ASIAN JOURNAL OF BIOMEDICAL & PHARMACEUTICAL SCIENCES

e-ISSN: 2249-622X

RESEARCH ARTICLE

Biochemical Studies of In Vitro Glycation of Human DNA

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Received:
13th Oct 2012
Received in revised form:
29th Oct 2012
Accepted:
8st Nov 2012
Available online:
15th Nov 2012

OPEN ACCESS

Online ISSN 2249–622X
http://www.jbiopharm.com

ABSTRACT

Glucose can covalently bind to DNA by non-enzymatic process often termed as glycation. Glycation of DNA is of special interest due to its possible influence on the functionality of DNA and overall effect on gene expression. Glycation of DNA leads to formation of advanced glycation end products (AGEs). These AGEs may accumulate with time, overwhelming the enzymatic defense against glycation leading to loss of viability and gradually to cellular aging. In our study, different concentrations of glucose were incubated with human DNA for varying time periods. The reaction product was characterized by UV spectroscopy, nitroblue tetrazolium (NBT) reduction assay, melting temperature and electrophoretic study. Oxidation induced by glycation of DNA was also analyzed by scavenging studies. It was found that ultraviolet (UV) spectroscopic analysis of Glycated DNA shows hypochromicity, indicates the formation of advance glycated end products. Amadori product content was found high in glycated samples. Thermal denaturation of glycated DNA shows increase in Tm value by 4 ^{o}C and change in banding pattern of native and glycated adduct on agarose gel electrophoresis. The quenching effect of glutathione (GSH) provides clue for generation of free radicals during glycation. The DNA modified with D-glucose may be one of the etiological pathogenic factors for diabetes mellitus.

KEYWORDS: AGE, glycation, diabetes mellitus.

1. INTRODUCTION

Advanced glycated end products (AGEs) are molecules formed from the non-enzymatic reaction of reducing sugar with free amino group of proteins, lipids and nucleic acids [1]. DNA is subjected to continuous damage by oxidation and various other processes. DNA modification by glycation has been generally overlooked in these processes. This is, perhaps, an oversight since oxidative stress that gives rise to oxidative damage of DNA [2] is associated with accumulation of cellular glycating agents that also increase the glycation of DNA. Glycation of DNA gives rise to characteristics nucleotide adducts some of which have been found to increase in oxidative stress [3]. This is not surprising since glucose and other saccharide derivative fragments to form glyoxal and methyl glyoxal in the early stages of glycation reaction [4]. The presences of nucleotide AGEs are associated with increased mutation frequency, DNA strand breaks and cytotoxicity. The effect of AGEs formation on DNA reported to date is the induction of unusual rearrangements [5]. In mammalian cell evidences has been found that AGEs formation on DNA may be responsible for insertion containing repetitive sequences of Alu family that has been found to disrupt human gene [6]. The cellular protection against oxidative damage to DNA is achieved by enzymatic and non-enzymatic antioxidants [7] that neutralize or detoxify reactive oxygen species. Nucleotide glycation and related effects are expected to be the most marked in disease associated with accumulation of glycating agents to high concentration in diabetes and uremia [4]. The decline in DNA repair mechanism and persistence of lesion in DNA with increased age [8] may also exacerbate the effect of nucleotide glycation.

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2. MATERIALS AND METHODS

Agarose gel electrophoretic unit and Rocker shaker 100 were from GENIE, India. The 5415R centrifuge was from Eppendorf, Germany and UV-1700 spectrophotometer was from Shimadzu Co., Japan. Gel documenting system was from Uvitech, India. D-glucose was purchased from Heinz, India and agarose from SRL, India. All other chemical used in protocol were of analytic grade.

2.1. Isolation of Human DNA

Blood was collected from healthy voluntary donors from the OPD of SBS PGI College, Dehradun following approval by the ethical committee. Genomic DNA was isolated by the Proteinase K- Buffer method [9] from human blood.

2.2. Glycation of DNA

For the preparation of glycated products, DNA (100µg/ml) was incubated at 37°C with D-glucoseat different concentrations (50 mM, 100 mM, 150 mM and 200 mM) under sterile conditions for 0-30 days.

2.3. Determination of Amadori Products in DNA

Amadori products (fructosamine) in DNA were determined by the Nitroblue tetarazolium reduction assay [10] after some modification. The DNA sample (100µg/ml) was mixed with 100 mM sodium carbonate buffer (pH 10.8) containing 0.25 mM NBT and incubated for 5 hr at 37°C. Absorbance was read at 525 nm and the contents of Amadori products were determined using an extinction coefficient of 12640 $cm^{-1}m^{-1}$ for monoformazan (considering that 1 mole monoformazan is formed upon the reduction of NBT by 1 mole of fructosamine).

2.4. UV Spectroscopic Studies

UV spectra were recorded on a UV-240 spectrophotometer (Shimadzu, Japan). The UV spectra were taken between 200-400nm. The decrease in absorbance was calculated by using the following equation:

Hypochromicity = (Absorbance of native sample - Absorbance of glycated sample)/ Absorbance of native sample x100

Thermal denaturation of native and glycated DNA under identical conditions was evaluated by a temperature scan from 30°C to 95°C at an increment of 1°C/min on a Shimadzu UV-240 spectrophotometer equipped with a temperature programmer and controller assembly. The change in absorbance at 260 nm was recorded and melting temperature (Tm) of the samples calculated [11].

2.5. Agarose Gel Electrophoresis

The change in electrophoretic pattern of native DNA and glycated DNA was observed on 1% agarose gel at 40 mA in TAE buffer (40 mM Tris acetate, 2mM EDTA, pH 8.0). The gels were stained with ethidium bromide (1mg/ml) and visualized under UV light.

2.6. Treatment of Glycated DNA with Scavengers

Free Radical scavengers such as catalase (20µg/ml), GSH (10 mM) and mannitol (10 mM) were added to glycated DNA of 100 mM glucose concentration incubated for 0- 30 days. Samples were modified as described above and absorbance was measured at 260 nm.

3. RESULTS

3.1. AGEs modification of DNA

DNA was isolated from human blood and its purity was found to be pure (A_{260}/A_{280}=1.7). The characterization of native and glycated DNA has been shown in Table 1

Parameters	Native DNA	Glycated DNA
$A_{260/280}$	1.7	1.32
Melting Temperature (Tm)	81°C	85°C
% Hypochromicity	-	51%
Amadori products (nmol/mg DNA)	3	8.6

TABLE 1: Characterization of Native and Glycated DNA (100 mM)

The isolated DNA was then modified with different glucose concentrations to form glycated DNA. The changes induced in DNA by modification were analyzed by UV spectrophotometer. UV spectra analysis of 100 mM glucose concentration showed change in DNA spectra as a result of glycation. The marked hypochromicity of 51% and peak shift of 5 nm were exhibited in modified 100 mM glycated DNA as evident from UV spectral analysis (Fig.1).

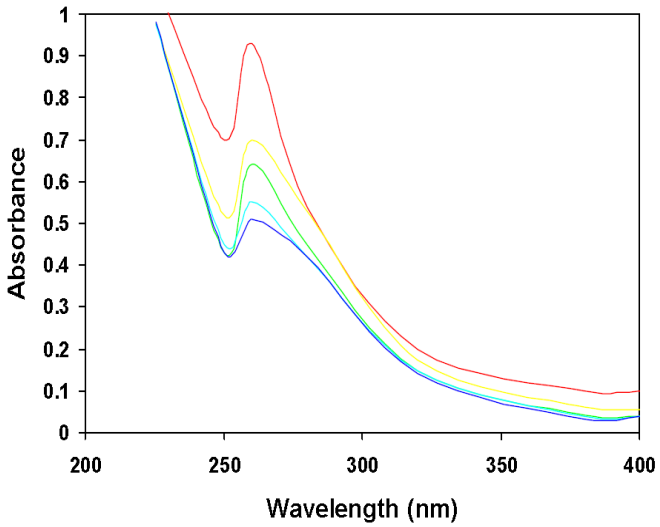

Figure 1: UV absorption of Native DNA (-), DNA incubated with 50 mM (-), 100 mM (-), 150 mM (-) and 200 mM (-) glucose.

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3.2. Assay of Glycated DNA

For the measurement of early glycated products in DNA, we performed the NBT reduction assay. It is a simple method and with some modifications proved to be relevant for analysis of glycated DNA. Glycation proceedes in two main stages, the first one including the formation of aldimines (schiff's base), which are then transformed in the second stage into more stable Amadori products. The adducts irreversibly bound to DNA are designated as advanced glycated end products. Amadori product content of native DNA is 3 nmol /mg DNA while as that of glycated DNA on increasing the incubation time content increases , but after two weeks content remains almost same which is 8.6 nmole/mg DNA.

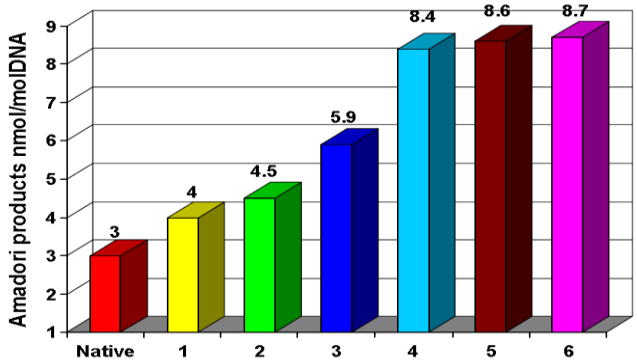

Figure 2: Formation of Amadori products during incubation of glycated DNA with NBT for two days (1), four days (2), one week (3), two weeks (4), three weeks (5) and four weeks (6)

3.4. Thermal Melting profile

The denaturation of DNA takes place due to disruption of hydrogen bonds between the complementary nitrogenous base in the two strands. Melting profile of native and glycated DNA was analyzed between 30°C and 95°C. Tm of native DNA was found to be 81°C while that of glycated DNA was found to be 85°C.Increase of 4 °C in the Tm coupled with the late onset of melting in case of glycated DNA signifies the greater thermal stability, as compared to their native analogue (Fig. 3). The late onset of melting in the case of glycated DNA is indicative of an unstable helix as a consequence of modification

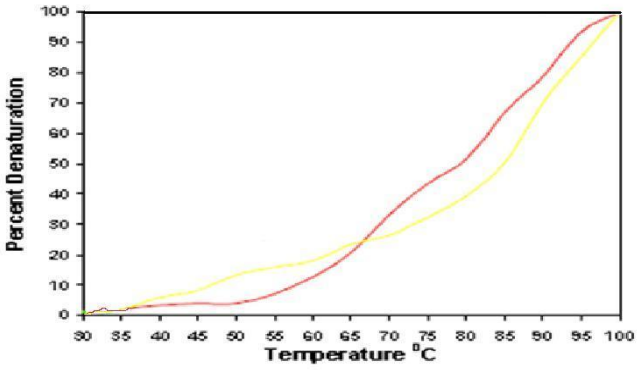

Figure 3: Thermal denaturation profile of native DNA (-) and glycated DNA (-).

3.5. Agarose gel electrophoresis

Electrophoretic pattern of native and glycated DNA was studied on a 1% agarose gel (Fig. 5). The band of native DNA was found to be more distinct and intense as compared to that of glycated DNA. A diminished or less intense band of glycated DNA indicates cross-linking of glucose moieties in the double stranded helix which results in the formation of heavily glycated products.

3.6. Treatment with free radical scavengers

Treatment of glycated DNA with scavengers like GSH, catalase and mannitol causes quenching of free radicals generated during glycation. Since oxygen radicals which may be superoxide or hydroxyl radicals are generated during glycation of DNA. GSH and catalase provides cellular protection against oxidative damage to DNA. GSH and catalase are known scavenger of superoxide radical. Mannitol a specific quencher for hydroxyl radical had no effect on radical generation. GSH shows maximum scavenging effect in our study (Fig. 4).

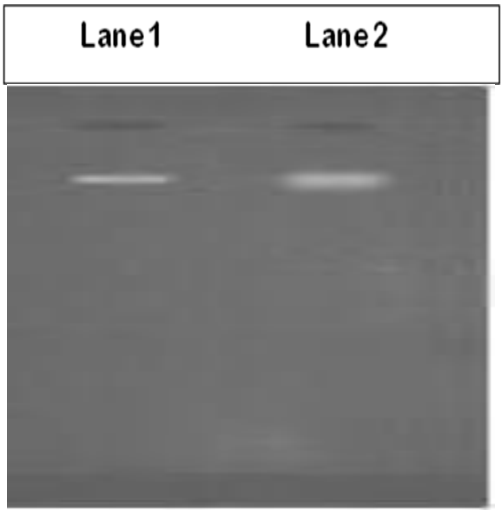

Figure 4: Agarose gel electrophoresis of native DNA (lane 1) and glycated DNA (lane 2).

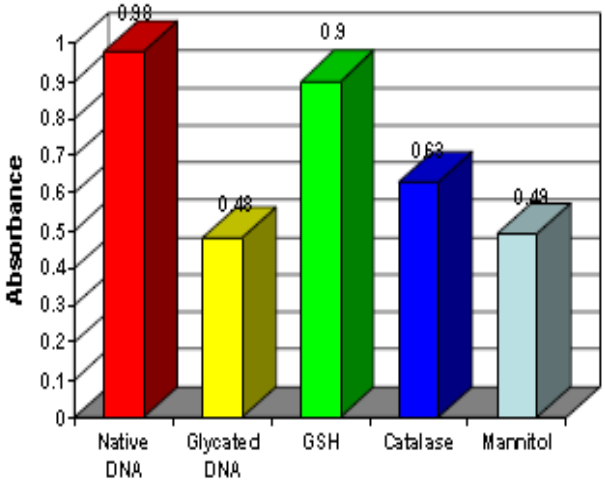

Figure 5: Effect of various free radical scavengers on glycated DNA.

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4. DISCUSSION

Glycation is the non-enzymatic addition or insertion of sugar molecules into DNA, protein and lipids that occur in biological molecules. The glycation of DNA give rise to characteristic nucleotide adducts, some of which have been found to increase in oxidative stress [3]. The glycation causes damage to DNA which is associated with mutagenesis, carcinogenesis and is also considered to be a pathogenic factor for diabetes mellitus [12]. The isolated DNA was incubated with different concentrations of glucose for various time intervals showing marked hypochromicity of 51% and shift in λmax as evident from UV spectral analysis. Previously, it was suggested that glycation of DNA resulting in the form of nucleotide AGEs is associated with increase in mutation frequency and cytotoxicity [13]. DNA glycation may contribute to the toxicity of several clinical cytotoxic anti tumor agents and over expression of enzymatic anti-glycation defense is associated with multi drug resistance in major classes of tumors [13]. Improved understanding of DNA glycation may give guidance on decreasing the risk of tumor associated with dietary factors [13].

For the measurement of early glycated products in DNA, we have performed NBT reduction assay, originally developed for quantification of fructosamine in serum glycosyl proteins [10]. This assay is applicable to nucleic acid as per se are devoid of reducing functionality. However, in addition to glycation, reducing moieties are likely to appear in the cellular DNA by other chemical or enzymatic reactions. Aldehydicapurinic/apirimidinidic (AP) sites can be directly induced in DNA by reactive oxygen species [14] or caused by glycosylase enzymes upon removal of oxidized bases [15]. Previous groups have reported that Amadori product formation in glycated samples is higher than in native [10]. We have also measured Amadori products of native and glycated samples. Our data substantiates the earlier findings. The hydrogen peroxide so produced during glycation may be converted to highly reactive hydroxyl radical via Fenton reaction. Oxygen radicals are generated in the process of auto oxidation of sugar [16, 17]. The cellular protection against the oxidative damage in DNA is achieved by enzymatic and non enzymatic antioxidants [18]. The presence of superoxide was detected by reduction of NBT [19]. The increase in absorbance of glycated DNA at 560 nm suggests the presence of superoxide radical. The superoxide formation is suggested to be involved in the Millard reaction of DNA with sugar [20]. Treatment of glycated DNA with scavengers like GSH, catalase and mannitol causes quenching of radicals generated during glycation. GSH showed maximum scavenging effect in our study which is in accordance to the previous findings that the depletion of cellular antioxidant GSH led to increased binding of glucose derivatives to DNA [21]. Glycation damage to DNA is associated with mutagenesis and carcinogenesis, although cellular protection against glycation is functioning, the mutagenic potential is low. DNA glycation is covalent modification of DNA and will damage the function of DNA (replication and transcription) as well as many alkylating agents. In addition it would be expected that glycated contributes to site specific radical DNA damage [22]. Improved understanding of DNA glycation may give guidance on decreasing the risk of tumors associated with dietary factors.

5. ACKNOWLEDGEMENTS

Authors are grateful to the Department of Biochemistry and management of SBS Post Graduate Institute of Biomedical Sciences, Balawala, Dehradun for the continued support during this study. RA is also thankful to the administration of Oman Medical College, Sohar for their support and encouragement during preparation of this manuscript.We also declare that there is no conflict of interest among authors.

6. REFERENCES

1. Fu M X, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product Nepsilon-(corboxymethyl) lysine, is a product of both lipid peroxidation and glycoxidation reactions. J Biol Chem. 1996; 271: 9982-9986.
2. Ames BN. Dietary carcinogens and anticarcinogens. Science. 2003; 221: 1256-1263.
3. Mistry N, Podmore I, Cooke M, Butler P, Griffiths H, Herbert K, Lunec J. Novel monoclonal antibody recognition of oxidative DNA damage adduct, deoxycytidine-glyoxal. Lab Invest. 2003; 83(2): 214-250.
4. Thornalley PJ, Langborg A and Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J. 1999; 344: 109-116.
5. Lee AT, Cerami A. In vitro and in vivo reactions of nucleic acids with reducing sugar. Mutat Res. 1990; 238: 185-191.
6. Bucala R, Lee AT, Rourke L, Cerami A. Transposition of an Alu-containing element induced by DNA-advanced glycosylation endproducts. Proc Natl Acad Sci USA. 1993; 90(7): 2666-2670.
7. Koschinsky T, He CJ, Mitsuhashi T, Bucala R, Liu C, Buenting C, Heitmann K, Vlassara H. Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in

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diabetic nephropathy. Proc Natl Acad Sci USA. 1997; 94: 6474-6479.

8. Mullaart E, Lohman PHM, Berends F, Vijg J. DNA damage metabolism and aging. Mutat Res. 1990; 237: 189-210.
9. Ahmad R, Rasheed Z, Kaushal E, Singh D, Ahsan H. Biochemical evaluation of human DNA-lysine photoadduct treated with peroxynitrite. Toxicol Mech Methods. 2008; 18(7): 589-95.
10. Johnson RN, Metcalf PA, and Baker JR. Fructosamine: a new approach to the estimation of serum glycosyl protein. An index of diabetic control. Clin Chim Acta, 1982; 127: 87–95.
11. Ashok BT, Ali R (1998) Binding of human anti-DNA autoantibodies to reactive oxygen modified DNA and probing oxidative DNA damage in cancer using monoclonal antibody. *Int J Cancer*, 78, 404-09.
12. Thornalley PJ. Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems - role in ageing and disease. Clin Lab. 1990; 45: 263-273.
13. Murata-Kamiya N, Kamiya H and Kasai H. Glyoxal, a major product of DNA oxidation, induces mutations at G: C sites on a shuttle vector plasmid replicated in mammalian cells. Nucleic Acid Res. 1997; 25: 1897-1902.
14. Breen AP and Murphy JA. Reaction of oxyl radicals with DNA. Free Rad Biol Med. 1995; 18: 1033-1077.
15. Rosenquist TA, Zharkov DO and Grollman AP. Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc Natl Acad Sci USA. 1997; 94: 7429-7434.
16. Kashimura N, Morita J, Komana T. Autoxidation and phagocidal action of some reducing sugar phosphates. Carbohydrate Res. 1979; 70(1): C3-C7.
17. Thornally PJ. Monosaccharide autoxidation in *health* and disease. Environ Health Perspect. 1985; 64: 297-309.
18. Sies H. Antioxidant in Disease Mechanism and Therapeutic Strategies, 1996. Academic Press, San Diego, CA.
19. Nakayama T, Kimura T, Kodama M, Nagata C. Generation of hydrogen peroxide and superoxide anions from active metabolites of naphthalamines and aminoazo dyes: its possible role in carcinogenesis. Carcinogenesis. 1983; 4: 765-769.
20. Morita J and Kashimura N. The Maillard reaction of DNA with D-fructose-6-phosphate. Agric Biol Chem. 1991; 55: 1359-1366.
21. Shires TK, Tresnak J, Kaminsky M, Herzog SL and Truc-Pharma B. DNA modification in vivo by derivatives of glucose: enhancement by glutathione depletion. FASEB J. 1990; 4: 3340-3345.
22. Samuni A, Aronovitch J, Godinger D, Chevion M, Czapski G. On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism. Eur J Biochem. 1983; 137: 119-124.

Conflict of Interest: None Declared

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