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Chronic Vitamin C or E Supplementations Impair Insulin Sensitivity and Increased the Diabetogenic Effect in Rats.

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ABSTRACT:

The classical risk factors of diabetes are unable to explain the endemic raise in diabetes. In spite of the well established actions of vitamins as antioxidants, the association studies between antioxidant vitamin status and its ameliorative effects in diabetes has no conclusive results at all and the interventional studies have no favorable effect of vitamin supplementation, and other studies indicated a possible link between the increased incidence of T2DM in antioxidant supplemented populations. To show role of chronic antioxidant vitamin (C and E) supplementation for the increased risk of T2DM in rats. In the present study, rats were divided into three groups: control group which were given distilled water only, Vitamin C supplemented group which were orally supplemented with vitamin C (100, 200,500 mg/kg) and Vitamin E supplemented group which were orally supplemented with vitamin E (50,100,200 mg/kg). Chronic supplementation of rats with antioxidant vitamins C and E caused significant elevation in the fasting blood glucose, insulin and HOMA which indicated a diabetogenic effect. Also, pancreatic tissues showed elevated GLUT 2, glucokinase and NRF2 and decreased UCP2 upon supplementation with antioxidant vitamins. It could be concluded that the chronic supplementation with antioxidant vitamins have diabetogenic effect through many mechanism that may involve pancreas and peripheral tissues.

Keywords: Diabetes, Glucose sensing, Insulin resistance, UCP2, antioxidant, Vitamin C, Vitamin E

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder differentiated by high blood glucose levels and modified lipid metabolism caused by inappropriate insulin action or insulin secretion. Worldwide, more than half a billion people—men, women, and children of all ages—have diabetes; in the next 30 years, that figure is expected to more than quadruple to 1.3 billion people.[1] recognizing the etiology and the early detection of type 2 diabetes are keys to prevention. Diabetes requires a large economic load as direct costs (medical) and indirect costs (premature mortality, productivity loss, and the negative impact of diabetes on nations' products.[2]

Oxidative stress has an important role in the progression of diabetes mellitus and its complications.[3] Hyperglycemia leads to increase oxidative stress, which leads to the deterioration of the master processes; insulin signaling and insulin production that impaired during diabetes. Also, antioxidant mechanisms are impaired in diabetic patients, which may lead to oxidative stress. [4]

Several studies have examined the potential involvement of dietary antioxidants, such as vitamins, in improving the condition of diabetes and in inhibiting the development of complications of diabetes. Studies conducted on the efficiency of antioxidant therapy in the treatment of T2DM have raised doubts about the usefulness of antioxidant supplements. A previous report showed that the use of antioxidants increases death from all causes. For T2DM patients, taking antioxidant supplements may even impair their disease conditions due to the further dampening of ROS signaling by exogenous antioxidants.[5] The authors concluded that self-described antioxidants may lead to T2DM over decades. But this hypothesis needs to be confirmed by empirical and clinical studies. So, this study was designed to evaluate the possible effects of chronic supplementation with antioxidant vitamins (C and E) on some proteins involved in glucose sensing mechanisms in the pancreas, including; glucose transporter 2 (Glut2), glucokinase, uncoupling protein 2 (UCP2) and nuclear factor-erythroid-2-related factor 2(Nrf2).

Animals and Methods:

Animals

The present study was carried out on seventy male albino rats 2 months old, weighing between 100-120 g at the beginning of the experiments. Rats were obtained from animal house of Medical Research Institute. Animal were housed 5 per cage at 23 ^{o}C on a 12-hour light/dark cycle and provided with a commercial diet and tap water ad libitum.

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Ethical statement

The current protocol was approved by Alexandria University-Institutional Animal Care and Use Committee (AlexU-IACUC, Approval number: AU01223111234). All experiments fulfill the guidelines of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and the recommendations of Egypt's guide for the care and use of laboratory animals. All efforts were made to curb the distress of rats during the experimental period. By using best practice for commonly used procedures, such as blood sampling and feeding rats with a consistent supply of food and water, and simultaneously clean their cages, which can enormously improve animal welfare

Experimental design:

All experiments were conducted in accordance with the guide for the care and use of lab animals. The rats were assigned to one of the following groups:

- Control group: The rats (n=10) were given distilled water only for 6 months.
- Vitamin C supplemented groups: The rats were orally supplemented with freshly prepared aqueous solution of vitamin and were sub-divided into three subgroups (10 rats each) supplemented with 100, 200 or 500 mg/Kg of vitamin C daily for 6 months.
- Vitamin E supplemented groups: The rats were orally supplemented with vitamin E prepared in olive oil and were sub-divided into three subgroups (10 rats each) supplemented with 50, 100 or 200 mg/Kg of vitamin E daily for 6 months.

The weekly changes in fasting blood sugar levels were assayed in tail vein using Glucometer (Accu-chek) and body weight were recorded to adjust the doses of vitamins. On day 180 after supplementation, animals were scarified by cervical dislocation. The abdomens were opened and the blood samples were collected to obtain plasma for assessment of glucose, insulin and lipid profile (triglycerides, cholesterol, LDL-cholesterol and HDL-cholesterol) using available commercial kits. The whole pancreases were removed, put on ice, weighed and were used to determine their levels of Glut2, glucokinase, Nrf2, UCP2 and glutathione (GSH).

Assay of Nrf2 in the pancreatic nuclear extract:

The nuclear content of pancreatic cells was extracted using active motive nuclear extraction kit after isolation of cells of pancreas using collagenase digestion.[6] The Lysis Buffer used in the extraction contained phosphatase inhibitors to prevent dephosphorylation of transcription factors during the extract preparation and the assay.

The pancreatic tissue content of the Nrf2 was assayed in the nuclear extract using TransAM@ Nrf2 ELISA kit (Active Motive, USA). The Kit contained a 96-well plate on which has been immobilized oligonucleotide containing the ARE consensus binding site (5´-GTCACAGTGACTCAGCAGAATCTG-3´). The active form of Nrf2 contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used to detect Nrf2 recognizes an epitope on Nrf2 protein upon DNA binding. Addition of an HRP-conjugated secondary antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry.

Assay of Glut2 in the pancreatic tissues:

Membrane fractions of pancreatic tissues were prepared by a modification of the procedures of Wang *et al.* (1997).[7] Tissues were homogenized in 25 mM Tris-HC1 (pH 7.4), 10 mM $MgC1_2$ and 0.25 M sucrose then centrifuged at 720 × g for 30 min. The resulting supernatant was centrifuged at 15000 × g for 90 min. The supernatant was discarded and the pellet resuspended in 25 mM Tris-HC1, pH 7.4 containing 10 mM $MgC1_2$ (All steps were performed at 4°C).

The pancreatic content of Glut2 was assayed using immunoassay kit (EIAab, China) for the quantitative determination of rat Glut2 concentrations. Briefly, standards or samples were added to the appropriate microtiter plate wells (pre-coated with an antibody specific to Glut2) then a biotin-conjugated polyclonal antibody preparation specific for GLUT2 was added. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB substrate solution was added to each well. Only those wells that contain GLUT2, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm.

Assay of glucokinase (GK) and uncoulping protein 2 (UCP 2) in pancreatic extract:

The pancreatic content of GK and UCP2 were assayed using immunoassay kit (EIAab, China) for the quantitative determination of rat GK and UCP2 concentrations according to the manufacturer's instructions

Assay of pancreatic contents of total reduced and oxidized glutathione:

Glutathione (GSH) and glutathione disulphide (GSSG) were assayed using the method of Griffith[8] which depends on the oxidation of GSH by 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2-nitrobenzoic acid (TNB). Oxidised GSSG is reduced enzymaticly by the action of glutathione reductase and NADPH to regenerate GSH. The rate of TNB formation is monitored at 412 nm and is proportional to the sum of GSH and GSSG present in the sample.

Statistical analysis:

All data are presented as mean±SD. The Kolmogorov-Smirnov test was used to verify the normal distribution of the studied variables. A one-way analysis of variance (ANOVA) with Post Hoc test (LSD) was performed on each normally distributed variable to compare the mean values of different groups. For abnormally distributed variables the Mann–Whitney-U tests were used to evaluate the statistical significance of the differences between groups. *t*-test was used to compare the mean values of rats supplemented with different vitamins at corresponding dose. Differences were considered significant at $P<0.05$. All statistical analyses were performed using SPSS statistical software version 18 (SPSS, Chicago,IL).

Results:

Glucose homeostasis parameters

The results of fasting blood glucose (FBG) are shown in Figure (1). Supplementation of rats with vitamin C showed duration -dependent increase in FBG level. This increase was significant as early as the first month of supplementation with 500 mg/kg vitamin C while lower doses (100 and 200 mg/kg) showed significant elevation from the second month of supplementation (Figure 1A).

The rats supplemented with vitamin E showed biphasic change in FBG levels. During the first three months, the levels of FBG showed duration-dependent decrease. This decrease became significant after 1 month (with dose of 200 mg/Kg) and 2 months (with doses of 50 and 100 mg/Kg). From the 5th month of supplementation, the levels of FBG showed significant increases with all doses with the highest level observed in the rats supplemented with 200mg/Kg vitamin E (Figure 1B).

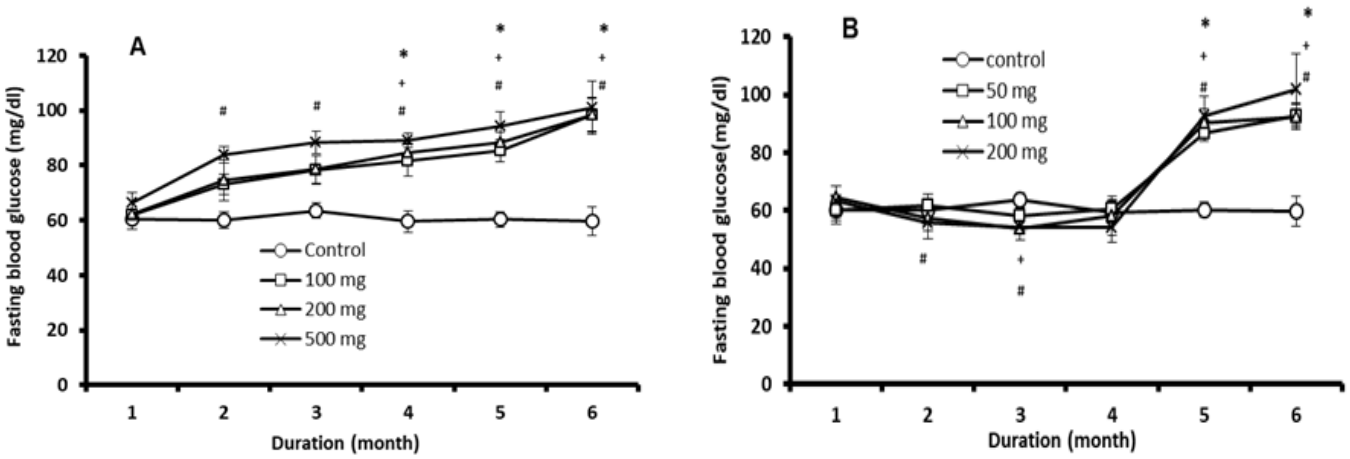

Figure (1): The change in fasting blood glucose level during 6 months follow-up period in control rats and rats supplemented with different doses of vitamin C (A) and vitamin E (B). Each point represent mean of 10 measurements±SD (*, +, # represent a significant difference between rats supplemented with low, intermediate and high vitamin doses compared to control rats respectively, Using ANOVA, p<05)

At the end of supplementation period, we observed that all the rats supplemented with different doses of vitamin C and E showed significantly higher FBG levels than control rats, there were no significant differences between rats supplemented with different doses of vitamin C and E except that the highest dose of vitamin E showed a significant difference from lower doses (Table 1).

At the end of follow-up period, insulin levels were significantly increased in all groups of rats supplemented with different doses of vitamin C and E compared to control rats except for the 100mg/kg vitamin E supplemented group that showed no significant change (Table 1).

The insulin resistance index calculated as HOMA-IR showed a pattern of change similar to insulin level. It was significantly increased in all groups of rats supplemented with different doses of vitamin C and E compared to control rats except for the 100mg/kg vitamin E supplemented rats that showed no significant change. There were no significant differences in HOMA-IR between vitamin C and E supplemented rats except for the 100mg/kg vitamin E supplemented group that showed a significantly lower HOMA-IR compared to the corresponding dose of vitamin C supplemented group (Table 1).

Lipid profile:

Regarding serum lipid profile, there were no significant differences in the plasma levels of triglyceride (TG) of vitamin C and E supplemented groups from control group. Also, no significant differences were observed between the rats supplemented with vitamin C and E. The rats supplemented with vitamin C showed significant dose-dependent decline in total cholesterol (TC) level. The doses of 200 and 500 mg/kg showed significantly lower TC level compared to control rats (Table 1).

The rats supplemented with vitamin E showed different pattern of changes in TC according to the dose. The low dose (50 mg/kg) showed no significant change while the intermediate dose (100 mg/kg) showed significantly lower TC level than control value and low dose. In contrast at the highest dose (200 mg/kg) the TC level was significantly higher compared to control rats, lower doses (50 and 100 mg/kg) and vitamin C groups even at the highest dose (500mg/kg) (Table 1).

All groups of rats supplemented with different doses of vitamin C and E showed significantly lower HDL-C than control rats in a dose-dependent manner, especially in the rats supplemented with vitamin E which showed significantly lower level compared to rats supplemented with vitamin C at highest dose (Table 1).

The level of LDL-C showed significant dose-dependent decline in the rats supplemented with vitamin C. The lowest dose of vitamin C showed non-significantly lower level while the intermediate and high doses of vitamin C showed significantly lower LDL-C level compared to control value. The supplementation of rats with vitamin E showed different patterns of changes according to the dose. At lower doses (50 and 100 mg/kg) the LDL-C level significantly decreased in a dose dependent manner compared to control. At the highest dose (200 mg/kg) the rats showed significantly higher LDL-C level compared to control rats and rats supplemented with the highest vitamin C dose (Table 1).

Table (1): The change of glucose homeostasis parameters and lipid profile in control rats and rats supplemented with different doses of vitamin C and vitamin E for 6 months.

	Control rats	Vit C Supplemented rats			Vit E Supplemented rats		
		100 mg/Kg	200 mg/Kg	500 mg/Kg	50 mg/Kg	100 mg/Kg	200 mg/Kg
FBS (mg/dl)	60±5	98±6*	99±6*	101±10*	92±4*	93±5*	102 ±12^{*,a,b}
Insulin (µIU/ml)	7.4±1.77	10.4±2.50*	11.2±1.03*	14.4 ±2.28^{*,a,b}	10.6±2.8*	7.2 ±2.03^{a,c}	14.1 ±2.90^{*,a,b}
HOMA	1.04±0.46	2.47±0.059*	2.67±0.22*	3.59±0.71^{*,a,b}	2.20±0.73*	1.64 ±0.45^{*,a,c}	3.56 ±0.95^{*,a,b}
TG (mg/dl)	39.9±1.8	40.8±8.4	40.7±6.0	41.57±5.7	42.1±6.3	41.2±5.6	42.2±6.1
TC (mg/dl)	129.2±4.3	134.5±4.8$_{*}$	124.3 ±3.6^{*,a}	111.3±6.2^{*,a,b}	129.2±5.1*	105.3±11.3^{*,a,c}	136.6 ±5.4 $_{a,b,c}$
HDL-C (mg/dl)	37.3±1.1	36.3±1.32	34.4·±9.4^{*,a}	34.8±1.6^{*,a}	35.6±1.9*	33.2 ±2.2^{*,a}	30.1 ±1.5^{*,a,b,c}
LDL-C (mg/dl)	96.8±11.7	90.0±5.4	81.8 ±5.4^{*,a}	68.2±5.6^{*,a,b}	98.0±5.3^{*,a}	85.1 ±6.0^{*,a,c}	63.9±11.9 $_{a,b,c}$

*Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats (p<0.05), a: significantly different from rats supplemented with the low dose of each vitamin (p<0.05), b: significantly different from rats supplemented with the intermediate dose of each vitamin (p<0.05) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test (p<0.05)*

Pancreatic glucose sensing parameters

The rats supplemented with different doses of vitamin C and E showed a significantly higher pancreatic GK content compared to control rats. Rats supplemented with vitamin C at doses of 100 and 200 mg/kg showed relatively similar elevation in pancreatic GK content, about 2-fold control value while at the dose of 500 mg/kg the rats showed greatly higher pancreatic content of GK; about 4-fold its level in the control rats (Figure 2).

The vitamin E supplementation results in significant dose-dependent increase in the pancreatic content of GK, with the highest content observed in the rats supplemented with 200mg/kg (4-fold control rats). The rats supplemented with 50 and 100 mg/kg vitamin E showed significantly higher pancreatic GK content compared to vitamin C supplemented rats (Figure 2).

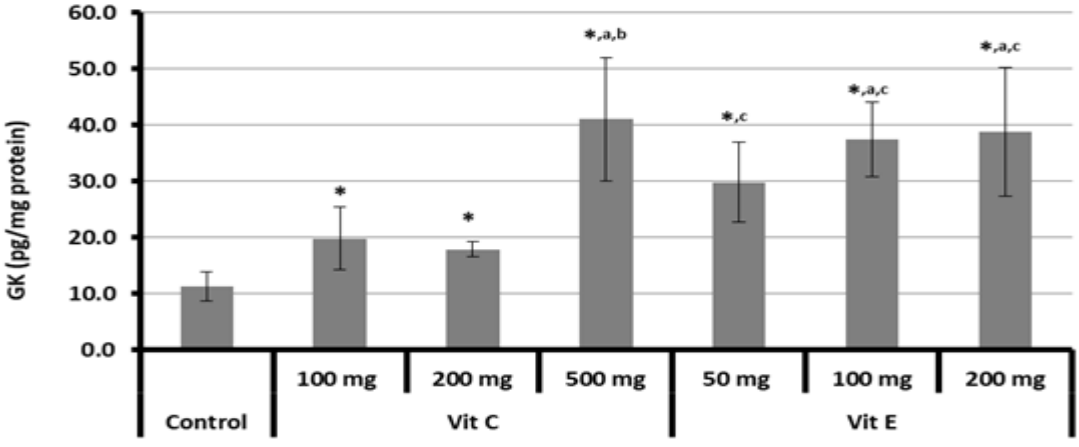

Figure (2): The change in pancreatic GK content after 6 months supplementation with different doses of vitamin C and vitamin E. Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats (p<0.05), a: significantly different from rats supplemented with the low dose of each vitamin (p<0.05), b: significantly different from rats supplemented with the intermediate dose of each vitamin (p<0.05) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test (p<0.05).

The level of pancreatic Glut2 showed no significant change in the rats supplemented with vitamin C at doses of 100 and 200 mg/kg, while at the highest dose (500 mg/kg) its level was significantly elevated to be about 2-fold control value (Figure 3). The supplementation of rats with vitamin E showed significant dose-dependent elevation in Glut2 content in the pancreas compared to control rats. The highest level observed in the pancreas of rats supplemented with 200 mg/kg vitamin E (2.3-fold control value) (Figure 3).

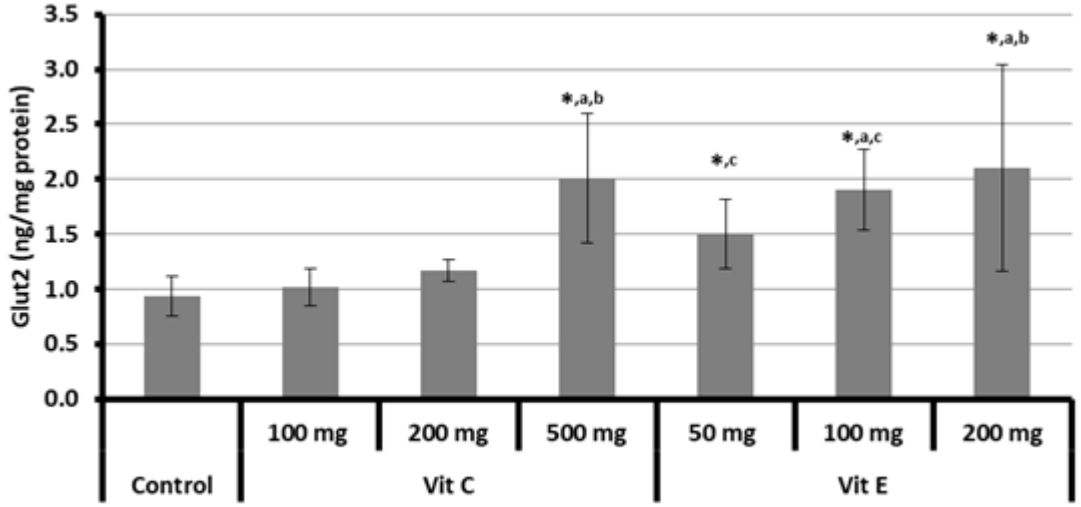

Figure (3): The change in pancreatic Glut2 content after 6 months supplementation with different doses of vitamin C and vitamin E. Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats ($p<0.05$), a: significantly different from rats supplemented with the low dose of each vitamin ($p<0.05$), b: significantly different from rats supplemented with the intermediate dose of each vitamin ($p<0.05$) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test ($p<0.05$)

The supplementation of rats with vitamin C at doses of 100 and 200 mg/kg showed a dose-dependent decline in the pancreatic UCP2, while at dose of 500 mg/kg the hepatic UCP2 was the same as that observed at the lowest dose (100 mg/kg) (Figure 4). The rats supplemented with vitamin E showed a dose-dependent decline in the pancreatic UCP2 content. However, the lowest dose (50 mg/kg) showed a significant increase in the UCP2 compared to control rats, while the higher doses (100 and 200 mg/kg) significantly decreased its level compared to control rats (Figure 4).

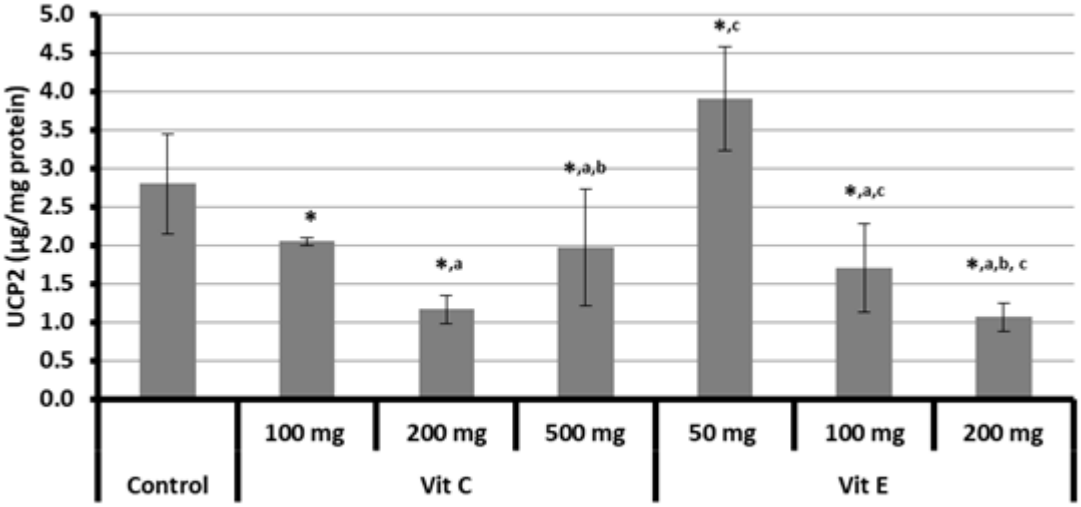

Figure (4): The change in pancreatic UCP2 content after 6 months supplementation with different doses of vitamin C and vitamin E. Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats ($p<0.05$), a: significantly different from rats supplemented with the low dose of each vitamin ($p<0.05$), b: significantly different from rats supplemented with the intermediate dose of each vitamin ($p<0.05$) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test ($p<0.05$).

Pancreatic redox parameters:

It was clear that, the supplementation of rats with vitamin C at doses of 100 and 200 mg/kg had no significant effect on the pancreatic nuclear Nrf2 level while at the dose of 500 mg/kg the level greatly elevated to be about 6.6-fold control value (Figure 5). On the other hand, vitamin E supplementation resulted in significant increase in the pancreatic nuclear Nrf2 level compared to control at all doses in a dose-dependent manner with the highest level observed in the rats supplemented with 200 mg/kg vitamin E which showed about 6.7-fold control (Figure 5)

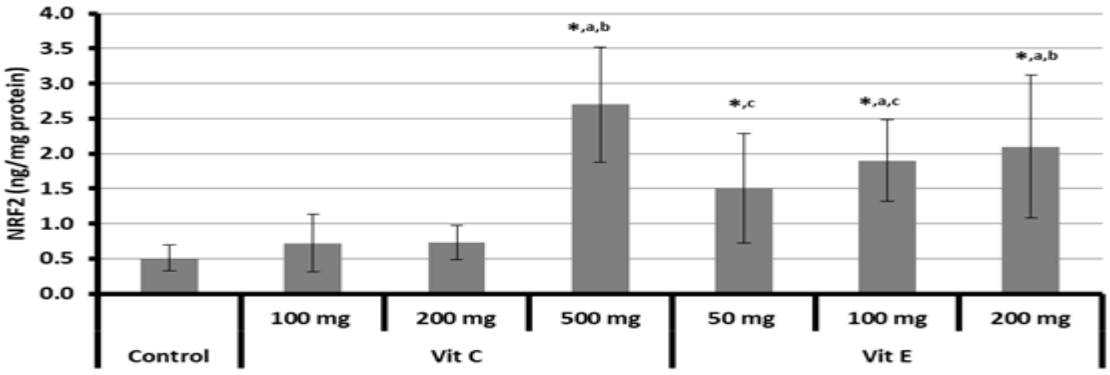

Figure (5): The change in pancreatic Nrf2 content after 6 months supplementation with different doses of vitamin C and vitamin E. Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats ($p<0.05$), a: significantly different from rats supplemented with the low dose of each vitamin ($p<0.05$), b: significantly different from rats supplemented with the intermediate dose of each vitamin ($p<0.05$) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test ($p<0.05$).

The chronic supplementation of rats with vitamin C and E significantly increased the pancreatic levels of total and reduced GSH compared to control rats especially at the higher doses 200 and 500 mg/kg for vitamin C and 100 and 200 mg/kg for vitamin E (Table 2). The increase of total and reduced GSH showed a dose-dependent pattern with both supplementations; however vitamin C supplementation showed significantly higher level of GSH than vitamin E (Table 2).

On the other hand, the chronic supplementation with vitamin C showed no significant change in the pancreatic levels of GSSG except at the dose of 100 mg/kg which showed significant decrease (Table 2). The rats supplemented with vitamin E showed higher levels of GSSG in the pancreatic tissue compared to control which became significant only with the dose of 100 mg/kg. All rats supplemented with vitamin E showed higher GSSG levels compared to rats supplemented with corresponding doses of vitamin C (Table 2).

The rats supplemented with vitamin C showed significantly higher hepatic GSH/GSSG ratio compared to control. Also, vitamin E supplemented rats only at the highest dose showed significantly higher ratio than control (Table 2).

Table (2): The change of pancreatic content of total, reduced and oxidized glutathione in control rats and rats supplemented with different doses of vitamin C and vitamin E for 6 months.

	Control rats	Vit C Supplemented rats			Vit E Supplemented rats		
		100 mg/Kg	200 mg/Kg	500 mg/Kg	50 mg/Kg	100 mg/Kg	200 mg/Kg
Total GSH (nmole/mg protein)	189.3±16.0	206.4±35.4	249.17±60.0 ^{*,a}	287.4±30.04 ^{*,a,b}	198.5±29.2	237.6±38.4 ^{*,c}	273.5±66.2 ^{*,a}
Reduced GSH (nmole/mg protein)	174.8±17.2	194.2±34.2	224.4±48.2 *	272.3±30.14 ^{*,a,b}	182.4±29.4	218.6±37.6 *	256.9±66.2 ^{*,a,b}
GSSG (nmole/mg protein)	7.6±0.72	6.12±1.8 *	7.3±0.83 ^{a}	7.07±0.82	8.0±1.10 ^{c}	9.5±1.05 ^{*,a,c}	8.2±1.20 $^{b.c}$
GSH/GSSG	23.05±3.4	34.6±12.4 *	30.6±6.5 *	39.2±6.5 ^{*,b}	23.0±5.0 ^{c}	22.9±3.5 ^{c}	31.7±9.8 ^{*,a,b,c}

*Data presented as Mean ± SD and n of rats in each group =10. *: significantly different from Control rats ($p<0.05$), a: significantly different from rats supplemented with the low dose of each vitamin ($p<0.05$), b: significantly different from rats supplemented with the intermediate dose of each vitamin ($p<0.05$) and c: significantly different from rats supplemented with the vitamin C at the corresponding dose by t-test ($p<0.05$)*

Correlation studies:

The serum insulin level is positively correlated with: pancreatic glucokinase (r=0.375, p=0.003 , Figure 6A), Glut2 (r=0.376, p=0.003,Figure 5B), tGSH (r=0.375, p=0.021 , Figure 6C) and Nrf2 (r=0.601 , p=0.000 , Figure 6D) of rats supplemented with vitamin C. In the same rats, pancreatic Nrf2 is positively correlated with HOMA-IR (r=0.601, p=0.001, Figure 6E) and glucokinase (r=0.878, p=0.001, Figure 6F).

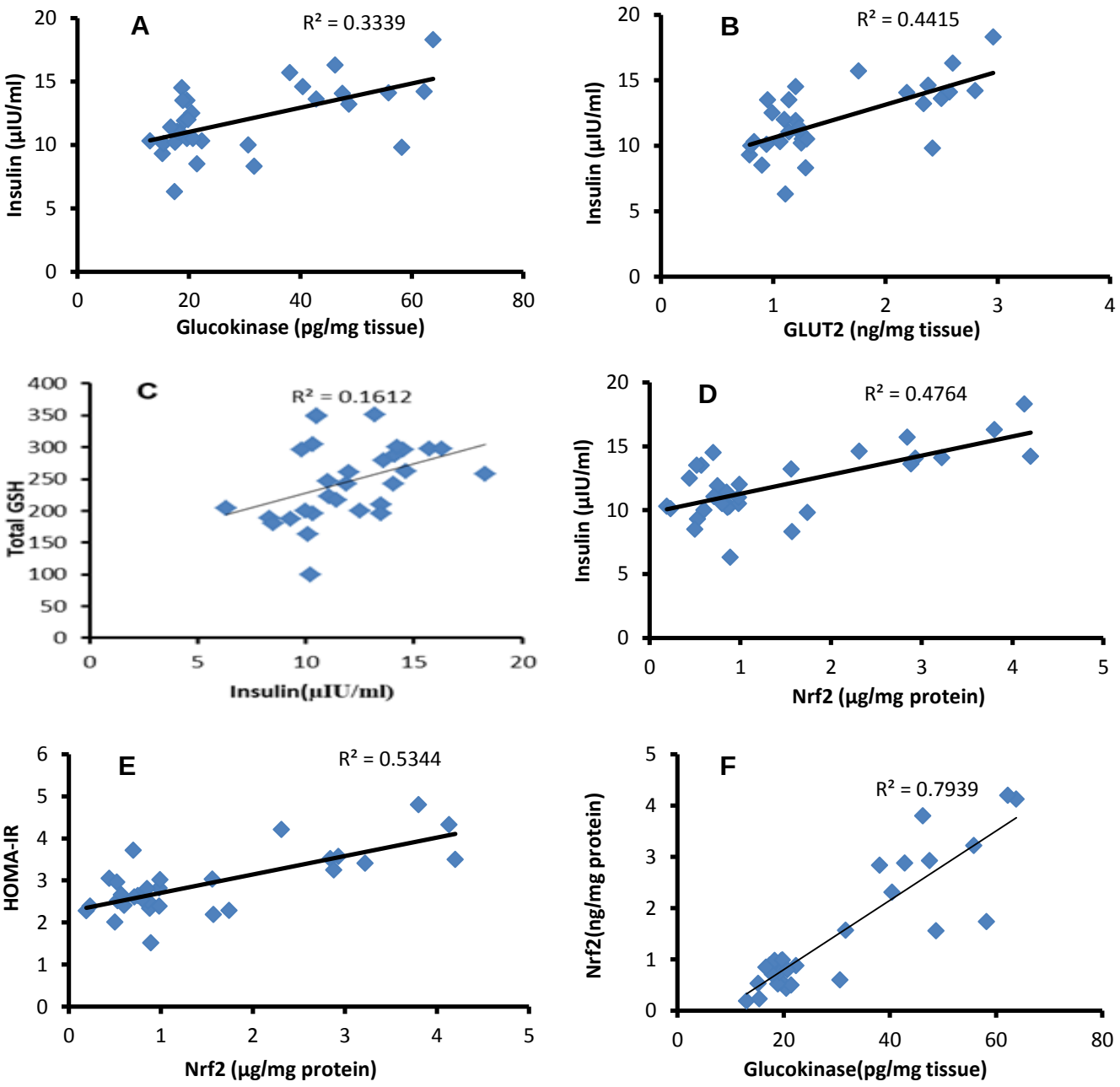

Figure (6): The correlations obtained in vitamin C supplemented rats.

In vitamin E supplemented rats, similar pattern of correlations are also observed, insulin is positively correlated with pancreatic glucokinase (r=0.383, p=0.036, Figure 7A) and Nrf2 (r=0.397 , p=0.030 , Figure 7B). In the same rats, pancreatic Nrf2 is positively correlated with FBG (r=0.416, p=0.022, Figure 7C) , HOMA-IR (r=0.545 , p=0.002 , Figure 7D), glucokinase (r=0.736 , p=0.000 , Figure 7E) and Glut2 (r=0.903, p=0.000, Figure 7F).

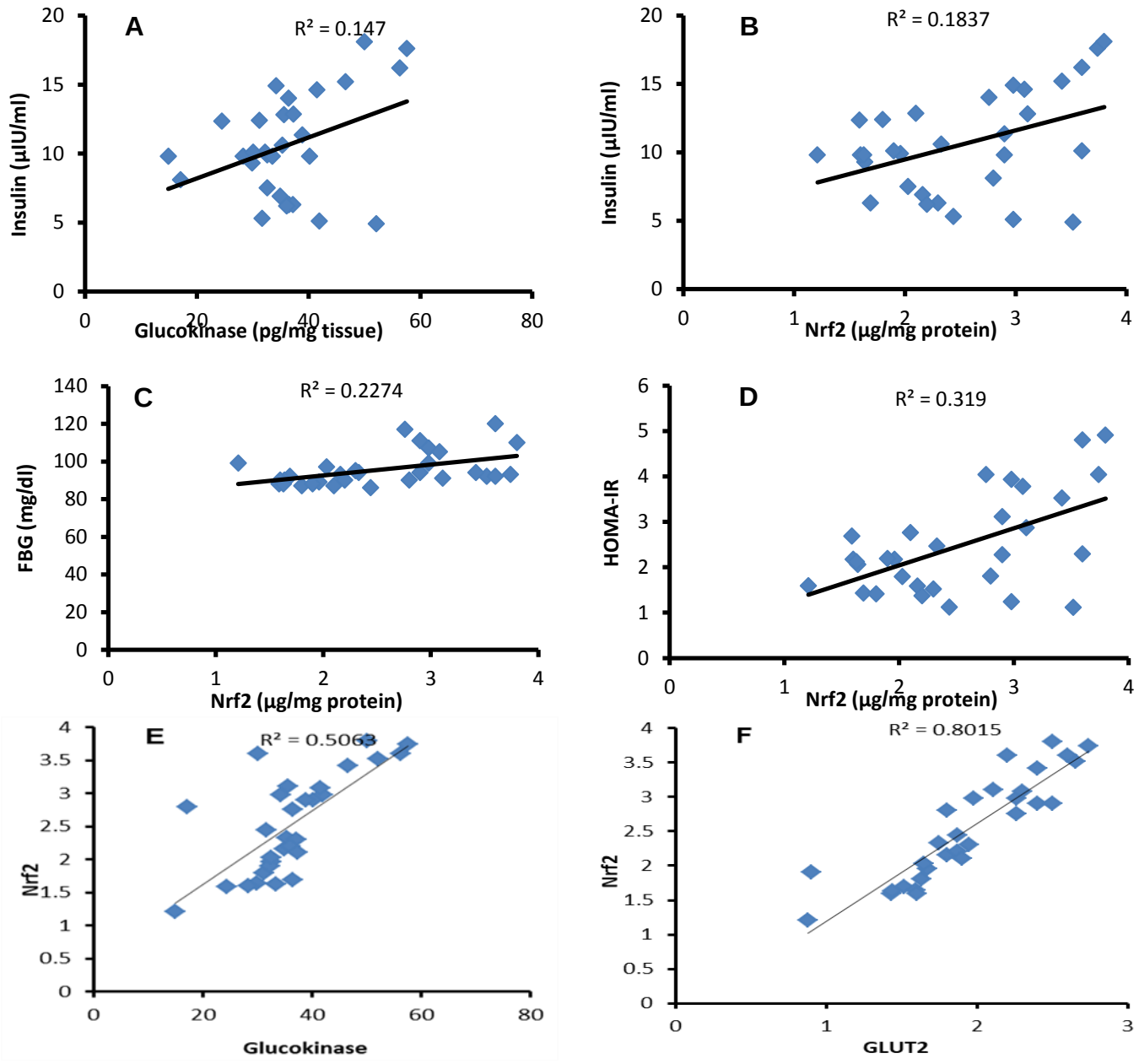

Figure (7): The correlations obtained in vitamin E supplemented rats.

Discussion:

From the present study, it is evident that the long-term supplementation of rats with vitamin C for 6 months resulted in significant duration and dose-dependent increase in fasting blood sugar compared to control. However, vitamin E showed significant decline in the blood glucose level during the early 4 months of supplementation after which the glucose level significantly elevated which became significant from the 5th month at all used doses with the highest level associated with the highest vitamin E dose; 200 mg/Kg. However, no hyperglycemia was detected, these results may imply that the chronic supplementation of normal rats with antioxidant vitamins has a diabetogenic effect as indicated by significant elevation in the fasting blood glucose level.

The mechanism(s) of this diabetogenic effect of antioxidant vitamins is still unclear, however some hypotheses have been proposed last years. The normal glucose and insulin homeostasis is based on glucose metabolism in β-cells of pancreas by what is called Glucose-Stimulated Insulin Secretion (GSIS). The glucose sensing apparatus in the β-cell is composed of low affinity glucose transporter (Glut2) and low-affinity glucose phosphorylating enzyme glucokinase.[9] The classical scheme of GSIS depends on the production of ATP molecules by cytoplasmic and mitochondrial metabolism of glucose.[10] The ratio of ATP/ADP increases as processing of the glucose increases, leading to closure of K_{ATP} channels causing depolarization of the plasma membrane and opening of Ca^{2+} channels. The entry of Ca^{2+} triggers exocytosis of the insulin granules. However, the precise signals that couple glucose metabolism to insulin secretion are still incompletely understood. Recently, many studies suggested that reactive oxygen species (ROS) derived

from mitochondrial glucose metabolism are potential metabolic signals that facilitate insulin secretion.[11] One of the proposed hypotheses to explain the diabetogenic effect of antioxidants suggested that over supplementation may inhibit GSIS from pancreas through over-activation and/or expression of the antioxidant systems in pancreatic tissues which scavenge and diminish the physiological levels of hydrogen peroxide involved in GSIS. The increased insulin level in the vitamin C and E treated rats provides exclusion of this hypothesis. The rats supplemented with vitamins especially vitamin C showed significant elevation in the insulin level compared to un-supplemented control rats however, they showed dose-dependent increase in fasting blood glucose level. This may suggest that the vitamin C and E induce diabetogenic phenotype through a mechanism that is not related to insulin secretion but may be related to insulin action.

We suggested that the increased insulin level may be caused by the increased glucose sensing by pancreatic cells because of increased levels of Glut2 and glucokinase. This assumption may gain support from the correlation studies which indicated that, the insulin level is positively correlated with pancreatic Glut2 and glucokinase in the rats supplemented with vitamin C and E. The increase in insulin level together with increased blood glucose may indicate a status of insulin resistance.

In support of the previous assumption, the rats chronically supplemented with different antioxidant vitamins (C and E) showed a dose-dependent increase in HOMA-insulin resistance index which indicates an insulin resistance state in these rats. Therefore, insulin is unable to act properly on resistant tissues and this resulted in poor glucose disposal, so β-cells initially compensated for insulin resistance by increasing insulin secretion till β-cell exhaustion and apoptosis could occur. Multiple organs contribute to the development of peripheral insulin resistance, with the major contributors being skeletal muscle, liver, and adipose tissue. [12]

On the other hand, the level of pancreatic UCP2 showed down regulation in the rats chronically supplemented with vitamin C, however, vitamin E supplementation significantly upregulated the pancreatic UCP2 level at lowest dose (50 mg/Kg) while, the higher doses (100 and 200 mg/Kg) significantly downregulated its level. Studies have implicated UCP2 in the inhibition of insulin secretion and action through inhibition of ATP production and free fatty acid (FFA) metabolism and also highlighted the major role played by UCP2 in the control of reactive oxygen species (ROS) formation.[13]

UCP2 have been implicated in the pathogenesis of type 2 diabetes mellitus and its chronic complications . [14] The documented low UCP2 in the pancreas may be involved in increasing insulin secretion. An inverse relationship between suppression of GSIS and UCP-2 expression have been demonstrated by *in vitro* study.[15] Moreover, rat clonal beta cell line (INS-1E) genetically modified to overexpress UCP2 gene showed increased survival after treatment with the H_2O_2. Similarly, oxidative stress-prone beta cells tried to overcome H_20_2 toxicity by inducing UCP2 mRNA. 16 Also, in another study it was established that endothelial cells of retinal cells incubator with high glucose levels, in fact even regulate UCP2 expression for protection from ROS damage derived from glucotoxicity.[17] On the other hand, UCP-2 deficient mice had increased ATP production and GSIS.[18]

In line with the results of the present study it was documented that, in human the mean postprandial plasma glucose was elevated in the vitamin C-supplemented subjects compared with subjects given placebo with no change in insulin level.[19] Vitamin C is structurally similar to glucose and can replace it in many chemical reactions, and thus is effective in prevention of non-enzymatic glycosylation of proteins. [20] The main difference between rats and human with respect to vitamin C is that; most mammals including rats can produce vitamin C in the liver; however, humans and other primates are unable to synthesize ascorbic acid due to the lack of functional L-gulono-lactone oxidase enzyme catalyzing the final step in the biosynthesis and, therefore, rely completely on a dietary supply of vitamin C.[20]

The transport of vitamin C as dehydroascorbic acids (DHA) occurs by facilitated diffusion, enabling transport along a concentration gradient. This gradient is maintained as DHA is reduced to ascorbic acid (ASC) immediately after crossing the membrane. [21] DHA, but not ASC,[22] is transported by facilitated diffusion through four glucose transporters (GLUT 1–4), although with varying affinities and efficiencies.[23] The Glut-transport of DHA is competitively inhibited by glucose, e.g., excess glucose in plasma or intestine will block the receptor-binding site and, subsequently, decrease Glut-facilitated DHA transport and vice versa. From this and on the basis of the research of others,[24] it would be anticipated that the rate of glucose entry into peripheral tissues, such as the brain, muscle, or liver, would be reduced in vitamin C supplemented rats, because of competition for membrane transport through glucose transporters, thereby increasing blood glucose level. We should note that this is not the case in diabetic individuals who suffer from hyperglycemia, because high glucose level inhibits vitamin C absorption and may cause vitamin C deficiency. In this case, the administration of vitamin C has beneficial effects on diabetic status. These should explain the reported significant reduction in serum fasting blood sugar, LDL-cholesterol, glycated hemoglobin (HbA1c) as well as serum fasting insulin after consumption of 1000 mg/ day of vitamin C in T2DM patients.[25] Forghani et al[26] also, showed a significant decrease in serum HbA1c and LDL levels in patients supplemented with 1000 mg/day of vitamin C for 6 weeks.

In addition, vitamin C acts as a regulator of catabolism of cholesterol to bile acid and has been demonstrated to be an important factor in lipid regulation.[27] Also, vitamin C showed beneficial effects on lipids in human. [27] This is in accordance to our data which indicated significant down-regulation of serum total cholesterol and LDL-cholesterol levels in the rats supplemented with vitamin C for 6 months while no significant change could be detected in the triglycerides level. The same rats showed also a decreased level of HDL-cholesterol which may considered as a risk for metabolic syndrome and T2DM. In line with these results it was reported that 2 g/day of vitamin C for 90 days, showed a significant decrease in total cholesterol in T2DM patients**.**[28]

During the planning for this study it was expected that, the antioxidant vitamins supplementations could enhance the antioxidative capacity of the pancreatic β-cells which in turn could scavenge the physiological levels of ROS that are involved in the GSIS which would expectedly result in decline in the insulin secretion. Notably, the supplementations caused a significant dose-dependent elevation in the Nrf2 (especially with vitamin E), the transcription factor which act as a master regulator of all antioxidant genes. Also, the supplementation of vitamins caused a significant dose-dependent elevation of glutathione system, but unexpectedly caused a significant-dose dependent elevation of insulin level. These confused observations may be solved by the data of glucose sensing parameters in pancreatic tissues including Glut2 and glucokinase which showed up-regulation by chronic supplementation with antioxidant vitamins. Although antioxidants may function as negative regulators for ROS, ROS- mediated signal transduction is relatively resistant to ROS scavenging. This assumption is supported by the evidence that ROS-mediated signal transduction occurs in normal cells which usually contain considerable amount of antioxidants. [29]

It was documented that, high glucose concentrations activate Nrf2 signaling in cardiomyocytes.[30] In response to glucose exposure, cardiomyocytes lacking Nrf2 have slightly higher generation of ROS and more apoptosis, as well as greater functional deficits in contractility than wild type.[30] These studies point to a role of Nrf2 in mitigating oxidative stress generated during hyperglycemic conditions *in vitro*. However, there is little information regarding how Nrf2 affects glucose handling under normal conditions *in vivo*. The results of this study indicated that, the pancreatic Nrf2 levels are positively correlated with serum insulin, HOMA-insulin resistance index and pancreatic glucokinase in normal rats supplemented with vitamin C for 6 months. Also, rats supplemented with vitamin E showed similar correlation pattern as the pancreatic Nrf2 levels are positively correlated with serum insulin, HOMA-insulin resistance index, GLUT2, pancreatic glucokinase and also with fasting blood sugar. These patterns of correlation may imply that, the Nrf2 may actively be involved in the enhanced secretion of insulin in the normal rats supplemented chronically with antioxidant vitamins and contribute to the elevated HOMA-index in those animals.

The supplementation of rats with vitamin E during the first 4 months showed no significant increase in the fasting blood glucose, but the significant elevation in fasting blood glucose was observed after 5 and 6 months of supplementation. In accordance with these data another study reported that, the vitamin E supplementation reduces blood sugar levels and improves insulin sensitivity and the associated features of insulin resistance in overweight individuals during the initial 3 months of the study after which these effects were diminished.[31] The increased glucose sensing and insulin sensitivity may be involved in the initial effect of vitamin E indicated by the dose-dependent elevation in Glut2 and glucokinase content in the pancreatic tissue. Vitamin E may improve insulin action by improving the chemical-physical state of plasma membranes resulted from a decrease in oxidative stress. [32]

Many studies data suggested that α-tocopherol is not only an antioxidant but also a regulator of gene expression through its binding to nuclear receptors. The data indicate that α-tocopherol modulates the expression of sterol response element–binding proteins (SREBP-1 and SREBP-2).[33] SREBP-1c plays a key role in glucose-stimulated Glut 2 gene expression. The Glut2 promoter is activated by SREBP-1.[34] Also, it was reported that, vitamin E induces Nrf2 expression . [35]These stated facts may explain the dose-dependent inductive action of vitamin E on the pancreatic level of Glut2 and Nrf2 which in association with increased glucokinase activity increase glucose sensing, stimulate GSIS and insulin release.

The effect of vitamin E on glucose homeostasis and related variables was not maintained during the final 2 months of the study. There is no obvious cause for this observation. However, we cannot exclude the possibility that in the present study extending the period of supplementation may have contributed to the deterioration in insulin sensitivity and associated with insulin resistance as indicated by increased insulin resistance index with the highest dose used (200 mg/Kg). These results suggest that vitamin E in excess, not vitamin E itself, may contribute in increasing the risk of insulin resistance. This underscores the U-shaped risk response curve of vitamin E intake. It's possible that taking extra vitamin E overcomes the natural balance. Perhaps excess vitamin E has a negative effect on the redox and metabolic status.

According to these results, the increased fasting blood glucose and insulin resistance with chronic antioxidant vitamins supplementation might be explained by the effect of high antioxidant vitamins on the insulin signaling in peripheral tissues. Binding of insulin to its receptor initiates the insulin signaling cascade, which is accompanied by a burst of hydrogen peroxide that acts as a second messenger.[36] High activity of GPx, which removes hydrogen peroxide, might thus interfere with insulin signaling. For example, transgenic mice overexpressing GPx developed insulin resistance, hyperglycemia, hyperinsulinemia, and obesity, also a strong correlation was noted between increased erythrocyte GPx activity and mild insulin resistance in pregnant women.[37] By contrast, knockout of GPx improved insulin-induced glucose uptake and insulin resistance in mice.[37] In line with these studies our study indicated that, antioxidant vitamins supplementation result in a dose-dependent elevation of total and reduced glutathione which is the main co-substrate of GPx in liver tissue. The positive correlation between hepatic glutathione level and serum insulin level may support the previous explanation especially in vitamin C supplemented rats. The diabetogenic effect of antioxidant vitamins especially vitamin E may be explained by other mechanisms that could need further supporting evidence. Numerous studies have focused on the insulin antagonistic protein tyrosine phosphatase 1B (PTP1B).[38] It was documented that the supplementation of rats with vitamin E causes up-regulation of the PTP1B.[39] An increased PTP1B activity may promote diabetes by reducing insulin signaling at the receptor level. These effects could impair the insulin signaling at different levels in the peripheral tissue.

Conclusion

The results of the present study provide experimental evidence that the chronic supplementation of rats with antioxidant vitamins can impair the insulin sensitivity and cause diabetogenic effect. On the other hand, vitamin E did not interfere with glucose homoeostasis as vitamin C did when used for short-term.

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Conflict of interest

No conflict of interest is declared

6. References

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