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A comparative study on the role of Lysine and BSA in Glycation-induced damage to DNA

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Abstract

Glycation is a nonenzymatic process in which carbonyl groups of sugar react with the amino group of proteins and nucleic acids. This process leads to the formation of many deleterious products which are commonly called as advanced glycation end products (AGEs). These products have been implicated in various pathophysiological conditions like retinopathy, diabetes, Parkinson's, Alzheimer etc. In the present study, effect of glycation on the structure of nucleic acids (plasmid and genomic DNA) has been studied. Glucose and methyl glyoxal (MG) caused damage to plasmid DNA in the presence of lysine and/or $FeCl_3$. When plasmid pBR322 DNA was incubated with BSA and MG, it showed gradual increase in the structural alteration with corresponding increase in incubation. Similarly, genomic DNA was also damaged in the presence of lysine, MG and $FeCl_3$. These results indicate the glycoxidative damage of nucleic acids and role of metal ions in the catalysis of the process. This work also indicates the differential potential of lysine and BSA in glycation-induced damage of DNA.

Keywords: Glycation, Maillard products, DNA damage, Free radicals, Sodium azide, Aminoguanidine.

1. Introduction

Glycation is a biochemical process wherein carbonyl groups of sugars interact with amino groups of other macromolecules like nucleic acids, proteins and lipids. This process is nonenzymatic and can be divided into two stages: early and advanced glycation. In the early glycation stage, carbonyl groups and amino groups combine and lead to the formation of Schiff bases and Amadori products. The intermediates of this stage are formed rapidly and they are reversible in nature. The accumulated Amadori products undergo complicated chemical rearrangement reactions (oxidations, reductions and hydrations) and finally lead to the formation of advanced glycation end products (AGEs) (Stitt **2001**).

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This process takes place in days, weeks or months and it is irreversible. AGEs are very stable and accumulate inside and outside the cells and interfere with the function of macromolecules (Goldin *et al.* **2006**).

The early and advanced glycation end products are continuously synthesized in the body even at the normal glucose levels. However, the deleterious effect of these products is observed due to their consequential accumulation after sometime and when the level of glucose increases above the normal in the blood. Glycated products interfere with the homeostasis and have been associated with various diseases such as diabetes, cataract, Alzheimer's, dialysis related amyloidosis (DRA), atherosclerosis as well as ageing (Ahmad **2005**). However it is still not clear that which AGE(s) is/are involved in these complications and what is the mechanism. A large amount of reactive oxygen species (ROS) is produced as a result of subsequent

modifications of Amadori products and AGEs and this includes superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), alkoxyl (RO), peroxyl (ROO), hydroxyl radicals (OH) and hypochlorous acid (HOCL) (Ahmad *et al.* **2014**).

Formation of Schiff's bases and Amadori production are indications of glycation reaction. Many of these products have been shown to be potentially harmful to the biomolecular structure and cellular integrity. Several scientific groups have tried to characterize the process involved in the formation of AGEs but the exact mechanism of Amadori products formation and also their conversion to AGEs is still not known. Therefore, it has still not been possible to develop a drug which can be a total cure for glycation and its related disorders. Also, there are very few reports available in the literature as far as damage to DNA by sugar metabolites is concerned. There may be several routes by which nucleic acids are damaged during the process of glycation. Formation of an adduct between DNA and glycation products as well as reactive oxygen species induced damage are the two possible mechanisms. While the former could be a direct process involving an interaction between carbonyl groups and the amino groups of the nucleic acids, the latter will be an indirect effect due to reactive oxygen species produced as a result of early and advanced glycation products. Maillard reaction product can cause DNA strand breakage and act as mutagen (Hiramoto *et al.* **1997**). Glycation of DNA can have multiple effects such as strand breaks, unwinding of the double helix, mutations and formation of DNA-protein and nucleotide-nucleotide cross-links (Pun and Murphy **2012**).

In the present study, the effect of Maillard reaction products on the structure of DNA (both plasmid and genomic) has been analyzed. DNA was also incubated with BSA and MG to induce glycation and analyze the structural alteration in the nucleic acids. Results suggest the role of sugars and their derivatives in the differential damage to DNA. MG and lysine caused more damage to the DNA as compared to MG-BSA combination.

2. Materials and methods

2.1. Materials: Plasmid pBR322 DNA, glucose, L-lysine, Tris Buffer, and ferric chloride were obtained from Merck India. Agarose, BSA, methyl glyoxal, and sodium azide were procured from Sigma Aldrich. All the other chemicals used were of high analytical grade.

2.2. Generation of Maillard Products: A mixture of 0.5 g of glucose and 0.5 g of lysine was added to 5 ml of water. The solution was neutralized at pH 7.0 and made up to 10 ml with water. The solution was heated at 100 °C for 3 hrs or until the solution turned to reddish brown. This Maillard product was used for the analysis of DNA damage by glycation.

2.3. DNA damage by Maillard reaction of glucose-lysine mixture: A mixture of 8 µl of the solution containing Maillard product, 1 µl of 1 M phosphate buffer (pH 7.4), and 1 µl of solution containing 500 ng plasmid pBR322 DNA was incubated at 37°C overnight. The reaction was stopped by freezing at -20 °C (Kikugawa *et al.* **1994**).

2.4. In vitro glycation of plasmid pBR322 DNA by MG and lysine: 500 ng of plasmid pBR322 DNA in 100 mM potassium phosphate buffer at pH 7.4 was incubated for 3 hrs at 37 °C with lysine (20 mM) and MG (20 mM) in the presence and absence of $FeCl_3$ (100 µM), aminoguanidine (10 mM) and sodium azide (250 mM) (Suji and Sivakami **2007**). The reaction was stopped by freezing at -20 °C.

2.5. In vitro glycation of genomic DNA by MG and lysine: Genomic DNA (0.346µg) was incubated with lysine (20 mM), MG (20 mM) and $FeCl_3$ (100 µM) at 37°C for 5 days. Samples were collected at an interval of 24 hours and stored at -20 °C.

2.6. Glycation of plasmid pBR322 DNA by MG and BSA: Plasmid pBR322 DNA (500 ng) was incubated with BSA (10 mg/ml), MG (20 mM) and NaN_3 (250 mM) at 37 °C for 10 days and 21 days. The glycated sample was stored at -20 °C.

2.7. Agarose Gel Electrophoresis of glycated DNA (plasmid and genomic) sample: 20µl of samples were mixed with 4µl gel loading dye (6X) and loaded on to 1 % Agarose gel. Agarose gel electrophoresis was performed at 80 V till the dye band ran 3/4th of the gel length. Subsequently, the gel was stained with ethidium bromide (1.5 ml of 1 mg/ml EtBr in 300ml tank buffer) for 20 min. The gel was visualized under UV trans-illuminator and bands analyzed with the help of control.

3. Results

3.1. Effect of Maillard product on plasmid pBR322 DNA: The aqueous solution of the glucose and lysine (pH adjusted to 7) was heated at 100 °C for 3 hours. The solution turned to reddish brown. Supercoiled plasmid pBR322 DNA was incubated with Maillard product (8 µl) for overnight at 37 °C and subjected to gel electrophoresis. It can be observed that supercoiled pBR322 (form I) was damaged partially and generated open circular form (form II) (Figure 1; Lane 2). The Maillard product at higher concentration (8 µl) effectively converted a supercoiled form into an open circular form and a linear form (form III) (Figure 1; Lane 3).

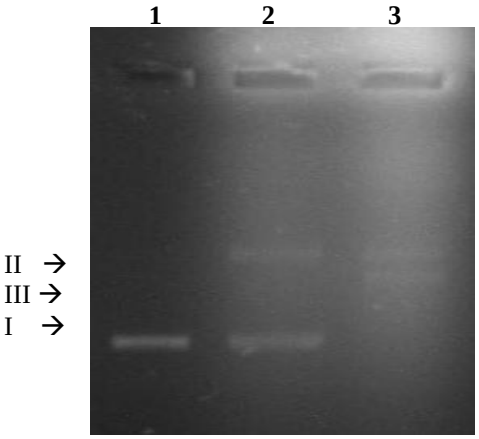

Fig 1 Plasmid DNA damage by Maillard product. Maillard product was obtained by heating the mixture of glucose (0.5 %, w/v) and lysine (0.5 %, w/v) at the 100°C for 3 hrs. pBR322 (500 ng) was incubated with different amount of Maillard product (4 µl and 8 µl) at 37°C for overnight. Lane 1: pBR322 alone; Lane 2: pBR322 + Maillard product (4 µl); Lane 3: pBR322 + Maillard product (8 µl). I: supercoiled pBR322; II: partially damaged and generated open circular form; III: open circular and linear form of pBR322. (I – supercoiled, II – open circular and III – linear)

3.2. Effect of Metal-catalyzed glycation on DNA (pBR322) damage: The incubation of plasmid pBR322 DNA with MG and lysine caused strand breaks partially (Figure 2; Lane 2). Presence of Fe^{3+} in the system enhanced DNA strand breakage and resulted in increased amount of open circular form (Figure 2; Lane 3). Strand breakage was inhibited by sodium azide (Figure 2; Lane 5 and 8). Aminoguanidine did not lead to strand breakage in the absence of $FeCl_3$ (Figure 2; Lane 6). However, it resulted in the strand breakage in the presence of $FeCl_3$ similar to the Lys-MG- $FeCl_3$ combination (Figure 2; Lane 7).

3.3. Plasmid pBR322 DNA damage by BSA and MG: Incubation of DNA with BSA and MG for 10 days induced DNA strand breakage as shown by the decrease in the amount of supercoiled form I and concomitant increase in open circular form II (Figure 3 A; Lane 4). NaN_3 (30 mM) showed partial inhibitory effect on DNA damage (Figure 3A & B; Lane 4). DNA damage increased when duration of incubation with BSA and MG was increased to 21 days (Figure 3 B; Lane 3). This increased incubation led to conversion of supercoiled to linear form as compared to open circular after 10 days treatment. White fluorescent patches present on the gel may be indicative of presence of AGEs.

3.4 Genomic DNA cleavage during the Glycation of Lysine by MG: DNA was incubated with lysine, MG and $FeCl_3$ at 37 °C for 5 days. 20 µl samples was taken out from the tube after 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs and kept at -20°C. As can be seen from the Figure 4, DNA was damaged by lysine-MG system and DNA damage was increased with increase in incubation time (Figure 4; Lane 2 to 6).

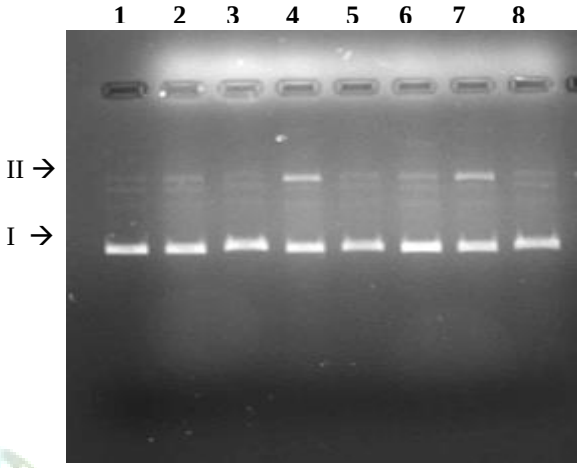

Fig 2 Metal-catalyzed glycation-induced DNA damage. pBR322 DNA (0.5 µg) was incubated with lysine (20 mM), MG (20 mM), $FeCl_3$ (100 µM), aminoguanidine (10 mM) and sodium azide (250 mM) in combinations (mentioned in lane description) at 37 °C for 3 hrs. Lane 1: pBR322 alone; Lane 2: pBR322 + Lys (20 mM) + MG (20 mM); Lane 3: Lane 2 + NaN_3 (250 mM); Lane 4: Lane 2 + $FeCl_3$ (100 µM); Lane 5: Lane 4 + NaN_3 (250 mM); Lane 6: Lane 2 + AG (10 mM); Lane 7: Lane 4 + AG (10 mM); Lane 8: Lane 7 + NaN_3 (250 mM). (I – supercoiled, II – open circular)

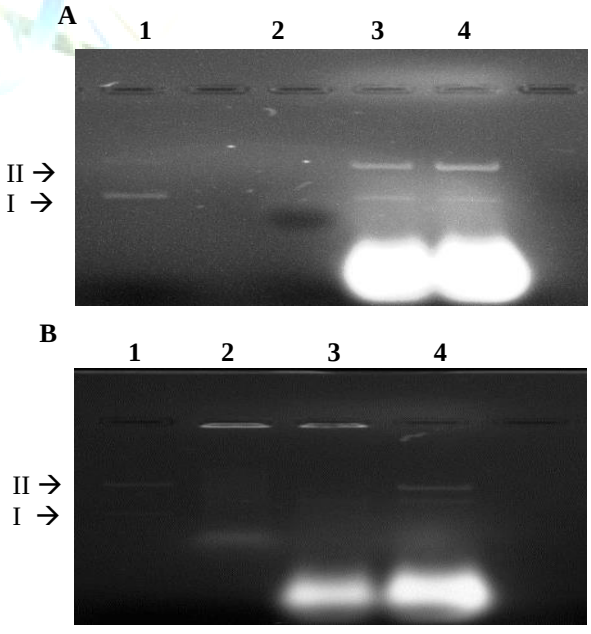

Fig 3 DNA damage by BSA and MG. pBR322 DNA (0.5 µg) was incubated with BSA (10 mg/ml), MG (20 mM) and NaN_3 (250 mM) at 37 °C for 10 days.

(A) and 21 days (B). Lane 1: pBR322 alone; Lane 2: BSA alone; Lane 3: pBR322+ BSA + MG; Lane 4: Lane 3 + NaN_3 (B) and dialyzed in 10 mM phosphate buffer for overnight at 4 °C. Lane 1: pBR322 alone; Lane 2: BSA alone; Lane 3: pBR322+ BSA + MG; Lane 4: Lane 3 + NaN_3. ((I – supercoiled, II – open circular)

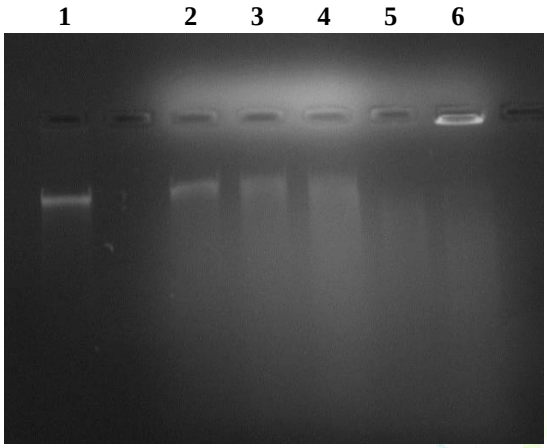

Fig 4 Genomic DNA damage by lysine, MG and $FeCl_3$. Genomic DNA (0.346µg) was incubated with lysine (20 mM), MG (20 mM) and $FeCl_3$ (100 µM) at 37°C for 5 days. Lane 1: DNA alone; Lane 2: DNA + Lys (20 mM) + MG (20mM) + $FeCl_3$ (100µM) (after 24 hrs); Lane 3: Lane 2 - after 48 hrs; Lane 4: Lane 2 - after 72 hrs; Lane 5: Lane 2 - after 96 hrs; Lane 6: Lane 2 - after 120 hrs.

4. Discussion

Glycation leads to generation of early and advanced glycation end products. These products have been implicated in altering the structure of biomolecules including lipids, proteins and nucleic acids. The application of synthetic and natural products in the treatment of glycation related diseases has contributed to the knowledge of damage mechanism. The mechanism of damage is exactly not known, however cross linking, aggregation and generation of free radicals have been assumed to play significant role in the damage process.

The model system to study the glycation *in vitro* is incubation of lysine and methyl glyoxal for several hours (Yim *et al.* **1995**). Proteins or nucleic acids are added to the incubation mixture to study the effect of glycation on their structure. Glycated samples are analyzed by different methods including spectrophotometric and electrophoretic techniques. In the present study, plasmid pBR322 DNA was used as standard to analyze the effect of glycation on the conformation of nucleic acids (Ali *et al.* **2014**; Kang **2003**; Levi and Werman **2001**; Pischetsrieder *et al.* **1999**).

Initially the effect of Maillard product, generated as a result of boiling the mixture of glucose-lysine in aqueous system, was checked on the structure of plasmid pBR322 DNA. Maillard product damaged the supercoiled DNA and resulted in the formation of both open circular and linear DNA. The gel showed lot of white fluorescent patches in the lanes which may be due to the presence of AGEs, some of which are highly fluorescent.

The results presented in this work suggest that DNA strand breakage was induced by co-incubation of lysine and MG. The damage of plasmid pBR322 DNA increased in the presence of ferric ion (Fe^{3+}). Trace metal such as iron, which is present in biological systems may react with H_2O_2 to produce hydroxyl radical and then induce DNA strand breakage. These results also suggest that the reaction of MG-mediated DNA breakage may be caused by traces of transition metals which undergo Fenton reaction and lead to the production of free radicals (Fenton **1894**). Inhibition of glycation by antioxidants has been established and many of these antioxidants act as radical trapping agents (Khalifah *et al.* **1999**). Previously it has been shown that MG, Lys, and $FeCl_3$ alone had no effect on the integrity of supercoiled DNA (Ali *et al.* **2014**). When sodium azide, a hydroxyl radical scavenger, was added in the reaction mixture it was found that the free radical could not induce damage to DNA. Therefore, it can be concluded that damage to DNA is mainly due to the generation of free radicals during the late stage of glycation reactions. There are several other reports which suggest this mechanism of metal-induced free radical mediated damage to DNA (Kang **2003**; Suji and Sivakami **2007**). The effect of aminoguanidine, a potent dicarbonyl scavenger, was also checked on the DNA damage by dicarbonyls. It caused the strand breakage in the presence of Lys, MG and $FeCl_3$ which was very similar to the pattern observed with the DNA incubated in the presence of Lys, MG and $FeCl_3$ without AG. It could be due to the fact that AG is a known dicarbonyl scavenger and it produces free radicals in the presence of Fe. Suji and Sivakami (**2006**) have shown in that AG and Fe caused strand breakage of the DNA due to their involvement in the generation of H_2O_2.

Experiments were also designed to compare the damage to DNA in the presence of BSA. It was observed that DNA was damaged significantly in the presence of BSA and MG. However, a free lysine caused more damage to DNA than BSA and it also required less time than BSA. This can be due to higher reactivity of free lysine as compared to bound lysine in BSA.

In this study the damage to genomic DNA was caused by late stage glycation model system comprising of lysine, MG and $FeCl_3$. The damage of genomic DNA increased with increase in duration of treatment

probably due to increase in the formation of free radicals. However, the pattern of damage to plasmid and genomic DNA is different. In case of plasmid pBR322 DNA, the damage is marked by a change in the conformation from supercoiled to nick to linear. On the other hand when genomic DNA was treated with Lys, MG and Fe, it showed a smear on the gel and the extent of smearing increased with increase in incubation time. It can also be observed that genomic DNA took much longer time to show the damaging effect of glycation system. The present study shows the higher glycating and DNA damaging activity of MG in accordance with earlier reports of Rabbani and Thornalley (**2014**).

5. Conclusion

This study indicates the differential role of free amino acid (lysine) and amino acid residues in protein (BSA) in glycating and damaging the DNA structure. It was also observed that plasmid DNA was more susceptible to oxidative stress than genomic DNA. We also report the high glycating and DNA damaging activity of methyl glyoxal in the present study.

6. Acknowledgement

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7. Conflict of interest statement

We declare that we have no conflict of interest.

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