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Glycoxidative damage to human DNA: Neo-antigenic epitopes on DNA molecule could be a possible reason for autoimmune response in type 1 diabetes

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Advanced glycation end-products (AGEs) are known to be mutagenic, diabetogenic and vascular disease risk factors. Methylglyoxal (MG) is a dicarbonyl species that reacts with biological macromolecule (proteins, DNA and lipids) to give AGEs. Nonenzymatic glycation of MG with lysine (Lys) in the presence of copper (Cu2+) is reported to generate reactive oxygen species (ROS) capable of causing DNA damage. We show that DNA modification in MG–Lys–Cu2+ system results in the generation of strand breaks, base modification, hyper-chromicity and increased fluorescence intensity. Superoxide generation in the MG–Lys system was found to be significantly higher when compared with that in the MG and Lys alone. Moreover, n-penicillamine and pyridoxal phosphate significantly inhibited the formation of glycation products. The presence of a major DNA glycation adduct, N2-carboxyethyl-2′-deoxyguanosine (CEdG), was detected by high performance liquid chromatography (HPLC) and confirmed by nuclear magnetic resonance (NMR). As reported earlier, modified DNA (MG–Lys-Cu2+–DNA) was highly immunogenic in experimental animals. Furthermore, induced anti-MG–Lys–Cu2+–DNA antibodies were effective probe for detecting glycoxidative lesions in human genomic DNA of type I diabetes patients. Our results clearly imply that interaction of MG–Lys and Cu2+ leads to the formation of AGEs and also the production of potent ROS, capable of causing DNA damage, thereby playing an important role in diabetes mellitus.

Keywords: advanced glycation end-products / diabetes / DNA / glycation / methylglyoxal

Introduction
Reactive carbonyl species (RCS) are potent mediators of cellular carbonyl stress originating from endogenous chemical processes such as glycation and lipid peroxidation. These are heterogeneous group of carbonyl activated by α, β-unsaturation as in 4-hydroxyynenal and acrolein, α-oxo-substitution as in glyoxal and methylglyoxal (MG) and β-oxo-substitution as in malondialdehyde (Thornalley 1996). Among all of the reactive carbonyls produced, MG and glyoxal are exclusively responsible for the glycation reaction. Glycation is the nonenzymatic addition of free reducing sugars into biological macromolecules such as DNA, proteins and lipids (Bucala et al. 1985; Sengupta and Swenson 2005). Initiation of glycation occurs by the formation of acid-labile Schiff base adducts, which undergoes Amadori rearrangements into more stable products known as early glycation products. The early glycation products undergo slow transformation to yield the irreversible advanced glycation end-products (AGEs) (Akhter et al. 2013). Glycation results in the production of free radicals, which play important roles in the development of cancer, diabetes, arthritis, heart disease, cataract, atherosclerosis and neurodegenerative disorders (Schleicher et al. 1997; Baynes 2001; Ahmad, Ahmad, et al. 2011; Mustafa et al. 2012; Raheem et al. 2013). Several interrelations have been shown between oxidative stress and AGEs. Reactive oxygen species (ROS) generated in oxidative stress can in turn accelerate the AGE formation (Ahmad, Akhter, et al. 2013).

Among all dicarbonyl intermediates formed in the glycation reaction, MG is a potent and most reactive AGE precursor, forming adducts on arginine, Lys, cysteine and deoxyguanosine (dG) residues. Furthermore, it has been shown that DNA can be glycated in vitro yielding N2-carboxyethyl-2′-deoxyguanosine (CEdG) as a major product. In vitro, nucleobases and ds-DNA react with sugars in a similar way as proteins (Lee and Cerami 1987; Knerr and Severin 1993; Singh et al. 2001). The exocyclic amino group of 2′-deoxyguanosine is particularly prone to glycation, leading to the formation of N2-carboxyethyl, N2-carboxymethyl, as well as cyclic dicarbonyl adducts (Ochs and Severin 1994). The CEdG is a stable reaction product, formed from a variety of glycating agents, such as glucose, ascorbic acid, glyceraldehyde, dihydroxyacetone (DHA) and MG (Larisch et al. 1997; Frischmann et al. 2005). Apart from in vitro DNA glycation, under hyperglycemic condition the glycation of DNA occurs in vivo and is considered to be a pathogenic factor for diabetes (Levi and Werman 2003; Dutta et al. 2005).
The present study aimed to glycate human DNA by MG and Lys in the presence of Cu\(^{2+}\). Glycation induced structural perturbations in the DNA macromolecule were characterized by agarose gel electrophoresis and various spectroscopic techniques. The DNA-AGEs generated in the MG-Lys–Cu\(^{2+}\) system was confirmed by antiglycation study. Formation of CEdG, a major adduct of DNA glycation, was confirmed by high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) studies. We propose one of the possible reason for type I diabetes autoimmunity by utilizing the induced antibodies against glycated human DNA in experimental rabbits (Ahmad et al. 2011). These experimentally induced antibodies have been used as an immunochromical probe to detect glycoxidative damage in the lymphocyte DNA isolated from type I diabetic patients.

**Materials and methods**

Human placental DNA, MG, anti-rabbit IgG alkaline phosphatase conjugates, p-nitrophenyl phosphate, Tween-20, Protein A-agarose (2.5 mL prepacked column), nuclease S1, superoxide dismutase (SOD), ethidium bromide, agarose and dialysis tubing were purchased from Sigma Chemical Company, Saint Louis, MO. Polystyrene microtiter flat bottom enzyme linked immunosorbent assay (ELISA) plates (maxisorp) were from Nuncl (Denmark). Pyridoxal phosphate (PLP) was from Calbiochem, Billerica, MA. DHA was from Merck, Germany. Lysine was from Sisco Research Laboratory, Pallikaranai, Tamil Nadu. HbA1C kit was purchased from Bio-Rad, Hercules, CA. All other reagents/chemicals were of the highest analytical grade available. All the ethical requirements were adhered to for this study.

**Purification of human placental DNA**

Commercially available human placental DNA was purified free of proteins and single stranded regions with slight modifications (Habib et al. 2009). DNA (2 mg/mL) was dissolved in 0.1 × saline sodium citrate buffer, pH 7.3 and extracted with an equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered container and shaken for 1 h. The aqueous layer containing DNA was separated from organic layer and re-extracted with equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered by adding one-tenth volume of 200 mM ethylene diamine (30 mM sodium acetate containing 30 mM zinc chloride, pH 5.0) and treated with nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was stopped by adding one-tenth volume of 200 mM ethylene diamine tetra acetic acid (EDTA), pH 8.0. Nuclease S1 treated DNA was extracted twice with chloroform-isoamyl alcohol and finally precipitated with two volumes of chilled ethanol. The precipitate was dissolved in the required buffer. Purity of DNA was confirmed by A260/A280 ratio which was in the range of 1.8–2.0.

**Modification of human placental DNA**

Human placental DNA was modified by MG and Lys in the presence and absence of Cu\(^{2+}\) as described previously (Ahmad et al. 2011). 37.8 μM of human DNA was thoroughly mixed with MG (40 mM), Lys (40 mM) and Cu\(^{2+}\) (300 μM) in 10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and incubated at 37°C for 24 h followed by extensive dialysis against phosphate buffer saline (PBS) to remove unbound constituents.

**Spectroscopic analysis**

The ultraviolet absorption profile of native human placental DNA and modified DNA was recorded in the wavelength range of 200–400 nm on Shimadzu UV-1700 spectrophotometer. Fluorescence emission profiles of native and modified human placental DNA were recorded on Shimadzu RF-5301 spectrofluorometer. Native and modified DNA was excited at 370 nm and emission profile was recorded at 450 nm.

**Agarose gel electrophoresis**

0.8% agarose in tris acetate EDTA buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 50°C and then poured into the gel tray and allowed to solidify at room temperature. Native and modified human placental DNA were mixed with 1/10 volume of sample buffer (0.125% bromophenol blue, 30% Ficoll-400, 5 mM EDTA) in 10 × electrophoresis buffer) and loaded in the wells of 0.8% agarose gels (Shahab et al. 2012). Electrophoresis was done for 2 h at 40 mA and the gels were stained with ethidium bromide (0.5 μg/mL), and viewed under UV light.

**Nuclease S1 digestibility**

Native human placental DNA and its modified analogue were characterized by nuclease S1 digestibility assay (Matsuo and Ross 1987). One μg each of native DNA and the modified DNA in acetate buffer (30 mM sodium acetate, 1 mM zinc sulphate, 100 mM sodium chloride, pH 4.6) were treated with nuclease S1 (20 unit/μg of DNA) for 30 min at 37°C. The reaction was stopped by adding one-tenth volumes of 200 mM EDTA, pH 8.0. The samples were subjected to agarose gel electrophoresis and viewed by illumination under UV light after ethidium bromide (0.5 μg/mL) staining.

**Measurement of superoxide anion**

The generation of \(\text{O}_2^–\) in the reaction mixture was determined by cytochrome-c reduction assay (Beauchamp and Fridovich 1971). A reaction mixture contained MG (40 mM) and Lys (40 mM) and 100 μM cytochrome-c in 10 mM phosphate buffer (pH 7.4). The reduction rate was determined as the increase in absorbance at 550 nm for 10 min at room temperature. Absorbance was taken at the interval of every 1 min.

**AGE inhibition study**

AGE inhibition was performed using a carbonyl trapping compound, d-penicillamine (1 mM) and PLP (10 mM) on human placental DNA. The modifications were studied by incubating DNA (25 μg/mL) in 10 mM sodium phosphate buffer, pH 7.4 containing MG (40 mM), Lys (40 mM) and Cu\(^{2+}\) (300 μM) at 37°C for 24 h. Percent DNA quenching was calculated from absorbance at A260 nm. AGE inhibition was further confirmed from absorbance at A330 nm.

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Synthesis of the CEdG
The synthesis of CEdG was carried out as described by Seidel and Pischetsrieder (1998), with slight modifications. Briefly, 50 mg of deoxyguanosine suspended in 1 mL of 100 mM phosphate buffer (pH, 7.4), was incubated with 100 mg of DHA at 70°C in a shaking water bath. It got dissolved at 70°C in the course of reaction after 24 h. CEdG was isolated by preparative HPLC using 50 mM ammonium acetate buffer solution and methanol as eluents.

1H NMR of the synthesized CEdG
1H NMR spectra were recorded on a Bruker DRX-300 MHz FT NMR spectrometer. All samples of NMR spectroscopy were first lyophilized and prepared using dimethyl sulfoxide (DMSO) as a solvent. The chemical shifts in parts per million (ppm) are expressed with respect to tetra methyl silane as a reference.

HPLC of native and modified human DNA
An Agilent 1100 capillary HPLC System equipped with a Synergi C18 analytical column was used for HPLC analysis of native and modified analog of human placental DNA. General chromatographic conditions were as follows: C18 column (2 mm × 150 mm with 4 μm particle size); eluent A, 5 mM aqueous ammonium acetate buffer, pH 7; eluent B, acetonitrile (CH3N) gradient solution—the CH3N concentration was raised from 0 to 4.0% in the first 5 min; from 4.0 to 6.5% over 30 min; held at 6.5% for 5 min, and then raised to 90% to wash residual material off the column at a constant flow rate of 500 μL/min.

Isolation of DNA from human lymphocytes
Blood samples were collected in EDTA vials from different type I diabetes patients and healthy individuals after prior consent. Lymphocyte DNA was isolated as per the manufacturer’s instructions. To 500 μL of Qiagen protease, 5 mL of blood was added and then buffer AL (6 mL) was mixed, followed by vigorous shaking. Mixture was incubated at 70°C for 10 min. Then 5 mL of ethanol (98%) was added to the sample and mixed by inverting the tube, followed by vigorous shaking. The solution was then carefully transferred onto QIAamp Maxi column placed in a 50 mL centrifuge tube and was centrifuged at 1850 × g for 3 min. Filtrate was discarded and 5 mL of Buffer AW2 was added and again centrifuged at 4500 × g for 1 min. Again 5 mL of AW2 buffer was added and centrifuged at 4500 × g for 15 min discarding the filtrate. Then 600 μL of buffer AE was poured directly onto the membrane of the column which was later incubated for 5 min, and centrifuged at 4500 × g for 2 min. Eluent containing DNA was reloaded onto the membrane of the column, and was again incubated for 5 min, and centrifuged at 4500 × g for 5 min. Eluent containing DNA was air dried and dissolved in PBS, pH 7.4. Absorbance of DNA solution was monitored at 260 and 280 nm to ascertain its purity and concentration.

Direct binding ELISA for the detection of autoantibodies
ELISA was carried out on flat bottom polystyrene plates as described earlier (Moinuddin et al. 2012; Shahab et al. 2013). Briefly, microtiter wells were coated with 100 μl of 2.5 μg/mL of DNA (in tris buffer saline (TBS), pH 7.4) and incubated for 2 h at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the plate, devoid of antigen, served as control. The test-plate wells were emptied and washed thrice with tris buffer saline—tween 20 (TBS-T) to remove the unbound antigen. Unoccupied sites were blocked with 150 μL of 1.5% nonfat dry milk (in TBS, pH 7.4) for 4–5 h at 4°C followed by single wash with TBS-T. In direct binding ELISA, sera were directly added into antigen-coated wells and incubated for 2 h at 37°C and overnight at 4°C respectively. The wells were emptied and extensively washed with TBS-T. Anti-immunoglobulin G alkaline phosphatase conjugate was added to each well and incubated at 37°C for 2 h and then the plates were washed thrice with TBS-T followed by a single wash with distilled water. Para-nitrophenyl phosphate was added and the developed color was read at 410 nm on a microplate reader. The results were expressed as mean of difference of absorbance values in test and control wells (Atest − Acontrol).

Detection of glycoxidative lesions in human DNA by enzyme-linked immunosorbent assay
ELISA was performed as described elsewhere with slight modifications (Khan et al. 2007). In order to detect glycation induced lesions in diabetic subjects, DNA was isolated from human lymphocytes of diabetes patients and experimentally induced anti-MG–Lys–Cu2+–DNA IgG was used as a probe to detect glycoxidative DNA lesions. Immune complexes, formed by incubating a fixed amount (40 μg) of anti-MG–Lys–Cu2+–DNA IgG with increasing concentration (0–20 μg/mL) of lymphocyte DNA, were coated on microtiter plates, already coated with MG–Lys–Cu2+ modified human DNA (2.5 μg/mL).

Estimation of HbA1C
Kit purchased from of Bio-Rad was used for the estimation of HbA1c in diabetes sera using HPLC based D-10 HbA1c program. In this procedure, sample is automatically diluted and injected into the analytical cartridge. The D-10 device delivers buffer gradient of increasing ionic strength to the cartridge, and the hemoglobin are separated on the basis of their interactions with the cartridge material. The separated hemoglobin pass through the flow cell of the photometer and the absorbance is recorded at 415 nm. The software performs reduction of raw data collected from each analysis. Two-level calibration is used for quantitation of HbA1c values. A sample report and chromatogram is generated for each sample. The HbA1c area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak area from the A1c peak area. The normal HbA1c reference range of the kit used in this study was 4.2–6.1%.

Statistical analysis
Results are expressed as mean ± SEM. All statistical calculations were performed using the SPSS software (version 16). A P-values of <0.05 was considered as statistically significant. Linear correlation analysis using absorbance difference as dependent variables was also performed.
Results and discussion

Glycation of human placental DNA

Human placental DNA was glycated by MG and Lys in the presence and absence of Cu²⁺. Pilot experiments were run to work out the incubation time and optimum concentration of MG, Lys and Cu²⁺ required to modify the DNA. Human DNA (37.8 μM) was incubated with MG, Lys and Cu²⁺ along with respective controls for different time intervals (3, 6, 12 and 24 h) at 37°C. Maximum hyperchromicity at 260 nm was observed at 24 h incubation as further incubation did not result in any change in the hyperchromicity. Therefore, our studies were performed on human DNA incubated for 24 h with 40 mM each of MG and Lys in the presence and absence of copper sulphate (Cu²⁺, 300 μM) in PBS.

A characteristic peak at 260 nm was observed with native human DNA in UV-Vis spectroscopy. However, upon modification with MG and Lys, 68% hyperchromicity was observed in the DNA peak. Moreover, as reported previously (Ahmad et al. 2011), in MG + Lys + Cu²⁺ modified DNA, the observed hyperchromicity in the DNA peak was 75.5% (Figure 1). This shows extensive damage to the human DNA, possibly leading to the formation of single strand breaks and base modifications. However, when MG, Lys and Cu²⁺ were individually incubated with human DNA under identical conditions, no significant change in UV-Vis spectrum was observed. Furthermore, MG–Cu²⁺ and Lys–Cu²⁺ systems also did not show any detectable change in UV-spectral profile. The change in hyperchromicity of MG–Lys and MG–Lys–Cu²⁺ system could be attributed to the destabilization of hydrogen bonds and modification of nitrogenous bases which result in the destruction of chromophoric groups through attack on the sugar-phosphate back bone.

Interestingly, a new additional peak was obtained at ~330 nm, when human DNA was incubated with MG–Lys and MG–Lys–Cu²⁺ systems. This could be attributed to the generation of DNA-AGEs. MG–Lys–Cu²⁺ derived DNA-AGEs show the highest absorbance, suggesting Cu²⁺ enhanced AGEs formation. UV absorbance between 320 and 370 nm can be used to judge the onset of AGE formation that are derived from the reaction of DNA with the modifier (MG–Lys–Cu²⁺). An earlier study (Schmitt et al. 2005) has also reported an extra peak in the 320–330 range, due to the formation of glycation induced AGEs, thus supporting our findings.

DNA-AGEs are formed spontaneously by the reaction of carbonyl compounds such as MG both in vitro and in vivo, after a complex cascade of dehydration, condensation, fragmentation, oxidation and cyclization reactions (Schleicher et al. 2001). The detection can be based on the fluorescent properties of AGEs (Monnier and Cerami 1981). The detection can be based on the fluorescent properties of AGEs (Monnier and Cerami 1981). The detection can be based on the fluorescent properties of AGEs (Monnier and Cerami 1981). The detection can be based on the fluorescent properties of AGEs (Monnier and Cerami 1981).

**Fig. 1.** Ultraviolet absorption spectra of native human DNA (—); human DNA modified with 300 μM Cu²⁺ (——); 40 mM Lys (open triangle); 40 mM Lys + 300 μM Cu²⁺ (filled triangle); 40 mM MG (open circle); 40 mM MG + 300 μM Cu²⁺ (filled circle); 40 mM MG + 40 mM Lys (open square) and 40 mM MG + 40 mM Lys + 300 μM Cu²⁺ (filled square).

DNA-AGEs were characterized by an increase of 76.2% in the fluorescence intensity in the case of glycated DNA when compared with to the native form (Figure 2).

**Fig. 2.** Fluorescence emission spectra of native human DNA (—) and modified human DNA with 40 mM MG + 40 mM Lys + 300 μM Cu²⁺ (——).

Agarose gel electrophoresis of native and MG–Lys–Cu²⁺ glycated human DNA

Appreciable evidence for generation of strand breaks (single and double) in the human DNA as a consequence of MG–Lys–Cu²⁺ modification was gathered by gel electrophoresis on 0.8% agarose. The gel pattern (Figure 3A) shows an increase in the mobility of glycated DNA with increasing incubation time (Lanes 2–5). Maximum mobility was observed at 24 h incubation, and further incubation did not have any consequential effect on DNA migration pattern. The increase in mobility may be due to the generation of single stranded breaks by glycation induced intermediates or free radical generation or both, which
may result in the formation of small size DNA showing faster mobility compared with the native DNA of Lane 1. Earlier studies have demonstrated that the structural alteration in DNA following damage by various agents result in the generation of single strands in the DNA molecule (Shishido and Ando 1974; Yamasaki et al. 1997). Therefore, generation of single strand breaks was analyzed by nuclease S1 digestibility assay. Native and modified human DNA were digested with nuclease S1 (20 units/μg DNA) for 30 min and electrophoresed on 0.8% agarose to visualize the generation of single strand breaks. The controls were native and modified DNA samples, untreated with nuclease S1. Modified DNA showed decrease in fluorescence intensity following nuclease S1 digestion. On the other hand, nuclease S1 treated and untreated native DNA showed almost identical electrophoretic migration pattern and fluorescence intensity (Figure 3B). The results show substantial digestion of modified DNA by nuclease S1, while native DNA remained unaffected. These observations clearly demonstrate sufficient distortions (formation of single strand breaks) in the helical structure of DNA upon MG–Lys–Cu$^{2+}$ treatment, rendering it susceptible to digestion by single strand specific nuclease S1.

**Quantitation of superoxide anion**

Amino acids (Lys and arginine) treated with MG have been reported to give electron paramagnetic resonance signals (Pethig and Szent-Gyorgyi 1977), indicative of the formation of free radicals (‘OH and O$_2^•$). Several reports (Bucala et al. 1991; Simpson et al. 1992) have shown that glycated protein generated superoxide anions, which initiated lipid peroxidation. During incubation of MG with Lys, the formation of superoxide anion was gradually increased in a time-dependent manner (Figure 4A). The result suggests that DNA strand breakage by the MG–Lys–Cu$^{2+}$ system may be dependent on the formation of superoxide anion in this system. Moreover, the production of superoxide radical gradually inhibited by increasing concentration of SOD, further confirming the formation of superoxide radical (Figure 4B). The decrease in the superoxide formation was statistically highly significant for 500 units of SOD ($P < 0.001$).

**Antiglycation study**

Antiglycation activity of d-penicillamine and PLP was assessed in vitro via UV-Vis spectroscopic measurements. The extra peak at 330 nm in UV-Vis spectroscopy, as reported earlier, is due to the formation of DNA-advance glycation end-products (Ahmad et al. 2011). Quenching of this peak was exploited to see their antiglycating effect from absorbance at A$_{330}$ nm. d-penicillamine (1 mM) and PLP (10 mM) showed remarkable inhibition of 55 and 73% respectively in DNA modification as analyzed at 260 nm; the characteristic wavelength maxima for DNA. However, at 330 nm d-penicillamine and PLP showed still higher inhibition of 69 and 85%, respectively (Figure 5). The decrease in the percent modification from PLP and pyridoxamine at 260 and 330 nm were found statistically significant ($P < 0.001$) when compared with original modification of the glycation reaction. The enhanced inhibition of 330 nm peak represents inhibition of AGEs formation, clearly indicating the formation of AGEs in the MG–Lys–Cu$^{2+}$ modified human DNA.

**Fig. 3.** (A) Agarose gel electrophoresis of native and modified human DNA. Lanes 2–5: DNA samples treated with MG–Lys (40 mM each) and Cu$^{2+}$ (300 μM). Lane 1: Native human DNA. Lane 2: Modified human DNA with 3 h incubation. Lane 3: Modified human DNA with 6 h incubation. Lane 4: Modified human DNA with 12 h incubation. Lane 5: Modified human DNA with 24 h incubation. (B) Nuclease S1 digestibility of native and modified DNA. Lane 1 contains native DNA, while Lane 2 contains native DNA treated with nuclease S1 for 30 min; Lane 3 contains modified DNA and Lane 4 contains modified DNA treated with nuclease S1 for 30 min. Electrophoresis was carried out on 0.8% agarose gel for 2 h at 30 mA.
Characterization of CEdG by HPLC

Synthesis of the standard, CEdG was performed as described earlier (Seidel and Pischetsrieder 1998). After final preparation, CEdG was isolated by preparative HPLC using 50 mM ammonium acetate buffer solution and methanol as eluents. The elution of CEdG was obtained at a retention time of 14.399 min when UV detector was used for the experiment (Figure 6A). However, deoxyguanosine gave elution at a retention time of 9.1 min.

NMR of CEdG

For structural assignment of the compound, the CEdG peak was isolated by HPLC and then subjected to $^1$H NMR analysis. Resonance signals can be identified and characterized in $^1$H NMR spectrum of CEdG (Figure 6B), recorded in DMSO-d$_6$. $^1$H NMR (400 MHz, DMSO-d$_6$, 20°C) assignment for CEdG:

- $\delta$ 10.6 (s, 1H, N1-H)
- $\delta$ 7.93 (s, 1H, C8-H)
- $\delta$ 6.76 (d, 1H, C2-NH)
- $\delta$ 6.12 (dd, 1H, C1′-H)
- $\delta$ 5.3 (d, 1H, C3′-OH)
- $\delta$ 4.89 (s, 1H, C5′-O-H)
- $\delta$ 4.36 (m, 1H, C2-NH-C)
- $\delta$ 4.18 (q, 1H, C2-NH-C)
- $\delta$ 3.81 (m, 1H, C3′-H)
- $\delta$ 3.5 (ddd, 2H, C5′-H$_2$)
- $\delta$ 2.64 (ddd, 1H, C2′-H)
- $\delta$ 1.39 (d, 3H, C2-NH-CH$_3$)

The $^1$H NMR analysis indicated a -CHX-CH$_3$ group bound to the purine (guanine) ring. The chemical shifts ($\delta$), 4.18 ppm (q, 1H, C2–NH–C) and 1.39 ppm (d, 3H, C2–NH–CH$_3$), respectively, is attributed to the carboxyethyl group of the standard CEdG.

Characterization of native and MG–Lys–Cu$^{2+}$ glycated human DNA by HPLC

Figure 7 shows the representative HPLC chromatograms of acid hydrolyzed samples of native and modified human DNA. Well-defined peaks at retention time 4.467, 7.332 and 8.727 min were observed in native DNA. However, in the case of modified DNA, these peaks shifted to 6.748, 8.455 and 12.399 min, respectively, suggesting considerable change in the DNA bases. The extra peak at a retention time of 14.249 min in the acid hydrolysate of modified DNA is characteristic of N2-(1-carboxyethyl)-2-deoxyguanosine (CEdG) adduct. This is in conformity with the standard CEdG results wherein also when deoxyguanosine was exposed to DHA, a distinct peak at retention time of 14.399 min was observed (Figure 6A). The CEdG adduct is a marker of glucose and MG-induced DNA glycation.
Glycation adducts of DNA may have a potential as biomarkers, since all nucleated cells contain the same DNA content and should reflect the relative level of MG in the target tissue. Reaction of double-stranded DNA with MG or glucose in vitro primarily produces $N^2$-carboxyethyl-2'-deoxyguanosine (CEdG), suggesting it to be the likely major adduct formed in vivo (Papoulis et al. 1995). This implicates that CEdG is a useful biomarker for monitoring oxoaldehyde-induced stress in response to enhanced glycolytic flux or environmental exposure to MG. The standard adduct, CEdG, formed by preparative HPLC was further proved by nuclear magnetic resonance analysis. The HPLC performed for native and glycated DNA showed remarkable difference in peak retention times. The extra peak in modified DNA is in accordance with the standard
CEdG results. However, native DNA did not show any peak at this retention time, suggesting sufficient distortion in the DNA structure has caused it to form the glycation adduct, CEdG, which might be used as a biological marker for early detection of diabetes.

Fig. 7. (A) Representative HPLC chromatogram of acid hydrolysate of native human DNA. (B) Representative HPLC chromatogram of acid hydrolysate of modified human DNA.

Detection of autoantibodies against MG–Lys–Cu\(^{2+}\)–DNA in diabetic subjects

In order to analyze the role of glycated DNA in eliciting immune response in diabetic patients, the presence of autoantibodies in the diabetic subjects against MG–Lys–Cu\(^{2+}\)–DNA...
was probed. The pilot study was performed to screen out the positive sera samples from both type I and type II diabetic subjects. Our study comprised 40 serum samples each of type I and type II diabetes mellitus patients (totally 80 sera samples). Control serum samples from age- and sex-matched individuals were obtained from 20 normal healthy subjects. All sera were diluted to 1:100 in TBS-T and subjected to direct binding ELISA on solid phase, separately coated with equal amounts of native human DNA and MG–Lys–Cu$^{2+}$–glycated human DNA. Out of 40 sera from type I diabetes, 27 (67.5%) showed higher binding with the MG–Lys–Cu$^{2+}$–DNA, whereas in type II diabetes, only 10 (25%) out of 40 showed enhanced binding with the MG–Lys–Cu$^{2+}$–DNA when compared with the native form (Figure 8). The serum samples that showed enhanced binding (more than double the background) were considered for positive sera having antibodies against glycated DNA. In case of type I diabetes mellitus patients we observed high statistical significance ($P < 0.001$) in the binding of glycated human DNA when compared with the native analog; however, in type II diabetes patients, the binding was moderately significant in statistical terms ($P \leq 0.05$) when compared with native human DNA. Moreover, the 10 sera that showed enhanced binding in type II diabetic sera were having secondary complication of nephropathy. These results points toward that most type I diabetic subjects (67.5%) and some type II (25%) (only secondary complication samples) showed the presence of autoantibodies against MG–Lys–Cu$^{2+}$–DNA.

**Glycoxidative lesion detection**

As reported earlier, female rabbits immunized with MG–Lys–Cu$^{2+}$ modified human DNA elicited high titer immunogen-specific antibodies (Ahmad et al. 2011). The experimentally induced anti-MG–Lys–Cu$^{2+}$–DNA IgG was used as an immunochip probe to detect the glycoxidative lesions in the lymphocyte DNA isolated from the blood of type I and II diabetes patients. After the initial screening of the type I and II diabetic sera for the presence of autoantibodies, the detection of glycoxidative lesion was executed only for the sera displaying very high absorbance in direct binding ELISA. Therefore, 12 type I and 8 type II sera were screened out from 40 sera samples of each group. The binding pattern of the DNA isolates was quite revealing; DNA of type I diabetes patients produced appreciable inhibition in the binding of experimentally induced anti-MG–Lys–Cu$^{2+}$–DNA antibodies. Inhibition of anti-MG–Lys–Cu$^{2+}$–DNA IgG by lymphocyte DNA from type I diabetes patients was recorded in the range of 58.2–72.4%. However, type II diabetes subjects exhibited moderate inhibition in the range of 41.3–54.4%. The inhibition of anti-MG–Lys–Cu$^{2+}$–DNA-IgG by lymphocyte DNA from healthy individuals was quite low (Table I). Moreover, the average inhibition for type I diabetes was 67.03%, while for type II subjects it was recorded to be meager 46.91%.

Significantly high recognition of the lymphocyte DNA from type I diabetes patients by the experimentally induced antibodies against the modified DNA is a clear indicator of epitope sharing between the human DNA modified in vitro by MG–Lys–Cu$^{2+}$ and the lymphocyte DNA of type I diabetes patients. This leads to the conclusion that MG–Lys–Cu$^{2+}$ generates neoepitopes on the lymphocyte DNA molecule that are recognized as “alien” or nonself by the immune system resulting in the autoantibody generation in diabetes type I patients. Significantly high recognition of the lymphocyte DNA from type I diabetes patient by the experimentally induced antibodies against MG–Lys–Cu$^{2+}$ modified human DNA forms an evidence toward the involvement of modified bases and single strand regions in disease pathogenesis.

**Correlation between HbA1c and anti-glycated-DNA autoantibodies**

Table II shows the glycemic parameters in diabetic subjects based on relative absorption to native DNA and DNA-AGEs.

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**Table I. Detection of glycoxidative lesions in the DNA samples isolated from type I and II diabetes mellitus patients and normal healthy individuals**

<table>
<thead>
<tr>
<th>Sera group</th>
<th>Number of samples tested</th>
<th>Maximum percent inhibition at 20 μg/mL</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I diabetes</td>
<td>12</td>
<td>70.2, 60.5, 58.2, 61.7, 69.6, 65.4, 68.7, 68.9, 71.4, 67.3, 70.1, 72.4</td>
<td>67.03 ± 5.35%</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>8</td>
<td>41.3, 44.2, 46, 54.4, 43.6, 46.5, 48.3, 51</td>
<td>46.91 ± 4.67%</td>
</tr>
<tr>
<td>Normal human</td>
<td>5</td>
<td>30.4, 25.6, 20.85, 29.6, 24.3</td>
<td>26.15 ± 3.93%</td>
</tr>
</tbody>
</table>

Binding of anti-MG–Lys–Cu$^{2+}$ modified DNA IgG to DNA isolated from lymphocytes of type I and II diabetes patients and normal healthy individuals was ascertained by competitive inhibition ELISA. The microtiter plates were coated with MG–Lys–Cu$^{2+}$ modified human DNA (2.5 μg/mL); DNA isolated from lymphocytes of patients with type I and II diabetes and healthy individuals were used as inhibitors. DNA isolates from diabetic subjects showed significantly higher inhibition than normal human DNA ($P < 0.001$).
There was significant difference in fasting and postprandial blood sugar and HbA1c (P < 0.015, P < 0.005, P < 0.001, respectively) but no significant difference in disease duration. Moreover, linear correlation analysis was performed using absorbance difference as dependent variable and HbA1c %, nephropathy and lipids as independent variables (Table III). It was found that the absorbance difference was significantly related to HbA1c (r = 0.486, P < 0.001), nephropathy (r = 0.239, P < 0.003) and serum triglycerides and very low density lipoprotein-cholesterol (VLDL-cholesterol) (r = 0.186, P < 0.001 and r = 0.190, P < 0.001, respectively).

Conclusion
The products of nonenzymatic glycation and oxidation of DNA, the DNA-AGEs, accumulate in a wide variety of environments. MG, an offshoot product of carbohydrate and protein metabolism is involved in the formation of AGEs by reacting with free amino groups of Lys and arginine, which are present at the active sites in most of the enzymes. The AGEs were more pronounced in the presence of Cu2+. Thus, the MG–Lys–Cu2+ system can be very deleterious for macromolecules like DNA, proteins etc. Our results suggest that the cumulative action of these species produce AGEs and potent oxidants as well, which cause damage to the DNA molecule. Moreover, similar pattern of perturbations was found in DNA isolates from type I diabetes patients, indicating the generation of neo-antigenic epitopes on DNA molecule that could be a possible reason for autoimmune response in type I diabetes.

In the present study, higher binding of serum antibodies with MG–Lys–Cu2+ glycated-DNA as against native DNA in diabetic patients with HbA1c of 10.2 ± 2.8% compared with those with HbA1c of 7.6 ± 1.2% (P < 0.001) indicates that the effects of DNA glycation are marked in diabetes. The results are in line with an earlier report (Bucala et al. 1984), who have shown that like protein, nucleic acids too can suffer nonenzymatic modification by reducing sugars and persuade structural and organizational changes analogous to those reported for nonenzymatic browning of proteins. The DNA glycation marker, N2-carboxyethyl-2′-deoxyguanosine, has been reported in kidney and aorta of diabetic and uremic patients (Li et al. 2006). In view of the immunogenic nature of AGEs, it may not be out of context to speculate that persistence of comparable structures in vivo can initiate and/or propagate autoimmune response (Turk et al. 2001). In our study, the presence of anti-DNA-AGEs autoantibodies in 67.5% of type I diabetic sera may be a consequence of auto-immune response against persistent DNA-AGEs. Anti-DNA-AGEs autoantibodies were also correlated with diabetic nephropathy. Findings from the present study indicate that DNA-AGEs and autoantibodies against glycated DNA are correlated with HbA1c and microvascular complication like diabetic nephropathy and may help as supplementary biomarkers for valuation of chronic glycaemia. Nevertheless, a comprehensive, potential and prospective study is required to establish efficacy of DNA-AGEs and its autoantibodies in the assessment of glycemic control and its relationship with microvascular hitches in diabetic subjects.

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Conflict of interest
None declared.

Abbreviations
AGEs, Advanced glycation end-product; CEdG, N2-carboxyethyl-2′-deoxyguanosine; Cu2+, copper; DHA, dihydroxy acetone; DHA, dihydroxyacetone; EMG, exponentially modiﬁed Gaussian; Lys, lysine; MG, methylglyoxal; PBS, phosphate buffer saline; PLP, pyridoxal phosphate; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Implication of DNA glycation in diabetes mellitus


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