Original Article

Effect of Ascorbic Acid on Glycation Level in Diabetic Condition

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ABSTRACT

Background: Diabetes is a serious metabolic disorder that results in significant morbidity and mortality. Diabetes is characterized by hyperglycaemia resulting in various short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular changes.
Objective: In this study, effect of ascorbic acid on glycation was investigated.
Design of Study: Experimental Study.
Place of Study: Allied Hospital and National Hospital, Faisalabad.
Materials and Methods: Studies were performed by using normal and diabetic plasma. Samples were incubated for 5 weeks at 37 ^{o}C temperature and varying concentrations of glucose and vitamin C. Two glycation assays (Thioarbituric acid and Periodate) were used to measure and compare the glycation level.
Results: The results indicated that increase in glycation was observed from 1st to 3rd week of incubation and it was decreased after 5th week due to the formation of advanced glycation end products. With three concentrations of ascorbic acid variable responses were observed however, it was observed all three concentrations were responsible to increase glycation.
Conclusions: Ascorbic acid will facilitate glycation in hyperglycaemia condition and Periodate borohydride proved itself more reliable and sensitive glycation assays than TBA test.

Key Words: Glycation, Diabetes, Maillard reaction, Ascorbic acid, TBA, Periodate

INTRODUCTION

Diabetes is a serious metabolic disorder with micro and macrovascular complications that results in significant morbidity and mortality[1]. According to WHO projection, the prevalence of diabetes is likely to increase by 35%. Currently there are over 150 millions diabetics worldwide and this is likely to increase to 300 million more by the year 2025 [2]. Chronic hyperglycemia during diabetes causes glycation of body protein that in turn leads to secondary complications affecting eyes, kidney, nerves and artery[3]. The glycation process, otherwise known as the Maillard reaction, is divided into three key stages: the early reactions resulting in the formation of a Schiff base and Amadori products, the rearrangements of these chemical groups and the final reactions forming the classical Maillard browning products or now known as AGEs [4]. The *in vivo* formation of non-enzymatic glycated compounds was first detected in 1969 from studies on chromotagenic mobilities of fast moving, minor hemoglobins from diabetic patients, in particular HbA1C, now routinely used as a clinical tool in the management of glycaemic control in diabetic patients [5]. Hemoglobin and serum albumin may undergo a slow non-enzymatic glycation, mainly by formation of a Schiff base between ε-amino groups of lysine or arginine residues and glucose molecules in blood [6].

There is a novel class of enzymes found in *Aspergillus* called amadoriases, was found to catalyze the deglycation of Amadori products. Most recently, human fructosamine-3- kinase (FN3K) has been identified which phosphorylates fructoselysine (Fl) residues on glycated proteins, to Fl-3- phosphate and lead to its spontaneous decomposition, thereby reversing the nonenzymatic glycation process at an early stage[7]. Now it is the need of time to develop or isolate new compounds either from plants or synthetically to control diabetes and other age accelerating diseases. The first compound which has been extensively studied *in vitro* and *in vivo* to be a powerful inhibitor of glycation and AGE formation is aminoguanidine (AG) [8]. A derivative of vitamin B_6, (pyridoxamine) also inhibits the post Amadori steps of the Maillard reaction by sequesting catalytic metal ions and blocking oxidative degradation of Amadori intermediates[9]. The major aim of this study was to investigate effect of ascorbic acid on glycation in normal and diabetic human plasma and compare the sensitivities of Thiobarbituric Acid (TBA) and Periodate method.

MATERIALS AND METHODS

Effect on glycation was monitored by Ascorbic acid (vitamin C).

Selection of conditions and concentrations

Four concentrations ($G_{1=}$500 m*M*, $G_{2=}$250 m*M*, $G_{3=}$50 m*M and* $G_{4=}$5.5 m*M*) of glucose were used. Plasma fractions were collected from diabetic patient's blood (Type II) and from normal/ healthy male and female volunteers. Samples were stored at -20 ºC until use. Normal and diabetic plasma samples were diluted to the range having protein concentration up to 20 mg/mL. Two glycation assays were performed to measure glycation level. Total proteins (Biuret method [10]) were estimated before and after dialysis before carrying out experiments. Three concentrations of ascorbic acid ($I_{1=}$ 10 m*M*, $I_{2=}$ 5 m*M* and $I_{3\ =}$ 1 m*M*) were used for experimental studies.

Selection of Combinations

To study the effect of ascorbic acid with normal (P_N) and diabetic (P_D) plasma sixteen combinations were made and all were placed at 37 ºC at same time for five weeks (Table -1).

Glycation of Plasma

All plasma combinations were incubated with four concentrations of glucose (in PBS) at 37ºC for 1-5 weeks.

Plasma samples were dialyzed to remove free glucose after incubation as free glucose is the major hindrance in estimation of glycation level and AGE production. Glucose was again estimated after dialysis to confirm that concentration of glucose is decreased or not.

Glycation of plasma with Ascorbic acid

Plasma samples were also incubated with all glucose and three inhibitors concentration at 37ºC for 1-5 weeks.

Thiobarbituric Acid (TBA) Colorimetric Technique

TBA technique was used for the determination of both enzymatic and non-enzymatic glycation[11]. This method is based upon the reaction between fructose – amino acids and week acid, yielding 5- hydroxymethyl furfural (HMF).

Non enzymatic glycation was determined as follows.

NE Glycation = (C Glycation + E Glycation) - E Glycation

(NE = Non Enzymatic, E= Enzymatic, C= Collective)

Periodate Borohydride Assay

This test is based on the production of Formaldehyde by periodate oxidation of cis-diol, aminol, ketol or ketoamine structures. Two moles of formaldehyde are produced from hexose sugar. The amount of formaldehyde produced is quantified as the fluorescent adduct formed by condensation of formaldehyde with acetyl acetone and ammonia. Fructose is used as standard as it is structurally analogous to the ketoamine form of the glycogroups[12,13]

Table 1: Different combinations for Plasma glycation inhibition

S#	Combinations for Normal/ Diabetic Plasma	S#	Combinations for Normal/ Diabetic Plasma
1	$G_1 + P_{N/D}$	9	$G_3 + P_{N/D}$
2	$G_1 + P_{N/D} + I_1$	10	$G_3 + P_{N/D} + I_1$
3	$G_1 + P_{N/D} + I_2$	11	$G_3 + P_{N/D} + I_2$
4	$G_1 + P_{N/D} + I_3$	12	$G_3 + P_{N/D} + I_3$
5	$G_2 + P_{N/D}$	13	$G_4 + P_{N/Ds}$
6	$G_2 + P_{N/D} + I_1$	14	$G_4 + P_{N/D} + I_1$
7	$G_2 + P_{N/D} + I_2$	15	$G_4 + P_{N/D} + I_2$
8	$G_2 + P_{N/D} + I_3$	16	$G_4 + P_{N/D} + I_3$

RESULTS

Effect of ascorbic acid on glycation level with normal human plasma

The results after the measurement of glycation level and effect of ascorbic acid on glycation measured by both TBA and Periodate borohydride assays with normal human plasma are given in Figure 1&2.

At 37 ºC temperature, glycation was given maximum with G_1 (5.616 mole/mole) and was minimum with G_4 (2.540 mole/mole) after 3rd and 1st week respectively when measured by TBA. Instead of glycation inhibition ascorbic acid facilitated glycation and increased it, this increased was maximum by I_1 (10 m*M*) followed by I_2 (5 m*M*) and glycation inhibition was only seen by I_3 (1m*M*) concentration of ascorbic acid (Figure-1).

Same trend of glycation acceleration was also measured by Periodate borohydride assay but more sensitively. With this assay, 500 m*M* (G_1) glucose concentration showed maximum glycation i.e. 5.906 mole/mole after 3rd week of incubation and 5.5 m*M* (G_4) give minimum i.e. 3.986 mole/mole after 1st week of incubation. Again I_3 (1m*M*) was the only concentration who showed inhibition (Figure-2).

Effect of ascorbic acid on glycation level with diabetic human plasma

Effect of ascorbic acid on glycation with diabetic human plasma was measured by TBA (Figure 3).

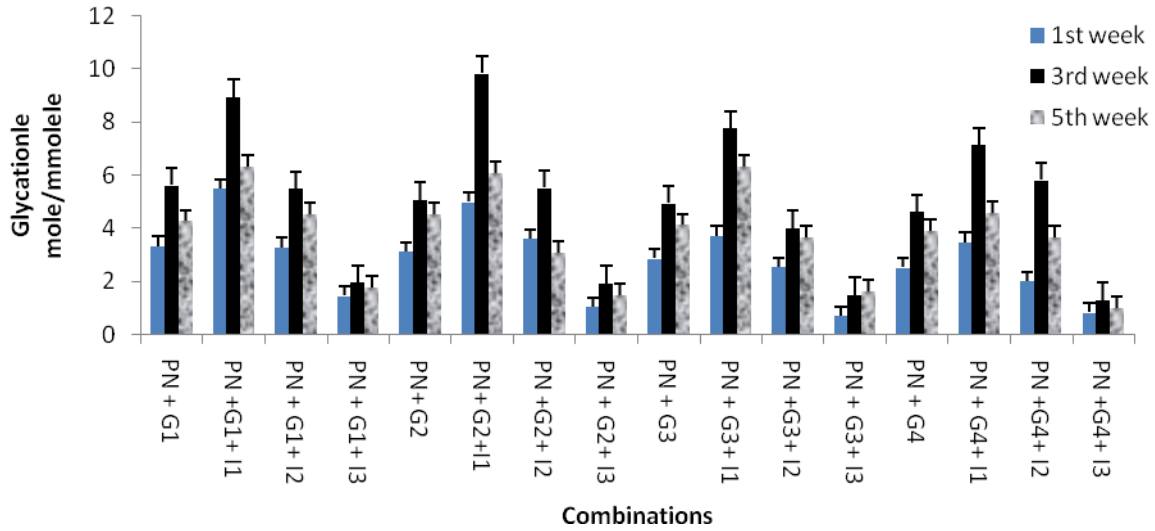

Figure 1: Effect of vitamin C on glycation level with TBA in normal human plasma

Normal plasma protein ($P_N \approx 20$ mg/mL) was incubated with all glucose concentrations (G_1= 500 m*M*, G_2= 250 m*M*, G_3= 50 m*M* & G_4= 5.5 m*M*) and Ascorbic acid as an inhibitor with three concentrations (I_1= 10 m*M*, I_2= 5 m*M* & I_3 =1m*M*) in 0.075 *M* PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 ºC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein).

***** Values were the average of experiments carried out at n= 3

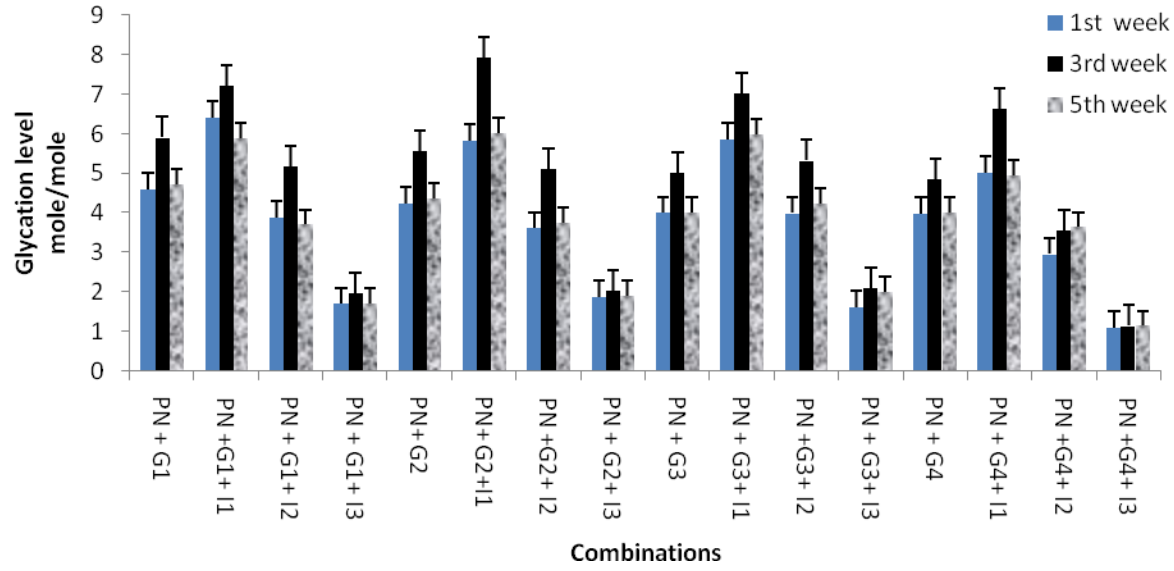

Figure 2: Effect of Ascorbic acid on glycation level with Periodate borohydride assay in normal human plasma

Normal plasma protein ($P_N \approx 20$ mg/mL) was incubated with all glucose concentrations (G_1= 500 m*M*, G_2= 250 m*M*, G_3= 50 m*M* & G_4= 5.5 m*M*) and Ascorbic acid as an inhibitor with three concentrations (I_1= 10 m*M*, I_2= 5 m*M* & I_3 =1m*M*) in 0.075 *M* PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 ºC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein).

***** Values were the average of experiments carried out at n= 3

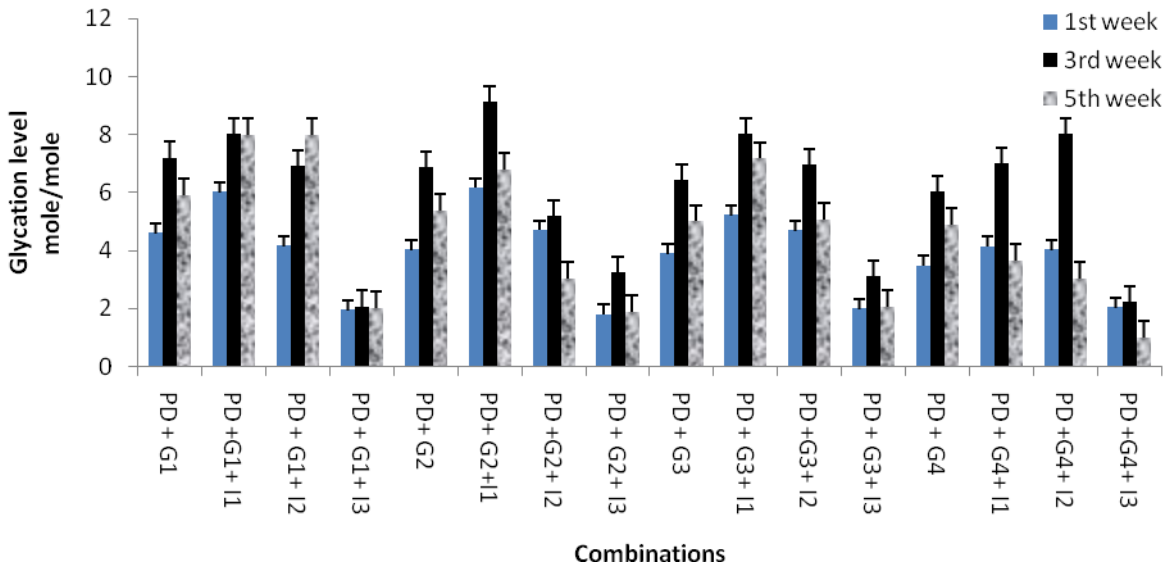

Figure 3: Effect of Ascorbic acid on glycation level with TBA in diabetic human plasma

Diabetic plasma protein ($P_D \approx 20$ mg/mL) was incubated with all glucose concentrations (G_1= 500 m*M*, G_2= 250 m*M*, G_3= 50 m*M* & G_4= 5.5 m*M*) and Ascorbic acid as an inhibitor with three concentrations (I_1= 10 m*M*, I_2= 5 m*M* & I_3 =1m*M*) in 0.075 *M* PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 ^oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein).

***** Values were the average of experiments carried out at n= 3

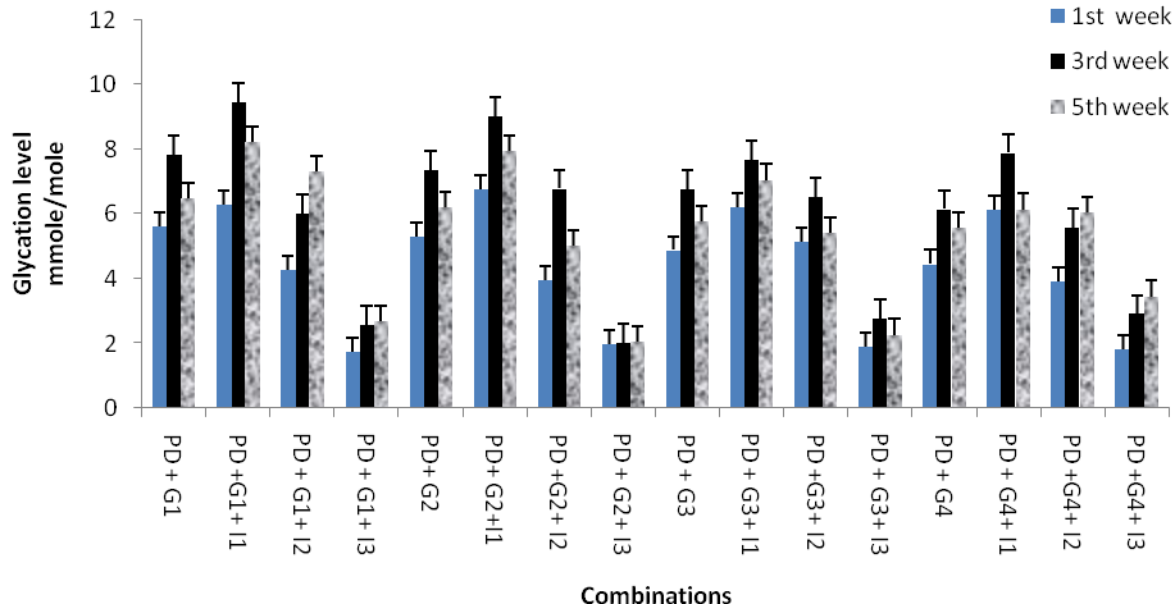

Figure 4: Effect of Ascorbic acid on glycation level with Periodate borohydride assay in diabetic human plasma

Diabetic plasma protein ($P_D \approx 20$ mg/mL) was incubated with all glucose concentrations (G_1= 500 m*M*, G_2= 250 m*M*, G_3= 50 m*M* & G_4= 5.5 m*M*) and Ascorbic acid as an inhibitor with three concentrations (I_1= 10 m*M*, I_2= 5 m*M* & I_3 =1m*M*) in 0.075 *M* PBS (pH 7.4, 0.1% Sodium Azide) and reaction mixtures were incubated at 37 ^oC for 5 weeks at the same time. Samples were analysed after 1st, 3rd and 5th week and glycation level was measured in mole\ mole (glucose\ protein).

***** Values were the average of experiments carried out at n= 3

Glycation level at 37 ^{o}C temperature, was given maximum with G_1 (500 m*M*) having value 7.195 mole/mole when measured by TBA and was minimum with G_4 (5.5 m*M*) with 3.466 mole/mole after 3rd and 1st week respectively. Again with ascorbic acid, instead of glycation inhibition, increased in glycation was maximum by I_1(10 m*M*) even it was almost double as compare to glucose concentrationthen. I_2 (5 m*M*) also activator of glycation only I_3 (1m*M*) was the concentration of ascorbic acid who inhibited glycation but not so actively (Figure-3).

When glycation level was measured by Periodate borohydride assay in diabetic human plasma it was observed, 500 m*M* (G_1) glucose concentration showed maximum glycation i.e. 7.838 mole/mole after 3rd week of incubation and 5.5 m*M* (G_4) give minimum i.e.4.453 mole/mole after 1st week of incubation. Again I_3 (1m*M*) was the only concentration who showed inhibition (Figure-4).

DISCUSSION

The results after the measurement of glycation level and effect of ascorbic acid on glycation measured by both TBA and Periodate borohydride assays with normal and diabetic human plasma indicated that ascorbic acid facilitate glycation in hyperglycaemia condition. Our results go against previous findings who quantified Amadori products and AGEs by thiobarbituric acid colorimetry and intrinsic fluorescence respectively. The inhibitors included ascorbic acid, tocopherol, pyridoxal, niacinamide, sodium selenite, selenium yeast, and carnosine showed significant correlation between the inhibition of glycation and the inhibition of AGE formation. They observed serum protein glycation was decreased with an average of 46.8% by ascorbic acid[14]. There is a report that also presents the phenomenon of non-enzymatic glycosylation and observed vitamin C had lowering effects on non-enzymatic glycation[15]. However, the data evaluated by few scientists argued our results as they studied non enzymatic glycation and AGEs formation by glucose and/or ascorbate, *in vitro*. The incubation of 100 m*M* D-glucose and 10 m*M* L-ascorbate with lens proteins resulted in an increasing incorporation over 3 weeks, ascorbate was 18-fold more reactive with lens proteins than glucose. Protein crosslinking was not obvious with 250 m*M* glucose but ascorbate caused extensive crosslinking even at 3.0 m*M*. On a molar basis, ascorbate was 70 fold more active than glucose and 100 fold more active than fructose in the cross-linking assay[16]. Our results were also favored by a study, that proteins are subject of post-translational modification by sugars and their degradation products *in vivo*, the process is often referred as glycation. According to them L-Dehydroascorbic acid, an oxidation product of L-ascorbic acid, is known as a potent glycation agent[17]. Our results are inline by a report which explains AGEs such as carboxy methyl lysine (CML) and carboxy ethyl lysine (CEL) require oxidative fragmentation of the carbon skeleton of glucose, but may also be formed from other hexoses, glycolytic intermediates and ascorbic acid[18].

In this study two glycation assays were used to measure glycation level and it was found Periodate borohydride assay is more sensitive than TBA method. Gallop, [12] quantified the glycation level of hemoglobin with Periodate borohydride method and it was further adapted by using a microplate reader with sensitivity enhancement (0.1 mg protein). They concluded this technique should be of particular importance for investigating the role of glycation of membrane proteins in the pathogenesis of diabetes.

It was also clear from the results that in most of experiments maximum glycation was in 3rd week of incubation. In the 1st week it was increased and became maximum in 3rd week and that was again decreased in 5th week. At low glucose level, glycation was low which is in accordance to previous findings [19]. The results of present study were also in accordance with few studies where it was found that glucose concentration and incubation time are the most clinically relevant variables affecting the extent of glycation. Characteristic to diabetes, increased level of glucose concentration cause the level of amadori products on protein to rise[20,21].

CONCLUSION

It was reported from our findings, ascorbic acid facilitates glycation in hyperglycemia condition and Periodate borohydride is more sensitive and efficient method for measuring glycation level.

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