Structural and immunological characterization of Amadori-rich human serum albumin: Role in diabetes mellitus

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Abstract

Proteins modifications in diabetes may lead to early glycation products (EGPs) as well as advanced glycation end products (AGEs). Whereas no extensive studies have been carried out to assess the role of EGPs in secondary complications of diabetes, numerous investigators have demonstrated the role of AGEs. Early glycation involves attachment of glucose on ε-NH₂ of lysine residues of proteins leading to generation of the Amadori product (an early glycation species). This study reports the structural and immunological characterization of EGPs of HSA because we believe that during persistent hyperglycemia the HSA, one of the major blood proteins, can undergo fast glycation. Glucose mediated generation of EGPs of HSA was quantitated as Amadori products by NBT assay and authenticated by boronate affinity chromatography and LC/MS. Compared to native HSA changes in glycated-HSA were characterized by hyperchromicity, loss in fluorescence intensity and a new peak in the FTIR profile. Immunogenicity of native- and glycated-HSA was evaluated by inducing antibodies in rabbits. Results suggest generation of neo-epitopes on glycated-HSA rendering it highly immunogenic compared to native HSA. Quantization of EGPs of HSA by authentic antibodies against HSA-EGPs can be used as marker for early detection of the initiation/progression of secondary complications of diabetes.

Introduction

Non-enzymatic glycation is a multi-step process, involving chemical modification of reactive amino groups in proteins by reducing sugars and carbohydrate derived reactive carbonyls. Early glycation involves the reaction of glucose with N-terminal and lysyl side chain amino groups to form Schiff's base (an aldimine) which through acid-base catalysis undergoes rearrangements to form more stable early glycation product known as Amadori product [1]. After Amadori, the reactions become more varied and complicated leading to the formation of advanced glycation end products (AGEs),¹ that interact with various AGE receptors, thereby playing an important role in the long-term sequelae of diabetes. On the other hand, the Amadori-modification is structurally distinct from those associated with AGEs and Amadori-modified proteins operate through receptors different from that of AGEs [2,3].

The emphasis of research in recent years has been on AGEs, little attention has been paid on the characterization of early glycation products. However, it was only recently that the role of Amadori-glycated proteins has come into consideration [4]. Studies have shown that both Amadori product as well as Amadori-derived AGEs are involved in the secondary complications of diabetes [5]. Furthermore, Amadori adducts have been shown to be important contributors in the development of various diabetic complications such as microangiopathy [6]. Amadori adduct (fructosyl lysine) in type 1 diabetic patients was also found to be independently associated with retinopathy and early nephropathy [7]. In addition to the implications of Amadori adduct in the pathogenesis of diabetic micro and macro-vascular complications, glucose metabolism in skeletal muscles is likely to be affected by Amadori adduct [8].

The most abundant protein in human serum, prone to glycation is albumin [9]. Glycation induced structural alterations has profound impact on the functional properties of albumin [10]. Changes in HSA structure and conformation by Amadori product formation strongly affects its anti-oxidant properties and foster acquisition of pro-oxidant activity in presence of copper [11,12]. It has been reported that the drug binding property of HSA may be altered at various stages of diabetes as the extent of glycation and type of modifications are varied in this protein [13-15]. Non-enzymatic glycation of HSA occurs at multiple sites. Evidence suggests that ε-amino groups of lysine residue in human serum albumin are preferred locus for early glycation [13,16]. As glycation primarily occurs at intra-chain lysine residues, lysine rich proteins like HSA

References

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   E-mail address: kalam76@rediffmail.com (K. Alam).
2. Abbreviations used: AGEs, advanced glycation end products; HSA, human serum albumin; NBT, nitroblue tetrazolium; DTPA, diethylene-triaminepentaacetic acid; IgC, immunoglobulin C.
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Fig. 1. Ultraviolet absorption spectra of native HSA (-) and HSA modified with 12.5 mM (-•-) and 25 mM (-••-) and 50 mM (-•••-) glucose.

Fig. 2. Electrophoretic pattern of native HSA (lane 2 & 6) and HSA incubated for one week with 12.5 mM, 25 mM and 50 mM glucose (lane 3, 4 and 5, respectively). Samples were electrophoresed on 10% SDS-PAGE for 4 h at 50 V.

Fig. 3. Fluorescence emission profile of native HSA (-) and HSA glycated with 50 mM glucose (---). The samples were excited at 295 nm.
having 59 lysine residues could be used as a marker for early glycation induced changes.

In the present study, HSA was incubated with varying concentration of glucose for one week to generate Amadori-rich glycated HSA. Structural changes were investigated by UV, fluorescence and FT-IR spectroscopy as well as by SDS-PAGE. The Amadori product was assayed by NBT and authenticated by boronate affinity chromatography and LC/MS using furosine as standard. Antigenicity of native and glycated-HSA was probed in rabbits. Binding specificity of the induced antibodies was assessed by inhibition ELISA. Presence of autoantibodies against native and glycated-HSA in the sera of type 1 diabetic patients was also investigated.

**Materials and methods**

Human serum albumin (HSA), m-aminophenylboronic acid, anti-rabbit/anti-human IgG-alkaline phosphatase conjugate, p-nitro-phenyl, Protein A-agarose CL-4B pre-packed column, Tween-20 and Freund's complete & incomplete adjuvants were purchased from Sigma Chemical company (St. Louis, USA). Furosine was obtained from Polypeptide Laboratories (Strasbourg, France). D-glucose and sodium borohydride (NaBH₄) were obtained from Merck (Darmstadt, Germany). Nitroblue tetrazolium (NBT) was purchased from SRL Chemicals (India). Polystyrene flat bottom microtiter plates and modules were from Nunc (Roskilde, Denmark). All other chemicals and reagents used were of highest analytical grade available.

**Preparation of Amadori-HSA**

Glycated HSA containing high level of Amadori products was prepared according to the method of Cohen and Hud [17]. Briefly, HSA (10 μM) was incubated with different concentrations of glucose (12.5, 25 and 50 mM) in 20 mM phosphate buffered saline (PBS, pH 7.4) in presence of 0.01 mM diethylene-triaminepentaacetic acid (DTPA) at 37 °C under sterile conditions in capped vials for one week. At the end of incubation, the samples were extensively dialyzed against phosphate buffer (pH 7.4) to remove excess glucose. Equal concentration of HSA dissolved in the same buffer and kept under identical conditions served as control.

**Reduction of glycated samples with NaBH₄**

Glycated samples were reduced with NaBH₄ in PBS, pH 8.0, as described previously [18]. Slow addition of 1 N HCl was used to remove excess borohydride.
Characterization of Amadori product (Fructosamine) by NBT assay

Content of Amadori product, measured as fructosamine in the dialyzed preparations, was evaluated by NBT assay [19]. Native and glycated samples (100 μl each) were added to 96-well microtiter plates in duplicate. One hundred microlitre of NBT reagent (250 μmol/l in 0.1 mol/l carbonate buffer, pH 10.35) was added to each well and incubated at 37 °C for 2 h. The color was read on a microplate reader at 525 nm. Amadori products were determined using an extinction coefficient of 12640 cm⁻¹ mol⁻¹ for monoformazan [20].

Boronic affinity chromatography

Presence of Amadori products in glycated samples was assessed on boronic affinity column following the procedure described earlier [21].

Spectroscopic analysis

UV absorption characteristics of native and glycated samples were recorded in the wavelength range of 190–400 nm on a Shimadzu UV-1700 spectrophotometer using quartz cuvette of 1-cm path length. Fluorescence analysis of native and glycated samples was done on Shimadzu (RF 5301-PC) spectrophotometer. The samples were excited at 295 nm and the emission intensities were recorded in the range of 300–500 nm. Possible presence of the AGEs in the glycated samples was verified with AGE-specific fluorescence at 440 nm after excitation at 370 nm [22]. FT-IR measurements of native and glycated samples were carried on Shimadzu FT-IR spectrophotometer (8201-PC) in the spectral range of 400–4000 cm⁻¹. Samples to be analyzed on FT-IR spectrophotometer were first lyophilized and prepared as KBr pellets.

Liquid chromatography–mass spectrometry (LC/MS)

HSA glycated with glucose was subjected to LC/MS studies for adduct identification. Prior to loading, the samples were hydrolyzed as described previously [23]. The hydrolysates were filtered through a medium grade filter paper. Reversed-phase separation was carried out on an Agilent 1100 series capillary HPLC system equipped with a synergi C₁₈ analytical column (2 x 250 mm with 5 μm particle size). The chromatographic conditions were as follows; 0.4% acetic acid (Solvent A), 0.2% acetonitrile (Solvent B) each containing 2% formic acid. Gradient elution protocol was as follows; 0% to 2% solvent B in the first 5 min, from 2% to 6% solvent B over 19 min, 6% to 80% solvent B in 11 min, and then raised to 80% to wash the residual material off the column at a constant flow rate of 0.5 ml/min. Mass spectrometric analysis was then carried out on Micromass Quattro Ultima Triple Quadrupole Mass spectrometer interfaced to an Agilent 1100 capillary HPLC system. The mass spectrometer was operated in a positive ion mode and full scan mass spectra were recorded in 0–400 m/z range.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of native and glycated samples was performed as de-
Electrophoresis was done at 80 V for 4 h at room temperature and the bands were visualized after silver staining.

**Immunization schedule**

According to a previously described protocol [25], white New Zealand female rabbits were immunized with native and glycated-HSA. Briefly, rabbits (n = 4; two each for native and glycated-HSA) were immunized intramuscularly at multiple sites with 100 μg of antigen emulsified with an equal volume of Freund’s complete adjuvant. Animals were boosted with the same amount of antigen in Freund’s incomplete adjuvant at weekly intervals for 6 weeks. To evaluate antibody titer, test bleeds were performed after alternate booster dose. After an appropriate level of antibody was reached, the animals were finally bled and the separated sera were heated at 56 °C for 30 min to inactivate complement proteins.

**Enzyme linked immunosorbent assay**

Titre of induced antibodies was monitored by direct binding ELISA which was carried out on polystyrene plates as described earlier [26]. Antigenic specificity of the antibodies was ascertained by competition ELISA [27]. Percent inhibition was calculated using the formula:

\[
\text{Percent inhibition} = 1 - \left( \frac{A_{\text{sample}}}{A_{\text{baseline}}} \right) \times 100
\]

**Purification of IgG**

Immunoglobulin G (IgG) was isolated from preimmune and immune sera on Protein A-agarose pre-packed column [28]. The homogeneity of isolated IgG was assessed on 10% SDS–PAGE.

**Band shift assay**

For the visual detection of antigen antibody interaction and immune complex formation, gel retardation assay was performed [28]. Immune complex was prepared by incubating constant amount of glycated-HSA with varying amounts of affinity purified IgG in PBS for 2 h at 37 °C and overnight at 4 °C. After incubation, the samples were electrophoresed on 10% SDS–PAGE for 4 h at 80 V. The bands were visualized using silver nitrate staining.

**Blood collection from human subjects and sera separation**

Blood was collected from voluntary donors with a history of type 1 diabetes attending Rajiv Gandhi center for diabetes and endocrinology, J.N. Medical College, Aligarh Muslim University, India. Sera from normal healthy subjects served as control. A detailed history on demographics and cumulative clinical and laboratory manifestations during the period of disease were noted in every patient. Our study protocol was approved by the Institutional Ethics Committee. Sera, separated from the blood samples, were decomplemented by heating at 56 °C for 30 min and stored in aliquots at −20 °C with 0.1% sodium azide as preservative.

**Statistical analysis**

Data are given as mean ± SD. Statistical significance of the data was determined by student’s t-test (stataographies, origin 6.1). A p value of <0.05 was considered statistically significant.

**Results**

**Characterization of Amadori-rich glycated HSA by physicochemical methods**

HSA (10 μM) was incubated with 12.5, 25 and 50 mM glucose at 37 °C for one week, sufficient time for Amadori product formation [29,30]. The content of Amadori product in glycated-HSA samples was determined by NBT assay which is a reliable parameter for early glycation products. The yield of Amadori product measured as fructosamine was 27.4, 51.3, 94.4 nmol/mg, respectively (Fig. 1a). Higher yield of Amadori product was seen in HSA glycated with 50 mM glucose. Under identical conditions, native HSA gave almost negligible fructosamine concentration of 2.3 nmol/mg. Moreover, after reduction with NaBH₄, the level of fructosamine in glycated samples decreased to 12.2, 21.3 and 46.1 nmol/mg respectively (Fig. 1b). The formation of Amadori product (fructosyl lysine) measured as furosine was further confirmed by HPLC (data not shown).

**Boronate affinity chromatography for Amadori product**

HSA incubated with different concentrations of glucose was passed through boronate column and the results are given in Fig. 2. Percentage of Amadori bound by the matrix increased with glucose concentration. Native HSA did not show any retention on the boronate column.

**Ultraviolet analysis of native and glycated-HSA**

Glycation-induced structural changes in the samples were evaluated by UV absorption spectroscopy (Fig. 3). Native HSA gave characteristic peak at 280 nm, whereas in glycated samples with increasing concentration of glucose, an increasing hyperchromic effect was observed. Highest modification reached at 50 mM of glucose, which showed a marked hyperchromicity (58.2%) as compared to native analog.

**Electrophoretic pattern of native and glycated-HSA**

Fig. 4 shows the migration pattern of native and glycated samples in 10% SDS–PAGE. As compared to native HSA, glycated samples showed appreciable change in electrophoretic mobility with concomitant increase in band intensity. Moreover, HSA incubated with 50 mM glucose showed maximum change in electrophoretic mobility accompanied with an increase in band intensity. Thus, on the basis of higher level of Amadori content as detected by NBT, boronate binding, increased hyperchromicity and significant change in electrophoretic migration pattern of HSA modified with 50 mM glucose, we chose it for further characterization.

**Fluorescence analysis of native and glycated-HSA**

Tryptophan specific fluorescence of native HSA and HSA glycated with 50 mM glucose was measured by spectrophotometer. Both native as well as glycated-HSA were excited at 295 nm. Native HSA showed a strong emission peak at 340 nm because of its single tryptophan residue (Trp 214). Glycation induced structural change in glycated-HSA was evident from 43.2% loss in intensity (Fig. 5). Furthermore, the glycated-HSA did not show AGEs specific fluorescence at 370 nm (λex) and 450 nm (λem) (data not shown).

**FTIR spectra of native and glycated-HSA**

FTIR spectra recorded for both native (Fig. 6a) and glycated-HSA (Fig. 6b) were analyzed on the basis of shape and frequency of the amide I and II bands and other characteristic group present in their structures. Amide I band (arising from C=O stretching) in native HSA showed a peak at about 1660 cm⁻¹ which was consistent with the absorption peak of α-helix. This band at 1660 cm⁻¹ shifted to 1651 cm⁻¹ in glycated-HSA. The amide II band (originating from N-H bending vibrations of peptide groups) at 1543 cm⁻¹ in native HSA shifted to 1532 cm⁻¹ in glycated-HSA. A new peak at 1711.2 cm⁻¹ corresponding to keto (C=O) group of Amadori product was observed in the spectra of glycated-HSA. Moreover, in native HSA the band at 3209.1 cm⁻¹ related to the stretching vibration of the OH group shifted to 3309.1 cm⁻¹ in glycated-HSA.

**Detection of furosine in native and glycated-HSA by LC–MS**

Furosine, a known gold standard of Amadori adduct was evaluated in the acid hydrolysate of glycated-HSA. Fig. 7a–c shows mass
Antigenicity of glycated-HSA

Native HSA injected into rabbits induced moderate antibody response with a titre of 1:6400 as revealed by direct binding ELISA (Fig. 8a). On the other hand, glycated-HSA was found to be a potent immunogen and induced high titre antibodies (>1:12,800) (Fig. 8b). Under identical conditions, pre-immune serum included as control, showed negligible binding with either of the immunogens.

IgG was purified from preimmune and immune sera of native HSA as well as glycated HSA by affinity chromatography on Protein-A Sepharose column. The purified IgG eluted as a single symmetrical peak. SDS-PAGE of purified IgG under non-reducing conditions showed a single homogeneous band. Direct binding ELISA of purified anti-glycated HSA IgG and anti-HSA IgG showed strong binding with their respective immunogens. However, negligible binding was observed with pre-immune IgG (data not shown). The results indicate that the immunogen binding property of purified IgG was intact even after giving harsh conditions during purification procedures.

Antigenic specificity of anti-glycated HSA antibodies was further ascertained by inhibition ELISA (Fig. 9). A maximum of 89.3% inhibition in antibody binding was observed when glycated-HSA (the immunogen) was used as inhibitor. An inhibitor concentration (immunogen) of only 3.5 µg/ml was required to achieve 50% inhibition in the antibody binding. Native HSA used as inhibitor showed a maximum of 48.2% inhibition, whereas NaBH₄ reduced glycated HSA resulted in 69.4% inhibition in antibody binding. The anti-glycated HSA IgG exhibited binding with glycated forms of BSA, IgG, histone and PLL (poly-L-lysine). The antigen binding characteristics of anti-glycated HSA antibodies has been summarized in Table 1.

Visual detection of antigen–antibody interaction was monitored by gel retardation assay. The formation of immune complex between glycated HSA and anti-glycated HSA IgG was ascertained from appearance of high molecular weight immune complex with retarded mobility (Fig. 10a). It clearly indicates that, on increasing the amount of anti-glycated HSA IgG there was an increase in the formation of high molecular weight immune complex which resulted in retarded mobility and corresponding decrease in unbound antigen. Native HSA incubated with the same amount of anti-glycated HSA IgG did not show high molecular weight immune complex or decrease in the intensity of the unbound antigen under identical experimental conditions (Fig. 10b).

Detection of autoantibodies against glycated-HSA in type 1 diabetes sera by enzyme immunoassay

Serum samples of type 1 diabetes were divided into two groups on the basis of presence or absence of secondary complications. The demographic and clinical characteristics of normal and diabetic subjects included in the study are presented in Table 2. Fifty sera from type 1 diabetes and twenty one sera from normal human subjects were tested for binding to native, glycated and NaBH₄ reduced glycated-HSA. Seventy three percent (16 of 22) of type 1 sera with secondary complications showed enhanced binding with glycated-HSA as compared to native and NaBH₄ reduced glycated-HSA (Fig. 11). Furthermore, only sixty four percent (18 of 28) of type 1 sera without secondary complications, showed enhanced binding with glycated-HSA forms of HSA as compared to the native form. Whereas, type 1 diabetic group (without secondary complications), out of 28 samples tested, only 18 samples had enhanced binding.
with glycated-HSA. Normal human sera did not show appreciable binding with coated antigens.

**Discussion**

Non-enzymatic glycation of macromolecules has been linked to pathogenesis of various complications of diabetes. Although Amadori-glycated proteins are the major glycated modifications, most studies so far have focused on the characterization of AGEs and their possible role in assessing diabetic complications, whereas only a few studies have highlighted the role of early glycation products or Amadori products. The predominant form of glycated proteins in plasma exist as Amadori products rather than the more labile Schiff base form [31]. Furthermore, concentration of Amadori-glycated proteins is at least 2% of serum proteins, whereas the concentrations of AGE-equivalents are less than 0.01% [32]. It has been found that Amadori-modified albumin is an independent and potent trigger of molecular mediators contributory to diabetic complications and various diabetic complications are ameliorated upon inhibition of albumin glycation [33,34]. Against this backdrop, the present study describes the structural and immunological characterization of Amadori-rich glycated HSA.

The formation of Amadori-HSA was confirmed by NBT reduction which is a specific and reliable parameter for Amadori products [35,36]. The extent of Amadori adduct in glycated-HSA samples was further evaluated on boronate affinity matrix. The species retained on the boronate matrix speaks of concentration adduct formation is facilitated in a short period of time.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum % inhibition at 20 μg/ml</th>
<th>Concentration for 50% inhibition (μg/ml)</th>
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<tr>
<td>ROS-glycated phenylalanine</td>
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</table>

which indicates that the glycated samples are rich in Amadori products. Moreover, glycoxidation and crosslinking was inhibited by DTPA which is known to inhibit the development of AGE-specific florescence in proteins without significantly affecting the glycation of proteins [35,36]. The formation of Amadori adducts in glycated-HSA samples was further evaluated on boronate affinity matrix. The species retained on the boronate matrix speaks of Amadori product. It may be plausible to think that at high glucose concentration adduct formation is facilitated in a short period of time.

**HA samples modified with 12.5, 25 and 50 mM glucose exhibited hyperchromicity of 14.3%, 38.3% and 58.2% respectively. The observed hyperchromicity could be due to modification of aromatic amino acids or changes in the microenvironment of aromatic amino acids. It has been reported that glycation induced AGE-specific absorbance in proteins and their unfolding leading to cross linking and aggregation are responsible for a change in conformation of protein [37]. In our case, the onset of AGE formation in the glycated samples was ruled out by the absence of absorbance between 300 and 400 nm. SDS-PAGE of glycated samples showed noticeable concentration dependent decrease in electrophoretic mobility with concomitant increase in band intensity, which might be due to the addition of glucose moieties with the amino groups of the protein at Amadori stage. Moreover, no dimer or polymer formation was observed, which clearly indicate the absence of any cross-linked structure. Late glycation of HSA results in crosslinked dimers [11].

Early stages of glycation occurs primarily on the e-amino groups of lysine residues [13]. Glycation of lysine residues might play an important role in altering the tryptophan micro-environment. As HSA contains only one tryptophan residue (Trp-214), the loss in fluorescence intensity observed in glycated-HSA could be ascribed to the alteration of tryptophan-214 micro-environment as a result of glucose modification.
Fig. 10. BND shift assay of anti-glycated-HSA IgG binding to (a) glycated HSA and (b) native HSA. Electrophoresis was performed on 10% SDS-PAGE for 4 h at 80 V. (a) Glycated HSA (25 μg, Lane 1) was incubated with 10, 20, 30, and 40 μg anti-glycated HSA IgG, respectively (lanes 2–5) for 2 h at 37 °C and overnight at 4 °C. (b) Native HSA was incubated with 10, 20, 30, and 40 μg anti-glycated HSA IgG, respectively (lanes 2–5) under identical conditions. Lane (8a and b) contains anti-glycated HSA IgG alone.

FT-IR spectral analysis clearly reflects the position and intensity of characteristic bands in native and glycated-HSA. It is well established that the FT-IR spectrum of proteins exhibit a number of amide bands, which represent different vibrations of peptide moieties [38]. Amide I and II constitute two major bands of the protein infrared spectrum. Amide I band is mainly associated with the C=O stretching vibration of the peptide linkage and corresponds mainly to α-helix structure. Amide II band, in contrast, derives mainly from N—H in-plane bending and from C—N stretching vibration [38]. The shifting and decrease in intensity of amide I and II bands indicate structural changes in intramolecular bonding and may come from the impact of early glycation on the overall protein conformation. Additional evidence to support the effect of early glycation comes from the appearance of a new peak at 1711.2 cm⁻¹ corresponding to the carbonyl group of ketones in glycated-HSA which confirmed the presence of Amadori product in modified HSA [39]. The vibrational mode due to OH stretching lies in the region 3100–3600 cm⁻¹ [40]. In glycated-HSA an increase in absorption in the stretching vibration region of OH groups could be due to the attachment of glucose moieties to HSA. Moreover, in the FT-IR spectra of glycated-HSA low intensity absorption peak was observed at 1082.7 cm⁻¹ originating from C—C=O stretching vibration of sugar moieties, since carbohydrates (glucose) have strong absorption bands in this region [41] it might be due to glucose bound to HSA. Furosine is the gold standard of early glycation reaction [23,42,43] and its positive detection in the glycated-HSA by LC–MS suggests definite presence of Amadori adduct.

Immunogenicity of native and glycated-HSA was evaluated by inducing antibodies in female rabbits. Glycated-HSA was found to be a potent stimulus inducing high titre antibodies. Native HSA induced low titre antibodies. Our results suggest glycation-induced structural alteration in HSA, presenting potential neo-epitopes that lead to substantially enhanced immunogenicity of glycated-HSA as compared to native HSA. Antigenic specificity of affinity purified anti-glycated HSA IgG reiterated that the antibodies preferentially recognized the modified epitopes on glycated-HSA. Notable feature of the anti-glycated HSA IgG was that the maximum inhibition in antibody binding was caused by glycated-HSA followed by NaBH₄-reduced glycated HSA and least with the native HSA. The reduction of glycated-HSA with NaBH₄ causes structural alteration of Amadori product. More stable hemiketal furanose or pyranose ring structure is formed by the cyclization of the straight chain of Amadori adduct [44]. Reduction of Amadori product by NaBH₄ leads to the elimination of the keto group. This

Table 2
Demographic profile and clinical features of normal and diabetic subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Normal subjects</th>
<th>Type 1 diabetes with secondary complications</th>
<th>Type 1 diabetes without secondary complications</th>
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</tr>
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<td>Age (years)</td>
<td>39 ± 3</td>
<td>54 ± 8</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>9:12</td>
<td>7:15</td>
<td>16:12</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.2 ± 0.04</td>
<td>8.1 ± 1.5</td>
<td>7.2 ± 1.3</td>
</tr>
<tr>
<td>BMI</td>
<td>21.7 ± 3.9</td>
<td>25.5 ± 5.2</td>
<td>26.3 ± 4.6</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>107 ± 6.8</td>
<td>361 ± 32.4</td>
<td>326 ± 21.3</td>
</tr>
</tbody>
</table>
results in shifting the existing equilibrium between the linear configuration and the furanose or pyranose ring structures more. Therefore, reduced inhibition of antibody binding by NaBH₄ treated glycated-HSA as compared to its non-reduced counterpart suggests epitope recognition on Amadori product. Our results are in full agreement with earlier reports that the ring structure of Amadori-HSA is essential for the recognition of the induced antibodies [45]. The induced antibodies also exhibited polyspecificity with respect to antigen binding as determined by inhibition assay. The binding of induced antibodies was tested against native, glycated and ROS forms of BSA, IgG, histone and PLL. The induced antibodies showed variable degree of recognition of the glycoconjugated forms of the above mentioned proteins. Since PLL is a homopolymer of lysine residues and BSA, IgG, histone are lysine rich proteins, the observed cross-reactivity could be due to glycation of lysine residues. These results indicate towards the epitope sharing between Amadori rich glycated-HSA and glycated forms of PLL, BSA, IgG and histone.

Gel retardation data further substantiated the preferential recognition of glycated-HSA over native HSA by anti-glycated HSA antibodies. The formation of high molecular weight immune complex and appreciable decrease in the unbound antigen observed with glycated HSA, compared to native HSA, shows that the major autoantibodies are directed against the modified epitopes.

Amadori products of HSA are found to be immunogenic and Type 1 diabetic patients with retinopathy and nephropathy have higher Amadori albumin levels than those without it [45,46]. The possible role of native and modified forms of HSA (reduced and non-reduced) was also investigated in type 1 diabetic patients. Serum autoantibodies in type 1 diabetic patients with secondary complications showed preference for Amadori-HSA, indicating a causal role of Amadori-glycated proteins in the pathogenesis of diabetic secondary complications. Therefore, the systemic rise in the level of autoantibodies against Amadori-HSA could emerge as additional authentic biomarker for assessing secondary complications in type 1 diabetes.

The present study has identified the modifications and structural changes in HSA (lysine rich protein) induced by glucose in a specified time period. Based on our data we conclude that the glycated lysine residues in Amadori-HSA present unique antigenic determinants, rendering it highly immunogenic. Our results demonstrate that the neo-epitopes generated on HSA by Amadori modification might play a role in the induction of autoantibodies in type 1 diabetic patients, especially those with secondary complications. Hence, inhibition of Amadori-glycated albumin may be a target for reduction of diabetic vascular complications.

Acknowledgments

Authors are thankful to the Department of science and Technology (DST) for instrument facilities created with the financial help from DST under its FIST programme.

References


Table 3

<table>
<thead>
<tr>
<th>Sera</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>21.0</td>
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<tr>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>4</td>
<td>25.2</td>
</tr>
<tr>
<td>5</td>
<td>26.1</td>
</tr>
<tr>
<td>6</td>
<td>24.9</td>
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<td>7</td>
<td>26.4</td>
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</tr>
<tr>
<td>15</td>
<td>22.4</td>
</tr>
<tr>
<td>16</td>
<td>21.1</td>
</tr>
</tbody>
</table>

Mean ± SD 26.2 ± 3.8%  61.08 ± 4.9%  43.9 ± 4.3%
Physicochemical analysis of structural changes in DNA modified with glucose

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Department of Biochemistry, Faculty of Medicine, A.M.U., Aligarh 202002, U.P. India

ABSTRACT

Reactions of reducing sugars with free amino groups of proteins can form advanced glycation end products (AGEs). While the formation of nucleoside AGEs has been studied in detail, no extensive work has been carried out to assess DNA Amadori and DNA advanced glycation end products. In this study, we report biophysical/chemical characterization of glucose-induced changes in DNA, as well as DNA Amadori and DNA advanced glycation end products. Glucose treated DNA exhibited hyperchromicity, decrease in melting temperature, and enhanced emission intensity in a time dependent manner. Formation of DNA Amadori product and DNA advanced glycation end products, mainly CEdG (N^c-carboxyethyl-2'-deoxyguanosine), were the major outcome of the study.

1. Introduction

The free amino groups of proteins can non-enzymatically react with the carbonyl group of reducing sugars such as glucose to generate Schiff base. Within a relatively short period of time the Schiff base reaches equilibrium with more stable but still reversible Amadori products. The Amadori products can then undergo a series of further rearrangements and dehydration to form irreversible stable end products [1,2]. It is well established that proteins are readily glycated in vitro and in vivo [3]. The first glycation product to be detected in vivo was glycated-hemoglobin (HbAic), the Amadori product of HbA [4]. AGEs accumulate on serum proteins and in various tissues, particularly during aging, diabetes and renal failure [5]. Elevated AGE levels contribute to the development of diabetes and uremic complications, such as atherosclerosis, nephropathy and retinopathy [6,7].

Analogous to proteins, glycation may also affect DNA. In vitro, nucleobases and DNA react with sugars in a similar way as proteins [7-9]. The exocyclic amino group of 2'-deoxyguanosine is particularly prone to glycation reactions leading to the formation of N^c-carboxyethyl, N^c-carboxymethyl, N^c-(1-carboxy-3-hydroxypropyl) and N^c-(2-carboxy-3,4,5-trihydroxy-pentyl) modifications as well as cyclic dicarbonyl adducts [9-12]. The two diastereomers of N^c-carboxyethyl-2'-deoxyguanosine (CEdG<sub>2</sub>) are stable reaction products that are formed from a variety of glycating agents such as glucose, ascorbic acid, dihydroxyacetone (DHA) or methylglyoxal [11,13]. First evidence that CEdG is formed in vivo was put forward by Schneider et al. [14]. Later on, the presence of CEdG in human urine was confirmed by immunofluorescence chromatography coupled with HPLC-diode array detector (DAD) [15]. Using the same method, CEdG was also detected in the genomic DNA of human smooth muscle cells and bovine aorta endothelium cells in vitro [15]. Furthermore, nuclear DNA glycation has been observed in the kidney cells from patients with diabetic nephropathy and in the aorta of diabetic and non-diabetic hemodialysis patients. Moreover, CEdG has also been detected in human kidneys and aorta by immunohistochemistry with the monoclonal CEdG antibody. Thus, CEdG is the only DNA-AGE which has been detected in vivo so far [16]. Nucleic acids can be directly modified by reducing sugars such as glucose 6-phosphate. The resulting modified DNA shows increased mutational events in bacteria and eukaryotic cells [17-19]. Furthermore, CEdG was identified as a major glycation product of DNA under physiological conditions. This chemically defined modification induces damage in DNA such as strand breaks, which are responsible at least in part for the observed reduction of the transformation rate. Furthermore, DNA AGEs are potentially genotoxic compounds and could be directly linked to alterations in the DNA structure and functionality. CEdG, selectively introduced into the DNA, destabilized the N-glycosidic bond between carboxyethylguanine and the sugar-phosphate DNA backbone, leading to the specific loss of the modified guanine (depurination). Consequently, the occurrence of single-strand breaks was observed in CEdG-modified DNA leading to mutations [12,20]. The transformation of bacterial cells by glycated plasmids resulted in an increased mutation frequency [20] caused by insertions, deletions, as well as multiple species [17]. Likewise, single-base substitutions and the transposition of an Alu-containing element were observed, when DNA, which was pretreated with glyoxal or sugars, was transfected into mammalian cells [21,22]. In vivo, it has been shown that 3-deoxyglucosone
(a glucose degradation product) induces embryonic malformation and teratogenicity, an effect that may be related to DNA AGEs [23]. The CEDC in a gene may lead to a reduced transcription and to a loss of gene function due to mis-sense or nonsense mutations [24]. In this study, we have studied the glucose induced structural changes in native DNA by physicochemical methods as well as quantitative estimation of DNA Amadori products and DNA advanced glycation end products in glucose-modified DNA.

2. Materials and Methods

2.1. Chemical

Analytical grade \( \text{D}-\text{glucose} \) and dihydroxyacetone (DHA) were purchased from E. Merk, Germany. Calf thymus DNA, dialysis tubing, ethidium bromide, sodium azide and agarose were purchased from Sigma Chemical Company, USA. Chloroform, methanol, isopropanol, sodium carbonate, sodium bicarbonate, sodium monobasic and dibasic salts were purchased from Qualigen, India. Nitroblue tetrazolium (NBT) was purchased from Hi-Media, India.

2.2. DNA glycation

Commercially obtained calf thymus DNA was purified [25] and glycated in 0.2 M phosphate buffer (pH 7.0) containing 0.02% sodium azide, as described earlier [26]. Briefly, native DNA \((25\, \mu\text{g/ml})\) was incubated with \(130\, \text{mM} \text{D}-\text{glucose} \) (final volume 3 ml) and for one, two, three and four weeks at \(37\, ^\circ\text{C}\) along with native DNA as control. After incubation, excess glucose was removed by extensive dialysis against \(10\, \text{mM} \) sodium phosphate buffer (pH 7.4) containing 100 mM sodium chloride. Levels of glycation and structural changes in DNA were analyzed.

2.3. UV-visible and fluorescence spectroscopy

UV-visible spectra were recorded in the wavelength range of 200-600 nm on spectrophotometer (model UV-1700; Shimadzu Corporation, Japan). Fluorescence emission spectra were recorded on spectrophotofluorometer (model RF-5301; Shimadzu Corporation, Japan). Native and glycated-DNA samples were excited at 290 and 400 nm respectively and emission profiles were recorded.

2.4. Circular dichroism (CD) measurement

CD studies were carried out on spectropolarimeter (model J-810; Jasco Inc., Great Dunmow, UK) in a cell of 1 cm path length at 265-6 in 220-400 nm range. The base line was corrected with PBS (pH 7.4) and the DNA was used at a concentration of \(37.8\, \text{mM} \). Molar ellipticities \( [\theta] \) were calculated in terms of base pair concentration according to the following equation:

\[
[\theta] = \frac{\theta}{100d}
\]

where \(\theta\) (theta) is the measured ellipticity (mdeg), \(c\) is the molar concentration of DNA (in terms base pair), and \(d\) is the path length \((\text{cm})\).

2.5. Thermal denaturation studies

Thermal denaturation [27] of native and glycated-DNA was evaluated under identical conditions by a temperature scan from \(30\, ^\circ\text{C}\) to \(95\, ^\circ\text{C}\) at an increment of \(1.0\, ^\circ\text{C}\) min on spectrophotometer attached with a temperature programmer and controller assembly. The increase in absorbance at \(260\, \text{nm}\) was taken as a measure of helix denaturation. The process was characterized by determining the percent DNA in denatured state as a function of temperature [28].

2.6. Agarose gel electrophoresis and nuclease S1 assay

The change in electrophoretic migration pattern of native and glycated-DNA was observed on 0.8% agarose gel which was electrophoresed at 30 mA for 2 h in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). Bands were visualized under UV light after staining with EtBr. Native and glycated-DNA were incubated with nuclease S1 (20U/\mu\text{g DNA}) for 30 min at \(37\, ^\circ\text{C}\) and the reaction was terminated by adding 200 mM EDTA, pH 8.0 and then electrophoresed under the same conditions as described above [27].

2.7. High-performance liquid chromatography (HPLC) and LC-MS

HPLC analysis of native and glycated-DNA samples was performed with an Agilent 1100 capillary HPLC system (Palo Alto, CA) equipped with a Synergi C18 (Synergi, USA) analytical column. C18 column \((2\, \text{mm} \times 150\, \text{mm}; \text{particle size } 4\, \text{\mu m})\); eluent A \((5\, \text{mM} \text{aqueous ammonium acetate buffer}, \text{pH 7})\), eluent B \((\text{acetonitrile gradient solution})\). The acetonitrile concentration was raised from 0 to 4.0% in first 5 min; from 4.0 to 6.5% over 30 min; held at 6.5% for 5 min, and raised to 90% to wash residual material off the column.

---

Fig. 1. UV absorption profile of native DNA (-) and one (-O-), two (-D-), three (-A-) and four weeks old (-4-) glycated-DNA.

Fig. 2. Fluorescence emission profile of native DNA (-) and one (-O-), two (-D-), three (-A-) and four weeks old (-4-) glycated-DNA. Samples were excited at 290 nm.
Fig. 3. Fluorescence emission spectra of native DNA (−) and one (−○−), two (−□−), three (−△−) and four weeks old (−▲−) glycated-DNA. Samples were excited at 400 nm.

at a constant flow rate of 500 µl/min. DNA bases were detected by diode array detector (DAD) at 254 nm. LC×MS analysis of CEIG standard was performed using Micromass Quattro Ultima Triple Quadrupole Mass Spectrometer (Beverly, MA) interfaced to an Agilent Capillary HPLC system.

2.8. Determination of DNA Amadori products

Amadori products in glycated-DNA samples were determined by NBT reduction test [29,30]. The DNA samples (100 µl) were mixed with 1 ml of 100 mM sodium carbonate-bicarbonate buffer, (pH 10.8) containing 0.25 mM nitroblue tetrazolium (NBT) and incubated at 37 °C for 45 min. The color (absorbance) was read at 525 nm against distilled water and content of DNA Amadori products (nM/ml) was determined by multiplying absorbance by the coefficient 12.64, typical of monoformazan.

2.9. HPLC-electrospray ionization mass spectrometry

(HPLC×ESI-MS)

HPLC×ESI-MS study requires an orthogonal time-of-light (TOF) mass spectrometer (Beverly, MA, USA) equipped with standard electrospray ionization (ESI) device, PE Sciex’s TurboSpray (TIS), and atmospheric pressure chemical ionization (APCI) sources. The LC system includes the Agilent 1100 capillary HPLC unit (Palo Alto, CA) equipped with a 100-position autosampler, a DAD, a thermostatic column compartment, and a micro-vacuum degasser. The mass spectral data were collected at positive ion polarity. Nitrogen was used as the nebulizer, heater, and collision gas. The Sciex heater was set to 350 °C, and the spray tip potential was adjusted at 4000 V. Under these conditions, the MS system was then operated in full scan (m/z 40–1000). Spectra acquisition was performed in Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>Absorbance at 525 nm</th>
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<tr>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>2</td>
<td>0.020</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
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<td>6</td>
<td>0.175</td>
</tr>
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<td>7</td>
<td>0.200</td>
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<tr>
<td>8</td>
<td>0.251</td>
</tr>
<tr>
<td>9</td>
<td>0.177</td>
</tr>
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</table>

Table 1: Effect of incubation time on generation of NBT reactive early glycation product of DNA.
Fig. 7. (A) Agarose gel electrophoretic pattern of native and glycated-DNA. Native DNA (lane 1), one week old glycated-DNA (lane 2), two weeks old glycated-DNA (lane 3), three weeks old glycated-DNA (lane 4) and four weeks old glycated-DNA (lane 5). (B) Agarose gel electrophoretic pattern of nuclease S1 treated native and glycated-DNA. Native DNA (lane 1), nuclease S1 treated native DNA (lane 2), one week old glycated-DNA (lane 3) and nuclease S1 treated one week old glycated-DNA (lane 4). (C) Native DNA (lane 1), nuclease S1 treated native DNA (lane 2), two weeks old glycated-DNA (lane 3) and nuclease S1 treated two weeks old glycated-DNA (lane 4). (D) Native DNA (lane 1), nuclease S1 treated native DNA (lane 2), three weeks old glycated-DNA (lane 3) and nuclease S1 treated three weeks old glycated-DNA (lane 4). (E) Native DNA (lane 1), nuclease S1 treated native DNA (lane 2), four weeks old glycated-DNA (lane 3) and nuclease S1 treated four weeks old glycated-DNA (lane 4).
Fig. 8. ESI-MS analysis of native DNA (a) and one week old glycated-DNA (b) hydrolysate following HPLC separation.

every 2 s, and a total of 10 spectra were collected. All ions measured by MS were in the [M+H]^+ form.

3. Results

3.1. UV analysis of glucose-modified DNA

The DNA samples exhibited 38.6±78% hyperchromicity (λ_max 260 nm) depending on the time of incubation (Fig. 1). Additional peaks at 320 and 380 nm were observed on increase in incubation period.

3.2. Fluorescence studies on glucose-modified DNA

DNA (non-fluorescent) incubated with glucose for 1-4 weeks was excited at 290 nm and 400 nm and emission profiles were recorded to ascertain the formation of Amadori and advanced glycation end products. When excited at 290 nm, modified samples for 1-4 weeks showed gradual increase in emission fluorescence intensity (λ_max, 450 nm) (Fig. 2). The observed fluorescence intensity in one, two, three, and four weeks old glycated-DNA samples was 72.7, 132.3, 264.2, 386.5 and 417.6 arbitrary units, respectively. When the same samples were excited at 400 nm, fluorescence intensity of one week old glycated-DNA sample was found to be decreased from 72.7 to 46.4 (A.U.) (Fig. 3) while two, three and four weeks old glycated-DNA samples showed increase in fluorescence intensity from 132.3 to 148.4; 264.2 to 286.5 and from 386.5 to 417.6 (A.U.), respectively.

3.3. Nitroblue tetrazolium (NBT) reduction assay for DNA

Amadori products

NBT reduction assay is specific for Amadori products and not for AGEs [32]. Generation of DNA Amadori products was confirmed from reduction of yellow NBT to purple monoformazan (Fig. 4). In quantitative terms, the Amadori content was determined to be 3.18 ± 0.07, 1.65 ± 0.12, 1.17 ± 0.08 and 0.83 ± 0.13 nM/ml in DNA samples modified by glucose for one, two, three and four weeks, respectively. Furthermore, stable glycated product appeared during one day incubation (Table 1).

3.4. Circular dichroism (CD) profile of glucose-modified DNA

The CD profile of native DNA exhibited a negative peak of -9.8 mdeg at 245 nm and a positive peak of +10.7 mdeg at 275 nm (Fig. 5). DNA modified by glucose for 1-4 weeks did show negative peaks at 245 nm but ellipticity values were found to be -6.5, -5.2, -4.0 and -3.4 mdeg respectively compared to -9.8 mdeg ellipticity of native DNA. Under identical conditions and at 275 nm positive peak the ellipticities of 1-4 weeks old glycated-modified DNA samples were found to be +8.8, +7.9, +6.7 and +6.0 mdeg, respectively.

3.5. Thermal denaturation of glucose-modified DNA

The melting temperature (T_m) of native DNA was found to be 86±6°C. However, T_m of DNA modified by glucose for 1-4 weeks was found to be 80, 78, 75 and 73±6°C, respectively (Fig. 6). The decrease in T_m to the extent of 6, 8, 11, and 13±6°C may be attributed to structural alterations in the DNA molecule as a result of glucose induced modification. Generation of strand breaks and base modification appears to be major structural change that has occurred during glycation which may be responsible for early onset of melting in modified DNA samples. The physicochemical characteristics of native and modified DNA samples are summarized in Table 2.

3.6. Agarose gel electrophoresis

Compared to native DNA, the electrophoretic migration pattern of the modified samples (0.8% agarose gel) indicates structural alteration/fragmentation. DNA modified by glucose for four weeks showed maximum mobility (Fig. 7a). Reaction of glycation induced intermediates and/or superoxide radicals with sugar-phosphate back bone of DNA might be responsible behind generation of strand breaks/small size DNA fragments showing faster mobility [34–36]. In order to confirm the generation of single strand breaks, samples were digested with nuclease S1 (20 μg) to see DNA fragment size for 30 min
Fig. 9. HPLC chromatogram of standard dG and CEdG (a) and two weeks old glycated-DNA (b), three weeks old glycated-DNA (c), and four weeks old glycated-DNA (d).

Table 2
Ultraviolet and thermal denaturation characteristics of native and glycated-DNA under described experimental conditions.

<table>
<thead>
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<th>Parameter</th>
<th>Native DNA</th>
<th>One week old glycated-DNA</th>
<th>Two weeks old glycated-DNA</th>
<th>Three weeks old glycated-DNA</th>
<th>Four weeks old glycated-DNA</th>
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<tbody>
<tr>
<td>Absorbance ratio ($A_{260}/A_{280}$)</td>
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<td>1.72</td>
<td>1.65</td>
<td>1.49</td>
<td>1.45</td>
</tr>
<tr>
<td>Melting temperature (°C)</td>
<td>86</td>
<td>80</td>
<td>78</td>
<td>75</td>
<td>72.5</td>
</tr>
<tr>
<td>Occurrence of duplex melting (°C)</td>
<td>68</td>
<td>62</td>
<td>53</td>
<td>46</td>
<td>44</td>
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</table>

and electrophoresed on 0.8% agarose gel. DNA modified by glucose for one, two, three and four weeks showed decrease in fluorescence intensity when treated with nuclease S1. However, nuclease S1 treated native DNA co-migrated with control native DNA and had almost similar fluorescence intensity (Fig. 7a–c). These observations clearly demonstrate glucose induced strand breaks in the DNA rendering it susceptible to digestion by nuclease S1.

3.7. Characterization of DNA Amadori products by ESI-MS

To confirm the generation of Amadori products, native and one week old glycated-DNA sample was hydrolyzed and subjected to ESI-MS. Fig. 8a and b shows the mass-spectral profiles of native and one week old glycated-DNA hydrolysate. The ion at $m/z$ 315.18 is consistent with a [Schiff base+H]+ molecule resulting from the condensation and dehydration reaction of guanine (Mr 151.13) with glucose (Mr 180.16). The result suggests presence of stable Amadori intermediates post-Schiff base rearrangement.

3.8. Synthesis and characterization of 1-carboxymethyl)-2-deoxyguanosine (CEdG) by HPLC and analysis of glucose-modified DNA

CEdG was synthesized in laboratory according to the procedure described previously [12]. CEdG was isolated from the final preparation by preparative HPLC using ammonium acetate buffer and methanol as eluents. The elution of CEdG was seen at a retention time of 14.26 min when UV detector was used. However, for deoxyguanosine (dG) elution, the retention time was 9.4 min (Fig. 9a).

Fig. 9b–d shows representative HPLC chromatograms of acid hydrolysate of two, three and four week's old glycated-DNA. In
case of native DNA and one week old glycated-DNA we did not observe any peak with a retention time of 14.26 min, thereby showing the absence of CEdG (figure not shown). However, two, three and four weeks old glycated-DNA showed peak with retention time of 14.061 min, 14.213 min, 14.224 min which shows the presence of \( N^2(1\text{-carboxyethyl})\text{-2-deoxyguanosine} \) (CEdG) as it matches with the retention time of standard CEdG. Furthermore, the CEdG peak was found to be gradually increasing with incubation time. The CEdG is a marker of DNA glycation formed with glucose and other reducing compounds.

The CEdG synthesized from dG was analyzed on mass spectrometer which showed a mass (m/z) of 338 significantly different from the 266 mass of dG (Fig. 10a). When native DNA and one-week-old glycated-DNA were analyzed under identical conditions they did not show mass value matching with CEdG (figure not shown). Furthermore, analysis of two, three and four weeks old glycated-DNA suggests CEdG formation and m/z value of 338 [24] was seen in all three cases (Fig. 10b-d).

4. Discussion

Preliminary studies with nucleic acids have demonstrated that amino groups of DNA bases can non-enzymatically react with reducing sugars. Furthermore, incubation of DNA with reducing sugars can generate chromophores and fluorophores with structural properties similar to advanced glycated protein products [19,31]. Although efforts to characterize structural and functional changes in proteins by glycation continue, fine studies on nonenzymatic glycation of eukaryotic DNA have received minimal attention. In this study, we have demonstrated that glucose can cause extensive damage to DNA structure leading to strand breaks and formation of DNA Amadori products and DNA advanced glycation end products. Hyperchromicity observed at 260 nm in glycated-DNA samples with reference to native DNA may be due to adduction-cum-free radical mediated damage to sugar-phosphate backbone followed by partial unfolding of double helix and more exposure of chromophoric bases [33,34]. Furthermore,
appearance of new peaks and/or increased absorbance between 300 and 400 nm indicates formation of DNA AGEs (mainly CεdG).

Increased absorbance in the 300-400 nm range [31] and its usefulness in glycation of DNA/nucleotide/proteins with reducing sugars has been well documented [37,38]. The systemic decrease in Tm values of modified-DNA samples could be attributed to strand breaks, unstacking and base modification that may lead to altered hydrogen bonding between base pairs [35,36]. Strand breaks were specifically confirmed from positive nuclease S1 action on glucose-modified samples compared to native DNA, which remained resistant to nuclease S1.

Amadori products in one-week-old glycated-DNA sample were evident from reduction of yellow color of NBT to purple monomer. Here it may be noted that the NBT reduction assay is negative for AGEs [32]. Earlier studies have suggested that the reduction of NBT may be due to generation of superoxide radicals and degradation intermediates of Amadori products [39–42]. The ESI-MS result further substantiated the presence of Amadori products in one-week-old glycated-DNA. Evidence in favor of fluorescence, HPLC and LC-MS [30,43]. This study demonstrates that glucose can induce structural changes and strand breaks in DNA. Moreover, glycation of DNA evolves from the formation of Amadori products finally culminating into DNA AGEs.

Acknowledgment

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References