

Full Length Research Paper

Inhibition of protein glycation and advanced glycation end products by ascorbic acid

Hira Zafar¹, Munir Ahmad Sheikh¹, Fatma Hussain* and Muhammad Arif Maan²

¹Department of Chemistry and Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad-38040, Pakistan.
²Punjab Medical College/District Head Quarter Hospital, Faisalabad, Pakistan.

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Advanced glycation end products (AGEs) formation is increased in diabetes mellitus, leading to microvascular and macrovascular complications. Recently, much attention has been focused on natural and synthetic inhibitors to delay the onset or progression of diabetes and its comorbidities. Ascorbic acid (AA) can react with proteins, including hemoglobin and possibly interfere with protein glycation process. An *in vitro* glycation model containing plasma from type 2 diabetic and non-diabetic healthy volunteers together with glucose as glycating agent was used to study antiglycation activity of AA. Samples with different concentrations of glucose and AA were incubated for five weeks at 37°C. Nonenzymatic glycation (NEG) was quantitated by thiobarbituric acid calorimetry and AGEs were measured by enzyme linked immuno-sorbent assay (ELISA). The NEG and AGEs levels were reduced by AA. Increasing the AA concentrations greatly diminished protein glycations, indicating dose-dependent effects of AA. Plasma NEG and AGEs were decreased with an average of 20 to 26% ($p < 0.05$) and 26 to 28% ($p < 0.05$). A significant correlation was found between the glycation inhibition and the inhibition of AGE formation ($p < 0.05$). The antiglycation role of AA is evident in the present study and it also indicates the possibility of inexpensive, relatively non-toxic vitamin therapy for the prevention and treatment of diabetic complications. It is plausible that AGEs inhibition by AA may also form the basis for future intervention strategies in both diabetic and non-diabetic individuals.

Key words: Diabetes mellitus, glycation, advanced glycation end products, hyperglycemia, ascorbic acid.

INTRODUCTION

Advanced glycation end products (AGEs) are a group of modified molecular species formed by nonenzymatic reactions of reducing sugars with proteins, lipids or nucleic acids. AGEs are generated in hyperglycemia and their accumulation is accelerated in diabetes (Lautenslager et al., 2011; Schalkwijk and Miyata, 2012).

AGEs can inactivate proteins or modify their biological activities, leading to microvascular and macrovascular complications in diabetes mellitus (Jack and Wright,

2012; Luers et al., 2012; Yamagishi et al., 2012). Glycation of extracellular matrix proteins and low-density lipoprotein with subsequent deposition in the vessel wall could contribute to inflammatory response and atheroma formation (Mohar et al., 2012; Yamagishi et al., 2012).

Due to huge premature morbidity and mortality associated with diabetes, extensive efforts have been made to mediate protein glycation (Malviya et al., 2010). Although both synthetic and natural products are used as glycation inhibitors (Peng et al., 2011), the most successful among these are aminoguanidine, aspirin, acetaminophen and ibuprofen (Yan et al., 2008).

The interrelationship between diabetes and various vitamins is characterized by a high degree of reciprocity. Chronic uncontrolled hyperglycemia can cause significant alterations in the status of these nutrients and conversely some micronutrients can directly modulate glucose homeostasis (Mooradian et al., 1994). The consumption

*Corresponding author. E-mail: fatmauaf@yahoo.com. Tel: +9241-9200161-170. Ext: 3309, 3313.

Abbreviations: AGEs, Advanced glycation end products; AA, ascorbic acid; NEG, nonenzymatic glycation; ELISA, enzyme linked immuno-sorbent.

Table 1. Different combinations used for the glycation inhibition study.

S/N	Combination
1	PN/D + G ₁
2	PN/D + G ₂
3	PN/D + G ₃
4	I ₁ + PN/D + G ₁
5	I ₁ + PN/D + G ₂
6	I ₁ + PN/D + G ₃
7	I ₂ + PN/D + G ₁
8	I ₂ + PN/D + G ₂
9	I ₂ + PN/D + G ₃
10	I ₃ + PN/D + G ₁
11	I ₃ + PN/D + G ₂
12	I ₃ + PN/D + G ₃
13	I ₄ + PN/D + G ₁
14	I ₄ + PN/D + G ₂
15	I ₄ + PN/D + G ₃

PN/D, Normal/diabetic plasma; G, glucose (G₁, 5.5 mM; G₂, 25 mM; G₃, 50 mM); I, inhibitor (I₁, 100 mM; I₂, 200 mM; I₃, 300 mM; I₄, 400 mM).

of foods high in ascorbic acid (AA) has been associated with lower risk of diabetes. AA status may influence glycemic control, protein glycation and the sorbitol pathway (Kositsawat and Freeman, 2011; Vinson and Howard, 1996). Reduction in protein glycation with AA supplementation is evident from animal and human studies (Gembal et al., 1994; Krone and Ely, 2004). While AA is the most commonly consumed dietary supplement, knowledge of AA as glycation inhibitor is imperative. In the present study, we investigated AA as a potent inhibitor of protein glycation and AGE formation *in vitro*.

MATERIALS AND METHODS

Sampling

Blood samples were collected from diabetic (type 2 diabetes mellitus) and normal healthy volunteers after written informed consent. Plasma were separated and collected as plasma diabetic (PD) and plasma normal (PN). Glycation and AGE measurements were done for both fractions before incubation. Human plasma was preferred over bovine albumin because it contains glycated proteins (enzymatic) and it is relatively economical. Two types of controls were used: bovine albumin as negative control; plasma (normal/diabetic) incubated with glucose as positive control.

Incubations

People with diabetes have depressed AA levels due to multiple factors. There is substantial evidence that diabetics may require supplemental ascorbic acid in order to achieve tissue saturation and maximal physiological function (Cunningham, 1988; Som et al., 1981). Excessive concentrations of AA (100 to 400 mM) than the

normal physiological range were used to assess supplementation effects. Plasma samples were incubated with glucose (5.5, 25 and 50 mM) and AA (100, 200, 300 and 400 mM) at 37°C for five weeks (Table 1). At the end of incubation period, glucose concentrations were measured and samples were dialyzed to remove free glucose. Free glucose is the major hindrance in estimation of glycation level. Post-dialysis, glucose was again estimated to confirm final glucose levels. Total proteins in all samples after dialysis were determined by biuret method (Gornall et al., 1949). Measuring protein before and after dialysis monitored sensitivity and validity of the method.

Glycation analysis

Thiobarbituric acid (TBA) colorimetry test was used for the determination of both enzymatic glycation (EG) and non-enzymatic glycation (NEG) as described by Furth (1988).

Non-enzymatic and enzymatic glycation (collective)

One milliliter dialyzed sample whose total protein is already estimated (10 mg/ml) was used. Three test tubes were arranged for reduced and three for non-reduced samples. 0.1 ml of NaBH₄ was added to reduced samples and 0.1 ml of 0.01 N NaOH was added to non-reduced samples. All the tubes were left for 30 min at 37°C. After half an hour, 1 drop of 1 N HCl was added in each test tube, followed by 0.5 ml oxalic acid. Tubes were capped and autoclaved for half an hour at 124°C (115 Lb /inch² pressure). Tubes were cooled to room temperature and placed in ice. In each tube, 0.5 ml chilled 40% trichloroacetic acid was added. Samples were centrifuged for 15 min at 15000 rpm. Supernatant (1.5 ml) was taken and 0.5 ml freshly prepared TBA was added. The samples were incubated at 37°C in water bath for 15 min and absorbance was noted at 443 nm.

Enzymatic glycation

For determination of enzymatic glycation, 0.1 ml NaOH (0.01N) containing 400 molar excess of NaBH₄ was used. After the reduction, the glycation level was determined by the same process as mentioned above. Non-enzymatic glycation was determined as follows:

$$\text{NEG} = (\text{NEG} + \text{EG}) - \text{EG}$$

AGEs analysis

Anti-AGE immunoglobulins (antibodies) were purchased commercially from Sigma. AGEs-BSA was prepared by using bovine serum albumin (BSA). Enzyme linked immuno-sorbent assay (ELISA) was performed by using alkaline phosphatase enzyme and para-nitrophenyl phosphate as a substrate, following the procedures of Turk et al. (1998) and Zhang et al. (2005) with slight changes according to laboratory conditions. Antigen was diluted to a final concentration of 20 µg/ml in phosphate buffered saline (PBS). The wells of the polyvinylchloride (PVC) micro-titer plate were coated with 50 µl antigen dilution per well. The plate was covered with an adhesive plastic and incubated for 2 h at room temperature.

The coating solution was removed and plate was washed twice by filling the wells with 300 µl PBS. The solutions or the washes were removed by flicking the plates over a sink. The remaining drops were removed by patting the plate over a paper towel. The remaining protein-binding sites in the coated wells were blocked by adding 300 µl of blocking buffer, 5% non-fat dry milk PBS, per well.

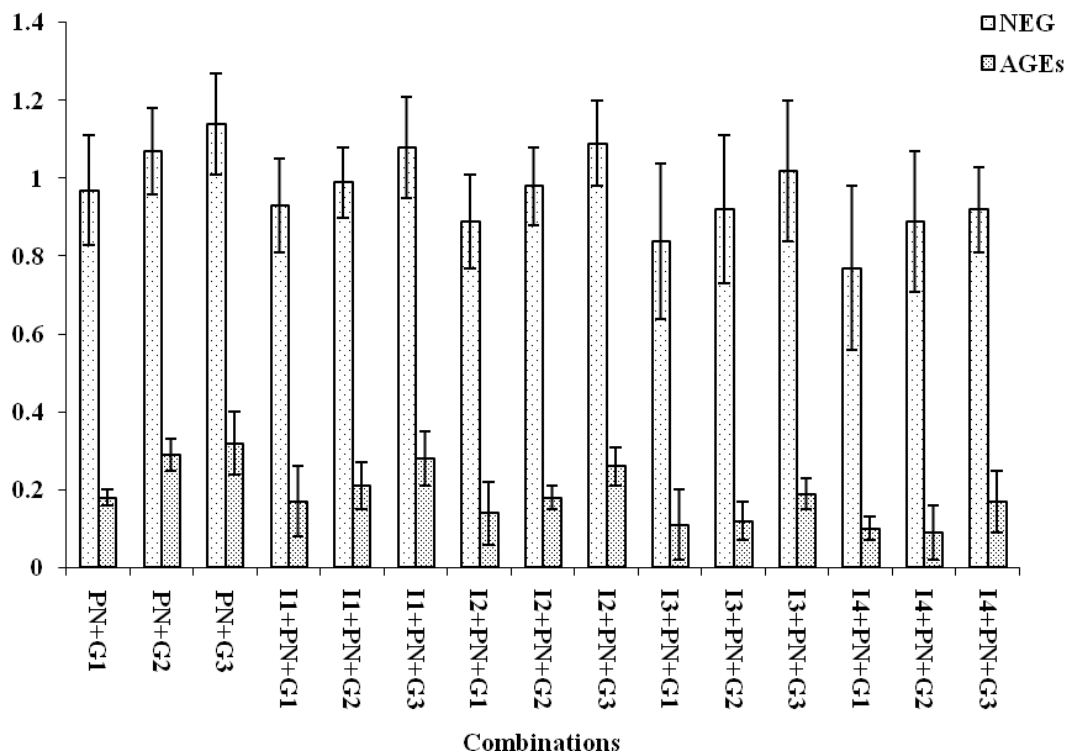


Figure 1. Effects of ascorbic acid on NEG and AGEs levels in normal plasma. PN, Normal plasma; G, glucose (G₁, 5.5 mM; G₂, 25 mM; G₃, 50 mM); I, inhibitor (I₁, 100 mM; I₂, 200 mM; I₃, 300 mM; I₄, 400 mM); NEG, mol.glucose/mol.protein; AGEs, unit/ml.

The plate was covered with an adhesive plastic and incubated for at least 2 h at room temperature or, if more convenient, overnight at 4°C. The plate was washed twice with PBS. 10-fold dilutions (1:100, 1:1000, 1:10,000, 1:100,000 and 1:1000,000) of plasma in blocking buffer were prepared and 50 µl of each dilution was added to an antigen coated well. The plate was covered with an adhesive plastic and incubated for 2 h at room temperature. The plate was washed four times with PBS and 50 µl of secondary anti-specie antibody conjugated to alkaline phosphatase was added, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Plate was covered with an adhesive plastic, incubated for 2 h at room temperature and washed four times with PBS. p-Nitrophenyl phosphate was dissolved at a concentration of 1 mg/mL in substrate buffer (1 M Di-ethanolamine, 0.5 mM MgCl₂, pH 9.8) and 50 µl substrate solution was added per well. Optical density (OD) at 405 nm was determined by an ELISA reader. An end-point measurement was performed after 1 h. Results were calculated as:

$$\frac{1 - (\text{Experimental OD} - \text{Background OD})}{\text{Total OD} - \text{Background OD}}$$

A 50% competition was defined as 1 unit of AGEs.

Statistical analysis

All data were expressed as mean ± SD of triplicate measurement. The degree of association between different variables was assessed by using Pearson's correlation coefficient (r). Student's t-test was performed by Statistical Package for the Social Sciences

(SPSS Inc. Chicago, IL, USA) software (version 15.0) with level of significance set at p < 0.05.

RESULTS AND DISCUSSION

Protein glycation leads to the formation and accumulation of toxic AGEs that can permanently alter the structure and function of body proteins (Wu et al., 2011). Vitamins can decrease the glycation process by binding with sugars or proteins (Davie et al., 1992). These findings led us to ascertain antiglycation potential of AA.

In vitro glycation inhibition was studied in normal and diabetic plasma using glucose as glycating agent. Glycation was quantitated by thiobarbituric acid colorimetry and AGEs were measured by ELISA. The TBA reaction can sometimes lead to misinterpretations of the results (Lapenna et al., 2001). However, being economical, it is preferred over costly chromatographical techniques. Previous studies using TBA method have indicated that glycated serum proteins are sensitive indicators of the degree of hyperglycemia in diabetes (Mcfarland et al., 1979). Antiglycation potential of AA is presented in Figures 1 and 2.

Negative control (BSA) exhibited nil NEG and AGEs measurements. In the case of positive controls, for PD, 3.71 ± 0.2, 3.84 ± 0.16, 3.97 ± 0.12 mol.glucose/mol. protein

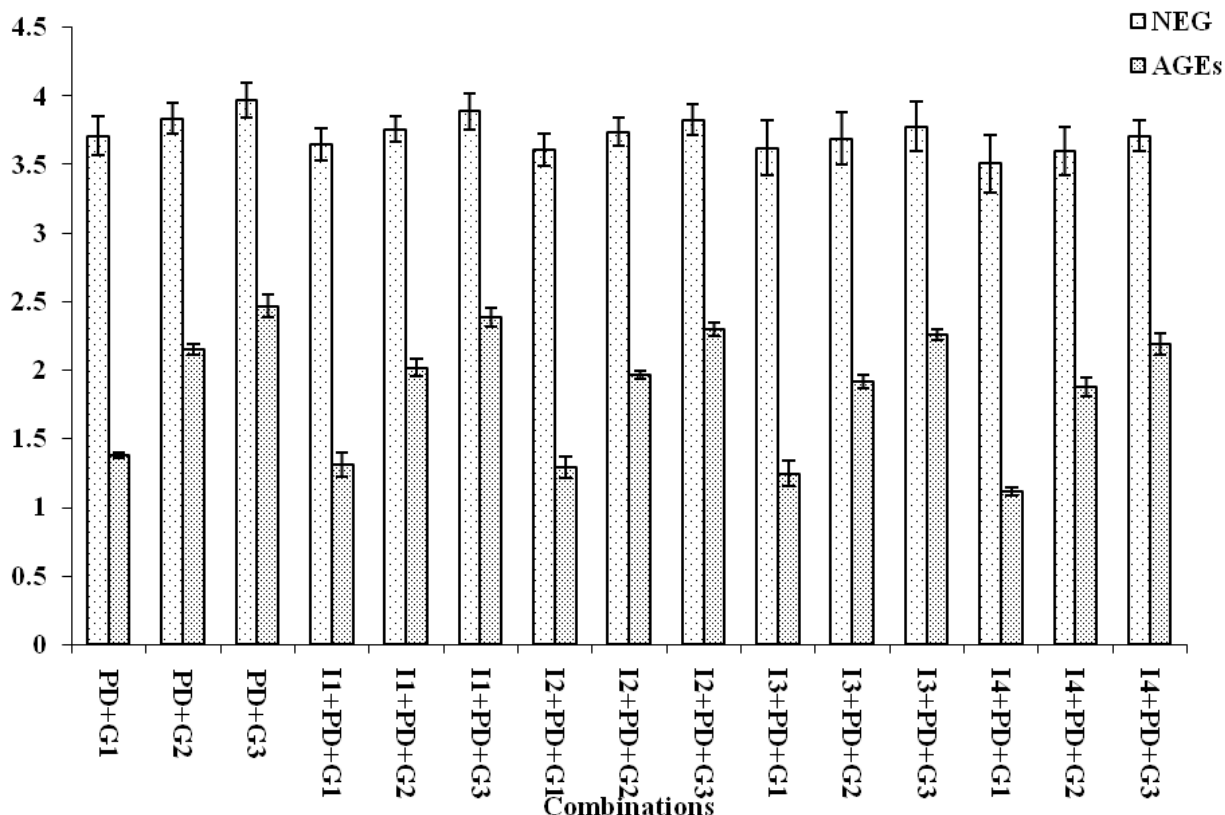


Figure 2. Effects of ascorbic acid on NEG and AGEs levels in diabetic plasma. PD, Diabetic plasma; G, glucose (G₁, 5.5 mM; G₂, 25 mM; G₃, 50 mM); I, inhibitor (I₁, 100 mM; I₂, 200 mM; I₃, 300 mM; I₄, 400 mM); NEG, mol.glucose/mol.protein; AGE, unit/mL

NEG and 1.38 ± 0.08, 2.15 ± 0.12, 2.47 ± 0.18 unit/mL AGEs levels were observed with 5.5, 25 and 50 mM glucose, respectively. Whereas, 0.97 ± 0.14, 1.07 ± 0.11, 1.14 ± 0.13 mol.glucose/mol.protein NEG and 0.18 ± 0.02, 0.29 ± 0.04, 0.32 ± 0.08 unit/mL AGEs levels were determined in PN samples with similar glucose concentrations. Incubation of 100 mM ascorbic acid with PD reduced NEG up to 6 to 8% (3.65 ± 0.21, 3.76 ± 0.27, 3.89 ± 0.19 mol.glucose/mol.protein) and AGEs up to 7 to 13% (1.31 ± 0.10, 2.02 ± 0.1, 2.39 ± 0.19 unit/mL) as compared to the positive controls. Percent reductions in NEG and AGEs by I₁ were more noticeable ($p < 0.05$) as compared to the controls. In PN, NEG and AGEs were 0.93 ± 0.12, 0.99 ± 0.09, 1.08 ± 0.13 mol.glucose/mol.protein and 0.17 ± 0.09, 0.21 ± 0.06, 0.28 ± 0.07 unit/mL with 100 mM ascorbic acid and 5.5, 25, 50 mM glucose, respectively. Noteworthy reductions (4 to 8%, $p < 0.05$) were observed in NEG and AGEs (1 to 8%, $p < 0.05$) in PN samples as compared to the controls. A significant correlation was found between the inhibition of glycation and the inhibition of AGE formation in both PD and PN samples ($r = 0.4$; $p < 0.05$).

Doubling the AA concentrations produced a profound decline in NEG (10 to 14%, $p < 0.05$) as well as AGEs (9 to 17%, $p < 0.05$) in PD samples with 3.61 ± 0.26, 3.74 ±

0.28, 3.83 ± 0.27 mol.glucose/mol.protein NEG and 1.29 ± 0.12, 1.97 ± 0.16, 2.3 ± 0.21 unit/mL AGEs. Similarly, I₂ reduced AGEs by 4 to 11% (0.14 ± 0.08, 0.18 ± 0.03, 0.26 ± 0.05 unit/mL) in PN samples as compared to controls. Protein glycation were significantly (5 to 9%, $p > 0.05$) decreased (0.89 ± 0.12, 0.98 ± 0.10, 1.09 ± 0.11 mol.glucose/mol.protein) by I₂. Decrease in AGEs correlated with decrease in NEG ($r = 0.69$; $p > 0.05$).

These results indicate that increase in AA concentrations produces a greatly diminished glycation and AGEs levels, whereas, the corresponding reduction was more apparent in diabetic samples as compared to the normal ones.

The glycation lowering effect in the present study could be due to the fact that the carbonyl group of AA competes with glucose for protein as described earlier (Davie et al., 1992).

Concentration-dependent inhibitory effects were more noticeable with 300 mM AA. A strong correlation existed between NEG and AGEs ($r = 0.58$; $p > 0.05$). Decrease in NEG also correlated with diminution in AGEs ($p < 0.05$). I₃ exhibited 3.62 ± 0.28, 3.69 ± 0.31, 3.78 ± 0.30 mol.glucose/mol.protein NEG and 1.25 ± 0.18, 1.92 ± 0.15, 2.26 ± 0.15 unit/mL AGEs in PD samples. Significant ($p < 0.05$) decline in NEG (9 to 19%) and

AGEs (13 to 23%) were noticeable from respective control measurements.

PN fractions with 300 mM AA showed 0.84 ± 0.2 , 0.92 ± 0.19 , 1.02 ± 0.18 mol.glucose/mol.protein NEG (13 to 15% decrease, $p < 0.05$) and 0.11 ± 0.09 , 0.12 ± 0.05 , 0.19 ± 0.04 unit/mL AGEs levels (7 to 17% decrease; $p < 0.05$). The data is partially in accordance with the findings of Vinson and Howard (1996). They quantitated amadori product by thiobarbituric acid colorimetry in normal subjects after ascorbic acid supplementation. Serum protein glycation was decreased at an average of 46.8% ($p < 0.01$). Contrary to these observations, Shoff et al. (1993) examined the relationship between glycosylated hemoglobin and intake of vitamins E, C and β -carotene in a population-based sample of middle-aged and older adults participating in the Beaver Dam Eye Study. In people without diabetes, energy-adjusted vitamin C intake was negatively associated with glycosylated hemoglobin (GHb).

Ascorbic acid demonstrates reduction in protein glycation both *in vivo* and *in vitro*. Binding to sugar or protein would inhibit AGE formation (Ceriello et al., 1992; Emekli, 1996; Zuwała-Jagięło, 2009). Conversely, Tarwadi and Agte (2011) investigated the effect of micronutrients on methylglyoxal-mediated *in vitro* glycation of BSA. Aminoguanidine showed highest inhibitory response for BSA glycation followed by quercetin, gallic acid and tannic acid. Promising antiglycation potential was seen for trolox, riboflavin, Zn and Mn as evidenced by decrease in the formation of AGEs and protein carbonyls. Ascorbic acid did not show significant antiglycation effect.

Optimal inhibitory effects were manifested by I_4 (400 mM). NEG were 3.51 ± 0.24 , 3.6 ± 0.23 and 3.71 ± 0.16 mol.glucose/mol.protein for PD samples. Their corresponding AGEs were 1.12 ± 0.08 , 1.88 ± 0.11 and 2.19 ± 0.13 unit/mL. Substantial decreases ($p < 0.05$) in NEG (20 to 26%) and AGEs (26 to 28%) as compared to the controls were assessed.

When 400 mM AA was used with PN, glycation and AGEs levels were 0.77 ± 0.21 , 0.89 ± 0.18 , 0.92 ± 0.11 mol.glucose/mol.protein and 0.10 ± 0.03 , 0.095 ± 0.17 , 0.25 ± 0.08 unit/mL, respectively. Although, decrease in NEG (18 to 22%) and AGEs (8 to 20%) in PN was significant ($p < 0.05$), it was less as compared to that in PD. In the case of I_4 , changes in AGEs were found to be associated with changes in NEG ($r = 0.75$; $p < 0.05$).

The data indicate dose-dependent antiglycation effects of AA in an *in vitro* model. As compared to the controls, various AA concentrations produced a significant inhibition of glycation and AGEs ($p < 0.05$).

Conclusion

It is likely that AGEs inhibition by AA may also form the basis for future intervention strategies in both diabetic

and non-diabetic individuals.

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