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Effects of Short-Term Streptozotocin-Induced Diabetes and Vitamin C on Platelet Non-Enzymatic Glycation

Saime Batırel[a] Ayşen Yarat[b] Nesrin Emekli[b]

[a]Medical Faculty, Istanbul Medipol University, and [b]Department of Biochemistry, Dentistry Faculty, Marmara University, Istanbul, Turkey

Key Words
Diabetes · Streptozotocin · Vitamin C · Platelet · Glycation

Abstract
Diabetes mellitus is one of the most prevalent metabolic syndromes worldwide. Glycation, a chemical modification of proteins with reducing sugars, indicates a possible explanation for the association between hyperglycemia and the wide variety of tissue pathologies. Non-enzymatic glycation (NEG) of platelet proteins is one of the key mechanisms in the pathogenesis of diabetic complications and may be significant in diabetic atherothrombosis. The aim of this study was to investigate the effects of streptozotocin (STZ)-induced short-term experimental diabetes on the glycation of platelets and to find out if vitamin C affected this glycation. A total of 40 male Wistar albino rats, 200–250 g, were randomly divided into 4 groups (2 diabetic and 2 control groups). The diabetic groups were made diabetic by intraperitoneal injection of STZ (65 mg/kg, citrate buffer pH 4.5). By daily intraperitoneal injection, 80 mg/kg vitamin C (Roche, Turkey) was administered until the end of the experiment. Blood glucose levels of the diabetic groups were significantly higher than those at day 0 and also higher than those of the non-diabetic control groups. The changes in total protein, NEG and vitamin C levels were not statistically significant. Although the differences among the groups were not statistically significant, vitamin C administration increased NEG levels in the diabetic group. The results of this study demonstrate that 8 days of STZ-induced short-term diabetes did not cause a significant increase in NEG of platelets. However, the effect of vitamin C on platelet NEG needs to be further investigated.

Introduction

Diabetes mellitus, one of the most prevalent metabolic syndromes worldwide, is characterized by hyperglycemia resulting in short-term metabolic changes in lipid and protein metabolism and in long-term irreversible vascular and connective tissue changes. These changes include diabetes-specific complications such as retinopathy, nephropathy and neuropathy, as well as macrovascular complications such as atherosclerosis, potentially resulting in heart disease, stroke and peripheral vascular disease [1]. Links between chronic hyperglycemia and the development of long-term diabetes-specific complications have been discovered but are not yet completely understood [1, 2].

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Prof. Dr. Nesrin Emekli, PhD
Department of Basic Sciences, Faculty of Dentistry
Marmara University, Nisantasi
TR–34365 Istanbul (Turkey)
Tel. +90 212 231 9120/111, Fax +90 212 246 5247, E-Mail nemekli@marmara.edu.tr

Glycation, a chemical modification of proteins with reducing sugars, indicates a possible explanation for the association [3, 4] between hyperglycemia and the wide variety of tissue pathologies. Research suggests that reducing sugars can react with the amino groups of long-lived proteins to produce non-enzymatic cross-links [5]. Formations of these cross-links occur as end stage products of the Maillard reaction; they are known as advanced glycation end products (AGEs) [3, 6].

AGEs are a class of complex, often unstable, reactive compounds formed in excess during aging and diabetes mellitus [5]. According to the 'glycation hypothesis', accumulation of AGEs alters the structural properties of tissue proteins and reduces their susceptibility to catabolism. It has been shown that the process of AGE formation is accelerated by hyperglycemia. Some of the protein alterations observed in diabetic patients resemble those in much older, non-diabetic patients, suggesting 'diabetes-induced early aging' [3–6].

Growing evidence suggests that platelets of diabetic patients are larger and hyperreactive, showing increased adhesion and aggregation, as well as increased platelet-dependent thrombin generation [7]. Several mechanisms may account for the increased platelet reactivity in patients with diabetes. Non-enzymatic glycation (NEG) of platelet proteins is one of the key mechanisms in the pathogenesis of diabetic complications and may be significant in diabetic atherothrombosis [8].

Vitamin C, the antiscorbutic vitamin, is found in a large variety of foods but particularly in fruits and vegetables. The 2 major forms, L-ascorbic acid and dehydro-L-ascorbic acid, are interconvertible through an oxidation reduction system which represents the fundamental basis for many of the biomedical roles of the vitamin [9].

Vitamin C is actively taken up in high concentration by secretory cells of the islets of Langerhans where it is believed to play a role in antioxidant defense and as an electron donor in the post-translational enzymatic peptidyl α-amidation of islet peptides including amylin and pancreatic polypeptide [10, 11]. In diabetes mellitus, the vitamin C metabolism is abnormal, and subjects have been shown to have low vitamin C and high dehydro-L-ascorbic acid concentrations in plasma [12].

The pathogenesis of atherosclerosis in diabetes has several potential contributors, which include increased intravascular thrombin generation and reduced fibrinolytic potential. Endothelial injury or plaque rupture with platelet adhesion and aggregation at the site of injury may be the critical event in producing morbidity and mortality from atherogenesis because most coronary events occur with less than one-third narrowing of the vessel lumen. Therefore, platelets may assume an important role in the signal event in atherosclerosis in diabetes [13, 14].

Consequently, the aim of this study was to investigate the effects of streptozotocin (STZ)-induced short-term experimental diabetes on the glycation of platelets and to find out if vitamin C affected this glycation.

Materials and Methods

A total of 40 male Wistar albino rats, 200–250 g, were randomly divided into 4 groups (2 diabetic and 2 control groups). They were kept at a constant temperature (22 ± 1°C) with 12-hour light and dark cycles. The diabetic groups were rendered diabetic at day 0 by intraperitoneal injection of STZ (65 mg/kg, citrate buffer pH 4.5) [15]. Two days after the STZ injection, blood glucose levels were determined. Rats with a glucose level <150 mg/dl were discarded from the experiment. After diabetes induction, 1 rat of the control and the diabetic group, respectively, was given 80 mg/kg vitamin C (Roche, Turkey) by daily intraperitoneal injection until the end of the experiment on day 8. Saline solution was given to the rest of the control and diabetic groups intraperitoneally. On day 8, cardiac blood samples were taken from all rats under ether anesthesia. All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Istanbul University, Institute of Experimental Medicine.

Blood glucose was measured by the Randox glucose kit (Randox, GL3981, UK).

Blood samples were centrifuged for 5 min at 123 *g*, and platelet-rich plasma was separated from the supernatant. Platelet-rich plasma was further centrifuged for 10 min at 492 *g* for the precipitation of the platelets, and the supernatant was used for the determination of vitamin C and glucose levels. The precipitate (platelet pellet) was washed 3 times with physiologic saline and then further diluted with 2 ml physiologic saline. This suspension was preserved for platelet count, glycosylation and protein determination. Platelets were counted using phase contrast microscopy [16].

NEG Assay

NEG of the platelet suspensions was assessed by the 2-thiobarbituric acid method [17]. The latter involved hydrolysing each 0.5-ml homogenate with 0.5 ml of 0.5 M oxalic acid in an autoclave for 1 h at 124 ± 1°C. To this, 0.5 ml 40% trichloracetic acid (w/v) was added, mixed, centrifuged at 1,500 *g* for 10 min and filtered using filter paper. Absorbance at 443 nm was recorded. Then, 0.75 ml of supernatant was incubated in 0.25 ml of 0.05 M 2-thiobarbituric acid at 37°C for 30 min. After standing for 15 min at room temperature, absorbance was again measured at 443 nm, and the differences between the first and second absorbances were calculated. The protein glycation values were expressed as nmol of fructose/mg protein. NEG values were also divided by the platelet cell counts in order to obtain the NEG/platelet ratio. Commercial fructose (Sigma, St. Louis, Mo., USA) was used as a standard.

Table 1. Mean levels of body weight and blood glucose at the end of 8 days

	Control (n = 10)	Control + vit C (n = 9)	Diabetic (n = 10)	Diabetic + vit C (n = 11)	p value (ANOVA)
Weight, mg	243.9 ± 36.2	243.3 ± 38.3	202.8 ± 39.7*	194.7 ± 36.2*	0.0086
Blood glucose, mg/dl	149.7 ± 19.4	124.8 ± 28.7	254.1 ± 115.2*	247.2 ± 101.8*	0.0011

Values are given as the mean ± SD. vit C = Vitamin C. * $p < 0.01$, significantly different from the control and control + vitamin C groups.

Table 2. NEG values of the platelet suspensions obtained from the 4 groups

	Control (n = 10)	Control + vit C (n = 9)	Diabetic (n = 10)	Diabetic + vit C (n = 11)	p value (ANOVA)
NEG, nmol fructose/mg protein	8.88 ± 4.92	5.84 ± 2.36	6.15 ± 3.91	10.88 ± 7.9	0.132
NEG/platelet, nmol fructose/mg protein/n platelets	1.1 ± 0.45	1.12 ± 0.57	1.16 ± 0.73	1.69 ± 1.04	0.226

Values are given as the mean ± SD. vit C = Vitamin C.

Protein Assay

Total protein levels of the platelet suspensions were determined according to the method of Lowry et al. [18]. In alkaline medium, proteins are reacted with cupper ions and then are reduced by folin reactive (phosphomolybdic-phosphotungstic acid). The absorbance of the blue-colored product at 500 nm was evaluated. Bovine serum albumin was used as a standard. The total protein level was expressed as % mg. Total protein levels were divided by the number of platelet cells in 1 mm^3 homogenate in order to obtain the protein/platelet ratio.

Determination of Vitamin C Levels

Vitamin C levels were determined by the dinitrophenylhydrazine method based on the production of a derivative of dehydroascorbic acid (DHAA) with dinitrophenylhydrazine to measure the DHAA content, and indirectly, to measure total ascorbic acid after the native ascorbic acid has been oxidized to DHAA [19, 20].

Statistical Analysis

Statistical analysis was carried out with the InStat statistics program using analysis of variance (ANOVA) and Student's t test. p values <0.05 were regarded as significant.

Results

The mean levels of the blood glucose and body weight for the 6 groups at the end of 8 days are shown in table 1. Prior to inducing diabetes (day 0), the groups were checked for differences in weight and blood glucose, but none were found. After 8 days of diabetes, the blood glucose levels of the diabetic groups were significantly higher than those at day 0 and also higher than those of the non-diabetic control groups. Vitamin C administration did not decrease the blood glucose level in the diabetic group (table 1).

Also, after 8 days of diabetes, body weights of the rats in the diabetic groups decreased significantly compared with those at day 0 and also with those of the non-diabetic control groups (table 1).

Total protein levels in the platelet suspensions were divided by the number of platelets in 1 mm^3 homogenate: they were found to be 0.144 ± 0.06, 0.182 ± 0.04, 0.202 ± 0.12 and 0.179 ± 0.11 mg/number of platelets in the control, control + vitamin C, diabetic and diabetic + vitamin C group, respectively. Differences between the groups were not statistically significant.

Vitamin C levels of the control, control + vitamin C, diabetic and diabetic + vitamin C group were 1.22 ± 0.34, 1.35 ± 0.38, 1.14 ± 0.51 and 0.99 ± 0.31 mg/dl, respectively. The mean vitamin C level of the diabetic + vitamin C group was lower than that of the control + vitamin C group; however, this difference was not statistically significant.

NEG levels of the platelet suspensions obtained from the 4 groups are given in table 2. Although the differences among the groups were not statistically significant,

vitamin C administration increased NEG levels in the diabetic group. NEG/platelet ratios are also given in table 2. Similarly, vitamin C administration insignificantly increased the NEG/platelet ratio in the diabetic group.

Discussion

The pathogenesis of diabetic complications continues to be a central issue in current diabetes research [2]. Type 2 diabetes mellitus increases atherothrombotic risk. Platelets in individuals with diabetes show increased activity at baseline and in response to agonists, ultimately leading to increased aggregation. The primary cause of mortality in the majority of patients with diabetes is atherothrombosis. Platelet hyperactivity (in combination with abnormalities in coagulation and fibrinolysis), which is characteristic in diabetes, undoubtedly is a contributing factor as platelets play a pivotal role in initiating and sustaining thrombi within vessels [21, 22].

Acute in vivo hyperglycemia results in platelet activation in type 2 diabetes. Furthermore, a link between hyperglycemic spikes and the incidence of ischemic events has been demonstrated [23]. Hyperglycemia also causes NEG of platelet membrane proteins resulting in changes in protein structure and conformation, as well as in alterations of membrane lipid dynamics [24]. This in turn can lead to enhanced expression of certain crucial platelet receptors, for instance, P-selectin and glycoprotein IIb/IIIa, thus altering platelet activity [25].

In the present study, we aimed to investigate the NEG of platelets in short-term acute hyperglycemia. Moreover, we aimed to investigate if vitamin C administration affected the level of this glycation in STZ-induced experimental diabetes. At the end of the 8-day experimental period, no significant difference was found among the 4 groups; however, the vitamin-C-administered diabetic group presented the highest NEG value, as well as the lowest plasma vitamin C levels.

Several studies have demonstrated that diabetes is associated with abnormalities in plasma vitamin C levels and ascorbate status [26, 27]. The loss of vitamin C in diabetes has been postulated to be due to the increased oxidative stress associated with the disease [28]. Benefits of vitamin C supplementation in humans with type 2 diabetes have been described. Additionally, it has been proposed that dietary measures to increase plasma vitamin C may be an important strategy to counter diabetes [29]. Clearly, vitamin C has several important biological functions that may be of benefit in these situations. It is conceivable that one of these roles is the maintenance of the functional integrity of biologically active proteins, such as insulin by inhibition of glycation [30].

However, in the literature, there are contradictory results on the effect of vitamin C on glycohemoglobin levels. Weykamp et al. [31] suggested that supplementation of non-diabetics with 750 or 1,500 mg of vitamin C daily for 12 weeks does not cause any interference with glycohemoglobin determinations by HPLC, electrophoresis, affinity chromatography or immunoassay, and does not reduce in vivo hemoglobin glycation. On the other hand, Ely [32] reported antagonism of hemoglobin glycation by ascorbic acid in animals and humans. To our knowledge, the effect of vitamin C administration on platelet glycation has not been studied before. Platelet hyperaggregability may be involved in the pathogenesis of diabetic micro- and macroangiopathy, and vitamin C has been suggested to reduce platelet aggregation [33]. Moreover, NEG of platelet membrane proteins has been reported to change platelet activity [25].

Based on the results of this present study, we may suggest that diabetes induced by STZ for 8 days did not cause a significant difference in terms of platelet NEG. However, among the 4 groups, the vitamin-C-administered diabetic group presented the highest NEG value. Accordingly, the oxidation of ascorbic acid has been reported to lead to the formation of several compounds which are capable of reacting with protein amino groups via a Maillard reaction [34]. The reaction by which ascorbic acid causes glycation and cross-linking of lens proteins displays a rigid requirement for the presence of oxygen and is inhibited by the presence of glutathione. Oxygen is required to oxidize ascorbic acid to DHAA and other products, i.e. the active glycating species [35].

In conclusion, although acute in vivo hyperglycemia results in platelet activation in type 2 diabetes, the results of this study demonstrate that 8-day STZ-induced short-term diabetes did not cause a significant increase in NEG of platelets. However, the effect of vitamin C on platelet NEG needs to be further investigated.

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