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Biophysical Changes in Red Blood Cells and Hemoglobin Components of Diabetic Patients

Sherif A. A. Moussa

Biophysics Laboratory, Biochemistry Department, Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Giza, Egypt

ABSTRACT

This paper designed to study the biophysical changes in red blood cells and hemoglobin as a response to diabetes millets. It was observed that glycosylation of both spectrin and hemoglobin increased in type-2 diabetes patients as compared to controls. Further, in type-2 diabetes, the spectrin glycosylation was more about three times than that of hemoglobin glycosylation. The percentage of red blood cells hemolysis of group II and III increased as compared with that of controls (group I) with the NaCl concentration decreased. The median corpuscular fragility (MCF), in groups II and III (0.46 and 0.48%, respectively) are greater than MCF in group I (0.45%). SDS-PAGE for the extracted hemoglobin showed that there is no change in molecular weight of hemoglobin in the three groups (about 66 kDa). The fractionation of hemoglobin was done, where HbA_{1a1} and HbA_{1a2} were eluted without any added NaCl in buffer, while HbA_{1b} was collected in buffer containing 0.05 M NaCl, HbA_{1c} was separated at salt concentration of 0.1 M in the buffer and finally, HbA_0 was eluted at 1.0 M NaCl concentration in the buffer. Methemoglobin formation by auto-oxidation was significantly higher from HbA_{1c} than that from HbA_0. Thermal denaturation at 62°C of HbA_{1c} and HbA_0 was studied; the extent of HbA_{1c} denaturation was significantly greater than that of HbA_0 denaturation, indicating higher thermolabile nature of HbA_{1c} than the non-glycated HbA_0.

Key Words: Osmotic fragility, RBCs, diabetes mellitus, methemoglobin, thermal denaturation.

Corresponding Author: Sherif A. A. Moussa
E-mail: sherifmoussa2006@yahoo.com

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INTRODUCTION

The red blood cell (RBC) biophysical features are of crucial importance for the cell behavior in blood flow. Through the processes of cell aggregation and deformation, RBCs determine blood viscosity and flow and hence, the effectiveness of blood circulation and maintenance of normal physiological conditions in the organism.

The central identifying feature of diabetes mellitus is chronic and substantial elevation of the circulating glucose concentration. The increased blood glucose stimulates non-enzymatic glycation of proteins namely, serum albumin *(Schleicher et al., 1993)*, α-crystallin *(Biemel et al., 2002)*, collagen *(Turk et al., 1999)*, low-density lipoprotein *(Stewart, et al., 1994)* and hemoglobin *(De Rosa, et al., 1998)*. The key step in the modification of proteins by glucose is Schiff base formation, followed by Amadori rearrangement *(Cohen and Wu, 1994)*. The Amadori product can then undergo oxidative cleavage, resulting in the formation of advanced glycation end products (AGEs) *(Giardino, et al., 1994)*. The first indication that a very simple chemical reaction between glucose and free amino groups on protein can lead to irreversible modification came with the characterization of hemoglobin A_{1c} (HbA_{1c}), which has the N-terminus of the β chain (valine) linked to glucose *(Cohen and Wu, 1994)*. HbA_{1c} concentration is proportionately increased in diabetic patients with ambient hyperglycemia and reflects the extent as well as management of diabetic condition *(Wolffenbuttel et al., 1996)*. Several reports have been made on glycation-induced structural and functional modification of hemoglobin *(McDonald et al., 1979; Svacina et al., 1990; Watala et al., 1992; Khoo et al., 1994; De Rosa et al., 1998; Peterson et al., 1998; Inouye et al., 1999; Kar and Chakraborti, 1999; 2001)*.

De novo oxidative damage, a result of increased protein glycosylation could participate in the mechanism, whereby diabetic erythrocytes may acquire membrane abnormalities *(Scwartz et al., 1991)*. Spectrin is a very important protein of erythrocyte membrane and a target for glycosylation and further oxidation, which might be responsible for increased number of poorly

deformable erythrocytes found among diabetic erythrocytes *(Lux, 1979; McMillan and Brooks, 1982)*. Enhanced glycosylation by elevated glucose concentration may induce the formation of oxygen derived free radicals through protein glycosylation, which releases early and late glycosylation end products, contributing to enhancement of oxidative stress *(Konukoglu et al., 2002)*. Both protein glycosylation and protein oxidation are biochemical alterations occurring in diabetes *(Resm et al., 2001)*. Under physiological conditions, auto-oxidation of glucose leads to hydrogen peroxide, reactive oxygen species and reactive ketoaldehydes, which modify the cellular proteins leading to their fragmentation by free radical mechanism. This protein fragmentation is inhibited by antioxidants confirming that tissue damage associated with diabetes has an oxidative origin *(Parthibhan et al., 1995)*. Evidence has accumulated indicating that the generation of reactive oxygen species (oxidative stress) may play an important role in the etiology of diabetic complications.

In this paper the author monitor the biophysical and biochemical changes in red blood cells such as osmotic fragility of RBCs, Separation of blood protein (hemoglobin) components and study the changes in hemoglobin and membrane protein (spectrin), auto-oxidation of different hemoglobin components.

MATERIALS AND METHODS

The present study was conducted on 35 subjects and they were divided into:

Group I: Five healthy controls,

Group II: Fourteen female non-insulin-dependant diabetic patients,

Group III: Sixteen male non-insulin-dependant diabetic patients.

ten ml blood was drawn in heparinized test tubes. Fasting plasma glucose level was measured by using commercial kits. Estimation of glycosylated hemoglobin was carried out by modified method of *Fluckiger and Winterhalter (1976)*. Spectrin was isolated by the method of *Coetzer and Zail (1982)*. Concentration of hemoglobin was measured by using commercial kits and concentration of erythrocyte spectrin was measured by Lowry method *(Lowry, 1951)*. Further, equal concentrations of spectrin and hemoglobin were adjusted and glycosylation status was determined by using fructose as external standard *(Parker et al., 1981)*.

Osmotic fragility of red blood cells:

Osmotic fragility of RBCs was determined as described by *Turgeon (1993)* at 540 nm using Spectronic 20D spectrophotometer (Milton Roy, USA) and the test was carried out within 30 min of collection. The blood of the three groups was added to serial dilutions of buffered NaCl, (pH = 7.4) at 27±2°C. The hemolytic percentage (% H) was calculated from the following equation:

% RBCs hemolysis at certain NaCl concentration =

$$\frac{A_1}{A_0} X100$$

Where A_1 is the absorbance of solution at certain NaCl concentration, A_0 is the absorbance of solution at 0% NaCl.

The median corpuscular fragility (MCF) was calculated which is the NaCl concentration at which 50% RBCs are hemolyzed.

Preparation of hemoglobin and separation of HbA_{1c} and HbA_0 from hemoglobin:

Human blood samples from patients of age group 45-50 years with non-insulin-dependent diabetes mellitus were collected in heparinized condition. Hemoglobin was isolated and purified from red blood cells (RBC) using Sephadex G-50 column chromatography *(Bhattacharyya et al., 1998)*. This eluted hemoglobin solution was applied to SDS-PAGE for determination of molecular weight *(Laemmli, 1970)*.

Hemoglobin solution was applied to Sephadex G-100 column (20 x 1.5 cm) pre-equilibrated with 50 mM phosphate buffer, pH 6.6. Fractions (0.5 ml) with increase of NaCl concentration in elution buffer from 0-1.0 M NaCl according to the method of *Cohen and Wu (1994)*. The absorbances of different hemoglobin fractions at 415, 540 and 670 nm were measured.

Autooxidation of HbA_0 AND HbA_{1c}:

Sterile-filtered HbA_0 or HbA_{1c} (40 µM) in 50 mM phosphate buffer saline (PBS), pH 6.6 was taken in sterile tubes and incubated at 4°C for 15 days. The aliquots were taken out aseptically every 24-h interval and the absorbances at 577 and 630 nm were measured. The extent of oxidation, as represented by methemoglobin formation, was estimated from the relation described by *(Winterbourn, 1987)*:

$$\text{(Met-Hb)}, \mu M = 279 A_{630m} - 3 A_{677m}$$

Thermal denaturation of HbA_0 and HbA_{1c}:

The sample of HbA_0 or HbA_{1c} (20 µM) in 100 mM phosphate buffer, pH 7.4 was incubated at 62°C in a water bath. At different time intervals, samples were withdrawn and chilled on ice immediately and centrifuged to remove denatured

protein precipitates. The absorbances of the supernatants at 523 nm were measured. The percentage of denaturation of protein was estimated according to the relation proposed by *Olsen (1994)*:

$$\text{Percentage of denaturation} = (A_0 - A_t)x\frac{100}{A_0}$$

Where A_0 and A_t are absorbances without incubation and with incubation for a particular time, t, respectively.

RESULTS

It was observed that glycosylation of both spectrin and hemoglobin increased in type-2 diabetes patients as compared to controls. Glycosylation status of both these proteins was similar in male and female patients. Further, in type-2 diabetes, the spectrin glycosylation was more about three times than that of hemoglobin glycosylation (Table 1).

Table 1: Plasma glucose, hemoglobin, glycosylated hemoglobin, spectrin glycosylation and hemoglobin glycosylation in different groups.

Parameter	Group I	Group II	Group III
Average age (years)	43.4±1.7	44.1±1.3	45.6±1.4
Fasting plasma glucose (mg/dl)	95.4±5.7	389.2±3.5	435.7±6.8
Hemoglobin (g/dl)	13.4±2.1	12.8±1.8	13.7±1.9
Glycosylated hemoglobin (%)	3.7±0.9	9.4±0.7	10.4±0.5
Spectrin glycosylation (nmole of fructose/mg spectrin)	9.3±0.6	29.8±1.2	29.7±1.3
Hemoglobin glycosylation (nmole of fructose/mg hemoglobin)	4.3±0.9	9.5±1.4	11.2±2.1

Figure 1 showed that the percentage of red blood cells hemolysis of group II and III was significantly increased as compared with that of controls (group I) with the NaCl concentration decreased. From (Figure 1) we can calculate the median corpuscular fragility (MCF), which is the NaCl concentration at which 50% of RBCs are hemolyzed. The MCF in groups II and III (0.46 and 0.48%, respectively) are greater than MCF in group I (0.45%).

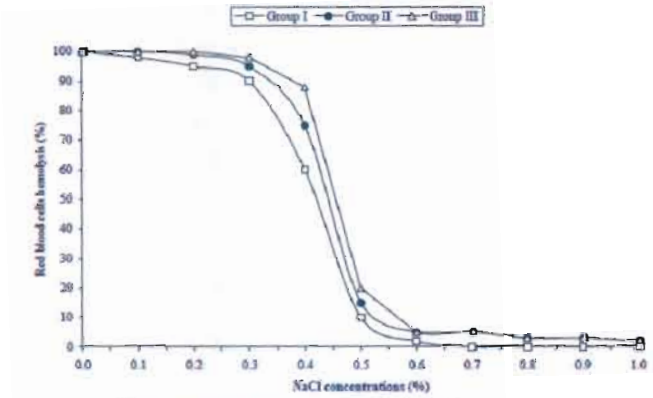

Figure 1: Shows the osmotic fragility of red blood cells in diabetic patients groups as compared to control.

Figure (2), SDS-PAGE for the extracted hemoglobin showed that there is no change in molecular weight of hemoglobin in the three groups (about 66 kDa). These indicated that the diabetes mellitus did not affect the molecular weight of hemoglobin, but affect the function.

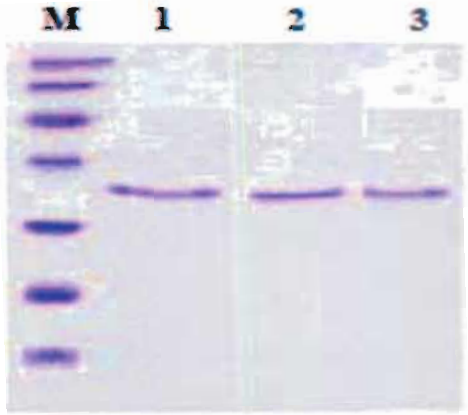

Figure 2: SDS-PAGE of the hemoglobin extracted from the three groups.

Fractions HbA_{1a1} (fractions 3-19) and HbA_{1a2} (fractions 20-42), containing β-N-fructose-1,6-diphosphate and β-N-glucose-6-phosphate, respectively were eluted without any added NaCl (Buffer 1). HbA_{1b} having β -N-carbohydrate was collected in the fractions 43-54 containing 0.05 M NaCl in the elution buffer (Buffer 2). HbA_{1c} was separated at salt concentration of 0.1 M (Buffer 3) (fractions 92-99) and finally, HbA_0 or nonglycated major human adult hemoglobin was eluted at 1.0 M NaCl concentration (Buffer 4) (fractions 101-106). A representative elution profile of the separated fractions is shown in (Figure 1), in which absorbances of different hemoglobin fractions at 415, 540 and 670 nm have been plotted against fraction numbers (Figure 3). HbA_{1c} and HbA_0 peak fractions were collected for this study.

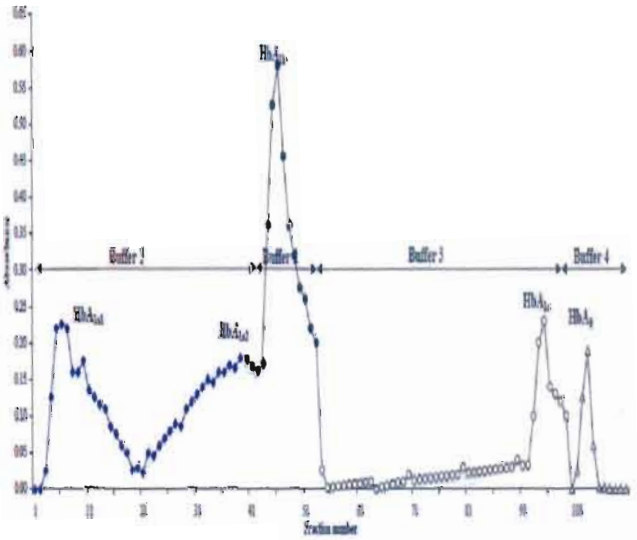

Figure 3: The elution profile of the hemoglobin fractions on sephadex G-100 cohumn chromatography using stepwise increase of NaCl concentrations (0-1.0M) in 50 mM phosphate buffer, pH 6.6. Buffer 1: No NaCl, Buffer 2:0.05 M NaCl, Buffer 3: 0.1 M NaCl and Buffer.

Figure 4 showed that the rate of methemoglobin formation was calculated from the absorbances at 577 and 630 nm according the aforementioned equation. Methemoglobin formation by auto-oxidation was significantly higher from HbA_{1c} than that from non-glycated protein HbA_0.

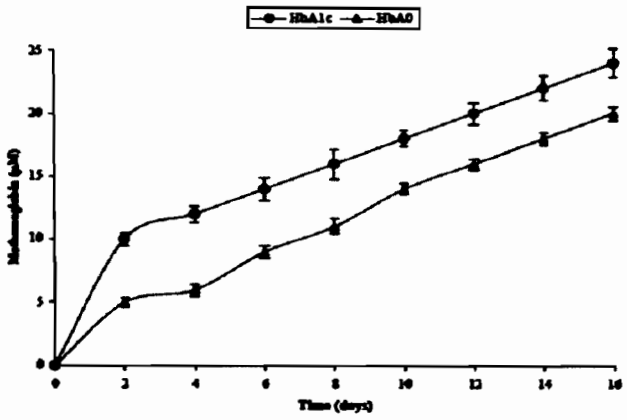

Figure 4: Auto-oxidation rate of HbA_{1c} and HbA_0 of differerent groups. The values are mean ± SD.

Thermal denaturation at 62°C of HbA_{1c} and HbA_0 was studied and the percentage of denaturation was plotted as a function of time (Figure 5). After 4 min, at each time point, the extent of HbA_{1c} denaturation was significantly greater than that of HbA_0 denaturation, indicating higher thermolabile nature of HbA_{1c} than the nonglycated HbA_0.

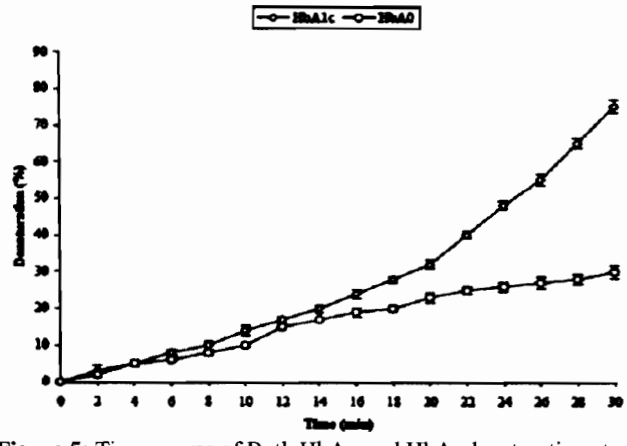

Figure 5: Time course of Both HbA_{1c} and HbA_0 denaturation at 62°C. Values are mean ± SD.

DISCUSSION

Diabetes mellitus is a group of metabolic disease characterized by hyperglycemia resulting from a defect in insulin secretion, insulin action or both. Increase in reactive oxygen species in diabetes mellitus is due to auto-oxidation of glucose, protein glycosylation and active polyol pathaway *(Gurler et al., 2000)*. The membrane protein glycosylation enhancement occurring in diabetes could be one of the reasons for lowered erythrocyte membrane fluidity in diabetes *(Lux et al., 1978)*.

It was observed that glycosylation of both spectrin and hemoglobin increased in type-2 diabetes patients. Further, in type-2 diabetes, the spectrin glycosylation was more about three times than that of hemoglobin glycosylation. This may be due to 7% of total amino acids as lysine in spectrin, which is believed to be the easily glycosylated *(McMillan and Brooks, 1982)*.

It is well known that, RBCs have to be squeezed and deformed to pass in blood capillary vessels of diameter smaller than that of RBCs itself, the degree of squeezing of RBCs depend mainly on their membrane elasticity. Therefore, the decrease of the RBCs membrane elasticity will lead to the increase of the blood capillary resistance for RBCs passage to the body cells for carrying normal metabolism and hence toxicity in some organs may occur *(Ali et al., 2003)*.

HbA_0 fraction was diluted with phosphate buffer immediately after separation to 0.1 M NaCl concentration. This was done to reconstitute back to tetrameric hemoglobin from dissociated subunits formed, if any, at high concentration of NaCl *(Bhattacharyya et al., 1998)*.

The rate of auto-oxidation of HbA_{1c} appears to be significantly higher than that of HbA_0. Similar results were obtained with NBT-induced auto-oxidation of HbA_0 and HbA_{1c} *(Kar and Chakraborti, 2001)* as well as non-glycated and glycated myoglobin *(Roy et al., 2004)*. In normal physiological condition, it is estimated that approximately 3% of hemoglobin is auto-oxidized in 24 h *(Nagababu et al., 2002)*. Equilibrium is generally maintained between the rates of formation of methemoglobin ($H\dot{b}^+A_0$) and its reduction to oxyhemoglobin (HbA_0). If HbA_{1c} induces the increased rate of auto-oxidation, the diabetic patients with increased level of HbA_{1c} might form more methemoglobin within erythrocytes. Also, increased formation of methemoglobin may promote hemichrome and finally Heinz body formation, which damage irreversibly the erythrocyte membranes *(Winterbourn, 1987 and Baliga et al., 1996)*.

Thermal stability of a protein is an important index for its structural integrity as well as its functional property. The fact that HbA_{1c} is more thermolabile than HbA_0 strengthens the view of structural modification of hemoglobin due to glycation. It has been reported that cytochrome P450, a heme containing thermolabile protein is degraded under certain pathological conditions and release its heme in the system to create oxidative stress *(Baliga et al., 1996)*.

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