Fluorescent Study of Human Blood Plasma Albumin in Diabetic Patients

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Abstract

Diabetes mellitus (DM) is a complex metabolic syndrome in which hyperglycemia, the primary clinical manifestation, contributes to the diabetic complications. Hyperglycemia favors protein glycation and, consequently, the production of advanced glycation end products (AGEs). Albumin is the largest

component of the plasma proteins, and glycated albumin has been reported as a potential glycation index in diabetes management. The aim of this study was to evaluate the utility of fluorescence spectroscopy of glycated albumin as a means for monitoring diabetes. We conducted a case-control study consisted of 93 patients (with and without vascular complications) and 58 population-derived, age-matched controls. Approximately 54% of the patients had vascular complications (nephropathies, retinopathies and neuropathies). The data presented in this work show that fluorescence spectroscopy can discrimin ate between control and diabetic patients. (P<0.0001) Besides, the fluorescence spectroscopy discriminates the diabetic patients without vascular complications and those with vascular complications (P<0.05). These results demonstrate that albumin fluorescent spectroscopy may offer a useful tool for monitoring diabetes.

Key words: Diabetes; Autofluorescence; Glycated albumin; Spectroscopy

INTRODUCTION

Diabetes mellitus (DM) is a common endocrine disorder that affects more than 100 million people worldwide.[1] DM is characterized by an increase in plasma glucose (hyperglycemia), which is caused by a lack of insulin, insulin resistance, or both.[2]

Hyperglycemia is still considered to be the principal cause of diabetes complications. Its deleterious effects are attributable to, among other things, the formation of sugar-derived substances called advanced glycation end products (AGEs).[3-6] Accumulated AGEs exert deleterious effects on the vascular wall, contributing to the development of micro- and macrovascular disease; these effects are particularly prevalent in type 1 diabetes and are generally accompanied by the pathogenic consequences of diabetes, including poor circulation to the extremities, retinopathy, nephropathy and coronary artery disease.[3]

The AGEs are a heterogeneous group of non-fluorescent and fluorescent compounds. The latter can be detected at an maximum excitation of 370 nm and maximum emission of 445 nm.[7-9]

Plasma proteins, including hemoglobin, lipoproteins and albumin, are especially susceptible to glycation because of their relatively low turn-over rate and the ability of sugars to accumulate within the blood.[10-11] Glycated hemoglobin (HbA1c) has been used as the gold standard parameter for monitoring diabetes. However, the use of HbA1c as an indicator of glycemic control over a 2–3 month period does not provide information on earlier changes in glycemic control or on various conditions affecting the lifespan of red blood cells.[12-13]

Glycated albumin has been reported as a potential alternative glycation index for diabetes management.[14] The turnover of serum albumin is more rapid (15–20 days) than that of hemoglobin; hence, glycated albumin is useful for the evaluation of short-term glycemic control (2–4 weeks) in diabetic patients.[13]

Several methods are presently employed in the isolation and quantification of glycated albumin, but the most uniform measurements are generally associated with immunoassays and the newer affinity chromatography methodologies employed by reference laboratories. [14-15]

Fluorescence spectroscopy is currently one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics. It was first used to study the natural 'autofluorescence' of human tissues by Alfano et al; 1984.[16] Natural tissue fluorophores include NAD-(P)H; FAD; structural proteins, such as collagen, elastin and their crosslinks; the aromatic amino acids tryptophan, tyrosine, and phenylalanine; and the porphyrins, each of which has a characteristic excitation wavelength with an associated characteristic emission. [17]

In the present work, we conducted a population-based case-control study to evaluate the potential of autofluorescence spectroscopy to detect alterations in the plasma levels of AGE-human serum albumin (AGE-HSA) in diabetic subjects.

MATERIAL AND METHODS

Biochemical and epidemiological data

Epidemiological data were acquired from medical records and interviews with individuals from 2009 to 2010.

The data on blood glucose levels (for both the diabetic and control group) were collected from the medical records, considering values up to three months prior to the date of collection.

The HbA1c levels of the diabetic individuals were collected from the medical records, considering values up to three months before the date of collection. For the control subjects, the levels were measured using a commercial kit (BioTécnica - Advanced Biotechnology – Varginha, Minas Gerais, Brazil).

Subject selection

This case-control study consisted of 93 patients and 53 population-derived, age-matched controls, all of whom were ethnic Brazilians. The study protocol was approved by the Ethics Committee of Federal University of São Paulo (UNIFESP), CEP 0278/09. At recruitment, written informed consent was obtained from each subject.

Sample acquisition and processing

The patients' samples were obtained from the Diabetes Clinic at UNIFESP between June and October of 2009, and the control samples were obtained from the Sleep Institute at the (UNIFESP) between December 2009 and May 2010.

A 7-ml blood sample was collected by venipuncture from a forearm vein, using tubes containing heparin or sodium fluoride to avoid clotting, and stored at 4 °C until processing.

The samples were processed at the Laboratory of Cellular and Molecular Biology of the Institute for Energy and Nuclear Research (IPEN), one or two days after the date of collection. The blood samples were centrifuged at 2500 rpm for 5 minutes, and the plasma was separated and stored at 20 °C.

Before spectroscopic analysis, the plasma samples were diluted (10-fold) in phosphate buffer and filtered through a 0.22-μm pore filter. The samples were wrapped in aluminum foil and immediately analyzed in duplicate.[18]

HbA1c quantification

The HbA1c analysis was performed using immunoturbidimetric methods with a commercial kit (BioTécnica–Biotecnologia Avançada–Varginha, Minas Gerais, Brazil).

Fluorescent spectral analysis

Fluorescence spectroscopy analysis was performed at the Center for Laser and Applications at the Institute for Energy and Nuclear Research.

The samples were excited at 370 nm with a 10-mm path length and studied on a Jobin Yvon Fluorolog-3 spectrometer (Longjumeau, France) with front-face collection geometry and a 0.2-nm resolution. The entrance and exit slits were sequentially adjusted at 5 mm.

Statistical Analysis

The normally distributed data are presented as means and standard deviations (SD) and the statistical analysis was performed using Student T test (parametric

analysis). The Mann-Whitney test was used for nonparametric analysis, with the data expressed as medians [25%–75%]. For nonparametric analysis with more than two groups we used the Student-Kruskal-Wallis One-way analysis of variance test, with data also expressed as median [25%-75%]. The gender comparison was performed using the Fisher exact test. Significance was set at $P<0.05$. The programs Excel 2007 and Sigmastat 1.0 were used to perform these analyses.

RESULTS AND DISCUSSION

Epidemiological data

A total of 93 diabetic patients were included in this study (30 men and 63 women, mean age=56 years). Approximately 54% of patients had vascular complications (nephropathies, retinopathies and neuropathies). Healthy control subjects (n=58) were recruited (21 men and 37 women, mean age=56 years. There were no significant differences between the groups regarding age and gender. The median blood glucose serum level and HbA1c of the diabetic group were almost 1.68 and 1.61 higher than that observed in control subjects. The baseline characteristics of the patient groups are summarized in Table I.

Table I: The baseline characteristics of the patients and controls.

Parameter	Diabetic (N=93)			Controls (N=58)			
	Average	Standard deviation	Min-max	Average	Standard deviation	Min-max	*P*
Age (years)	56.1	12.8	23 - 80	56	15	28 - 83	0.960*
Blood Glucose (mg/dL)	153	[121.5-198.3]	[63 – 427]	91	[86 - 93]	59– 99	0.0001**
HbA1c (%)	8	[7 – 10.3]	5.9 –	4.95	[4.3 –	2.8 - 6.2	0.0001*

			16.1		5.6]		*

*T test, **Mann-Whitney test.

Determination of HSA autofluorescence by fluorescence spectroscopy

HSA emission autofluorescence spectra were recorded for the 58 normal and 93 diabetic serum samples (Figure 1A), and the fluorescence spectra within the 400–550 nm range were analyzed. The spectrum consisted of a peak at approximately 455 nm, which is typical for albumin (Wong et al; 2002). Figure 1A indicates the fluorescence spectra for the diabetic and control groups. The median autofluorescence of the diabetic serum samples was 1.63 [1.35–2] million photons counted/second (MPCS), and that of the normal serum was 1.16 [0.99–1.45] MPCS (Figure 1B). There was a significant difference between the groups (P<0.001).

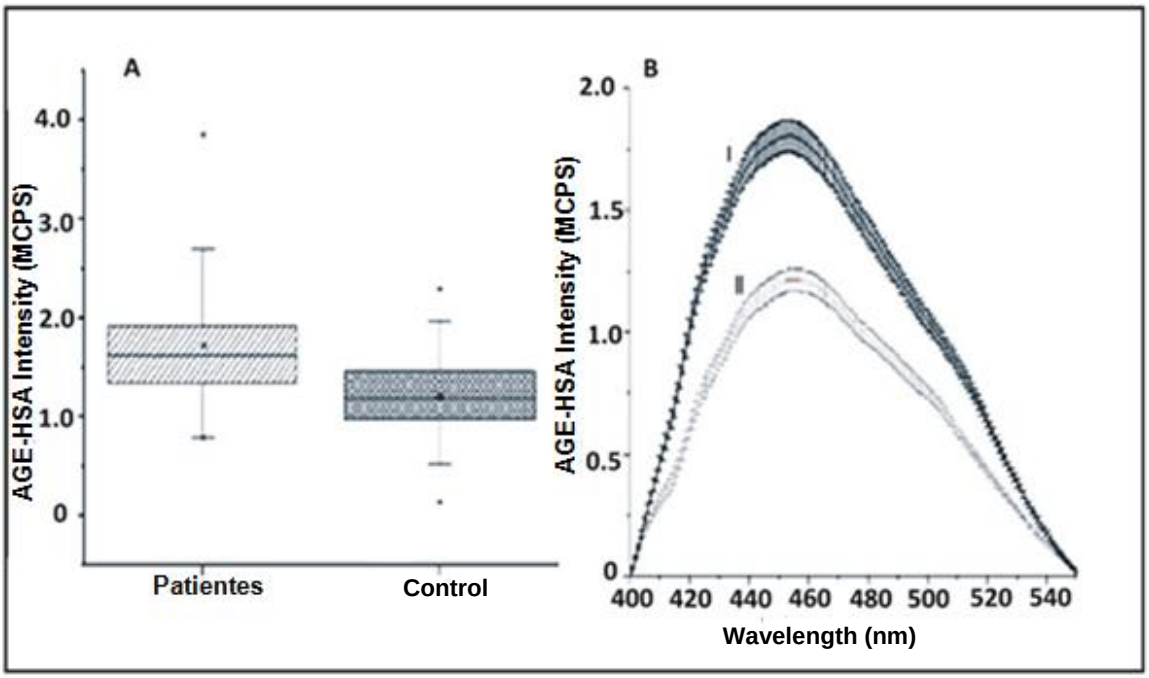

Figure 1: A: The AGE-HSA median emission intensities in the diabetic and control groups. $P<0.0001$ (Mann-Whitney test). B: Spectra of diabetic (I) and control (II) subjects.

The serum HbA1c levels of diabetics with and without vascular complications were compared in this work. As indicated in Figure 2, there was no significant difference in the HbA1c levels between diabetic patients with and without vascular complications ($P>0.05$). However, in the same subjects, the serum albumin emission autofluorescence was analyzed, and a significant difference was observed between diabetic patients without vascular complications and those with vascular complications ($P<0.05$) (Figure 3).

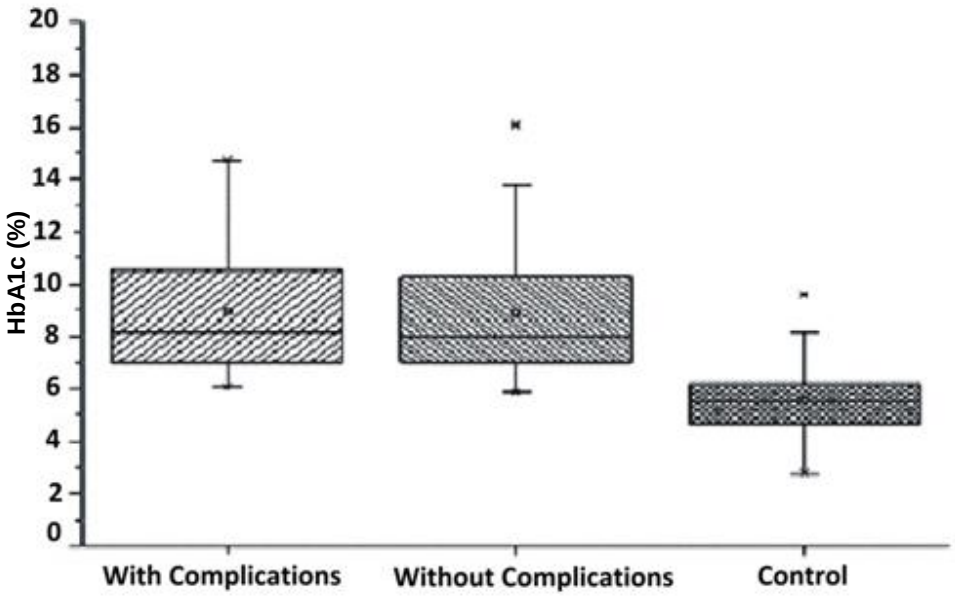

Figure 2 HbA1c median emission intensity in diabetic patients with and without vascular complications and in normal controls. The control values were significantly different from both the diabetic with complications and the diabetic without complications groups ($P<0.05$). The diabetic with complications group was not significantly different from the diabetic without complications group ($P>0.05$) (the significance was measured using the Kruskal-Wallis One-Way Analysis of Variance).

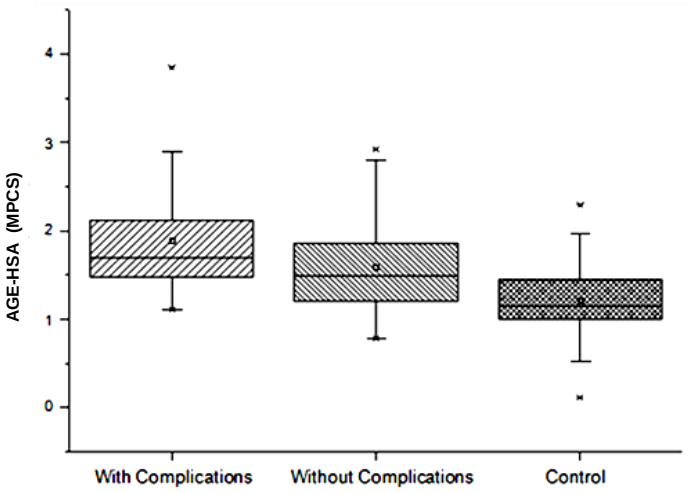

Figure 3 AGE-HSA median emission intensity in diabetic patients with and without complications. Control *versus* diabetic groups with and without complications; the diabetic group with complications *versus* the diabetic group without complications (P<0.05) (Kruskal-Wallis One Way Analysis of Variance).

DISCUSSION

By investigating the metabolic process, the disease can be diagnosed or monitored, and the autofluorescence of individual components can be used for both purposes. Albumin, the major component of plasma, is an autofluorescent molecule that can be used in the diagnosis of diseases.

Diabetes mellitus is a chronic disease, characterized by high plasma glucose levels, that requires long-term medical attention, both to limit the development of its devastating complications and to manage these complications when they do occur.

Our results show that the fluorescence emission spectra of all of the samples exhibited a peak at approximately 455 nm, which is typical for

albumin.[19] The fluorescent properties of serum albumin molecules are mainly due to their tryptophan residues, and these fluorophores are sensitive to biochemical alterations of the blood. The elevated levels of serum glucose in the diabetic patients lead to a significantly decreased intensity of fluorescence emission spectra. It is known that the interaction of glucose with HSA at high glucose concentrations results in the unfolding of HSA, which can explain the changes in the intrinsic fluorescence capacity of HSA in the diabetic patients.[20]

Vascular complications are one of the most serious consequences of diabetes and are responsible for most of the mortality observed in diabetic patients.[21] Consistent with the literature, the prevalence of diabetic complications in the present study was very high (54%), especially for microvascular complications (retinopathies and nephropathies). The high blood glucose concentrations promote the formation of AGEs, and these substances have been associated with endothelial cell injury and the formation of microaneurysms.[22]

HbA1c is an index of long-term glycemic control (2–3 months) and has been used as the gold standard parameter for monitoring diabetes.[13] However, the HbA1c levels were not able to discriminate between the diabetic patients with and without microvascular complication. This result could be explained by the fact that spectroscopy-based fluorescence determination has a much higher sensitivity than absorbance spectroscopy.[23] Additionally, fluorescence spectroscopy is more selective because only a small subset of absorbing molecules fluoresce and it has two spectral variables: the excitation and emission wavelengths.

The levels of glycated protein reflect the degree of hyperglycemia during a patient's life span. The turnover of serum albumin is more rapid (15–20 days) than the turnover of hemoglobin (90 days); hence, glycated albumin is useful for the evaluation of short-term glycemic control in patients with diabetes.[12-15,23-24]

In summary, this article describes an attempt to devise a simple, inexpensive, and easily repeatable method for diabetes monitoring based on native fluorescence spectral analysis of blood plasma.

Among instrumental techniques, fluorescence spectroscopy is recognized as one of the more sensitive. In fluorescence, the intensity of the emission of the sample is measured. The reason for the high sensitivity of fluorescence techniques is that the emission signal is measured above a low background level. This approach is inherently more sensitive than comparing two relatively large signals, as in absorption spectroscopy. The sensitivity of fluorescence techniques is up to 1000 times greater than that of absorption spectroscopy.

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