

Study on the Effect of Vitamin C on the *In Vitro* Albumin Glycation Reaction

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Abstract

Vitamin C, an antioxidant substance soluble in water, can react with amino groups of proteins to form schiff bases. As diabetes leads to glycation of various protein which has effect on structure and biochemical activity of them, the inhibition of this process seems very vital. For several years researches in this field have done their best to recognize the antidiabetic compounds.

The aim of this study is to determine the effects of vitamin C on albumin glycation *in vitro*. So in the presence of various concentration of vitamin C, albumin was glycated and evaluating using TBA method.

The results showed that vitamin C is statistically significant ($P < 0.05$) inhibit or decrease the reaction of albumin glycation. The findings of this research showed that vitamin C probably inhibit the reaction of glycation in decreasing complications occurring in diabetes.

Keywords: Albumin glycation; Protein glycation; *In vitro*; Vitamin C.

Introduction

Reducing sugars react non-enzymatically with proteins or nucleic acids to initiate post-translational modification known as non-enzymatic glycation (NEG). This reaction is one of the major complications of diabetes mellitus. The early stage of the non-enzymatic glycation of proteins leads to the formation of early glycation products (1). In this reaction, sugars condense with amino groups of macromolecules to form an adduct known as Amadori product (2). These compounds are collectively called advanced glycation end-products (AGEs).

Studies in past several years have implicated advanced glycation reaction in protein aging and the development micro and macrovascular complications, cataract and hepatocytes injury in diabetes (3-5). Early glycation products are known to occur in many proteins including hemoglobin (6), albumin (7) and lipoproteins (8). Both NEG and AGEs have been shown to be accumulated in various types of human tissues with long half-lives, such as collagen (9) and lens crystalline (10).

Vitamin C (ascorbic acid) is an antioxidant substance soluble in water which keeps most of metal cofactors in reduction status. In physiological conditions it appears in the form of dehydroascorbic acid and can react with amino groups and form schiff bases. Its further oxidation

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product, called diketogulonic acid shows the same reaction (11). Several studies have shown that vitamin C affects on glycation of proteins, for example in a research Akhilender showed that all forms of vitamin C can cause decrease in the process of glycation of various proteins such as collagen (12). Also, Davie et al (13) and Oian et al (14) reported that oral vitamin C inhibited the glycosylation of proteins. It seems that due to structural similarity to glucose, ascorbic acid can be replaced with glucose and affects the glycation of proteins. considering the importance of vitamin C on metabolism of different biomolecules such as proteins, it seems that the study of the effect of this substance on the process of different reactions of these molecules including glycation should be deeply taken into consideration. In this research the effects of different concentrations of vitamin C on in vitro albumin glycation have been studied.

Experimental

Materials

Ascorbic acid and all other chemical materials have been obtained from Merck company (Germany) and Sigma company (USA).

Methods

Test method Preparation and characterization of glycated albumin The glycated albumin was prepared according to the method of Monnier et al (15), with minor modification. Briefly, 0.15 g/ml of bovine serum albumin was dissolved with 0.1 M D-glucose in 0.3 M sodium phosphate buffer (pH=7.4). This solution was then sterilized by Millipore filtration (pore size 0.45 μ m) and incubated at 37°C for 72 h in room temperature in constant position. Unmodified bovine serum albumin (BSA) was prepared by incubation in 0.3 M sodium phosphate buffer without any D-glucose for 72 h. The samples were dialyzed against excess 0.01 M PBS (pH=7.4) to remove free glucose, and then applied to an endotoxin-absorption column (Pyrosep, Daicel Chemical, Japan). The column was equilibrated with 0.01 M PBS. 5 mg/ml of the samples were applied onto the column, and then the elution was begun at a flow rate of 0.4

ml/min. The fraction was monitored at 280 nm in a spectrophotometer.

Measuring the level of albumin glycation For approving the albumin glycation TBA test was used (16), as follows 1 ml of 20% TCA was added to the above solution and then centrifuged for 10 minutes at 300 rpm. The supernatant was discharged. This process was carried out twice. 1 ml phosphate buffer with the above specification and 0.5 ml 0.3 N oxalic acid were added to the sediment and put in boiling conditions in water bath. After the compound got cold to the lab temperature, 0.5 ml of 40% TCA was added to each sample. After centrifuging for 10 min at 3000 rpm, the supernatant was separated and 0.5 ml 5% M TBA was added to 1 ml of supernatant solution; then the whole was set in 40 degree water bath for half an h. At the end, the absorbance of the sample was measured at 443 nm. The colorimetric method with TBA is based on the hydrolysis of the glycated proteins using oxalic acid at 100°C yielding 5-hydroxymethyl furfural (5-HMF) which react with TBA. The absorbance was measured at 443nm. 5-HMF was used as standard and glycation of albumin was calculated and expressed as μ mol HMF per mg protein.

Preparing the stock solution of vitamin C vitamin C solution was prepared with the concentration of 1 mol/L in bidistilled water and used as stock solution.

Determining the effect of ascorbic acid on albumin glycation comparative studies between the effects of vitamin C on glycated albumin were performed according to the Sakat et al (17). After incubation of glycated albumin with the various concentration of vitamin C (0.1, 1 and 10 μ mol/L) at 37°C for 72 h, TBA method was carried out.

All of the experiment stages for each concentrations of ascorbic acid were done as triplet and for getting acceptable result the test was repeated.

Results and Discussion

In this research the effects of the various concentrations of ascorbic acid on albumin glycation were studied. Various concentrations of ascorbic acid (0.1, 1 and 10 μ mol/L) were

Table 1. Glycation of albumin ($\mu\text{mol HMF}/\text{mg protein}$) in presence of 100 mM glucose and in different incubation time at 37°C

Glucose	Time of incubation		
	24 h	48 h	72 h
0 (control)	5.02±0.46	21.9± 1.88	23.29± 1.92
100	31.13± 1.77*	53.23± 2.37*	123.25± 7.8*

The data are mean±SD of three separate triplicate experiments.

* $p < 0.05$ compared to the relevant control.

used in the test and using TBA method the level of albumin glycation was determined. The level of glycation of albumin was measured and the results are summarized in Table 1. As this table shows the albumin was glycated in the presence of glucose and the level of glycation was depended on time of exposure with glucose ($p < 0.05$). The results showed that vitamin C had potential inhibitory effect on albumin glycation, so that the concentrations 0.1, 1 and 10 $\mu\text{mol}/\text{L}$ had the inhibitory effects of 86.5%, 81.4% and 71.7% respectively (Table 2). The interaction of the different concentrations of vitamin C on inhibiting albumin glycation reaction showed that comparing concentrations of vitamin C in pairs would reveal each pair (0.1, 1 and 10 $\mu\text{mol}/\text{L}$) had statistically significant difference (Table 3).

This study confirmed that vitamin C affects the glycation of proteins. Vitamin C is a compound having two ionizable -OH group ($\text{pK}_{a1} = 4.25$, $\text{pK}_{a2} = 11.8$) that usually appears in the form of ascorbate anion in physiologic pH. Because of having carbonyl groups, it shows strong reaction with amino groups and consequently strongly reacts with amino groups of proteins (11). The mechanism of the effects of vitamin C on protein glycation is very complex. Some of the previous observations have shown that vitamin C can compete with glucose for binding to proteins and there by inhibit glycation of them. Also diketogulonic acid form can react with proteins and form

ketamine (7, 18). According to Price, Hunt and Khatami's findings vitamin C in different conditions $< 80\%$ inhibits protein glycation (19-21). Davie et al showed that vitamin C could also inhibit hemoglobin protein glycation (13). Also, Kim et al showed that glycated albumin induced cell death in cultured bovine retinal pericytes and antioxidants such as ascorbic acid reduced this cytotoxicity (22). We reported previously that vitamin C at concentrations of 0.1, 1 and 10 $\mu\text{mol}/\text{L}$ had the inhibitory action on cytotoxic effect of glycated albumin-metal ion transition system on rat hepatocytes, and at concentration 10 $\mu\text{mol}/\text{L}$ showed higher effect (23). Considering the fact that the concentrations of vitamin C used in the research are the same as vitamin C concentrations existing in plasma the inhibition of plasma albumin glycation reaction may confirm the possibility that we can use vitamin C for the treatment of diabetes mellitus. Because some of the chronic complications occurring in diabetes are thought to be due mainly to the further reactions of glycated proteins, using the inhibitors could have positive effects in reducing chronic complication occurring in diabetes (24, 25). The evidence have shown glycation is also involved in the normal process of aging (26) therefore long term vitamin C supplementation may even slow down this natural process.

In conclusion, this study has shown that vitamin C reduced the glycation of short-lived proteins such as albumin. Competition of vitamin

Table 2. Comparing the effect of vitamin C on inhibiting albumin glycation reaction in terms of different concentrations*

Vitamin C concentration	Level of inhibiting of albumin glycation (mean±SD)	Standard error of mean	F value	P value*
10 $\mu\text{mol}/\text{L}$	86.55±4.84	2.79	F= 12.771 (2,6)	P= 0.007 Significant
1 $\mu\text{mol}/\text{L}$	81.40±1.75	2.31		
0.1 $\mu\text{mol}/\text{L}$	71.69±3.68	1.01		

* One way analysis of variance

Table 3. Comparison of the different concentrations of Vitamin C in pairson inhibiting albumin glycation reaction in terms of different concentrations*

Comparing concentration (1)	Comparing concentration (2)	difference of (mean±SD)	P value*
VitaminC (10 µmol/L)	VitaminC (1 µmol/L)	-14.85±2.98	Significant P=0.04
VitaminC (10 µmol/L)	VitaminC (0.1µmol/L)	-9.7±2.98	P=0.04 Significant
VitaminC (1 µmol/L)	VitaminC (0.1µmol/L)	5.14±2.98	P=0.272 Non-Significant

* Tukey HSD test

C with glucose for reaction with protein amino groups appears to be the main explanation for this effect. However, further work is required to investigate in vivo reaction of vitamin C with protein amino groups. We suggest to perform these tests in vivo in order to specify whether vitamin C can be used therapeutically to delay or prevent the chronic complications occurring in diabetes.

These findings showed that vitamin C inhibited the reaction of albumin glycation and probably decreased complications occurring in diabetes

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