM) values for Hémorhéomètre and Ektacytometer following two hours' incubation of erythrocytes in hyperglycaemic buffer*

	Glucose concentration (mmol/l)				
	5	*p value*	*25*	*p value*	*50*
*Hémorhéomètre IF (%):**					
Diabetic erythrocytes (No of experiments = 17)	100 (6·5)	NS	100·7 (6·3)	<0·02	104·8 (8·0)
Normal erythrocytes (No of experiments = 17)	100 (5·2)	NS	103·4 (5·9)	<0·01	112·4 (8·1)
Ektacytometer EI max:					
Diabetic erythrocytes (No of experiments = 17)	0·56 (0·01)	NS	0·56 (0·01)	NS	0·56 (0·01)
Normal erythrocytes (No of experiments = 17)	0·58 (0·01)	NS	0·58 (0·01)	NS	0·57 (0·01)

*Results shown as a percentage in relation to the result at 5 mmol/l expressed as 100%.

Table 4 *Effects of inhibition of polyol pathway by Sorbinil (5 μg/ml) on filtration of erythrocytes from 17 healthy controls after incubation for two hours in hyperglycaemic buffer*

	Glucose concentration (mmol/l)				
	5		*50*		
		p value	*With Sorbinil*	*p value*	*Without Sorbinil*
Hémorhémètre IF (%)*	100 (3·5)	NS	101·1 (3·2)	<0·001	112·7 (3·9)

*Results for mean and SEM expressed as in Table 3.

added to erythrocytes from eight healthy controls and incubated for two hours in 50 mmol/l glucose there was no protective effect, IF increasing from 100% to 112·2% (SEM 6·6) without insulin and to 112·5% (6·3)% with insulin.

Incubation at the three glucose concentrations in the above experiments caused no appreciable change after two hours in erythrocyte mean cell volume, mean cell haemoglobin concentration, or erythrocyte morphology.

Discussion

In clinical studies of erythrocyte deformability filtration through 3 μm pores may be more sensitive to small changes in erythrocyte rheology than filtration through 5 μm pores,[29 30] provided that erythrocyte mean cell volume does not change.[22] It is also known that erythrocyte filtration techniques are highly sensitive to the presence of contaminating leucocytes in excess of $0{\cdot}025 \times 10^9$/l.[18] The conflicting results of previous erythrocyte filtration studies in diabetes[31-35] may therefore have been due to the use of 5 μm diameter pores or to a variable degree of leucocyte contamination of the test erythrocyte suspensions, or both.

We therefore filtered a pure suspension of washed erythrocytes through 3 μm diameter pores of both straight channel and tortuous channel type. No loss of filterability of diabetic erythrocytes through either type of membrane was found in the clinical study, even when poorly controlled patients were studied; this finding agrees with one previous study of 3 μm diameter straight channel pores.[36] Washed erythrocytes from diabetics in the steady state have also shown normal rheological behaviour in micropipette aspiration studies[37 38] and normal viscosity when measured at a high shear rate.[39] Our results show that unwashed diabetic erythrocytes elongate normally when sheared in the Ektacytometer over a range of osmolalities from 80 to 500 mmol/kg; this is again in agreement with a previous study.[40]

Our results were obtained using blood from 83 poorly controlled outpatient diabetics who had a mean blood glucose concentration of 12·2 mmol/l when venesected. We therefore studied the rheological effects of incubating erythrocytes in much higher concentrations of glucose. An appreciable reduction in 3 μm pore filterability was obtained at 50, but not at 25 mmol/l glucose, and the effect was greater for erythrocytes from normal subjects rather than from diabetics. Insulin had no rheologically pro-

tective effect in vitro, in contrast to a previous report.[2] Our in vitro incubations did not change the size, haemoglobin concentration, or shape of the erythrocytes. Alternative explanations for this loss of filterability of a pure suspension of washed erythrocytes include glycosylation of membrane protein,[41 42] or the intracellular accumulation of sorbitol via enhanced activity of the polyol pathway.[43] The effect of sorbitol accumulation on erythrocyte cytoplasmic viscosity is unknown, however, although normal erythrocytes incubated in glucose in vitro have been reported to have decreased filterability.[8] Our in vitro two hour incubation of normal erythrocytes in 50 mmol/l glucose resulted in a high intracellular concentration of sorbitol. When Sorbinil was added to the incubation buffer to prevent sorbitol accumulation there was no longer any appreciable loss of erythrocyte filterability. This substantiates the hypothesis that sorbitol accumulation has a rheological effect on erythrocytes, but, despite a 25 fold increase in sorbitol (to concentrations at least twice that found in poorly controlled diabetics) the loss of filterability through 3 μm pores was only 12%–13%. It would thus seem unlikely that sorbitol accumulation has an important role in the rheology of erythrocytes from poorly controlled outpatient diabetics, in whom we were unable to show any rheological abnormality by filtration or Ektacytometric methods.

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