



Characterization of Human Glycated DNA modified with Peroxynitrite

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Abstract:

Glycation is the non-enzymatic addition or insertion of sugar molecules into DNA, proteins and lipids that occur in biological molecules. The glycation of DNA gives rise to characteristic nucleotide adducts, some of which have been found to increase in oxidative stress. The glycation causes damage to DNA which is associated with mutagenesis, carcinogenesis and is also considered to be a pathogenic factor for diabetes mellitus. The present study was designed to evaluate the influence of glucose on DNA isolated from human blood and its modification with peroxynitrite. Modified, glycated DNA was characterized by UV spectroscopy, melting temperature and electrophoretic profile. Oxidation incurred by glycation of DNA and peroxynitrite was also analyzed by scavenging studies. In the present study peroxynitrite of glycated DNA leads to damage which may be due to nitration, deamination along with the formation of strand break and mutation. The modified DNA showed 18% hyperchromicity effect with the peak shift of approximately 5 nm to higher wavelength which may be due to alteration in normal helical structure and exposure of bases causes marked absorption in the UV range. Agarose gel electrophoresis clearly demonstrates that the instability in β -helix is as a result of peroxynitrite modification of glycated DNA. Additional evidence for the structural perturbation in DNA were further analyzed by thermal denaturation study. Peroxynitrite modification showed significant decrease in T_m which could be attributed to single strand generation.

Key Words: glycated DNA, peroxynitrite, human DNA

Introduction:

Glycation is the result of a sugar molecule, such as fructose or glucose binding to a protein, nucleic acids or lipid molecule without the controlling action of an enzyme sometimes called non-enzymatic glycosylation [1, 2]. Enzyme-controlled addition of sugars to protein or lipid molecules is termed as glycosylation [2]. Advanced glycated end products are

molecules formed from the non-enzymatic reaction of reducing sugars with free amino group of proteins lipids and nucleic acids [3].

The non-enzymatic reaction between reducing sugar and free amino group on DNA bases results in the formation of glycation and advanced glycation end products (AGEs). The cross linking of genetic material to glucose is one of the fundamental lesions produced in biological systems. The glycated form of DNA is a potent immunogen and may induce harmful immune response. The presence of AGE's on DNA causes unusual transpositional rearrangements. The AGE's contributes in the development of arthrosclerosis, kidney, vascular and neurological diseases in both diabetes and the aging process. The glycated substances may also be involved in the pathogenesis of Alzheimer's disease. Depletion of cellular antioxidant GSH leads to an increase in the binding of glucose derivatives to DNA. DNA can be cross linked with different substances, some of the nucleotide AGE's are N2-carboxymethyl oxyguanosine, 5-glycolyl deoxycytidine etc [4].

Previously, it was suggested that glycation of DNA resulting in the form of nucleotide AGEs is associated with increase in mutation frequency and cytotoxicity [4]. DNA glycation may contribute to the toxicity of several clinical cytotoxic anti-tumor agents. Over expression of enzymatic anti-glycation defence is associated with multi drug resistance in major classes of tumors. Improved understanding of DNA glycation may give guidance on decreasing the risk of tumor associated with dietary factors [4].

Peroxynitrite is reactive nitrogen species, formed by rapid reaction of highly reactive nitric oxide and superoxide anion (O_2^-). Peroxynitrite is a mutagenic agent, with the potential to produce nitration, nitrosation and deamination reaction on DNA bases. Due to the oxidizing property of peroxynitrite, it can damage a wide array of biomolecules in cells including DNA, lipids, proteins which lead to the oxidative stress. Peroxynitrite is a potent initiator of DNA strand breakage which is an obligatory stimulus for activation of nuclear enzyme, poly-ADP ribosyl synthase or polymerase (PARP)

excess activation of PARP can kill cell by depleting NAD⁺ and energy production which may be one of the mechanism by which cells with excess DNA damage are eliminated (which undergoes necrosis or apoptosis) [5, 6].

To counter the harmful effects of reactive species antioxidant defense mechanism operates to detoxify or scavenge these reactive species. Antioxidants are able to neutralize free radical and render them harmless. It has been suggested that the extent of damage caused by the free radical might be modified by three dietary intervention strategies: a) caloric restriction and thus a depression in free radicals arising due to normal metabolism; b) minimizing the intake of components that increase the free radicals such as poly-unsaturated fatty acids; c) supplementation with one or more antioxidants in diet.

A wide array of enzymatic antioxidants defence exists, including superoxide dismutase, glutathione reductase, catalase etc. The present study is designed to evaluate the influence of glucose on DNA isolated from human blood and its modification with peroxynitrite. Modified, glycosylated DNA was characterized by UV-spectroscopy, NBT reduction test, hydroxy radical, superoxide radical assay, melting temperature and electrophoretic profile. Oxidation incurred by glycosylation of DNA and peroxynitrite was also analyzed by scavenging studies.

Materials and Methods:

All chemicals used were of analytical grade. The instruments and apparatus were Eppendorf centrifuge 5415R (Germany), UV-1700 spectrophotometer (Shimadzu, Japan), agarose electrophoresis unit (Genei, India), gel documenting system (Uvitech, India).

1. Isolation of Genomic DNA from Human Blood:

i) **By Proteinase K- buffer method:** Briefly, 5.0 ml blood was collected in polypropylene tube containing 0.5 ml 2.7% EDTA. An aliquot of 1 ml EDTA blood was mixed with 200 µl lysis buffer (pH 7.5). After discarding the supernatant, proteinase K-buffer (pH 8), 10% SDS and 5 M NaCl were added. This was followed by the addition of phenol:chloroform (5:1). 70% alcohol was added after centrifugation for 5 min at 13000 rpm. Double distilled water was added to dissolve the DNA pellets and was stored at -20°C.

ii) **By Salting out method:** Briefly, 5.0 ml blood was collected in polypropylene tube containing 20 µl of 0.1 M EDTA. An aliquot of 0.5 ml EDTA blood was mixed with 800µl lysis buffer (pH 7.5). The supernatant was discarded and 500 µl of Tris-HCl (pH 8) was added to the pellet and vortexed. After discarding the pellet, the supernatant containing the isolated DNA was precipitated with 70% ethanol. The isolated DNA appears as a thread like structure which was spooled and dissolved in de-ionized water stored at -20°C.

2. Glycation of DNA:

For the preparation of glycosylated products, DNA was incubated with D-glucose (50 mM, 100 mM, 150 mM & 200 mM) under sterile conditions for (2,4,6) days and (1,2,3,4) weeks. DNA concentration was measured spectrophotometrically at 260 nm.

3. Modification of Glycosylated DNA by Peroxynitrite:

Glycosylated DNA was modified by peroxynitrite through the synergistic action of NO donor sodium nitroprusside (5 mM) and superoxide donor, pyrogallol (5 mM), in the presence of a chelator EDTA (5 mM). The reaction of glycosylated DNA with peroxynitrite was carried out for 3 hr at 37°C by the SNP/pyrogallol /EDTA (mixture ratio of 1:1:1). The absorbance of native and peroxynitrite DNA were read at 260 and 280 nm. A 260/280 ratio and change in λ max were recorded. Difference spectra of peroxynitrite modified glycosylated DNA was also plotted between 200-400 nm using glycosylated DNA as blank.

4. Thermal Denaturation:

Native, glycosylated DNA and peroxynitrite modified glycosylated DNA samples were subjected to thermal denaturation. All the samples were melted from 30°C-100°C and the absorbance at various temperatures was recorded at 260 nm. The % denaturation was calculated using the following equation.

$$\% \text{ denaturation} = (AT - A_{30}) / A_{\text{max}} - A_{30} * 100$$
where, AT = absorbance at the temperature T°C
A_{max} = maximum absorbance on the completion of denaturation.
A₃₀ = initial absorbance at 30°C

5. Agarose Gel Electrophoresis of Native, Glycated and Peroxynitrite modified DNA:

Electrophoresis was performed in horizontal agarose gel to determine the degree of modification in DNA. Agarose gel (1%) was dissolved in TAE buffer and the ethidium bromide solution (25 μ l) was added to it. The samples were loaded in different wells and electrophoresed at 30 mA for 3 hours. Gel was then visualized under UV light in Gel documentation system.

6. Treatment of Peroxynitrite Glycated DNA with Various Scavengers:

Catalase (20 μ g/ml), GSH (10 mM) and mannitol (10 mM) were tested for their antioxidant effect. The respective scavengers were added to glycated DNA and incubated for 10 min. Samples were modified as described above and absorbance was measured at to 260nm.

Results:

Modification of Glycated DNA by Peroxynitrite:

Glycated DNA (100 mM) was incubated with SNP/Pyrogallol and EDTA in equimolar ratio. UV spectra of glycated and modified samples was plotted between 200–400 nm. Glycated samples showed marked hyperchromicity (18%) after modification with peroxynitrite. The UV absorbance ratio of A260/280 of modified DNA was decreased to 1.2 from the usual 1.7 for native DNA (Fig. 1). Difference spectra of glycated DNA and peroxynitrite modified glycated DNA were analyzed between 200-400 nm. A marked hyperchromicity was observed over a wide range (Fig. 2).

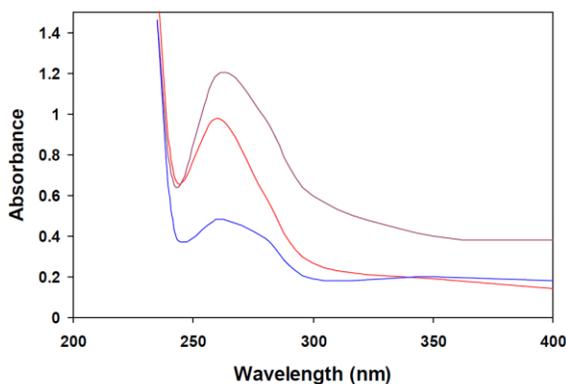


Fig. 1: UV absorption profile of native DNA (→), glycated DNA (→) and peroxynitrite-modified glycated DNA (→).

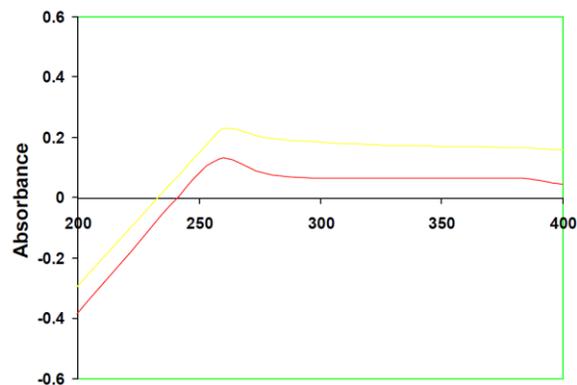


Fig. 2: Difference spectra of peroxynitrite-modified DNA (→) against glycated DNA (→).

Thermal Denaturation

The melting profile of native, glycated and peroxynitrite modified glycated DNA were analyzed between the temperature range of 30^oC-100^oC. Increase in absorbance at 260 nm was taken as a measure of helix denaturation. The process was characterized by determining the percent DNA in denaturated state as a function in increase in temperature and then computing melting temperature. The T_m of native DNA was found to be 81 ^oC and an increase of 5^oC was observed for glycated DNA. The T_m for peroxynitrite modified DNA was found to be 74^oC (Fig. 3). The properties of native and modified DNA have been summarized in Table 1.

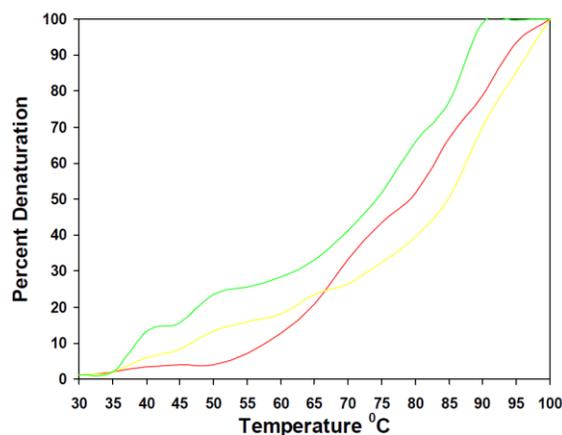


Fig. 3: Thermal denaturation profile of native DNA (→), glycated DNA (→) and peroxynitrite-modified glycated DNA (→)

Table 1: The physicochemical characteristics of native, glycated and peroxynitrite-modified DNA

Parameters	Native DNA	Glycated DNA	Modified DNA
A _{260/280}	1.7	1.3	1.2
Melting temperature (°C)	81	85	74
% Hyperchromicity	-	-	18
% Hypochromicity	-	51	-
Amadori products (nmol/mol DNA)	3	8.4	-

Agrose Gel Electrophoresis

Native, glycated DNA and peroxynitrite modified sample were run on 1% agarose. The sharp band was seen for native as compared to glycated DNA where as in peroxynitrite modified well no band was seen. This signifies the presence of single strand breaks due to the peroxynitrite modification

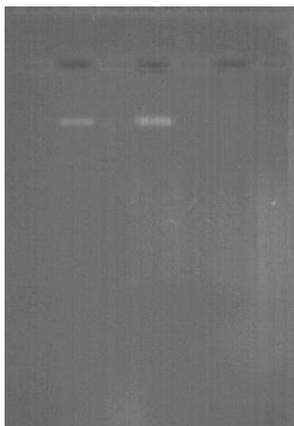


Fig. 4: Agarose gel electrophoresis of native DNA (Lane 1), Glycated DNA (Lane 2) and peroxynitrite-modified DNA (Lane 3)

Effect of Scavengers on Peroxynitrite Modified DNA

Scavenging effect of GSH, catalase and mannitol were analyzed by incubating the scavengers with peroxynitrite modified glycated DNA. GSH and catalase were found to show the best scavenging effect (Fig. 5).

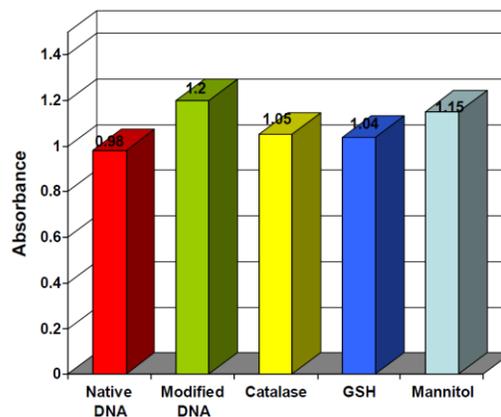


Fig. 5: Effect of various scavengers on peroxynitrite-modified glycated DNA

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 gives rise to characteristic nucleotide adducts, some of which have been found to increase in oxidative stress [7]. The glycation causes damage to DNA which is associated with mutagenesis, carcinogenesis and is also considered to be a pathogenic factor for diabetes mellitus [8].

In the present study peroxynitrite of glycated DNA leads to damage which may be due to nitration, deamination along with the formation of strand break and mutation [9]. The modified DNA showed 18% hyperchromicity effect with the peak shift of approximately 5nm to higher wavelength which may be due to alteration in normal helical structure and exposure of bases causes marked absorption in the UV range [10]. Agarose gel electrophoresis clearly demonstrates that the instability in β -helix is as a result of peroxynitrite modification of glycated DNA.

Additional evidence for the structural perturbation in DNA were further analyzed by thermal denaturation study. Increase in T_m and late onset of folding in case of glycated human serum albumin (HSA) signifies their greater thermal stability as compared to their native analogue [11]. Similar result was obtained with glycated DNA in our studies. Peroxynitrite modification showed significant decrease in T_m which could be attributed to the single strand generation [11]. Quenching studies were also performed to analyze the effect of various quenchers on peroxynitrite modified glycated DNA. GSH and catalase showed maximum scavenging

effect. The DNA damage profile caused by peroxynitrite is significantly different from that obtained with hydroxyl radical or singlet oxygen. In case of DNA modification, peroxynitrite can damage DNA by introducing oxidative modification in both nucleobases and sugars ^[12].

Conclusion:

- a) Isolated genomic DNA showed A260/280 ratio of 1.7 and a sharp band on agarose gel electrophoresis.
- b) The Glycation of DNA causes formation of nucleotide AGEs leading to the alteration in DNA analyzed by UV spectra and thermal denaturation characteristics.
- c) Amadori product content estimation confirmed the AGEs induced modification of DNA.
- d) Peroxynitrite induced damage to glycated DNA was analyzed by UV spectra and decrease in melting temperature.
- e) Scavenging effect of GSH provide clue for the generation of free radical during glycation.

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