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Effect of Vitamin C and/or Vitamin E on Kidney, Liver and brain Functions of Streptozotocin-Induced Diabetic Rats

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Abstract

Introduction: Diabetes Mellitus is one of the main threats to human health in the 21st century. **Purpose:** To evaluate the effect of vitamin C and/or vitamin E on liver, kidney and brain function of streptozoticin induced-diabetic rats. **Study Design:** One hundred and twenty male adult Sprague Dawley rats were divided into 6 groups (20 rats each), normal control, STZ-induced diabetic rat, STZ-induced diabetic rats treated with: antidiabetic drugs; vitamin C; vitamin E; vitamin C+E. **Material and Methods:** Blood samples were collected from all groups, Urea, creatinine, uric acid, total protein, Alanine/ aspartate transaminase and vitamin E were measured in plasma. **Results:** Urea, creatinine, uric acid, ALT, AST, vitamin E, LDH (in brain and liver) and MDA (brain) were significantly increased in STZ-induced diabetic rats. Treatment with vitamin C and/or E decrease significantly the increased level of the tested parameters and this may be due to the scavenging free radicals properties of vitamin C and/or E which prevents damage induced by hyperglycaemia; Also may be because vitamin C and/or vitamin E decreased lipid peroxidation and augmented the activities of antioxidant enzymes. **Conclusion:** Vitamin C and/or E may thus be a useful adjuvant therapy in the management of diabetes mellitus but it is better to use combination of the two vitamins rather than in single supplements to prevent the perturb antioxidant–proxidant balance.

Keywords: Diabetes Mellitus, Liver and Kidney Function, Vitamin E, Uric acid

Introduction

Diabetes mellitus (DM) is considered as one of the main threats to human health in the 21st century and the number of people with diabetes has increased worldwide **(1)**. Diabetes mellitus is characterized by abnormally high plasma glucose concentrations. Chronic hyperglycemia and the associated metabolic abnormalities are responsible for many disease complications, including damage to the blood vessels, eyes, kidneys and nervous system **(2)**.

Free radicals have been implicated in the pathogenesis of many degenerative diseases, including diabetes, atherosclerosis and cancer **(3)**. Diabetes has been considered to be associated with oxidative stress. Oxidative stress may cause oxidative damage of cellular membranes. Oxidative stress may cause oxidative damage of cellular membranes and changes in the structural and functional integrity of subcellular organelles and may produce effects that result in various complications in diabetic disease. Various studies have reported protective effects of antioxidant as vitamin C **(4)** against oxidative damage of diabetes. Vitamin C is an essential micronutrient that acts as a non-enzymatic, water-soluble antioxidant to prevent oxidative damage by free radicals. Vitamin C exerts a uricosuric effect that may be beneficial **(5)**. Vitamin C may reduce serum concentrations of uric acid that at high levels could become crystallized in the joint and kidney and lead to gout and kidney stones **(5,6).** However, the effect of vitamin C supplementation on serum uric acid levels has not been well documented.

Vitamin C is known as natural antioxidant. All known physiological and biochemical actions of vitamin C are due to its action as an electron donor. After vitamin C donates electrons, they turned into a free radical, ascorbyl radical or semidehydroascorbic acid which is relatively stable with a half-life of 10^{-5} seconds and is fairly uncreative. Ascorbate is therefore a good free radical scavenger due to its chemical properties **(7-9).** Vitamin C can recycle the lipid-soluble vitamin E by reducing alpha-tocopheroxyl radicals in membranes **(10).**

Vitamin E (α-tocopherol) is found in virtually all cell membranes, especially in the inner mitochondrial membrane, the site of the

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electron transport system **(11)**.Vitamin E is a lipid-soluble chain-breaking antioxidant which protects especially biological membranes from lipid peroxidation **(12).**

This article aimed to study the effect of vitamin C and/or E on biochemical parameters, such as liver enzymes, kidney parameters and malondialdehyde (MDA) level in normal and diabetic rats' organ.

Material and methods

Experimental animals

This study was approved by the high society of scientific ethic committee of NNI (**N**ational **N**utrition **I**nstitute) & GOTHI **(G**eneral **O**rganization for **T**eaching **H**ospitals and **I**nstitutes**)**.

One hundred and twenty (120) male Sprague Dawly rats aged 3 months, weighing 230±20 g were used in this study. All rats were housed in wire meshed cages. The animals were fed on a standard rat diet for 10 days for acclimatization and water was *ad libitum*. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, Missouri, USA) at a dose of 50 mg/ kg body weight. STZ was dissolved immediately before use in 0.05 mol/ L sodium citrate (pH 4.5). STZ-injected animals exhibited massive glycosuria and hyperglycemia within 2-3 days.

Blood was drawn from the tail vein and blood glucose was measured using Bionime, Rightest, GM 300). Rats were considered diabetic only if their fasting blood glucose levels exceeded 250 mg /dl **(13)**. Rat diet and body weights were also recorded on a weekly basis.

The standard rat chow diet (AIN-93 M diet formulated for adult rodents) was prepared **(14-15).**

Experimental design: Rats were divided into six groups (20 rats/ group) as follows:

1. Group 1: Control rats received standard normal diet.
2. Group 2: Diabetic rats (Diabetes was induced by a single intraperitoneal injection of streptozotocin, 50 mg/kg body weight).
3. Group 3: Diabetic rats treated with glibenclamide (600 µg/kg body weight in aqueous solution).
4. Group 4: Diabetic rats treated with vitamin C (1000 mg/kg b.w. /day I. p.).
5. Group 5: Diabetic rats treated with vitamin E (600 mg/kg b.w. /day I. p.).
6. Group 6: Diabetic rats treated with vitamin C+E (I. p.).

Blood Sampling

At the end of the experiments (6 weeks, 45 days), rats were fasted overnight, and then sacrificed, anesthetized under diethyl ether. Fasting blood samples were drawn and collected in 3 tubes, 2 of them with anticoagulant. They were kept at –80 °C.

Assay of Biochemical Parameters

Glucose was determined using Randox kit **(16)**. $HbA1_C$ was determined in whole blood using Stanbio kits procedure **(17)**. Plasma vitamin E was determined colorimetrically **(18).** Urea, creatinine and protein contents were estimated **(19, 20, 21)**. ALT and AST activities were measured spectrophotometrically **(22)**. Malondialdehyde was determined in brain according to the method of **(23).** Plasma lactate dehydrogenase (LDH) was determined in brain and plasma using kinetic endpoint kits **(24).**

Statistical analysis

The statistical significance of the data was calculated using the Student's t-test. Data were expressed as means ± SE for control and experimental animals. The data were analyzed using one way analysis of variance (ANOVA) followed by post hock Duncan's test using SPSS v 11 (statistical package for social sciences). The results were considered statistically significant if the $P < 0.05$.

RESULTS

Initial body weights (IBW) were comparable between all studied groups. However, final body weight (FBW) of all treated STZ-induced diabetic groups became significantly ($P<0.001$) lower than normal controls and significantly higher than untreated STZ-induced diabetic group ($P<0.001$, Table 1). Body weight gain (BWG) and % BWG in treated STZ-induced diabetic groups range was 27.1 (G 5)-38.5 (G 4) gm and 11.68 (G 5)-16.61 (G 4) % respectively.

Serum levels of glucose and blood $HbA1_C$ of STZ-induced diabetic groups were significantly higher than control group ($P< 0.001$) and decreased

significantly in all treated STZ-induced diabetic groups compared to diabetic group (P< 0.001) but still significantly higher than normal group (Table 2).

Plasma urea, creatinine and uric acid levels were significantly increased by (+105.05%, +173.56% and +52.24%; or 2.05, 2.74 and 1.52 X fold respectively) in STZ-induced diabetic rats as compared to controls. When STZ-induced diabetic rats were treated with vitamin C and/or E, a significant normalization of these parameters was observed, as compared to diabetic rats (Table 2).

Plasma ALAT and ASAT levels were significantly increased in diabetic rats as compared to controls. When diabetic rats were treated with vitamin C and/or E, a significant decrease of ALAT and ASAT was observed, as compared to diabetic rats (Table 2).

Plasma proteins levels were significantly decreased by (-20.35%) in diabetic rats as compared to controls. When diabetic rats were treated with vitamin C and/or E, a significant increase of protein was observed, as compared to diabetic rats (Table 2).

Vitamin E concentration of diabetic rats increased significantly in plasma compared with control group (+69.77%, P< 0.001, Table 3). When diabetic rats were treated with vitamin C and/or E, a significant decrease of ALAT and ASAT was observed, as compared to diabetic rats (Table 3).

Plasma and brain LDH and brain MDA were significantly increased in diabetic rats compared with control group.

DISCUSSION

The present study was designed to observe the effects of STZ-induced diabetes on the liver, kidney and brain function after STZ treatment.

Streptozotocin is a naturally occurring nitrosamide used to develop animal models of diabetes by exerting cytotoxic effect on pancreatic β-cells possibly by generating lipid peroxides and excess **r**eactive **o**xygen **s**pecies **(ROS)**, interfering with glucose transporter GLUT-2 and causing DNA damage either by alkylation or peroxynitrite formation **(25)**. The DNA strand breakage by streptozotocin activates poly ADP-ribose polymerase (PARP) and causes ATP depletion leading to cell death and drop in insulin level **(26)**. To assess therapeutic efficacy of vitamin C and/or E we choose glibenclamide, a member of sulfonylurea drugs used in treatment of type II diabetes. The mechanism of action of glibenclamide was reported to be inhibition of a K_{ATP} channel leading to depolarization of pancreatic β-cells and stimulation of insulin release **(27)**.

In the present study, diabetes induced significant weight loss **(28)** due to excessive breakdown of tissue proteins **(29)** as well as muscle wasting, dehydration and catabolism of fats **(30).** Administration of glibenclamide, vitamin C and /or vitamin E to diabetic rats minimized body weight loss which suggests interruption, at least partially, of the previously mentioned metabolic derangements.

Glycemic control manifested by serum glucose and HbA1c, showed significant decreased in diabetics treated with vitamin C and/or E compared to diabetics rats, which may suggest either sparing of more panctreatic islet cells with treatment, enhanced insulin sensitivity or insulin –like action of these drugs. Our results agree with **Evcimen *et al.*, (28).**

Vitamin C and E might enhance insulin release or sensitivity and might spared more pancreatic β-cells with more insulin availability. Also the hypoglycaemic action of combined vitamins C and E in diabetic rats may be due to increase of antioxidant enzymes expressions and/or activities, or due to inactivation of the circulating free radicals that quench nitric oxide (NO) before it reaches pancreatic β-cells, causing damage and/or death **(31)**.

Supplementation of vitamin E might alter insulin receptors in muscle or adipose tissue by increasing membrane motility. In addition, vitamin E may enhance glucose uptake by the diaphragm.

The Hypoglycemic effect of vitamin C and E was reported by many authors **(32, 33).** Vitamin C was reported **(34)** to stimulate insulin –like mechanism. Also, vitamin E might improve glucose metabolism by muscle cells and the circulation to the islets of Langerhans and other tissues **(35)**.

The significant decrease of HbA_{1C} in all treated diabetic groups can be attributed to amelioration of hyperglycemia as well as the free radical scavenging activity of vitamin C and E **(36, 37)**. Vitamin E is very effective in glycemic control, lowering HbA_{1C} levels **(38).** The result of this study disagrees with **Ble-Castillo *et al.*, (39)** where they found that vitamin E has no effect in glycemic control.

In the present study, the administration of vitamin C and/or E significantly decreased the significant increase of blood urea and creatinine level in diabetic rats and this may imply that vitamin C and/or E had adverse effect on kidney function. The significant increased level of urea and creatinine level in STZ-diabetic rats agree with **Suchitra *et al.*, and Campos *et al.*, (40, 41).**

According to **Kedziora-Kornatowska *et al.*, (42)** Lipid peroxidation increases in the kidney of diabetic animals; this might be due to decreases in antioxidant vitamins and enzymes. Oxidative stress has been suggested to play an important role in the pathogenesis of diabetic nephropathy in which oxidative stress increases and antioxidant status is reduced.

Oxidative stress is produced as a result of diabetic conditions and possibly causes a variety of tissue damage in patients with diabetes. Increased oxidative stress in the diabetic kidney may induce apoptosis, which may contribute to the development of diabetic nephropathy **(43).** The decrease in antioxidant defence, such as vitamin C, was also observed in patients with diabetic nephropathy **(44)**. The effects of several antioxidants administered at the onset of experimental diabetes have been reported to prevent diabetic renal injury **(45, 46)**. Antioxidant therapy may be beneficial in preventing the development of diabetic nephropathy.

Antioxidants might inhibit the development of diabetic nephropathy by suppressing apoptosis. Vitamin C plays a central role in the antioxidant defence system. Vitamin C has been shown to protect all classes of lipids from oxidation under a number of relevant types of oxidant stress. The uncharged form of vitamin C, dehydroascorbate, enters cells via a glucose transporter and is then converted back to ascorbate within these cells. Because dehydroascorbate and glucose compete for glucose transporters, the presence of hyperglycemia would work to exclude vitamin C from the cell and results in a decreased antioxidant capacity in some cell types that are dehydroascorbate-dependent such as renal tubular epithelial cells.

In diabetes, vitamin C exclusion from tubular epithelial cells, through competition of glucose and dehydroascorbate for a common transport mechanism, will deprive the cells of antioxidant ability and could lead to reactive oxygen species accumulation **(47)**. Both vitamin C and/or vitamin E decreased lipid peroxidation and augmented the activities of antioxidant enzymes studied in diabetic rat kidneys as well as decreased kidney weight. These results indicate the potential utility of antioxidant vitamins in protecting against the development of diabetic nephropathy **(45, 46).**

Uric acid level increased by about 52.24% in blood of our STZ-induced diabetic's rats compared with the normal control group. Uric acid, which is the end product of purine catabolism, also exerts antioxidative properties since uric acid is considered as plasma antioxidant and may participate to the defence against an oxidative stress by scavenging various ROS **(47, 48)**. Treatment with vitamin C and/or E stabilizes uric acid in plasma and protects it from oxidation **(49).**

The significant increased level of uric acid level in STZ-diabetic rats agrees with **Suchitra *et al.*, (40)** and disagrees with **Hfaiedh *et al.*, (50)** where

they found a significant decrease in alloxan-diabetic rats.

Nieto *et al.* (51) reported that an increase in the serum uric acid in the T2DM patients might reflect a compensatory mechanism to counter the occurred oxidative stress, while **Feig *et al.* (52)** stated that T2DM patients with high uric acid levels have a greater risk of developing cardiovascular diseases. Also **Corry *et al.* (53)** suggested that uric acid can induce oxidative stress in a variety of cells, including the vascular smooth muscle cells and thus, mediate the progression of cardiovascular disease **(54).**

Hyperuricemia may initiate or promote the progression of renal disease. Hyperuricemia was found to be associated with a significantly increased risk of renal insufficiency **(55).** Evidence for a possible causal link between hyperuricemia and renal disease comes from a remnant kidney model in rats, in which hyperuricemia induced systemic high blood pressure, proteinuria, renal dysfunction, and progressive glomerulosclerosis and interstitial fibrosis **(56).**

The mechanisms by which vitamin C reduced serum uric acid might be due to increased glomerular filtration and/or competition for renal re-absorption, i.e., vitamin C and uric acid are both reabsorbed via anion exchange transport at proximal tubules **(5)**. Possible reasons for an increase in glomerular filtration include an antioxidant effect that reduces microvascular ischemia in glomeruli and leads to increased blood flow at the site, dilation of afferent arterioles, and competition for re-absorption with ions such as sodium and potassium that exert osmotic effects.

Treatment with vitamin C significantly reduces serum uric acid concentration which might be beneficial in management or suppression of the progression of renal injury in diabetic rats.

Liver enzymes are used as markers of hepatotoxicity especially ALT, which is a more specific indicator for liver damage **(57)**. Our data reveal that the plasma level of ALT and AST increased significantly in STZ-diabetes, which means that STZ may have a toxic effect on the liver and diabetes may induce hepatic dysfunction, and treatment with vitamin C and/or E reduced ALT and AST activities. The hepatocellular injury is the trigger for the release (leakage) of these enzymes from the liver cytosol into the circulation.

A significant rise in serum AST and ALT activities in diabetic rats was found when compared with control group, which could relate to excessive accumulation of amino acids (glutamate and alanine) in the serum or plasma of diabetic rats as a result of amino acids mobilization from protein stores **(58)**. The higher levels of ALT and AST, may give rise to a high concentration of glucose. In other words, the gluconeogenic action of ALT and AST plays the role of providing new supplies of glucose from other sources such as amino acids. Following treatment with vitamin C and/or E, were significantly reduced ALT and AST activities (Table 3).

Vitamin E is capable of ameliorating the impaired hepatocellular function (ALT and AST) **(59)**.

The decrease in total protein might be due to microproteinuria, which is an important clinical marker of diabetic nephropathy **(60)** and it might also be due to a reduction in protein synthesis. The elevation of serum protein after vitamin E supplementation was probably due to decreased hepatic insulin resistance allowing insulin to stimulate the incorporation of amino acids into protein **(59)**.

Vitamin E is a non-enzymatic antioxidant. Vitamin E level was significantly increased in plasma of diabetic rats compared with normal controls which agree with **Sun *et al.*, and Seven *et al.*, (61, 62)** and disagree with **Wu *et al.*, (63)** where they found a significant decrease of vitamin E in diabetic patients and also disagree with **Peerapatdit *et al.*, and Young *et al.*, (64, 65)** where they found no change in diabetic rats. The increase in plasma vitamin E may be due to

hyperlipoproteinemia accompanying diabetes since most of vitamin E is carried by lipoprotein. Studies on the effect of vitamin E supplementation and deprivation in diabetes mellitus have been carried out in humans as well as on experimental animals **(66-68)**.

Lactate dehydrogenase (LDH) is a bi-directional cytoplasmatic enzyme present in essentially all major organ systems, capable of reversible formation of pyruvate and lactate in all eukaryotic and prokaryotic cells. The extra cellular appearance of LDH is used to detect cell damage or cell death. Due to its extraordinarily widespread distribution in the body, serum LDH is abnormal in a host of disorders. It is released into the peripheral blood after cell death.

Plasma and brain LDH were significantly increased in diabetic rats compared with normal control and these results agree with **El-Demerdash *et al.*, (69)** where they found a significant increase in plasma and brain of diabetic rats. Vitamin C and/or E treatment of diabetic rats cause significant decrease in LDH activity in plasma and brain tissue.

MDA (markers of lipids peroxidation) levels increased in brain of diabetic rats **(50, 70-72)** where diabetes produces oxidative damage in many regions of rat brain including the hippocampus and the control of diabetes is influenced by the adrenocortical function. Diabetes leads to long-term complications in the brain, such as increased risk of stroke and small vessel Disease **(73, 74)**.

Treatment of diabetic rats with vitamin C and/or E improved brain MDA level where a significant decrease was noticed compared to diabetic group. The beneficial effects of vitamin C and/or E could be attributed to improved antioxidant activity in the brain leading to reduction in membrane lipid peroxidation and also may be due to the scavenging free radicals properties of vitamin C and/or E which prevent brain from damage induced by hyperglycaemia **(75).**

Treatment of STZ-induced diabetic rats with vitamin E strengthened the anti-oxidative defence system by increasing membrane fluidity in the brain of STZ-induced diabetic rats **(76).** Vitamin E has a protective or therapeutic effect against the free radical injury and oxidative stress in the brain **(77).**

Conclusion

This study showed that liver enzymes, kidney parameters are elevated during STZ-induced diabetes. The treatment with natural antioxidant as vitamin C and/or E reduced the activities of some of these enzymes. Vitamin C and/or E may thus be a useful adjuvant therapy in the management of diabetes mellitus but it is better to use combination of the two vitamins rather than in single supplements to prevent the perturb antioxidant–proxidant balance.

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Table (1): Effect of treatment of STZ-induced diabetic rats with vitamin C and/or E on initial body weight (IBW), final body weight (FBW), Body weight gain (BWG) and %BWG

Rats	G No	IBW (g)	FBW (g)	BWG (g)	% BWG	Serum Glucose (mg/dl)	Blood HbA1c (g/dl)
Control	1	229.70±2.47	294.70±4.31	65.00±2.02	28.21±0.63	88.90±1.55	5.55± 0.10
Diabetic	2	234.60±2.12	208.60±2.07 [1]	-26.00±0.47 [1]	-11.09±0.14 [1]	258.10±2.18 [1]	8.09± 0.16 [1]
Glibenclamide	3	234.10±1.80	263.70±1.72 [1,2]	29.60±1.27 [1,2]	12.65±0.55 [1,2]	120.50±2.39 [1,2]	6.24± 0.12 [1,2]
Diabetic+Vit C	4	231.80±1.28	270.30±1.55 [1,2,3]	38.50±0.0.62 [1,2,3]	16.61±0.26 [1,2,3]	139.40±1.17 [1,2,3]	6.45± 0.0.08 [1,2]
Diabetic+Vit E	5	232.30±1.16	259.40±1.44 [1,2,4]	27.10±1.22 [1,2,4]	11.68±0.55 [1,2,4]	152.40±0.75 [1,2,3,4]	6.58± 0.09 [1,2,3]
Diabetic+Vit C & E	6	233.90±1.75	263.80±1.84 [1,2,4]	29.90±0.43 [1,2,4,5]	12.79±0.20 [1,2,4]	143.70±0.60 [1,2,3,4,5]	6.28± 0.08 [1,2,5]

***: superscript letters refer to group no., which are significant with; 1: G1, 2: G2, 3:G3, 4: G4, 5: G5; Significance of P < 0.001.**

Table (2): Effect of treatment of STZ-induced diabetic rats with vitamin C and/or E on urea, creatinine, uric acid, protein levels&ALT, ASTactivities.

	G No	Urea	Creatinine	Uric acid	ALT	AST	Protein
		(mg/dl)			(IU/L)		gm/dl
Control	1	39.80±0.66	0.87±0.04	2.01±0.04	48.00±1.08	49.30±0.85	6.93±0.04
Diabetic	2	81.61±1.31[1]	2.38±0.08 [1]	3.06±0.05 [1]	93.50±2.47 [1]	91.30±1.53 [1]	5.52±0.06 [1]
Diabetic+Glibenclamide	3	56.15±0.72 [1,2]	1.28±0.04 [1,2]	2.67±0.06 [1,2]	67.30±1.55 [1,2]	70.40±0.89 [1,2]	6.12±0.06 [1,2]
Diabetic+Vit C	4	59.95±0.75 [1,2,3]	1.32±0.03 [1,2]	2.49±0.06 [1,2,3]	63.40±1.09 [1,2]	69.90±0.64 [1,2]	6.17±0.07 [1,2]
Diabetic+Vit E	5	61.65±0.69 [1,2,3]	1.41±0.02 [1,2,3,4]	2.63±0.03 [1,2,4]	64.60±0.88 [1,2]	72.80±0.58 [1,2,3,4]	6.06±0.06 [1,2]
Diabetic+Vit C & E	6	48.89±0.44 [1,2,3,4,5]	1.13±0.02 [1,2,3,4,5]	2.39±0.02 [1,2,3,5]	56.30±0.82 [1,2,3,4,5]	54.63±0.36 [1,2,3,4,5]	6.39±0.05 [1,2,3,4,5]

***: superscript letters refer to group no., which are significant with; 1: G1, 2: G2, 3: G3, 4: G4, 5: G5; Significance of P < 0.001.**

Table (3): Effect of treatment of STZ-induced diabetic rats with vitamin C and/or E on Plasma Glucose, Blood HbA1c levels.

	G No.	Plasma Vitamin E (mg/L)	MDA Brain (mg/gm tissue)	LDH	
				Plasma IU/L	Brain IU/gm
Control	1	14.62±0.46	210.75±3.42	311.75±4.51	21.38±0.46
Diabetic	2	24.82±0.77 [1]	292.93±1.38 [1]	425.00±4.27 [1]	34.75±0.36 [1]
Diabetic+Gliben-clamide	3	19.35±0.45 [1,2]	266.93±1.38 [1,2]	360.37±1.65 [1,2]	28.12±0.44 [1,2]
Diabetic+Vit C	4	16.70±0.39 [1,2,3]	251.42±1.45 [1,2,3]	340.00±3.42 [1,2,3]	26.74±0.34 [1,2,3]
Diabetic+Vit E	5	16.49±0.22 [1,2,3]	267.07±1.36 [1,2,4]	348.60±5.08 [1,2,3]	27.51±0.19 [1,2]
Diabetic+Vit C & E	6	14.95±0.12 [2, 3,4,5]	248.85±1.78 [1,2,3,5]	322.58±2.90 [2, 3,4,5]	23.02±0.08 [1,2,3,5]

***: superscript letters refer to group no., which are significant with; 1: G1, 2: G2, 3: G3, 4: G4, 5: G5; Significance of P < 0.001.**