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Effect of Vitamin C Supplementation on Platelet Aggregation and Serum Electrolytes Levels in Streptozotocin-Induced Diabetes Mellitus in Rats

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Summary: Diabetes mellitus (DM) is a disease condition characterised by hyperglycemia; free radical and abnormal haematological indices. Vitamin C can reduce free radical generation and ameliorate adverse conditions of diabetes mellitus. The aim of the present study is to investigate the effect of vitamin C on platelet aggregation and electrolyte levels in Type 1 DM. Male Wistar rats were divided into four groups namely control, DM, DM +Vitamin C and Vitamin C groups. Rats were made diabetic with a single dose of streptozotocin (65 mg/kg) intraperitoneally. Vitamin C was administered orally to diabetic and normal rats at 200 mg/kg body weight for 28 days. Blood samples were analyzed for hematological parameters, platelet aggregation, and serum electrolyte levels. Blood glucose in DM+ Vitamin C group (9.9 ± 1.8 mmol/L) was significantly reduced ($p<0.01$) compared to DM group (32.2 ± 2.1 mmol/L) and significantly higher ($p<0.05$) than control (4.4 ± 0.8 mmol/L). Haemoglobin (Hb) concentration in DM group (12 ± 0.1 g/dL) was significantly reduced ($p<0.01$) when compared with control groups (14 ± 0.24 g/dL) and significantly increased ($p<0.05$) in the DM+vitamin C group (13.5 ± 0.5 g/dL) compared with the diabetic group. The mean corpuscular volume values in DM (68.66 ± 0.5 fL) and DM+vitamin C groups (68.11 ± 0.4 fL) were significantly higher ($p<0.01$) than the control (59.49 ± 0.5fL). Platelet count in DM group (523 ± 8.5 $x10^9$/L) was significantly raised ($p<0.01$) when compared to control (356 ± 6.2 $x10^9$/L) and significantly reduced ($p<0.01$) in DM+ vitamin C-treated group (385 ± 7.8 $x10^9$/L) compared with DM group. Platelet aggregation and serum sodium/potassium ratios was significantly reduced ($p<0.01$) in DM+vitamin C compared with DM group. These results suggest that oral vitamin C administration increases haemoglobin, reduced plasma glucose level, platelet count, serum sodium/potassium ion ratio and inhibits platelet aggregation in streptozotocin-induced DM in rats.

Keywords Diabetes mellitus, electrolytes, Haematological parameters, Platelet aggregation, Red cell indices, Vitamin C

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INTRODUCTION

Diabetes mellitus (DM) is one of the most common non-communicable diseases in the world (Ramakrishna and Jailkhani (2007) with a global prevalence of 8.8%. It is a disease that is characterized by vascular smooth muscle and endothelial dysfunction. Endothelial dysfunction plays an important role in the pathophysiology of atherosclerosis, leukocyte adhesion, endothelium platelet aggregation and vascular smooth muscle proliferation (Browne *et al*., 2003; Skrha *et al*., 2007). Haematological complication is a notable feature of diabetes mellitus and consists mainly of abnormalities in the function, morphology and metabolism of various blood cells (Comazzi *et al*., 2004).

Anaemia in DM is associated with erythropoietin deficiency and can occur early in diabetic neuropathy before the onset of advanced renal failure (Bosman *et al*., 2001). Abnormalities in fluid and electrolytes balance are common biochemical findings in DM (Obineche *et al*., 2006) and a reduction in plasma sodium and chloride ions in diabetic patients have been documented (Onwuliri *et al*., 2004). This is probably attributed to either loss, reduced intake/absorption or alterations in metabolism in diabetic condition. Vitamin C intake has many important biological functions such as increasing the white blood cell count and function (Iqbal *et al*., 2004, Hall *et al*., 2011), reducing arterial blood pressure and improving arterial stiffness in patients with type 2 diabetes (Mullan *et al*., 2002). Vitamin C has no adverse effect on serum electrolyte and may protect against atherosclerosis and hypertension (Eteng *et al*., 2006). Ascorbic acid supplementation increases hemoglobin concentration hematocrit level, red blood cell (RBC) count, serum and leucocyte ascorbate concentrations (Jaja *et al*., 2002) and primary defence

mechanisms against oxidative stress in DM (Alsaif, 2009).

DM is also associated with increased ex-vivo platelet aggregation and hypercoagulability of platelets (Skowasch *et al*., 2009), decreased haemoglobin concentration and anaemia (Ritz, 2006; Hasslacher *et al*., 2010). Vitamin C supplementation has been reported to improve erythropoietic activity, ascorbic acid status and electrolyte levels in healthy condition. Common sources of vitamin C are vegetables and fruits. However, data is lacking on the effect of vitamin C on haematological indices and electrolyte balance in diabetic condition. The purpose of this study was to determine the effects of vitamin C on haematological and serum electrolyte parameters in Type 1 diabetic condition. We hypothesized that administration of vitamin C would improve haematological indices, reduce platelet aggregation and restore electrolyte imbalance in Type 1diabetic rats.

MATERIALS AND METHODS

Chemicals

Vitamin C (L-ascorbic acid), streptozotocin, ethylenediamine tetra acetic acid (EDTA), dipotassium monophosphate, monopotassium diphosphate salts were obtained from Sigma Chemical Company, while formalin was purchased from BDH, Poole, UK. All the chemicals used were of pure analytical grade and prepared in deionised distilled water except streptozotocin which was dissolved in citrate buffer.

Experimental animals

A total of 24 male Wistar rats weighing between 170 to 180 g obtained from the animal house of Department of Physiology, College of Medical Sciences, University of Calabar, Calabar, Nigeria, were used for the study. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health after ethical approval was obtained from the Faculty of Basic Medical Sciences Animal Research Ethics Committee. The animals were randomly assigned into four groups of six rats namely control, DM, DM + vitamin C and vitamin C groups. They were kept in cages at room temperature of 29 ± 2ºC with a 12 hours light/dark cycle and had free access to water and rat chow.

Induction of diabetes mellitus

Type 1 DM was induced in two groups of experimental rats by intraperitoneal injection of streptozotocin (STZ) dissolved in citrate buffer (pH 4.5) at a single dose of 65 mg/kg body weight. Weight and age-matched control rats were injected with the citrate buffer. Blood glucose level and body weight were measured prior to STZ injection using an automated glucose analyzer (glucometer Acucheck mini plus, Roche, Germany) and weight balance respectively. DM was confirmed 48 hours after STZ injection in animals by the presence of blood glucose level greater than 10 mmol/L and glucosuria using clinistix (Bayer Diagnostics, Mannheim, Germany). Laboratory investigations were carried out in all groups of the animal after four weeks of diabetes mellitus induction. Vitamin C was administered orally to DM + Vit C and Vitamin C groups at a dose of 200 mg/kg body weight (Owu *et al*., 2006, 2012) orally for 28 days while rats in control and diabetic groups received placebo.

Collection of blood samples

Cardiac blood samples from all groups of animals were obtained for haematological analysis. Blood sample from each animal was collected into ethylenediamine tetraacetic acid (EDTA) tube and well-labeled non-heparinized sample tube. The former was used for blood cell count while the latter sample was allowed to stand for 3hours in iced water and centrifuged at 10,000g for 10 minutes. The serum was collected and stored at -20ºC until use. The RBC count, white blood cells count (WBC), packed cell volume (PCV), and hemoglobin concentration were determined using automatic blood cell counter (Hematology analyser KX-21N Sysmex, Deutschland GMBH, Germany).

Determination of platelet aggregation

From the blood sample drawn into the non-heparinized sample tubes, 0.25 ml of each sample was added to the 4.5 ml of buffered EDTA solution and 4.5 ml of buffered EDTA/formalin solution respectively. The blood was thoroughly mixed with each solution to give a volume dilution of 1:20. The samples were centrifuged at 200 g for 10 minutes using MSE centrifuge to obtain platelet-rich plasma (PRP) which was then used for platelet count. Platelets in the platelet rich plasma samples were counted using the light optical microscope and a hemocytometer following standard laboratory procedure. The counting chamber was the improved Neubauer and the power of magnification used was 40x. All counting was done within two hours after centrifuging the samples. Platelet aggregation was determined using the method of Wu and Hoak (1974) by finding the ratio of platelet count in buffered EDTA solution with the platelet count in Buffered EDTA/formalin solution. Serum sodium and potassium were estimated using flame photometer (Corning 410) while chloride ion (Cl^-) was estimated by mercuric thiocynate method using dialab kit. Bicarbonate ion (HCO_3^-) level in serum was determined using the titration methods.

Statistical analysis

The results obtained are expressed as mean ± Standard error of mean (SEM) and analysed using GraphPad Prism software version 5 (GraphPad Software, San

Diego, California, USA). One way analysis of variance (ANOVA) was used to compare means followed by Tukey's multiple comparison tests where F-value was significant. In all cases, *p* value less than 0.05 was considered statistically significant.

RESULTS

Blood glucose and body weight

Type 1 DM rats exhibited a significant ($p<0.01$) increase in blood glucose level compared with control (Table 1). Type 1 DM rats treated with vitamin C for four weeks showed a significant ($p<0.01$) reduction in blood glucose (9.9 ± 1.8 mmol/L) when compared to DM rats (32.2 ± 2.1 mmol/L) though the value was significantly ($p<0.05$) higher than control (4.4 ± 0.8 mmol/L). Vitamin C administration to normal rats did not alter the blood glucose level (4.6 ± 1.2 mmol/L) compared with normal control. There was a significant ($p<0.01$) decrease in final body weight in Type 1 DM rats (185 ± 5 g) when compared to the control (220 ± 8 g). The body weight of animals in DM + Vit C group (210 ± 8 g) was not significantly different from that in control and Vit C group (225 ± 6 g).

Blood cell counts

The haematological indices of the animals in control and DM+Vit C groups are presented Table 1. The mean red blood cell (RBC) count in DM +Vit C group (6.9 ± 0.44 $x10^{12}$/L) was comparable to the control group (7.9 ± 0.22 $x10^{12}$/L). The mean WBC counts in the control was 5.3 ± 0.93 cells/10^9/L and it was 5.9 ± 0.68 cells/10^9/L in DM group. In DM + Vit C group the WBC count was 3.8 ± 0.16 cells/10^9/L while it was 4.8 ± 0.77 $x10^9$/L in Vit C group. There was no significant difference in the WBC counts in DM group when compared to control. The platelet counts in DM group (523 ± 8.5 $x10^9$/L) was significantly ($p<0.05$) raised when compared with control (356 ± 6.2 x 10^9/L) and DM+Vit C group (385 ± 7.8 x 10^9/L). However, Vitamin C administration to diabetic group resulted in a significant ($p<0.05$) reduction in platelet count (385 ± 7.8 $x10^9$/L) when compared with the diabetic group. Administration of vitamin C did not cause any alteration in platelet count in Vit C group (369 ± 10.9 $x10^9$/L) when compared with control.

Haematocrit and haemoglobin concentration

The mean values of haematocrit and haemoglobin are presented in Table 1. The haematocrit values in Type 1 DM groups were similar to the values of the control group. Administration of vitamin C did not alter the haematocrit values when compared to the control group. The mean haemoglobin (Hb) concentration in the Type 1 DM group (12.0 ± 0.1 g/dL) was significantly reduced ($p<0.01$) when compared to control groups (14.0 ± 0.24 g/dL) whereas administration of vitamin C significantly improved ($p<0.05$) the Hb concentrations in the DM + vitamin C-treated groups (13.5 ± 0.5 g/dL) when compared to the DM group. There was a significant decrease ($p<0.01$) in mean corpuscular haemoglobin concentration (MCHC) in DM group when compared to the control group. However, vitamin C produced a significant increase ($p<0.05$) in MCHC value in DM + Vit C diabetic rats. The mean corpuscular volume (MCV) values in both DM and DM + Vit C-treated groups were significantly higher ($p<0.05$) than the control and vitamin C.

Platelet aggregation

In order to determine the extent of platelet aggregability in Type 1 DM, platelet aggregation was determined and the result is shown in Fig. 1. A significant decrease ($p<0.05$) in the platelet aggregation as depicted by high aggregation ratio was observed in DM + Vit C group when compared with control and DM groups. No significant difference was observed between the DM and control groups.

Electrolytes levels

Table 2 shows the mean serum levels of sodium, potassium, chloride and bicarbonate level. A significant increase ($p<0.05$) in serum level of sodium ion was recorded in the two diabetic groups compared to the control. Likewise, a significant increase

Table 1: Mean values of haematological indices and body weights in control and diabetic rats treated with vitamin C

Parameter	Control	DM	DM + Vit C	Vitamin C	F ratio
Body weight (g)	220 ± 8	185 ± 5**	210 ± 8	225 ± 6	6.7
Blood glucose (mmol/L)	4.2 ±0.7	32.2± 2.1**	8.9 ± 1.8†	4.5 ± 1.1	76.1
RBC count (□□10^{12}/L)	7.9 ± 0.22	6.7±0.68	6.9 ±0.44	7.1 ± 0.34	1.35
WBC count (□□10^9/L)	5.3 ±0.93	5.9 ±0.68	3.8 ±0.16	4.8 ± 0.77	1.62
Hb concentration (g/dl)	14 ±0.24	12 ±0.1**	13.5 ± 0.5a	13.8 ± 0.2	9.20
Haematocrit (%)	47 ± 1.1	46 ± 1.3	47 ± 1.6	47 ± 0.7	0.17
Platelet ($x10^9$/L)	356 ± 6.2	523±8.5**	385 ± 7.8†	369 ± 10.9	82.6
MCV (fL)	59.49±0.5	68.66±0.5**	68.11±0.4**	66.19±0.5*	71.1
MCHC (%)	29.78±0.8	26.09±0.5**	28.76±0.7 a	29.36±0.5	6.75

Results are expressed as mean ± standard error of mean, * = $p<0.05$ versus control; **=$p<0.01$ vs. control; † =$p<0.01$ versus DM group; a= $p<0.05$ versus DM; n= 6 in each group.

Table 2: Effect of oral administration of vitamin C on electrolyte levels of diabetic and control rats

Parameter	*Control*	*DM*	*DM + Vit C*	*Vitamin C*
Sodium ion (mmol/L)	127.3 ± 1.9	136.2 ±1.1*	138.4 ± 0.9*†	129.5 ± 2.1
Potassium ion (mmol/L)	4.7 ±0.8	3.5 ± 1.1	7.3 ± 1.1†	5.6 ± 0.4
Chloride ion (mmol/L)	106.0 ± 0.8	106.8 ±0.5	110.3 ± 3.1	102.7 ± 2.2
Bicarbonate (mmol/L)	21.3 ± 1.2	24.8 ± 1.2	16.8 ± 2.2*†	19.7 ± 1.2
Na^+/K^+ ratio	27.1± 1.1	38.9± 1.3	18.9± 0.9*†	23.1± 1.2*†

Results are expressed as mean ± standard error of mean. *=p<0.05 diabetic group compared with control, *=p<0.01 diabetic group compared with control † = p<0.05 DM + Vitamin C- group compared with DM group, n = 6 in each group.

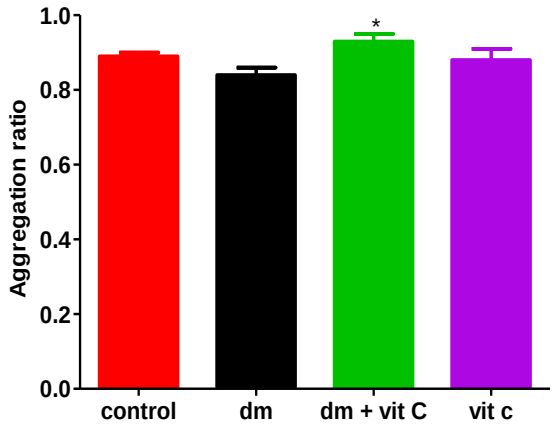

Figure 1: Platelet aggregation ratio in diabetes mellitus group and control treated with vitamin C. * = p< 0.05 compared to DM and control

(p<0.01) in potassium ion level was noted in the DM + Vit C group when compared with DM. The DM + Vit C group had a significantly lower (p<0.05) serum bicarbonate ion level than the DM and control groups. The mean serum chloride (Cl^-) levels were comparable in all groups of animals. The serum sodium/potassium ratio was significantly raised (p<0.05) in DM group when compared with the control. However, administration of vitamin C significantly reduced (p<0.05) this ratio in both groups supplemented with vitamin C when compared with DM and control.

DISCUSSION

The effect of vitamin C-oral administration on some haematological parameters was investigated in Type 1 diabetic male Wistar rats. Streptozotocin was used to induce Type 1 DM, a specific cytotoxic drug that destroys the insulin producing cells in the islets of Langerhans of the pancreas resulting in hyperglycaemia and loss of body weight. The decrease in body weight after diabetic induction is expected since this is one of the effects of Type 1 DM. Blood glucose level was elevated in DM though treatment with vitamin C reduced the noted hyperglycemia in the DM group. This observation confirms previous reports that vitamin C significantly reduced blood glucose level in experimental DM (Owu *et al*., 2006; Al-Shamsi *et al*., 2007).

This study has provided information on the haematological parameters such as RBC and WBC counts, haemoglobin concentration and haematocrit in Type 1 DM. Though there was a decrease in RBC, Hb and WBC in DM + Vit C group, the parameters were within the normal range reported for animals. The PCV was insignificantly higher in DM + Vit C group when compared to the control group.

Although the RBC values were within normal range, the decrease in Hb and MCHC in DM group when taken together reflects anaemia. Morales-Ramirez *et al* (1998) reported that Vitamin C increases the haematocrit through enhanced iron absorption (Atanasova *et al*., 2004) and as such help reverse anaemia. Inadequate production of red cells and some other formed elements have been reported in DM (Rabble *et al*., 1996; Thomas *et al*., 2004). In our results, we observed that the administration of vitamin C to Type 1 DM group significantly increased the haemoglobin and MCHC indices. MCV and MCHC are used to diagnose the types of anaemia (Davidson *et al*., 1981). The present study showed a high value of MCV and low MCHC that reflects macrocytic hypochromic anaemia. Following treatment of Type 1 diabetic rats with vitamin C, the macrocytic hypochromic anaemia as reflected by high MCV and low MCHC values was corrected.

Vitamin C did not significantly alter the WBC count in the treated groups. This is at variance with reports that it increased the total circulating white blood cell count in vitamin C-supplemented animals (Fraser *et al*., 1980; Field *et al*., 2002). Vitamin C significantly decreased platelet count and aggregation in Vitamin C supplemented diabetic group. This result is in agreement with previous studies (Wilkinson *et al*., 1999, Mullan *et al*., 2002) that reported a decreased platelet aggregation and arterial stiffness with vitamin C supplementation. However, Pignatelli *et al.* (2005) reported contrary that vitamin C did not affect platelet aggregation both *in vitro* and *in vivo* in healthy humans. Platelet aggregation, the clumping together of platelets in the blood is one of the underlying events that result in the formation of clot be involved in the genesis of diabetic microangiopathy (Barnett, 1993). Platelet aggregation and thrombosis play key roles in the progression of atherosclerosis and consequent

cardiovascular complications. When there is platelet aggregation, the aggregation ratio is less than one and conversely in the absence of aggregation, the ratio is close to one. The inhibition of platelet aggregation in this study further shows the beneficial role of vitamin C supplementation in its protective role against platelet activation in DM.

An elevated serum level of potassium ion and decrease in levels of sodium and chloride ions has been reported in DM (Onwuliri *et al*, 2004). There are also varied reports of effect of vitamin C on the serum electrolytes. While Al-Shamsi *et al*. (2006) reported significant alterations, Eteng *et al*, (2006) reported no adverse effect of vitamin C on serum electrolytes. We observed a significant variation within physiological limits in the serum electrolytes, with sodium being elevated with vitamin C supplementation. Such elevation had been reported and may be due to intracellular shift occasioned by osmotic diuresis which is a common feature in DM (Rao, 1992).

Various disorders such as renal failures, gastrointestinal diseases, DM and acidosis are characterised by electrolyte disturbances. Sodium-potassium ion (Na^+/K^+) ratio has frequently been used as a diagnostic tool to identify different disease conditions (Pak, 2000). DM has been reported to cause hyperkalemia both through acidosis and the reduced levels of insulin available to promote cellular uptake of potassium (Brink, 1999). Hyperkalemia may result from both a shift of the ion from the intracellular to the extracellular compartment and a decrease in the renal excretion of potassium (Carlotti *et al*., 2013). The hypokalemia reported in this study could arise from a drastic fall arising from the correction of acidosis. However, the elevated serum level of K^+ in DM + Vitamin C could not be easily discerned from this study and is subject to further investigation. The Na^+/K^+ ratio is a measure that compares the level of sodium and potassium ion in the body and a high ratio is associated with specific symptoms including acute stress, DM, heart disease and inflammation (Sjogren *et al*., 1998; Li *et al*, 2009). The high Na^+/K^+ ratio in the DM group in the present study is in agreement with previous studies (Cunningham, 1998; Farvid *et al*., 2004). Vitamin C supplementation in the diabetic group causes a significant reduction of the Na^+/K^+ ratio indicating that vitamin C can cause a beneficial reduction in Na^+/K^+ ratio.

Vitamin C has been reported to participate in mechanism for concerted glucose transport inhibition in cells (Castro *et al.*, 2008). Our result showed that blood glucose level was reduced in diabetic group treated with vitamin C and is in agreement with previously published data that showed improvement in glycaemic control with vitamin C supplementation (Afkhami-Ardekani and Shojaoddiny-Ardekani 2007, Hoffman *et al*., 2012). The beneficial effect of antioxidant on the beta cells, other target tissues and non-oxidative glucose metabolism (Dakhale *et al*., 2011) could be a possible mechanism of reduction of blood glucose by vitamin C in Type 1 diabetic condition. In addition, vitamin C has been reported to have anti-atherosclerotic effects in Type 2 DM by reducing hyper-coagulation of platelets (Gutierrez *et al*., 2013).

There are few limitations in this study. Even though vitamin C is water soluble and expected to be eliminated as such, this study did not quantify the urinary excretion and blood level of this vitamin. In addition, the study was relatively short duration. Despite these limitations, the results of this study showed that there was a reduced haematocrit and an increased platelet count while sodium ion concentration and serum Na^+/K^+ ratio were elevated in Type 1 DM. Oral administration of vitamin C to the Type 1 diabetic group resulted in an increase in haemoglobin concentration a reduction in sodium /potassium ion ratio. Vitamin C could be of immense importance in ameliorating the symptoms and preventing complications of DM considering the readily available dietary sources of vitamin C such as fruits and vegetable.

Conclusion

It is concluded that oral vitamin C administration increased haemoglobin, caused a decrease in plasma glucose level, platelet count, serum sodium/ potassium ion ratio and inhibits platelet aggregation in Type 1 DM in rats.

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