

## EVALUATION OF RED BLOOD CELL AGGREGATION IN DIABETES BY COMPUTARIZED IMAGE ANALYSIS

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**Abstract** Red blood cell (RBC) aggregation has been widely studied and its importance is well established in the rheology of microcirculation. RBC aggregation is a major factor responsible for the flow properties of blood. Increased RBC aggregation has been observed in several pathological states. Therefore, the measurement of erythrocyte aggregation is rheologically important for quantifying flow abnormality in pathological conditions. Normal RBC under low flow or at rest form rouleaux aggregates, while abnormal RBC aggregation may lead to the formation of irregular aggregate structures, which may be induced by cell-associated factors (reduced membrane sialic acid levels) but also by extracellular factors. The main objective of the present investigation was to study RBC aggregate morphology in diabetic patients, using direct microscopic observation and numerical processing of recorded digitized images. Blood samples were obtained from 20 diabetic patients and from 15 normal control subjects. The aggregate morphology was quantified by the so-called Aggregate Shape Parameter (ASP) defined as the ratio of the aggregate projected area to its square perimeter. ASP appeared significantly higher ( $p < 10^{-5}$ ) in diabetic patients ( $0.65 \pm 0.18$ ) than in normal controls ( $0.28 \pm 0.15$ ). This rheo-optical method based on the theoretical model for rouleaux aggregates provides a useful reference for measuring deviations of RBC aggregate morphology. Increased aggregation of RBC resulting from a decreased sialylation of glycoporphins may be an important factor in the development of vascular diseases and in the microcirculation impairment.

**Resumen** *Evaluación de la agregación eritrocitaria en diabetes por análisis computarizado de imágenes.*

La agregación eritrocitaria, juega un papel muy importante en el flujo sanguíneo, particularmente en la microcirculación. Su estudio cobra importancia en la medida que permita cuantificar anomalías circulatorias en diversas patologías. En condiciones normales los glóbulos rojos (GR) se agregan en estructuras cilíndricas denominadas *rouleaux*, mientras que en condiciones anormales, los agregados adoptan formas irregulares con tendencia morfológica esferoidal. Estas formas anómalas pueden ser inducidas por alteraciones celulares (disminución del contenido de ácido siálico de las glucoproteínas), o por factores extracelulares. El objetivo de este trabajo fue estudiar la morfología de los agregados eritrocitarios, comparándola entre 20 pacientes diabéticos y 15 individuos sanos, considerados grupo control. La agregación se cuantificó con un parámetro de forma de los agregados, denominado ASP (*Aggregate Shape Parameter*), definido como la relación entre el área proyectada de los agregados y el cuadrado de su perímetro. Los datos fueron obtenidos con muestras de suspensiones de GR en plasma autólogo, al 2% de hematocrito, las cuales fueron observadas con una cámara CCD y procesadas numéricamente con un procesador digital de imágenes (DIP), ambos acoplados a un microscopio invertido. Los pacientes diabéticos presentan valores de ASP ( $0.65 \pm 0.18$ ) significativamente mayores ( $p < 10^{-5}$ ) que los controles normales ( $0.28 \pm 0.15$ ). Un aumento de la agregación eritrocitaria representa un importante factor de riesgo en el desarrollo de patologías vasculares, con posible deterioro de la microcirculación. En ese sentido, el ASP provee una útil referencia para cuantificar alteraciones en la morfología de los agregados.

**Key words:** red blood cell aggregation, diabetes, vascular diseases

A large number of biological processes are connected with changes in the rheological properties of the body organs, fluids or cells concerned. Blood and red blood

cell (RBC) suspensions can be approximately described as concentrated dispersions of *Structural Units*, as RBC rouleaux and network of these rouleaux<sup>1</sup>. Both the aggregation and disaggregation of RBC play an important role in the pathophysiological behavior of blood circulation<sup>2</sup> in a large number of vascular disorders such as hypertension<sup>3,4</sup> and diabetes<sup>5,6</sup>. Evaluation of red blood cell aggregation was frequently extrapolated from blood viscosity measurements at low shear rates<sup>7,8</sup>. Relatively

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little data are available on the shear stress needed to break up the aggregated RBC in normal or pathological conditions. In contrast only few studies used direct quantitative methods to evaluate RBC aggregation<sup>9</sup>. In this study, we used an optical method for the quantitative determination of aggregate morphology, using computerized image analysis.

RBC aggregation is an important factor in blood viscosity at low shear rate. The role of aggregation as a potential risk factor of hyperviscosity will be now discussed. It is unlikely that rouleaux present a risk factor; unless, by the formation of tenacious networks leading to high blood viscosity. There may be a progressive transition of rouleaux superstructure into clumps and a spectrum of different morphologies of aggregates is feasible.

In diabetic patients, several hemorheological alterations have been described with important micro and macrocirculatory influence. One of the main alterations is an increased RBC aggregability, which has been attributed to the high levels of plasmatic fibrinogen frequently found in these patients. However, in diabetes, several modifications which have also been described in the structure of RBC membrane can contribute to the higher aggregability of these cells<sup>10</sup>.

The purpose of the present investigation was to study modifications in RBC aggregate morphology. This study has been carried out by analysing digitized microscopic images of RBC aggregates<sup>11</sup> and comparing them between healthy and diabetics subjects.

## Material and Methods

A total of 15 normal subjects and 20 diabetic patients, age  $56 \pm 4$  (mean  $\pm$  DS, ranging from 30 to 60 years) were studied. The patients were classified in Insulin Depend Diabetic ( $n = 7$ ) and Non Insulin Depend Diabetic ( $n = 13$ ). Regarding retinopathy, diabetic patients were classified according to its severity into two groups: proliferative retinopathy ( $n = 4$ ) and non proliferative retinopathy ( $n = 16$ ).

Human venous blood samples were taken and anticoagulated with heparin. Each sample was centrifuged to separate the plasma and to remove the buffy coat.

Samples were prepared by resuspending RBC at 2% hematocrit in autologous plasma. Aliquots of these suspensions were poured into an excavated slide and placed on the stage of an optical inverted microscope. RBC aggregate populations were microscopically observed for up to 5 minutes and recorded by a CCD camera coupled to a digital image processor. Digitized projected images of aggregates were analyzed for size, area and perimeter.

**RBC Aggregate Shape Parameter (AS).** The aggregation morphology is characterized by a dimensionless parameter called Aggregation Shape Parameter:

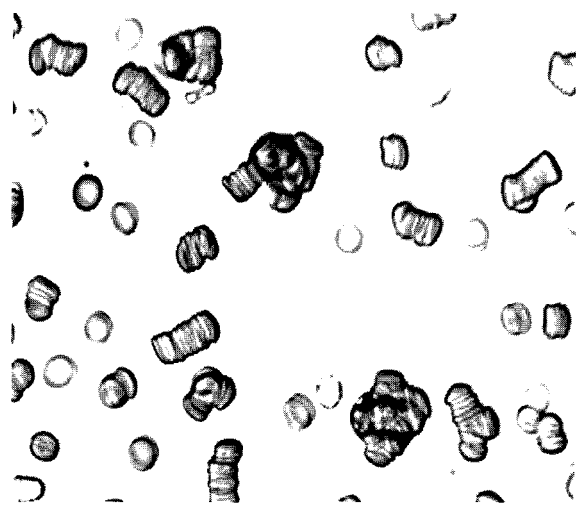
$$ASP = 4 \pi A/P^2$$

where A is the projected area of the aggregate and P is its perimeter.

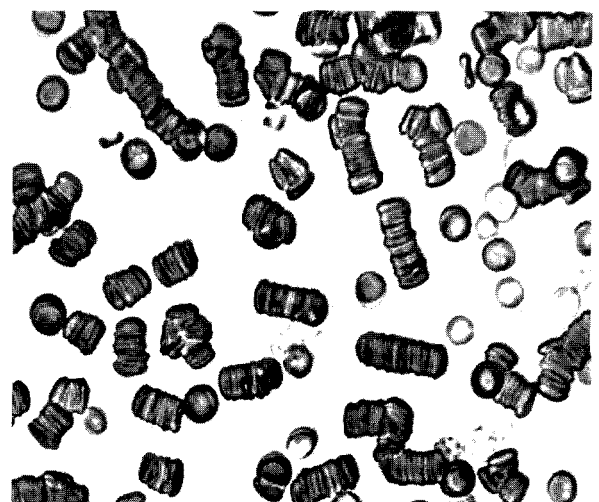
A circle, which is the projected area of sphere, has the maximal ASP value ( $ASP = 1$ ) while a cylinder shows a rectangular projected area. It is very simple to deduce that the longer the cylindrical aggregate, the smaller the ASP. It should be noted that the ASP is sensitive to small deviations of the

Table 1.— Average values of ASP in healthy and diabetic subjects (mean  $\pm$  SD)

Erythrocyte	ASP
Normal	$0.28 \pm 0.15$
Diabetics	$0.65 \pm 0.18$



A. spheroidal RBC aggregates in the sample from a DBT patient



B. Rouleaux formation in healthy control suspension

Fig. 1.— Digitized images of RBC aggregates (X 400) from two different samples. A. Diabetic patient, (DBT) and B. Healthy control

aggregate morphology from the normal rouleaux shape and rather insensitive to rouleaux beginning and stretching.

Results of ASP are expressed as mean  $\pm$  SD or median (range). Statistical differences were determined by using Mann-Whitney U-test.

## Results

Erythrocyte aggregation measured by ASP, appeared increased in the case of diabetic patients compared with normal control subjects (Table 1).

Digitized image of aggregated RBC revealed different rouleaux morphology for control and diabetic patients. The aggregates of diabetic patients take a spheroidal shape instead of the classical cylindrical shape (rouleaux), (Figure 1).

## Discussion

Several hemorheological alterations having important micro and macrocirculatory influence have been described in diabetic patients. One of the most important alterations encountered in impaired blood circulation was abnormally increased RBC aggregability.

Under normal conditions erythrocytes have a negative electrical surface charge, the magnitude of which affects profoundly their interaction with other erythrocytes and with biological surfaces. Some authors have demonstrated a decrease in erythrocyte glycoprotein sialic acid content in human diabetes<sup>12, 13</sup>. Decreased glycoprotein sialylation provides an explanation at the molecular level for increased erythrocyte aggregation and may be important in the pathogenesis of vascular disease in diabetes.

It is important to dispose of simple and satisfactory techniques to study and quantify RBC aggregation, because this phenomenon plays a major role in the rheological properties of blood and often appears altered in disease states.

This study presents ASP as a quantitative determination of RBC aggregate morphology, derived from the numerical process of digitized image of RBC aggregates. The parameter ASP, appeared significantly

higher ( $p < 10^{-6}$ ) in diabetic patients than in normal control subjects. It provides a useful reference for measuring deviations of RBC aggregate morphology from that of the rouleaux structure which characterizes normal aggregates while the formation of RBC clusters characterizes disease states.

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