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Optical Screening of Glycation Induced Structural Alterations in Serum Proteins of Diabetes Patients Using Spectroscopic Techniques

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***Abstract*— Non enzymatic glycation furiously give rise to its final product advanced glycation end products that contribute to various diabetes associated vascular complication in patients. The aim of this present research was to study the application of basic, fluorometric and spectroscopic techniques for determining glycation in serum of patients with diabetes mellitus. Blood samples from 50 type 2 diabetic patients and 20 healthy subjects were obtained to evaluate fasting plasma glucose, HbA1c, lipid profile, fluorescent advanced glycation end products by spectrophotofluorimetry, Ultraviolet spectroscopy, fructosamine content and attenuated total reflection Fourier transform infrared spectroscopy. Healthy subjects have significantly lower levels of serum advanced glycation end products specific fluorescence, and Ultraviolet absorption. Attenuated total reflection fourier transform infrared spectroscopy shows the alterations in secondary structure of serum proteins in diabetic subjects due to occurrence of glycation. Fasting plasma glucose level, HbA1c and lipid profile are found to be altered in patients with diabetes compared to healthy. Positive correlation was found in fructosamine and HbA1c among diabetes patients. A basic approach of fluorimetric and spectroscopic analysis to quantify AGEs in serum of diabetic subjects could be employed as broadcast tools to forecast diabetic complications, at a primary care stage.**

***Index Terms*— Advanced Glycation End-Products, Glycation, Diabetes Mellitus, Vascular complications, Spectroscopy**.

I. INTRODUCTION

Non enzymatic glycation is chemical rearrangement of Schiff base Amadori adduct that give rise to irreversible advanced glycation end products (AGEs). Sources of heterogeneous AGEs may be endogenous and exogenous. Endogenously formed AGEs follow Maillard browning reaction involving ketone or aldehyde moieties of reducing sugars and free primary amino residues of proteins. Further chemical rearrangement form stable and irreversible crosslink's of AGEs. Intracellular sources of sugar in the human body include glucose-6-phosphate and fructose that accelerate the initiation of AGEs formation. Oxidation specifically glycooxidation clubbed with glycation, results in AGEs formation, including pentosidine and Nε-[carboxymethyl]-lysine (CML). Thus AGEs can be found in diverse forms due to amendments in their structures with few modifications; among them all are not identified precisely. Apart from proteins, fatty acids reactive intermediate (3-deoxyglucosone and methylglyoxal) also slightly contribute to AGEs formation due to lipooxdation [1]. Exogenous AGEs can be formed due to heating of food that may be excreted through kidney later on [2]. AGEs are known to be a culprit in the development of various micro-vascular complications in diabetic patients [3-5]. The AGEs are involved in the pathogenesis of diabetic nephropathy due to alterations in the signalling pathways, cytokine expression and formation of the free radicals. Circulating levels of these AGEs have been found to be excreted in the urine in diabetic patients with renal insufficiency [6]. RAGE (receptor for advanced glycation end product) is localized on the surface of endothelial cells of peripheral and endonueurial blood vessels and it is believed that expression of these RAGE on binding with AGEs cause the complication of peripheral neuropathy [7]. Interaction of AGEs with RAGE depicts the possible coupling mechanism in microananginopathy with other micro-vascular complications in diabetes mellitus [8].

Presently unavailability of authentic universal method to quantification of AGEs in humans creating hurdles for clinicians to come up with complications of diabetes mellitus. However, several methods have been incorporated to understand the unifying mechanism of AGEs induced complications like fructosamine content, carbonyl content, thiobarbituric acid assay, lysine content, fluorescence, spectroscopy and electrophoresis. However, these methods are not globally accepted, but provide a bunch of information for AGEs induced alterations in the human body. A standard method to quantify food AGEs was designed by previously with CML via enzyme linked immunosorbent assay (ELISA) [2]. Most widely accepted research techniques for detection of fluorescent AGEs include measurement at excitation wavelength of λ295 nm with emission at λ340 nm in serum, however not implicated for clinical purpose.

In the present study, serum of diabetic and healthy subjects were evaluated for basic spectroscopic techniques for detection of glycated adduct and their induced complications

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in diabetes patients. Our results clearly indicate that although less precise and sensitive, these methods could be the possible approach at present to detect AGEs induced secondary complication in diabetes mellitus.

II. EXPERIMENTAL

A. *Ethical approval and permission*

All subjects were informed about the study procedure and they provided written informed consent. The study was approved by the local ethical committee of the J.N Medical College, Faculty of Medicine, Aligarh Muslim University, Aligarh (Govt. of India).

B. *Study design and patients*

This post hoc study includes a total of 50 (Type 2 diabetes mellitus) T2DM patients along with 20 healthy volunteers visited in Rajiv Gandhi Centre for Diabetes & Endocrinology, J.N Medical College, Aligarh Muslim University, Aligarh. The mean age+S.D selected was55.86+12.0 years. Venous fasting blood was collected from all subjects in vials for collection of blood serum and plasma, and total protein content was estimated according to previously described protocol [9].

Inclusion criteria

[1] Patients with T2DM with 5 year duration

Exclusion criteria

[1] T2DM with associated secondary complications.

[2] T1DM (Type 1 diabetes mellitus)

[3] GDM (Gestational diabetes mellitus)

C. *Standard Kits and reagent*

Serum lipid profile was done with standard biochemical kit (Avantor performance materials, Deventer,The Netherlands). HbA1c were estimated with standard kit (Recipe chemicals, Dessauerster 3, Gmbh, Germany), sodium chloride, Nitro blue tetrazolium dye were purchased from SRL Chemicals (India). All other chemicals and reagents used were of highest analytical grade available.

D. *Serum biochemistry*

Estimation of glucose has been performed in all samples with glucose estimation kit based on glucose-oxidase-peroxidase (GOD-POD) methods. Plasma samples of all patients were assessed for HbA1c measurement by standard kit (Recipe chemicals, Dessauerster 3, GmbH, Germany) at the termination of the experiment. Serum samples were processed for estimation of total cholesterol, High density lipoproteins (HDL), triglycerides, low density lipoproteins (LDL), very low density lipoproteins (VLDL) by colorimetric method (Avantor performance materials, Deventer, The Netherlands).

E. *Serum Fructosamine*

The content of fructosamine in the samples were estimated with nitro blue tetrazolium (NBT) assay in serum samples in 96 well plate slightly modified method [10]. Briefly 200 µl of NBT reagent (250 µmol/l in 0.1 mol/l carbonate buffer, pH=10.35) was added to each 20 µl serum samples contained in microplate well in triplicate, incubate at 370C for 2 hours. The colour develops was read on spectrophotometer at 525 nM. The initial formation of Amadori adduct were quantified using an extinction coefficient of 12640 cm-1 mol-1 for monoformazone.

F. *Serum UV absorption spectroscopy analysis*

Ultraviolet (UV) absorption spectra of 1:10 diluted serum sample were recorded samples were recorded in the wavelength range of 250-400 nm on Shimadzu UV-1700 spectrophotometer with quartz cuvette having 1 cm path length.

G. *Serum fluorescence spectroscopy analysis*

Quantification of advanced glycation end products (AGEs) performed as previously described protocol [11]. Briefly 100 µl serum samples were treated with tricholroacetic acid (TCA) (0.15M/L) for deprotonization. 200 µl chloroform was added, followed by centrifugation at 14,000 RPM for 1 min. 200 µl of supernatant was then placed in each microplate well, in triplicate. AGEs specific fluorescence was recorded at excitation wavelength of 295 nm with emission in range of 290-400 nm with Shimadzu (RF-5301-PC) spectroflurometer at 25+0.20C.

H. *Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)*

ATR-FTIR spectra of pooled sera of diabetic patients and healthy volunteer were analyzed on the basis of frequencies and vibrations of amide I and amide II bonds present in the serum protein. The spectra were carried out on Shimadzu FT-IR spectrophotometer (8201-PC) in the Infrared spectral range of 1200-2000 cm-1.

I. *Statistical analysis*

The results are represented as mean +S.D. A p value <0.05 considered to be significant. Correlation was found out in SPSS by using regression equation.

III. RESULTS

Glycemic control of diabetic patients reflects the mean fasting plasma glucose level as shown in Table I was significantly higher than healthy subjects. The HbA1c shows a marked increase of 8.39+1.14 in patients compared to healthy (5.98+1.08). Total cholesterol, triglycerides, LDL, HDL, VLDL also showed significant changes in diabetic subjects compared to healthy (Table I). Fructosamine content in diabetic subjects also shows marked significant increase compared to healthy (Table I). UV-absorption spectrophotometry results showed increase in absorption in serum of patients with diabetes mellitus compared to healthy (Fig I). Pentosidine specific fluorescence results from AGEs formation clearly demonstrate the marked increase in fluorescence intensity in serum of diabetic patients compared to healthy (Fig II). ATR-FTIR spectral analysis of sera of diabetic patients and healthy volunteer were demonstrated in Fig III. Amide I band (C=O stretching) showed peak in 1660 cm-1 in healthy individual which was consistent with the absorption peak of α-helix. This peak was shifted to 1651 cm-1in diabetic individual. The amide II band (N-H vibrations) shows peak at 1543 cm-1 in healthy individuals that has been shifted to 1532 cm-1 in diabetic individuals.

The findings of the present study showed significant positive correlation between fructosamine content and glycated haemoglobin % (HbA1c) in the diabetic group and found correlation coefficient 'r' to be +0.1473.

IV. DISCUSSION

Diabetes mellitus is one of the major threats, which endangers the millions of life over the world. It is manifested by persistence hyperglycaemia and insulin resistance being classified into type 1 (IDDM) ad type 2 (NIDDM) according to American diabetes association classification. Diabetic complications mainly include nephropathy, retinopathy, cardiovascular diseases, cerebrovascular, neuropathy, and peripheral vascular complications. The majority of diabetes research has been focused on cutting down hyperglycaemia induced micro vascular and macro vascular complications. On the other hand very little knowledge is available to understand the alterations in protein pool of serum upon glycation or hyperglycaemia induced modifications. Although in vivo AGEs have been implicated in worsening of diabetic associated complications, but still there is no better assay for their quantifications because of their complicated and diverse structures.

Previous studies have reported significant relation between AGEs and diabetic complications [12-14]. However, other methods are cumbersome and unfeasible to implicate in day to day purpose of primary care setting. In present study simple and basic techniques were used to find out the AGEs induced alterations. The present study shows interesting fact of increased AGEs specific fluorescence their by showing occurrence of AGEs upon glycation in hyperglyemic environment [15]. Fluorescent AGEs like pentosidine participate in fluorescence event. Persistent hyperglycaemia in diabetic patients gave rise to glycation that in turn modify various proteins. Among these modifications fluorescent AGEs formation being the major event that justifies the results of present study [16-18]. Protein pool at 280 nm showing a hyperchromic shift in diabetic patients, which may be attributed to surface exposure of chromophoric aromatic amino acids residues due to glycation induced modifications [19-20].

The ATR-FTIR spectra measurement of the sera of diabetic subjects shows the shifting of amide I and amide II peaks that shows the alterations in the secondary structures of serum proteins. The third peak of the carbonyl functional group has appeared showing the occurrence of glycation reactions of the serum proteins. Since IR spectroscopy measure the composite content of serum assuming the molecular and bonding alterations in disease process severely represented by amide regions of proteins. Although the spectroscopic techniques has not employed extensively for clinical purpose, however if implemented with clinical investigations may reveal a better scenario to understand the mechanism of AGEs well. The positive correlation among fructosamine content and HbA1c is the suggestive of glycation induced changes in haemoglobin protein and fructosamine content formed as a resultant of this reaction in early stages of glycation.

V. CONCLUSIONS

This study showed highly significant alterations in serum proteins structures in diabetic patients with prolonged hyperglycaemia. Significant increase in UV absorption and AGEs specific fluorescence in the sera of diabetic patients compared to healthy thereby showed formation of advanced glycation end products. ATR-FTIR spectra of diabetic patient's sera demonstrate the changes in amide I and amide II shifting thereby showing changes in serum protein secondary structures. The basic techniques employed in this study is useful to update the new method development in clinical field to detect the formation of glycated adduct. The basic spectroscopic techniques may be easily employed in the identification of glycation induced changes in protein structure and properties in diabetes mellitus.

VI. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

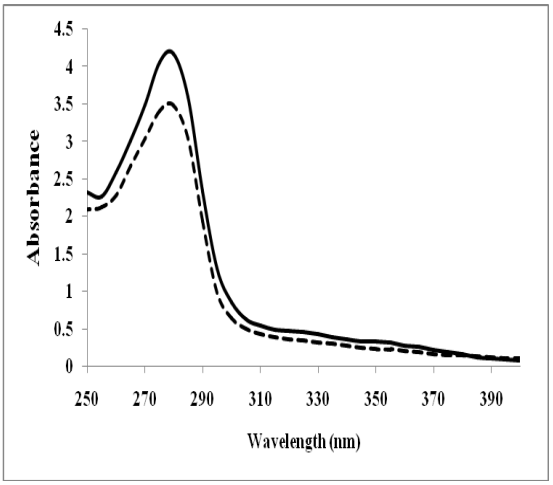

Fig 1: Ultra Violet absorption profile of diabetic sera (shown by dark black color) and healthy sera (shown by dotted line). The figure clearly showing maximum absorption in sera of diabetic patients compared to healthy subjects thereby showing the alterations in microenvironment of amino acids residues induced by glycation with same protein concentration. Abbreviation used nm= nanometer.

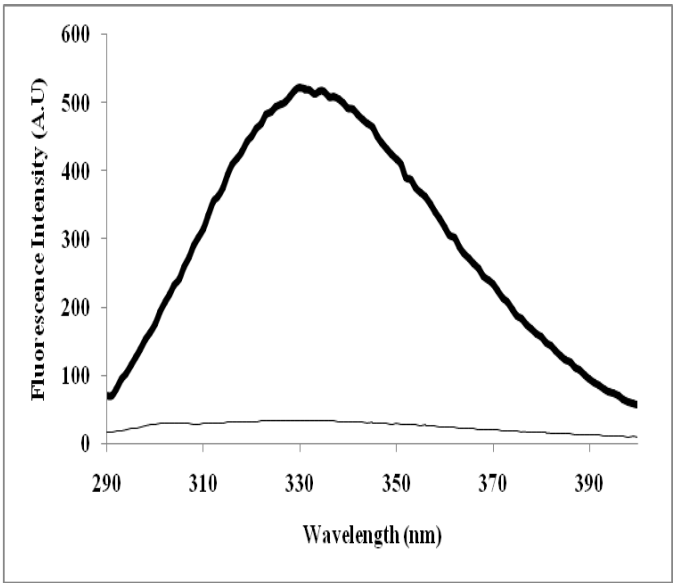

Fig 2: Advanced Glycation End Products specific fluorescence spectra of diabetic sera (shown by dark black color) and healthy sera (light black color). The fluorescence spectra recorded clearly deomstrates the formation of advanced glycation end products that shows maximum value of fluorescence intensity in diabetic subjects compared to healthy with same protein concentration. Abbreviation used A.U = Arbitary Units, nm= nanometer .

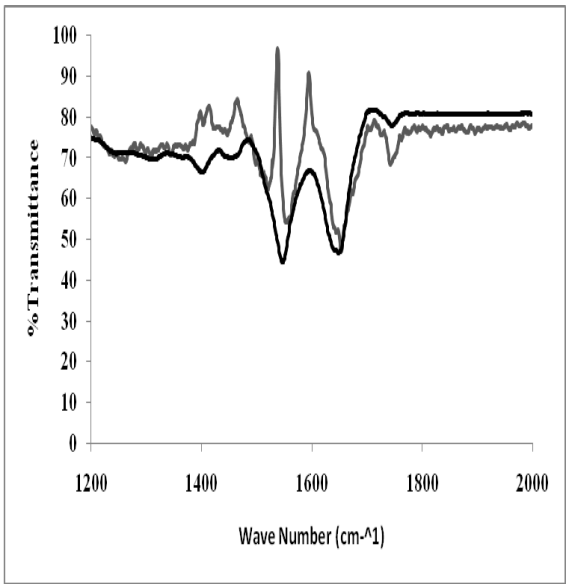

Fig 3: Attenuated total reflection fourier transform infrared spectroscopy spectra of healthy (dark black) and diabetic sera (grey) showing shifting of amide I and amide II peaks . The spectra recorded in figure clearly showing the shift in the peaks of amide I and amide II in the serum of diabetic subjects compared to healthy thereby providing evidence of change in secondary structure of proteins in serum with same protein concentration. Abbreviation used cm= centimetre.

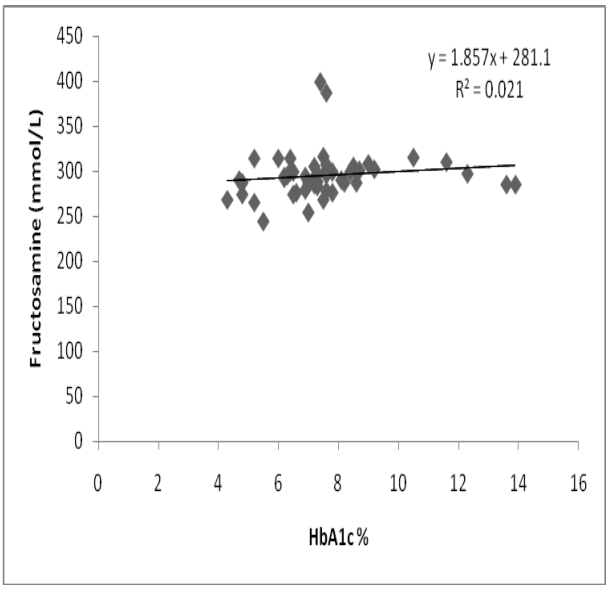

Fig 4: Correlation between glycated haemoglobin (HbA1c)% and serum fructosamine concentration in diabetic patients.

TABLE LEGENDS

TABLE I : Demonstrating the Serum Biochemistry of Healthy and Diabetic Patients. The Results Shown Represents Mean+S.D. A P Value <0.05 Considered to Be Significant and Clearly Showing Changes in Serum Biochemistry Parameters Compared to Healthy Subjects. Abbreviation Used (Mg/Dl= Milligram/Decilitre), N= Number of Subjects.

Parameters	**Healthy Subjects (n=20)**	**Diabetic Patients (n=50)**
Age (years)	55.86±12.0	56.23±9.63
HbA1c (%)	5.98±1.08	8.39±1.14
Fasting glucose (mg/dL)	85.85 ± 11.23	157.41 ± 38.93
Total cholesterol (mg/dL)	146.5 ± 23.22	169.3 ± 28.63
Triglycerides (mg/dL)	85.63 ± 24.44	164.23 ± 65.11
High density lipoproteins (mg/dL)	41.22 ± 7.9	36.52 ± 5.83
Low density lipoproteins (mg/dL)	89.63 ± 22.58	112.06 ± 26.14
Serum Fructosamine (mmol/L)	2.95 ±0.10	4.36 ±0.17

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