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Effects of Combined Treatment with Vitamin C and E on Endothelial Inflammation Biomarkers and Oxidative Stress in Diabetic Rats

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Abstract: The study was designed to investigate the effects of combined antioxidants of vitamins C [L-ascorbic acid] and E [DL-α-tocopherol] on endothelial inflammation biomarkers and oxidative stress in liver and kidney in streptozotocin (an i.p. single 40 mg kg^{-1} dose) induced diabetic rats. Concentrations of interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1(VCAM-1), lipid peroxidation product, thiobarbituric acid reacting substance (TBARS) and activities of glutathione peroxidase (GHP-Px), catalase (CAT) and superoxide dismutase (SOD) enzymes were compared in 3 groups of 10 rats each: control nondiabetic rats [group I], untreated diabetic rats [group II] and diabetic rats treated with vitamins C (200 mg $kg^{-1}day^{-1}$ i.p.) and E (100 mg $kg^{-1}day^{-1}$ i.p.) for four weeks [group III]). The rats in groups II had significantly ($p<0.05$) higher levels of blood glucose, Total Cholesterol (TC), triglycerides (TG), IL-6, TNF-α, ICAM, VCAM and TBARS than rats in group I. In addition, the rats in group II had significantly ($p<0.05$) lower activities of GHP-Px, CAT and SOD than group I. The treatment with vitamins C and E significantly ($p<0.05$) lowered blood glucose, TC, TG, IL-6, TNF-α, ICAM and VCAM levels by 36, 48, 34, 23, 23, 22 and 14%, respectively. Also, lowered TBARS levels and increased the antioxidant enzyme levels near to control values. The results verify the presence of oxidative stress in diabetes and suggest beneficial effects of combination vitamins C and E in combating the oxidative stress in this disease.

Key words: Vitamin C and E, diabetes, lipid peroxidation

INTRODUCTION

In clinical and experimental research, more attention has paid to study the role of antioxidant defense systems in prevention of the human diseases such as cancer, diabetes mellitus and cardiovascular pathologies (Vural *et al*., 2000). During the progression of these diseases, oxidative stress events occur, free radicals and Reactive Oxygen Species (ROS) are generated from the molecular oxygen. These free radicals and ROS are thought to contribute lipid peroxidation and protein degradation (Aksoy *et al*., 2003). Host survival depends on the cells and tissues ability to adapt or resist stresses and repair or remove damaged molecules and cells. Multiple enzymatic and non-enzymatic antioxidant defense systems which present in cells lead to inactivate those free radicals and reduce the amount of cellular oxidative damage that caused by them. While, synthetic antioxidants have potential health hazards. Now a days, the search of natural radical scavengers (antioxidants) has a great interest among the scientists (Bastianetto and Quirion, 2002; Kaliora *et al*., 2006).

Diabetic complications appear to be multifactorial in origin. High levels of glucose react with amine residues on proteins, lipids and nucleic acids to form Advanced Glycated End Products (AGEs). The AGEs were postulated to play a role in development of diabetic complications (Goh and Cooper, 2008). In addition, endothelial dysfunction accompanied by up regulation of inflammatory mediators which act as a major contributing factor in the pathogenesis of diabetic vascular complications (Nystrom *et al*., 2006; Sitia *et al*., 2010).

Many members of the immunoglobulin family of adhesion molecules; Intercellular Adhesion Molecules (Icams) and Vascular Cell Adhesion Molecules (VCAMs) are expressed in activated endothelium. Moreover, ICAM-1 and VCAM-1 are important in the adhesion of monocytes, lymphocytes and neutrophils to activated endothelium (Ulbrich *et al*., 2003). A previous study found that soluble adhesion molecule levels in diabetic patients were significantly higher than were those in healthy controls (Glowinska *et al*., 2005). Tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are associated with cardiovascular risk factors with prevalent coronary

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heart disease playing a role in triggering and perpetuation of atherosclerosis (Sowers and Melvin, 1999; Haddy *et al*., 2003).

Streptozotocin (STZ) is widely used to induce diabetes in experimental animals by causing selective destruction of pancreatic β-cells that secrete insulin (Aksoy *et al*., 2003). Hyperglycemia in diabetes results in excessive protein glycation and the production of reactive oxidants which lead to oxidative damage in organs (Brownlee, 2001). Shi and Vanhoutte (2009) found that the elevated levels of oxygen derived free radicals are the initial source of endothelial dysfunction in diabetes.

The main objective of this study was to evaluate the effects of combined antioxidant vitamins C and E supplementation on suppressing such damage caused by hyperglycemia in diabetic rats.

MATERIALS AND METHODS

Animals: Thirty male albino rats, weighting between 170 and 200 g, were used in the study. The animals were obtained from an animal house overseen by the Tanta University Faculty of Medicine, handling of the animals was approved by the local Ethical Committee for the Care and Use of Laboratory Animals. The rats were housed on 12 h light/dark cycle and were allowed free access to tap water and rat chow. The animals were divided into three groups of ten rats each.

Experimental design: Diabetes Mellitus (DM) was induced by an intraperitoneal (i.p.) injection of streptozotocin (40 mg kg^{-1} in freshly prepared citrate buffer pH 4.5) according to (Haidara *et al*., 2009). Control rats were injected with vehicle alone. The DM was verified 48 h later by measuring blood glucose levels (after an overnight fast) with the use of glucose oxidase reagent strips (LifeScan, Milpitas, CA, USA). Rats with a blood glucose level of ≥300 mg dL^{-1} were considered to be diabetic.

The study period lasted for 4 weeks, a period which has been proved to induce detectable diabetic complications in kidney, skeletal muscles, cardiac muscles and retina (Yechoor *et al*., 2002; Knoll *et al*., 2005).

The studied groups were as follows:

- **Group I:** Control non-diabetic rats
- **Group II:** Streptozotocin (STZ)-induced, untreated diabetic rats
- **Group III:** The STZ-induced, vitamins C and E-supplemented diabetic rats that were given vitamins daily by intraperitoneal injection (200 mg/kg/day vitamin C and 100 mg/kg/day vitamin E) 3 days after STZ treatment for 4 weeks. The optimal therapeutic dosage of vitamin C and E has not been established (Abdo *et al*., 1986; Landwehr, 1991). Based on studies in rats (Aksoy *et al*., 2005; Abbas and Sakr, 2013) we used doses of (200 mg/kg/day vitamin C and 100 mg/kg/day vitamin E) (i.p.) 3 days after STZ treatment for 4 weeks

Blood and organs sampling: At the end of 4 weeks, blood samples of the fasted rats were collected from the medial retro-orbital venous plexus immediately with heparinized capillary tubes (Heparinized Micro Hematocrit Capillaries, Mucaps) under light ether anesthesia (Sanford, 1954). Then the blood was centrifuged at 3000 rpm for 15 min to separate plasma for different biochemical assays. Then the animals were decapitated under ether anaesthesia and tissue samples (liver and kidney) were rapidly excised and stored at -20°C for subsequent biochemical assays.

Biochemical assays: Plasma glucose levels were measured using the oxidase method described by Trinder (1969). Total Cholesterol (TC) and triglycerides (TG) levels were determined using fully enzymatic techniques (Wahlefeld, 1974; Borner and Klose, 1977) on a clinical chemistry analyzer (HITACHI 737; Hitachi, Tokyo, Japan); intra- and inter-assay CsV were, 1.0 and 2.1 for TC measurement and 0.9 and 2.4 for TG measurement, respectively.

Interleukin-6 (IL-6) was determined by using commercially available ELISA kits according to manufacturers' instructions (R and D Systems, Minneapolis, MN, USA). The intra- and inter-assay CsV were 4.9 and 7.1%, respectively (Song and Kellum, 2005). Tumour Necrosis Factor-α (TNF-α) level was assessed by using commercially available ELISA kits according to manufacturer's instructions (TiterZyme EIA kit, Assay Designs, Inc., Ann Arbor, MI, USA). The intra-assay CsV were 4.5% for low and 3.6% for high concentration samples whereas the inter-assay CsV were 6.0% for low and 11.8% for high concentration samples (Zhang and Tracey, 1988).

Circulating levels of ICAM-1 and VCAM-1 were assessed by using commercially available ELISA kits according to manufacturers instructions (R and D Systems, Minneapolis, MN, USA). The intra- and inter-assay CsV were <10% (Peter *et al*., 1999; Witkowska and Borawska, 2004).

Thiobarbituric Acid Reactive Substances (TBARS) in tissue was estimated by a method that described by Ohkawa *et al*. (1979). The 0.2 mL of the tissue sample was

added to 0.2 mL of 8.1% Sodium Dodecyl Sulfate (SDS), 1.5 mL of 20% acetic acid solution (pH 3.5) and 1.5 mL of 0.8% TBA. The mixture was made up to 4.0 mL with distilled water and heated in a water bath at 90°C for 60 min. After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of n-butanol were added and shaken vigorously then were centrifuged at 4000×g for 10 min. The upper butanol layer was taken and its absorbance at 532 nm was read. TBARS level was expressed as mM/100 g tissue.

Activity of glutathione peroxidase (GSH-Px) was determined according to the method of Lawrence and Burk (1976). The assay mixture consisted of 2.0 mL of 75 mM phosphate buffer (pH 7.0), 50 mL of 60 mM glutathione, 0.1 mL of 30 units mL^{-1} glutathione reductase, 0.1 mL of 15 mM EDTA, 0.1 mL of 3 mM NADPH and the appropriate amount of tissue supernatant to a final volume of 3.0 mL. The reaction was started by the addition of 0.1 mL of 7.5 mM H_2O_2. The rate of change of absorbance during the conversion of NADPH to $NADP^+$ was recorded spectrophotometrically at 340 nm for 3 min. The GSH-Px activity for tissues was expressed as μmoles of NADPH oxidized to $NADP^+$ min^{-1} mg^{-1} protein.

Catalase (CAT) activity was measured according to the method of Aebi (1984). One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μmol of H_2O_2 in 1 min. In a cuvette containing 1.95 mL of a 50 mM phosphate buffer (pH 7.0), 0.05 mL of tissue supernatant was added. The reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 1 min. Using the reaction time (Δt) of the absorbance (A1 and A2), the following equation was generated to calculate the rate constant (k):

$$k = \frac{2.3}{\Delta T} \times \frac{\log A1}{A2}$$

The enzyme activity was expressed as k mg^{-1} protein.

Superoxide dismutase (SOD) activity was measured by the inhibition of pyrogallol autoxidation at 420 nm for 10 min according to the method of Marklund and Marklund (1974). The enzyme activity was expressed as U mg^{-1} protein, where 1U is the amount of enzyme required to bring about 50% inhibition of the autoxidation of pyrogallol. The assay mixture consisted of 1.8 mL of 50 mM Tris-HCl buffer (containing 10 mM EDTA), 0.1 mL of 6.0 mM pyrogallol and the diluted tissue supernatant to a final volume of 2.0 mL. The reaction was stopped by adding 0.05 mL of 1N HCl.

Statistical analysis: Data are presented as Mean±SD. The determinations were performed on 10 animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test (Stat View) and the significance was accepted at $p<0.05$.

RESULTS

Effect of combined treatment with vitamins C and E on plasma levels of glucose, total cholesterol (TC) and triglyceride (TG) in STZ-induced diabetic rats: Table 1 shows that plasma levels of glucose, TC and TG were significantly ($p<0.05$) increased in diabetic rats when compared with normal control rats. However, treatment of diabetic rats with combined vitamins C and E significantly ($p<0.05$) lowered plasma glucose (-36%), TC (-48%) and TG (-34%) when compared with untreated diabetic rats.

Effect of combined treatment with vitamins C and E on plasma levels of IL-6, TNF-α, ICAM and VCAM in STZ-induced diabetic rats: Table 2 shows that plasma levels of IL-6, TNF-α, ICAM and VCAM were significantly ($p<0.05$) increased in diabetic rats when compared with normal control rats. However, treatment of diabetic rats with combined vitamins C and E significantly ($p<0.05$) lowered plasma IL-6 (-23%), TNF-α (-23%), ICAM (-24%) and VCAM (-14%) when compared with untreated diabetic rats.

Effect of combined treatment with vitamins C and E on TBARS levels and antioxidant enzyme activities in STZ-induced diabetic rats: The STZ-induced diabetes showed a significant ($p<0.05$) increase of TBARS levels in the liver and kidney tissues when compared with the control group (Table 3). Combined treatment with vitamins C and E

Table 1: Effect of diabetes mellitus (DM) and combined vitamins C+E supplementation on plasma levels of glucose, total cholesterol (TC) and triglyceride (TG) in samples taken from the experimental animals at the end of the experimental protocol

Parameaters	Control	DM	DM+(Vitamin C and E)
Glucose (mg dL^{-1})	89.30±3.10	398.72±9.90*	254.57±5.20†
Total cholesterol (mg dL^{-1})	45.59±2.40	98.11±3.50*	50.04±2.90†
Triglycerides (mg dL^{-1})	37.64±1.00	149.35±9.10*	98.73±4.80†

Data are Mean±SEM, *$p<0.05$ compared with the control group, †$p<0.05$ compared with the diabetic untreated group

Table 2: Effect of diabetes mellitus (DM) and combined vitamins C+E supplementation on plasma levels of pro-inflammatory cytokines (IL-6 and TNF-α) and adhesion molecules (ICAM-1 and VCAM-1) in samples taken from the experimental animals at the end of the experimental protocol

Parameters	Control	DM	DM+(Vitamin C and E)
IL-6 (pg mL^{-1})	159.74±9.30	251.37±12.340*	192.87±11.80†
TNF-α (pg mL^{-1})	81.51±3.70	131.87±10.90*	100.69±5.50†
ICAM-1 (ng mL^{-1})	311.30±7.40	413.81±8.90*	322.64±7.60†
VCAM-1 (ng mL^{-1})	603.24±7.80	710.53±10.20*	611.18±7.50†

Data are Mean±SEM, *$p<0.05$ compared with the control group, †$p<0.05$ compared with the diabetic untreated group

Table 3: Effect of diabetes mellitus (DM) and combined vitamin C+E supplementation on tissue levels of TBARS and enzyme activities of glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) in liver and kidney from the experimental animals at the end of the experimental protocol

Parameters	Control	DM	DM+(Vitamin C and E)
Liver			
TBARS (nM mg^{-1} protein)	1.34±0.10	3.42±0.40*	1.47±0.20†
GSH-Px (U/mg protein/min)	460.04±10.30	301.67±12.90*	411.25±16.30†
CAT (U/mg protein/min)	0.85±0.03	0.44±0.03*	0.80±0.02†
SOD (U/mg protein)	6.16±0.50	3.46±0.30*	5.08±0.30†
Kidney			
TBARS (nM/mg protein)	1.84±0.08	4.52±0.18*	2.07±0.09†
GSH-Px (U/mg protein/min)	147.77±11.40	97.60±9.70*	139.56±10.80†
CAT (U/mg protein/min)	0.61±0.04	0.25±0.020*	0.49±0.03†
SOD (U/mg protein)	4.54±1.00	2.51±0.40*	4.03±0.20†

Data are Mean±SEM, *$p<0.05$ compared with the control group, †$p<0.05$ compared with the diabetic untreated group

produced a significant ($p<0.05$) decrease in lipid peroxidation compared with the diabetic group (Table 3). The STZ-induced diabetes also showed a significant ($p<0.05$) reduction of GSH-Px, CAT and SOD antioxidant enzyme activities in the liver and kidney tissues when compared with the control group (Table 3). In the combined vitamins C and E-treated diabetic group, the activities of GSH-Px, CAT and SOD were significantly ($p<0.05$) increased compared with the diabetic group, which reflects restoration of the antioxidant enzyme systems to near-normal values (Table 3).

DISCUSSION

In the current study, we examined the effects of combined administration of vitamins C and E on endothelial inflammation biomarkers and oxidative stress in STZ-induced diabetic rats. Four-week treatment with vitamin C (200 mg day^{-1}) combined with vitamin E (100 mg day^{-1}) lead to decrease in plasma levels of glucose, total cholesterol, triglyceride, proinflammatory cytokines IL-6 and TNF-α and adhesion molecules VCAM-1 and ICAM-1 in diabetic rats. Combined antioxidant treatment also cause improvement of the oxidative stress status through a significant ($p<0.05$) decrease in the oxidative stress marker TBARS and increase in the of antioxidant enzymes GSH-Px, CAT and SOD activities in the liver and kidney.

Previous studies of individual antioxidants C and E in humans and animals have shown inconsistent effects on insulin sensitivity and glycemic control. Some of them show improvements in diabetic treatment such as (Paolisso *et al*., 1995; Abdel-Wahab *et al*., 2002; Hamdy *et al*., 2009; Rafighi *et al*., 2013) and others didn't show any effect such as (Barbagallo *et al*., 1999). Drawbacks of these studies are that antioxidants do not work optimally individually but they serve as part of an antioxidant system where optimal protection against disease processes occurs with several antioxidants together as found in natural foods.

Regarding the antioxidants caused decrease in blood glucose level, we propose that the antioxidants act at several sites in the insulin metabolism pathways. For example, vitamin C supplementation increases antioxidant defenses in tissue (Sasazuki *et al*., 2008), inhibits insulin glycation in pancreatic beta cells in obese hyperglycemic rats (Abdel-Wahab *et al*., 2002), also it can potentiate insulin action and improves glucose uptake in humans (Paolisso *et al*., 1994). Furthermore, vitamin E protects against oxidative modification of insulin metabolism-mediating proteins such as glucose transporters and insulin kinases (Moorthi *et al*., 2006). Inhibition of free radical production and restoration of cell redox status with vitamins C and E might preserve insulin receptor structure and function and it can also improve insulin sensitivity in diabetic rats (Maziere *et al*., 2004; Haidara *et al*., 2010).

The lipid profile, which is altered in diabetes state (Betteridge, 1994; Giacco *et al*., 2010) is one of the main significant factors in development of cardiovascular diseases. Studies have shown that increase in plasma triglyceride and cholesterol levels may be act as a risk factor for the vascular disease (Shahar *et al*., 2003). Furthermore, the oxidative modification of LDL is an important step in the development of atherosclerosis (Bhakdi *et al*., 2004). This oxidation is initiated and propagated by free radicals where antioxidants become depleted (Kaviarasan *et al*., 2005). In this study, combined vitamins C and E supplementation can significantly ($p<0.05$) reduce the total cholesterol and triglyceride in diabetic rats when compared to untreated diabetic rats. This improvement in lipid profile in the present study is supported by previous studies done by Baydas *et al*. (2002), Ozkan *et al*. (2005), Alsaif (2009) and Badr *et al*. (2012). The possible explanations for that vitamins C and E have hypocholesterolemic effects is firstly because they can prevent LDL-cholesterol from oxidative damage and help in degradation of cholesterol. Secondly, it has been suggested that vitamin C is needed by the enzyme in the first step of bile acid synthesis (cholesterol-7, α-hydroxylase) by directing cholesterol towards bile acid synthesis and consequently it reduces its level in serum (White *et al*., 1994). Kaviarasan *et al*. (2005) reported that levels of total cholesterol, triglyceride, lipid peroxidation and glucose increased in hyperlipidemic patients with or without DM whereas there was decreased plasma concentrations of vitamin C, E and other antioxidants.

As a result of the above evidences, we can suggest that combined vitamins C and E supplementation improves the lipid profile in STZ-induced DM in rats by acting through cholesterol-7, α-hydroxylase to direct cholesterol into bile synthesis. Furthermore, scavenging of the free radicals, leads to decrease the oxidative damage to oxidized LDL-cholesterol.

The results of this study showed that proinflammatory cytokines IL-6 and TNF-α and adhesion molecules VCAM-1 and ICAM-1 were significantly ($p<0.05$) higher in diabetic than in control rats, indicating that diabetes causes overexpression of proinflammatory cytokines and adhesion molecules which may aggravate the inflammatory response and tissue injury (Ulbrich *et al*., 2003; Nolte *et al*., 2004). These findings are consistent with a previous study that ICAM-1 and VCAM-1 levels were elevated in diabetic patients (Glowinska *et al*., 2005).

Hyperglycemia may trigger a generalized vascular inflammatory process contributing to atherogenesis (Haffner, 1998; Ceriello *et al*., 2004). This process may involve increasing expression of proinflammatory cytokines such as IL-1b, IL-6 and TNF-α, possibly through an increase in oxidative stress status (Ceriello *et al*., 2004) or a decrease in antioxidant defense systems (Carr *et al*., 2000). Proinflammatory cytokines stimulate the expression of adhesion molecules such as VCAM-1 and ICAM-1 on vascular endothelium (Nystrom *et al*., 2006), which promote atherogenesis.

The effects of combined antioxidants vitamin C and E on early disease biomarkers such as proinflammatory cytokines or endothelial adhesion molecules in diabetic rats are not clear. We hypothesized that the antioxidants may work synergistically to combat the stimulus of diabetes-induced oxidative damage to the endothelium and may indirectly suppress high levels of IL-6, TNF-α, VCAM-1 and ICAM-1.

Previous studies in healthy adults (Van Dam *et al*., 2003) have shown that vitamin E supplementation suppresses VCAM-1 and ICAM-1 levels, whereas studies of uncomplicated type 1 diabetes mellitus patients treated with vitamin E did not show changes in VCAM-1 (Skyrme-Jones *et al*., 2001). Previous studies have shown reductions in lipid peroxidation (estimate of oxidative stress) and concurrent reductions in VCAM-1 (Neri *et al*., 2005) and ICAM-1 (Tousoulis *et al*., 2003) with supplementation of individual or combined antioxidants. Supplementation with antioxidants such as vitamins E, C, or β-carotene is purported directly to squench free radicals such as superoxide within the endothelium; to disrupt TNF-α intracellular signaling cascades or to reduce oxidative modification of LDL-C. All of these would otherwise initiate expression of endothelial adhesion molecules (Frei, 1999; Devaraj and Jialal, 2000).

In the present study, we analyzed liver and kidney TBARS level (as a marker of oxidative stress) and activities of antioxidant enzymes as the liver is the main metabolic organ and nephropathy is a common complication in diabetic patients. Since, high blood glucose is susceptible to oxidation, hyperglycemia causes high ROS production and on the other hand leads to high TBARS in tissues (Wolff and Dean, 1987; Brownle, 2001). We suggest a possible mechanism to explain why combined vitamins C and E supplementation may have an indirect effect in reducing TBARS level by their hypoglycemic and direct radical scavenging activities.

As shown in results, activities of antioxidant enzymes were deteriorated by STZ-induction. Combined vitamins C and E supplementation in diabetic rats protected to a certain degree, further deterioration of GSH-Px and CAT in liver and kidney. The antioxidant enzymes GPX, CAT and SOD are known to be inhibited in diabetes mellitus as a result of non-enzymatic glycosylation and oxidation (Kakkar *et al*., 1995). The positive impact of treatment with combined vitamins C and E on these enzymes observed in the present study could be explained by two possible mechanisms. First, the antioxidative effect of combined vitamins C and E may prevent further glycosylation and peroxidation of proteins by interacting with free radicals and hence, minimizing their noxious effects. Second, combined vitamins C and E may induce the protein synthesis of these enzymes which explains the observed elevated activity after treatment. In support of this view is the observation of Pawlowska-Goral *et al*. (2002) and Vimal and Devaki (2004), who found that polyphenolic substances such as flavonoids and vitamins increased the expression of SOD and GPX enzymes at the transcriptional level.

CONCLUSION

Short-term combined administration of vitamins C and E decreased plasma levels of glucose, total cholesterol, triglyceride, proinflammatory cytokines IL-6 and TNF-α and adhesion molecules VCAM-1 and ICAM-1 in diabetic rats. Combined antioxidant treatment also improved oxidative stress status through a significant decrease in the oxidative stress marker TBARS and an increase the activity of antioxidant enzymes GSH-Px, CAT and SOD in the liver and kidney. Therefore, this strategy may provide a therapeutic tool in cases of oxidative stress-induced cellular damage including diabetes. In general, prophylaxis with vitamins C and E may be valuable in reducing the risks of diabetes and its complications.

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