Hemoglobin structure at higher levels of hemoglobin A_1C in type 2 diabetes and associated complications

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Abstract

Background: Fourier transform infrared (FTIR) spectroscopy technique has not been used as a diagnostic tool for diabetes in clinical practice. This study was linked to structural changes in hemoglobin (Hb) in type 2 diabetes patients at higher levels of HbA_1C using FTIR spectroscopy.

Methods: Fifty-three diabetic patients from the Bahawal Victoria Hospital, Bahawalpur, Pakistan were categorized as group A ($6\% < HbA_1C < 7\%$; $n = 25$) and group B ($HbA_1C \geq 9\%$; $n = 28$). Another group (group N) of twenty blood samples was taken from healthy people from the Islamia University Bahawalpur, Pakistan. Data from all groups were collected from January 1, 2018 to March 31, 2019. The structure of Hb was studied by FTIR spectroscopy and impact of glucose on the fine structure of HbA_1C was estimated.

Results: Hb secondary structure erythrocyte parameters were altered by changing glucose concentrations. From FTIR spectra of all three groups it was found that Hb structure was slightly altered in group A, but significantly changed in group B ($P < 0.05$). There was an increase in β-sheet structure and a reduction in α-helix structure at elevated levels of HbA_1C (group B) in type 2 diabetes.

Conclusion: We suggest that higher level of glycation reflected by increased HbA_1C might be a contributing factor to structural changes in Hb in type 2 diabetic patients. FTIR spectroscopy can be a novel technique to find pathogenesis in type 2 diabetes.

Keywords: Hemoglobin; Spectroscopy; Hemoglobin A_1C; Spectrum

Introduction

Diabetes is a lifelong disease and according to the International Diabetes Federation 415 million cases of diabetes have been reported in year 2015 and these numbers are expected to be 642 million by 2040.[1] The pathological process for type 2 diabetes is not clear and is of great concern. According to the different health associations guidelines hemoglobin (Hb) A_1C (HbA_1C) levels for type 2 diabetic patients should be less than 7%. Hb is a very important constituent of red blood cells and changes in its structure affects the function of these cells. Controlling the level of HbA_1C minimizes the occurrence in diabetic patients of other diseases like oxidative stress and cardiovascular diseases.[2-12] It has been shown that blood glucose concentration in type 2 diabetes can cause cardiovascular morality.[13] Hyperglycemia can affect the blood vessels badly.[14]

There are many diseases which are related to protein misfolding such as type 2 diabetes and Alzheimer diseases.[15] Hb is an important erythrocyte protein and is affected by various factors. Environmental changes, pH deviations, temperature increases, and chemical modifications can cause functional changes in Hb.[6-8] For type 2 diabetes, α-helix sheet and β-sheet structure are thought to be most responsible misfolded proteins.[15] Moreover, disordered Hb in erythrocytes, which results from oxidative stress, accelerates the diabetic complications.[16-18] Fourier transform infrared (FTIR) spectroscopy has been used to identify the spectral changes of tissue and serum from diabetic patients. Scott *et al*[8] used FTIR for molecular structure of human saliva and FTIR spectroscopy has been used for the quantification of glucose for a long time.[19,20] Infrared spectroscopy has been found to be a useful technique in the laboratory either using attenuated total reflectance for liquid blood droplet or onto infrared windows for dried blood smear.[21-23] This study aimed

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to evaluate the influence of elevated levels of glucose on the structure of Hb.

Methods

Ethical approval

This research was approved by the Pakistan Medical Research Council which is an efficient ethic committee in Pakistan to monitor biomedical research. Signed consents from patients and control groups were obtained.

Sample preparation

Fifty-three diabetic patients were chosen from the Bahawal Victoria Hospital by signing the consent, having a mean age of 50.0 years. All the patients were classified into two groups, group A and group B. Group A contained 25 type 2 diabetic patients having HbA_1C level less than 7% but greater than normal range 6% (control group). The HbA_1C level in group B was higher which was ≥9% ($n = 28$). Other than these two groups, twenty blood samples from healthy volunteers from the Islamia University of Bahawalpur, were taken with the same age range and this group was named as group N. Data from all groups were collected from January 1, 2018 to March 31, 2019. Whole blood (1.5 mL) was taken and kept in ethylenediaminetetraacetic acid tube to prevent from coagulation. The data from the patients were based upon following inclusion factors: (i) Patients who were according to diagnostic criteria for diabetes were added (World Health Organization-1999); (ii) patients without chronic acute diabetic complications; (iii) patients without infectious diseases or cancer; (iv) patients without renal or digestive system diseases; (v) patients without hematological disorder; (vi) patients who are non-smokers, and (vii) patients who do not take antibiotics.

Preparation of hyperglycemic erythrocyte model

To measure the impact of glucose on erythrocyte parameters and Hb, the method proposed by Dong *et al*[24] was used for the preparation of erythrocytes from healthy adults. In brief, erythrocytes with different concentrations of glucose (8.5, 15.0, and 30.0 mmol/L) were incubated for 7 days in a phosphate-buffered saline (50.0 mmol/L) at 37°C under sterile conditions. In similar way, controlled erythrocytes were prepared and incubated without glucose. After taking out from incubator, samples were dialyzed for 50.0 mmol/L phosphate buffer (pH 7.4) to remove excess glucose. Later, samples were stored at 4°C for the next use.

Preparation of human Hb

To prepare Hb samples, 1.5 ml of whole blood was centrifuged at 4000 r/min ($16{,}020 \times g$) for 20 min and plasma was discarded. To wash the packed erythrocytes, 0.9% NaCl was used and again centrifuged at 4000 r/min for 10 min. Following the same method, these samples were washed three times. Pre-cooled distilled water in 10 mL quantity was added after washing in each per mL of red blood cells. After cooling at 4°C, this mixture was centrifuged at 12,000 r/min for 35 min. The supernatant part was filtered using 0.22 μm sterile membrane and later purified using gel filtration chromatography. To ensure purity greater than 95%, Hb fraction was correctly measured using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After dialyzing for several hours against distilled water, this pure Hb was freeze-dried and kept at –20°C. Hb from normal and other modeled diabetic erythrocytes was also prepared in the same way.

Detection of erythrocyte parameter of Hb

Morphology of erythrocytes was detected by microscopic static imaging technique. Hb was measured using FTIR spectroscopy.

FTIR spectroscopy

One milliliter of potassium bromide (KBr) pellets were prepared using 1.0 to 1.5 mg of freeze dried Hb. Hb was mixed in dry KBr with a mass ratio 1:100. Infrared spectra were obtained using Bruker, Tensor 27, Germany. Each spectrum was obtained for 64 sample interferograms and recorded in the range of 400 to 4000 cm^{-1} at 25°C. The spectra of water vapors and air were set as background and subtracted by the software automatically. Three spectra were taken for each of the sample and averaged by software OPUS 7 (Bruker, Karlsruhe, Germany). A function Savitsky-Golay smooth was used to denoise the spectrum. A software, peak Fit V4.0 (SeaSolve Software Inc., Framingham, MA, USA) was used to obtain derivative spectra to analyze the number of peaks in amide I region. After correcting the baseline, a fit was obtained in region 1700 and 1600 cm^{-1} for decomposing amide I band by Gaussian curve fitting. This amide I fit was correlated with an error factor of 0.005.

Statistical analysis

Data (normally distributed) of all the patients were displayed by mean ± standard deviation. The significance of three group comparison was calculated by analysis of variance and displayed by rank-sum test. The statistical analyses were done using the SPSS for Windows (SPSS Inc., Chicago, IL, USA). A P value less than 0.05 was considered statistically significant.

Results

Hyperglycemic erythrocytes model

Parameters of modeled hyperglycemic erythrocytes were largely altered with the increase in glucose concentration. The change in Hb structure was analyzed by FTIR spectroscopy and Figure 1 demonstrates the deformations in secondary structure of Hb contained in buffers having various glucose concentrations. The α-helix contents in Hb secondary structure were decreased at 15.0 and 30.0 mm glucose but increased at 8.5 mm. The β-sheet content was observed to increase in parallel with the glucose concentrations.

Morphology of samples

Figure 2 shows a comparison of normal, control and diabetic erythrocytes. It can be observed that the erythrocytes in normal group were oval in shape which

became nearly circular in groups A and B. The axis shortened due to structural changes in type 2 diabetes. These results showed a decrease in deformability in erythrocyte due to increase in rigidity.

Clinical characteristics

We compared the hyperglycemic, body mass index and fasting plasma glucose of three groups [Table 1]. The body mass index, HbA_1C and fasting plasma glucose were higher for group A and group B than group N. While comparing groups A and B, HbA_1C and fasting plasma glucose were significantly increased in group B than those in groups A and N, however, there was not any noticeable difference in body mass index of two groups.

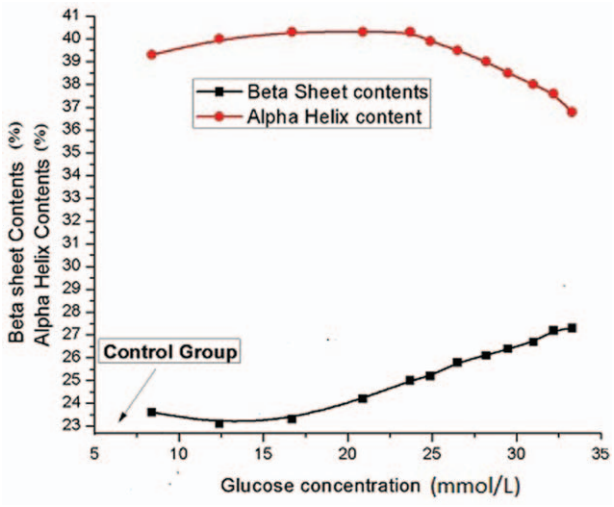

Figure 1: Variation in erythrocyte parameters with different glucose concentrations. Variation of erythrocyte parameters in glucose buffers at 8.5, 15.0, and 30.0 mmol/L compared with phosphate buffer saline. Figure shows an increase β-sheet structure and a decrease in α-helix structure with the increase in glucose concentration.

FTIR spectra of Hb

Figure 3 shows the spectrum of Hb in the region 4000 to 400 cm^{-1} of normal group N and diabetes (groups A and B). This figure shows the spectrum of Hb as different functional groups of protein. However, no difference was observed between the positions and bands of all three groups. The major difference was in the intensity among three groups, which altered the results largely. Conformal changes in Hb spectra were observed in amide bands such as Amide I, Amide II, and amide III bands at 1654, 1541, and 1312 cm^{-1}, respectively. Amide I (1700–1600) cm^{-1} band is further discussed for spectral changes in Hb at secondary level.

Intensity ratio comparison among three groups

The variation in quantity of all functional groups in blood samples could be observed by relative intensity ratios at different peaks. This intensity ratio could also be utilized to correct the uneven thickness and weighing mistakes in the sample pallets. As the FTIR spectra in the band above 3300 and below 1000 cm^{-1} were altered by absorption of H_2O and CO_2, respectively, absorption data of these two regions were not analyzed. In the region 3303 to 1000 cm^{-1}, there were not remarkable differences in relative intensity ratios of I_{3303}/I_{3306}, I_{1450}/I_{1396}, I_{1313}/I_{1254}, or I_{1166}/I_{10891} among three groups. At I_{2960}/I_{2874} and I_{1454}/I_{1541}, Hb level was

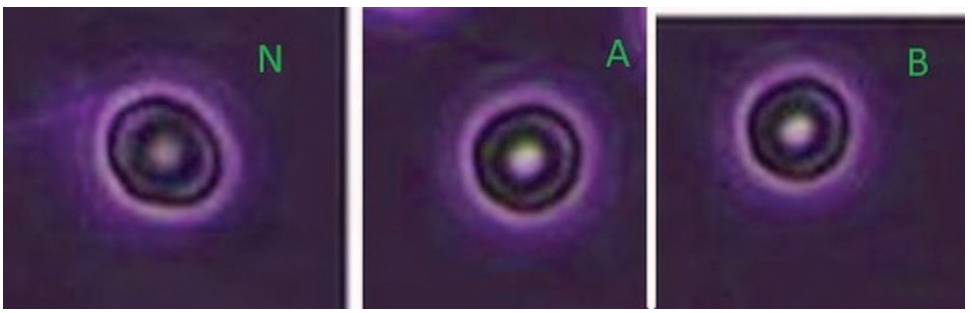

Figure 2: Morphology of erythrocytes. Morphology of erythrocytes in different groups such as normal (group N) and diabetic groups (groups A, B). Erythrocytes are oval in shape in groups A and N but shorten their axis and become almost circular in group B. Group N: Healthy controls; Group A: Patients with good glycemic control (6.0% < HbA_1c < 7.0% or 53 mmol/mol); Group B: Patients with higher hyperglycemia ($HbA_1c \geq 9.0\%$ or 75 mmol/mol).

Table 1: Clinical characteristics of the 53 diabetic patients (groups A and B) and 20 healthy controls.

Characteristics	Group N ($n = 20$)	Group A ($n = 25$)	Group B ($n = 28$)
Age (years)	52.18 ± 9.23	53.75 ± 13.54	55.17 ± 11.45
BMI (kg/m^2)	21.56 ± 1.05	24.18 ± 2.21	24.67 ± 2.19
FPG (mmol/L)	5.27 ± 0.62	7.21 ± 1.34	10.21 ± 1.58*
HbA_1C (%)	4.69 ± 0.31	6.05 ± 0.66	11.93 ± 2.10*

Data are shown as mean ± standard deviation. Group N: Healthy controls; Group A: Patients with good glycemic control (6.0% < HbA1c < 7.0% or 53 mmol/mol); Group B: Patients with higher hyperglycemia ($HbA_1c \geq 9.0\%$ or 75 mmol/mol). *$P < 0.05$, compared with groups A and N. BMI: Body mass index; FPG: Fasting plasma glucose; HbA_1C: Hemoglobin A_1C.

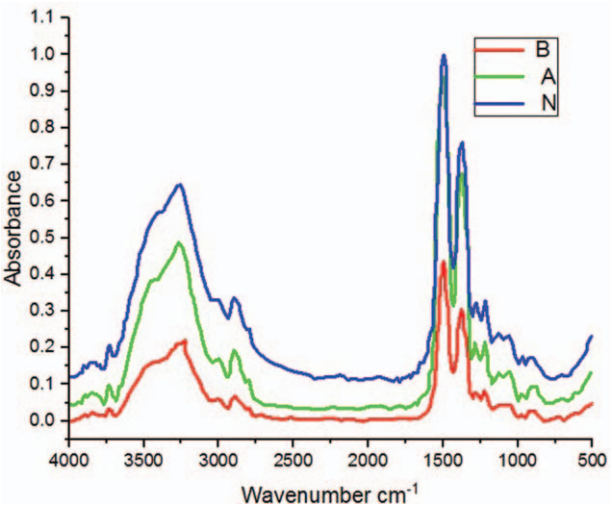

Figure 3: FTIR spectra of the 53 diabetic patients (groups A and B) and 20 healthy controls. FTIR spectra of groups A, B, and N with range 400 to 4000 cm^{-1}. The only difference is absorption intensity, which is at higher level for group N but lower for groups A and B. Group N: Healthy controls; Group A: Patients with good glycemic control (6.0% < HbA_1c < 7.0% or 53 mmol/mol); Group B: Patients with higher hyperglycemia ($HbA_1c \geq 9.0\%$ or 75 mmol/mol).

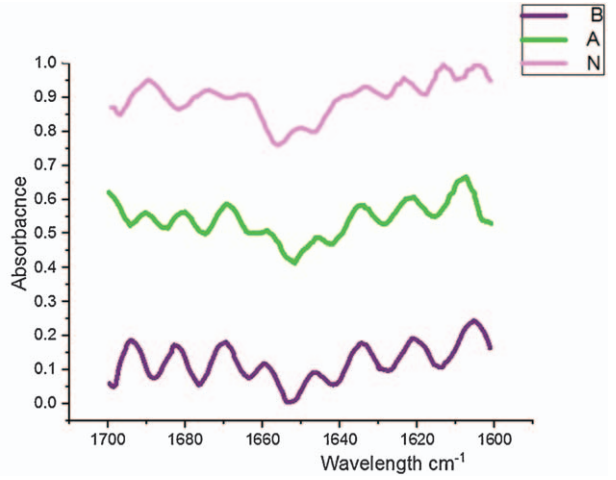

Figure 4: Derivative spectra in Amide I region. Amid I band shows secondary derivative FTIR spectra of all three groups A, B, and N. This secondary spectra show the variations in protein structure and shifting of peaks in all three groups. Group N: Healthy controls; Group A: Patients with good glycemic control (6.0% < HbA_1c < 7.0% or 53 mmol/mol); Group B: Patients with higher hyperglycemia ($HbA1c \geq 9.0\%$ or 75 mmol/mol).

slightly lower in group A than that in group N, but the difference was not statistically significant. While in group B these ratios were noticeably lower than those in group N and group A. We may suggest from these findings that structure of Hb in group B may have changed due to glycation.

Qualitative analysis of Amide I band

Amide I region had beta sheets, helices and other secondary structure of proteins in FTIR spectra [Figure 4]. We could observe the exact position of secondary structure by derivative spectra of these groups. In the secondary spectra, clear differences were observed in position, number, pattern, and signal intensity among three groups. The pattern peaks and signal intensity for group N located at 1609, 1645, 1670, 1682, and 1697 cm^{-1} were significantly different from group A and group B. The negative peak at 1609 cm^{-1} in group N was changed into positive peaks in both group A and group B. More ever peaks at 1670 and 1682 cm^{-1} in group A and B were shifted towards high intensities in group N. The peak at 1697 in group B showed a red shift with higher intensity in group N. At 1645 cm^{-1} there was an increase in the intensity of peaks with the increase in HbA_1C level among all groups.

Quantitative analysis of Amide I band

The Hb structure in amide I region was analyzed by the Gaussian curve fitting analysis. Average area of each secondary structure corresponds to various secondary structural constituents of Hb. All the secondary structures in amide I region were analyzed by curve fitted analysis as shown in Figure 5. While comparing the group N and group A, a slight change in α-helix and β-sheets was observed but this slight difference was not statistically important. There was a reduction in α-helix structures of group B, while β-sheets contents were significantly increased. Comparing group A and group B, group B showed an increase in β-sheets and a decrease in α-helix structures at HbA_1C level beyond 9%.

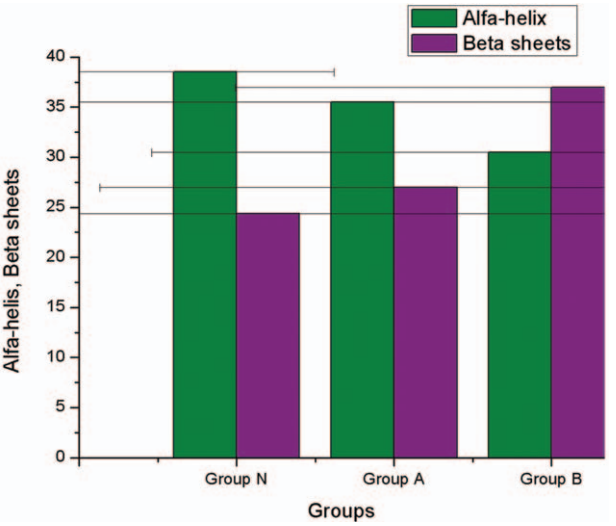

Figure 5: Secondary structure variations in three groups. Group N: Healthy controls; Group A: Patients with good glycemic control (6.0% < HbA_1c < 7.0% or 53 mmol/mol); Group B: Patients with higher hyperglycemia ($HbA_1c \geq 9.0\%$ or 75 mmol/mol). Figure shows variation in secondary structure of protein for groups B, A, and N.

Discussion

Type 2 diabetes mellitus is a complex disease which leads to a large number of disorders. Hyperglycemia is a cause for an increase in HbA_1C which reflects the patient's diabetes and risk factors for complications.

In this research we found changes in erythrocyte shape with different glucose concentrations in type 2 diabetes.[25] Moreover, a strong tendency for change also observed in secondary structure of Hb due to increased glucose concentrations. Thus, we suggest that increased glucose

may have an effect on Hb and erythrocytes. In our study, we evaluated the change in Hb structure in type 2 diabetes due to glycation. Our result showed that intensity ratios of Hb in group A were not significantly but slightly lower than those of group N, arguing that intra-molecular bonding in Hb is not altered at an HbA_1C level below than 7%. A controlled blood glucose environment may have a very little impact on Hb structure. However, group B showed significantly lower intensity ratios at I_{2960}/I_{2874} and I_{1654}/I_{1541} than group A and N. This decline in relative intensity may reflect the inter-molecular bonding changes which affect the structure of Hb. The peaks at 2961 and 2874 cm^{-1} are due to CH_3 stretching vibrations in Hb. In Amide I region peaks at 1654 cm^{-1} is due to C = O stretching in Hb and peak at 1541 cm^{-1} is because of C-N and N-H stretching vibrations. Our results resemble with Arif *et al*[26] who studied the type I diabetes serum of human with HbA_1C level greater than 9% and found a change in Hb structure. Our results are similar with Ye *et al*[25] who studied the impact of HbA_1C on Hb structure in diabetes type 2. They found a change in secondary structure of Hb at higher level of HbA_1C. An increase in glycemic level such as 285 mg/dL cause an increase in contents of β-sheets in diabetic patients.[25]

Our findings revealed that there was an increase in β-sheet structure and a reduction in α-helix structure at HbA_1C level above 9%. This might be due the reason that Hb in diabetes with higher level of HbA_1C, when exposed to glucose becomes chemically modified and might transform α-helix structure into β-sheets structures which cause an increase in unfolded form of protein. These changes in secondary structures lead to conformal changes in Hb.[17,18,27,28] An increase in β-sheet contents unfolds the native protein structures decreasing the structural firmness. Glycation of Hb cause conformal changes and affect the affinity of Hb for oxygen due to persistent hyperglycemic environment. Bose *et al*[29] proposed that structural modifications in Hb due fructose. Glucose and fructose both may interact with Hb to induce conformal changes and unfolding of Hb may cause degradation which might affect the affinity of Hb for oxygen. As Hb is an important constituent of erythrocytes and at higher level of HbA_1C, hyperglycemia may increase β-sheets contents in Hb. The resultant decrease in solubility of Hb may enhance the viscosity of components of their cells. These changes may decrease the deformability of erythrocytes in diabetic patients and reduce the erythrocytes flexibility. These changes may change the microcirculation, which cause diabetic complications by closing their flow in capillaries.[30-32] From these all, deformed structure and impaired function of erythrocytes might be linked with the development of vascular changes.[25,33,34]

Conclusions

The secondary structure of Hb was not altered in diabetic type 2 patients with good glycemic control, though alternation appears at higher levels of HbA_1C. We assume that an elevated level may be contributing to structural modification in Hb in diabetes. Deformation in Hb structure may be associated with pathological complications of type 2 diabetes.

Conflicts of interest

None.

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