Effects of Palm Vitamin E Against Oxidative Damage in Streptozotocin-induced Diabetic Rats

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Abstract
Palm vitamin E, which rich in tocotrienol is a highly effective antioxidant and able to reduce oxidative stress in various pathological conditions. Diabetes mellitus is often associated with increase in the production of reactive oxygen species together with reduction in antioxidants levels. In order to elucidate the antioxidative effects of palm vitamin E, we investigated the effects of 8 weeks supplementation with palm vitamin E on glycaemic status, malondialdehyde levels and DNA damage in Streptozotocin-induced diabetic rats. Alkaline Comet Assay assessed protective effects against DNA damage to peripheral blood. Streptozotocin diabetic rats were force fed daily with palm vitamin E (200mg/kg body weight), from day three of diabetes condition established. Following eight weeks of supplementation, palm vitamin E significantly improved fasting blood glucose and glycosylated hemoglobin. The increased in plasma malondialdehyde levels were also inhibited and the levels of oxidative DNA damage of peripheral lymphocyte was reduced. This suggested that palm vitamin E has the potential in improving glycaemic status and also the protective effects against oxidative damage by inhibiting the increased in malondialdehyde and preventing DNA damage in Streptozotocin-induced diabetic rats.

Keywords: Diabetes mellitus, palm vitamin E, antioxidant, oxidative Stress, DNA damage

Introduction
A considerable amount of evidence has suggested that oxidative stress plays an important role in the pathogenesis and complication of diabetes mellitus (DM). DM has often been associated with increased oxidative stress in vivo [1]. The increased in oxidative stress in diabetic patients occurred particularly in subjects with poor glycaemic control [1]. Studies on diabetic subjects have demonstrated that increase in the production of free radicals, as a result of hyperglycaemia, resulted in glycosylation of proteins and production of more reactive oxygen species (ROS) [1]. In addition, the oxidative stress is further exacerbated by the decrease in antioxidant enzymes activity, including superoxide dismutase (SOD), catalase, and glutathione peroxidase [2]. Under these conditions, damage can occur to cellular biomolecules such as lipid, protein, carbohydrate and DNA [3].

Lipid peroxidation products in plasma and cell membrane have been used as the main biomarkers of oxidative damage [4]. A broad range of oxidation products have been described [4], including lipid peroxides, which are the precursor for other reactive intermediates such as alkoyxyl radicals, and hydroxalkenalkenals formed in lipid peroxidation reaction, including malondialdehyde (MDA). A number of lipid peroxidation products mainly, MDA and 4-hydroxy-2-nonenal are known to interact with DNA [5]. Such interaction can lead to cytotoxicity, genotoxicity, and carcinogenecity [6].

Vitamin E is a highly effective antioxidant and able to reduce the oxidative stress in many pathological conditions including DM [7]. The protective effect of vitamin E on oxidative stress in DM may be mediated through inhibition of free radical formation. Palm vitamin E (PV) which contains tocopherol and mainly tocotrienol is also an excellent antioxidant [8]. PV has been used as a nutritional supplement and also has several potential benefits including therapeutic potential [8]. Some researchers have found that tocotrienol is more potent than tocopherol in preventing the oxidative damage in cell membrane [9].

Therefore the aim of the study was to evaluate the potential of PV in preventing oxidative damage in DM. In the presence study we investigated the effects of oral supplementation of PV on glycaemic status, lipid peroxidation and DNA damage on Streptozotocin (STZ)-induced diabetic rats.

Methods
Animals
Male Spraque Davey rats weighing 260-290 gram were supplied by Animal House of Universiti Kebangsaan

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Malaysia, which located at the Institute of Medical Research, Kuala Lumpur. Animals were housed two per cage. All animals were maintained on a balanced diet and water ad libitum without restricted. The study was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) no; Biomed/2001/Sit/17-May/047 and UKMAEC guidelines were followed, while using live animals.

Induction of Diabetes

Diabetes was induced after an overnight fast by single intravenous injection of STZ (50 mg/kg) (Sigma, St Louis, MO, USA), which was freshly dissolved in normal saline. Another group of rats, which only received saline injection, formed the non-DM group (NDM) (n=7). Three days later blood were withdrawn via tail vein and glucose concentration was measured by a strip-operated blood glucose sensor (Companion 2, Medisense Ltd., Birmingham, UK). Rats with blood glucose levels >15.0 mmol/l were included in the study and divided into two groups i.e. supplemented with PV (n=8) and not supplemented with PV (No Suppl) (n=8). PV was obtained from Malaysian Palm Oil Board. PV administered orally at a dose of 200 mg/kg/day daily throughout the feeding period of eight weeks and the chosen dose was based on the previous research [10]. The supplementation was started at day three after diabetic induction. No Suppl and NDM rats were left untreated. Food and water intake was recorded daily whereas body weight was recorded once a week.

Biochemical Analysis

After eight weeks of supplementation, rats were fasted overnight and blood was collected by cardiac puncture under deep anesthesia with diethyl ether. Blood was collected into tubes containing EDTA and kept on ice overnight and blood was collected by cardiac puncture. Biochemical parameters were analyzed by one-way ANOVA and distribution was verified by Shapiro-Wilk test. The resulting cell pellet was washed 3 times with phosphate-buffered saline (PBS) without Mg²⁺ and Ca²⁺. Washed pellet were then re-suspended in 200 µl of PBS and stored at 4°C in the dark (to minimize additional DNA damage), to be use on same day for the Alkaline Comet Assay.

The Alkaline Comet Assay procedure was done as described previously by Singh et al. (1988) [13] with a slight modification. 100 µl of 0.5% normal melting point agarose (Sigma) was pipetted onto frosted microscopic slides and allowed to solidify under coverslip, which was then carefully removed. 10 µl of lymphocyte were suspended in 80 µl low melting point agarose gel, rapidly pipetted onto the first agarose layer, and gently spread by placing a coverslip on top. This was allowed to solidify on an ice tray for 5 min. After removal of the coverslip, the slides were immersed in freshly prepared lysing solution (2.5 M NaCl, 100mM EDTA, and 10 mM Tris, with 1% Triton-100, pH 10) and incubated for 2 hour at 4°C. The slides were removed from the lysing solution, drained, and placed in horizontal gel electrophoresis tank. The tank was filled with fresh, cold electrophoresis solution (1mM EDTA and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali-labile damage before electrophoresis. Electrophoresis was conducted at 4°C for 20 min using 25V and current of 3000 mA. Following electrophoresis, the slides were washed (3 times) using neutralisation buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali. Finally, the slides were stained with 50 µl ethidium bromide (20 µg/ml Sigma USA).

Slides were stored in a lightproof box containing PBS moistened tissues and viewed at the following days. Observation were made using Leitz Laborlux fluorosence microscope (Nikon) equipped with an epifluorescence mercury lamp source (excitation filter 515, barier filter 590nm) and X40 fluorescence objective (numerical aperture 0.85) and data were analyzed using a specialized single-cell gel (SCG) image analysis programmed (TriTex Comet Score™ (free comet score))

The image analysis software provides a full range of densitometric and geometric parameters describing the complete comet, as well as the head and tail of DNA portions. Since the comet assay essentially reflects the displacement of fluorescence from the head to the tail in damage cells, we used % tail DNA, i.e. the percentage of total nuclear DNA that has migrated to the tail, and tail moment (% Tail DNA x length) as the parameter to quantify basal levels of DNA damage. Each slide was analyzed in duplicate and 50 cells per slide were scored.

Statistical Analysis

Statistical analysis was performed using SPSS statistical package version 11.0. All results were expressed as mean ± standard error of mean (SEM). Normality of distribution was verified by Shapiro-Wilk test. The parameters were analyzed by one- way ANOVA and
followed by LSD post hoc test to compare the difference between groups. The differences between groups were considered significant at p<0.05.

**Results**

Throughout eight weeks of study, water and food intake of No Suppl diabetic group were markedly increased when compared to NDM group (Figure 1A and 1B). Food and water intake, were calculated per cage, so that statistical analysis was not legitimate. As seen in Figure 1A and 1B, PV supplementation had considerable effect on food and water consumption. Diabetic rats exhibited significantly reduced body weight compared to NDM group. PV supplementation caused less body weight reduction and the final body weight of PV group was significantly higher than No Suppl group.

Throughout eight weeks of study, all diabetic rats showed persistent hyperglycemia. The palm vitamin E supplementation induced a significantly decrease in blood glucose and HbA1c levels of diabetic animals compared with No Suppl diabetic rats (Table 1). MDA levels, an indicator of lipid peroxidation, were significantly higher in Non Suppl than NDM group. Supplementation of diabetic rats with PV for eight weeks normalized plasma MDA level (Figure 3).

**Table 1:** Plasma glucose and HbA1c levels in the NDM, Non Suppl and PV groups after eight weeks of study

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<td>No Suppl</td>
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<td>PV</td>
<td>26.23 ± 0.65 * *</td>
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Data are the mean ± SEM. * P<0.001 compared with the NDM group; * P<0.01 compared with the No Suppl diabetic group.

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**Figure 1:** Mean Weekly of water (A) and food (B) intake in different group of rats. Food and water intake were calculated per cage (two animals in a cage), so that statistical analysis was not legitimate.

**Figure 2:** Rats body weight in different groups of study. * P<0.001 vs NDM group, * p<0.01 vs No Suppl diabetic group.

**Figure 3:** Plasma MDA levels in different groups of rats. * P<0.001 vs NDM group, * P<0.001 vs No Suppl diabetic group.

**Figure 4:** Images of comets in different groups of study.

* P<0.001 vs NDM group, * P<0.001 vs No Suppl diabetic group.
Figure 4 shows the images of lymphocyte DNA after Comet Assay was performed. The basal of endogenous DNA damage measured by the mean percentage Tail DNA of the lymphocytes in diabetic rats was significantly higher in No Suppl rats compared to NDM rats (p<0.001). PV supplementation significantly lowered the percentage of Tail DNA than No Suppl diabetic rats (p<0.01) (Figure 5). Tail moment, which is defined as the product of the tail length and the fraction of DNA in the tail were also used as the parameter to describe the extent of DNA damage. Diabetic rats showed significantly higher levels of tail moment as compared to NDM rats (p<0.001) and PV supplementation was also able to normalize the tail moment value.

STZ injection causes destruction to the β cell of pancreas and lead to reduction of insulin secretion in STZ induced diabetic rats. This mechanism cause increase in plasma glucose levels, which lead to diabetes condition. The diabetic rats also exhibit polyphagia due to failure of cells to get glucose as a source of energy. This may further increase in blood glucose levels. As an alternative, diabetic animals get their source of energy from catabolism protein and fat, which lead to the reduction in body weight. Hyperglycaemia also leads to polyuria and polydipsia. In the present study, all diabetic rats showed higher levels of glycaemic status, food and fluid intake as well as decrease in body weight, which also has been reported by previous researcher [16].

In the present study, after eight weeks of supplementation, PV has lower levels of FBG and HbA1c as compared with No Suppl diabetic group. This finding shows that PV has the ability to improve the glycaemic status in streptozotocin-induced diabetic rats. Improving the glycaemic status could be the reason for the improvement in body weight reduction, food and fluid intake as can be seen in the PV supplementation rats.

The extension in glycation of hemoglobin in red blood cell depends on long-term of blood glucose concentration. The percentage of glycated hemoglobin concentration correlates most strongly with the mean of blood glucose concentration assessed during the preceding 4-6 weeks. Therefore lowering the glucose concentration in plasma would also result in the reduction of HbA1c formation.

Vitamin E supplementation has been shown to decrease HbA1c and lipid peroxidation in type 1 diabetic patients [17]. The study also suggested that improvement of glycaemic status could be due to the improvement in glucose metabolism. Meanwhile, Luostarinen et al. (1995) had showed that vitamin E supplementation for four weeks was able to prevent hyperglycemia condition following fish oil intake in healthy volunteers [18]. Vitamin E supplementation increased the production of insulin and increase insulin: glucose ratio. The antioxidant effects of vitamin E could also protect β cell destruction due to the lipid peroxidation cause by fish oil [18].

PV is also an excellent antioxidant, which has been used as a nutritional supplementation and has several potential benefits including therapeutic potential [19]. Having antioxidant properties, PV would able to scavenge free radical that has been produce by STZ injection. Pancreas has low levels of antioxidant and easily attack by free radicals [20]. Chronic hyperglycemia induces oxidative stress on the pancreatic β cell, which cause cytotoxicity that might worsen the diabetic state [21]. Vitamin E is a major peroxyl radical scavenger in cell membranes. It interrupts or inhibits reactions of lipid peroxidation, which is the basis for its function as antioxidant [22]. Therefore, antioxidant supplementation may be effective in preventing pancreatic damage cause

Discussion

DM is associated with increased in oxidative stress in vivo. The elevated oxidative stress may be caused by increased levels of free radicals due to their increased production and/or decreases in the antioxidant defense system [14]. The increased oxidative stress together with poor metabolic control enhances lipid peroxidation in diabetic patients, which has been proposed to be associated with the etiology of diabetic complications [15].
Effects of palm VE in diabetic rats

shown to reduce H2 O2 -induced HO • generation and also as a free radical scavenger [31]. Vitamin E has been mediated through inhibition of free radical formation and E on oxidative stress induced DNA damage may be chromosomal damages. The protective effect of vitamin E has already been reported to have antioxidative effect [26]. Cumulative evidence had demonstrated that antioxidants can influence the reaction of the cells to due to free radical toxicity [27, 28]. The presence of antioxidants may ameliorate pathogenesis of diabetes complication due to free radical activity. Ihara et al. (2000) reported that vitamin E supplementation before the induction of diabetes by STZ could prevent the destruction of β cell and improved the glycemic status in of diabetes rats [23]. In our study, PV supplementation was started on day 3 after STZ induction. Even though the destruction of β cells has already taken place and caused chronic hyperglycemia, further destruction may be prevented by PV. This is probably through its antioxidant mechanism. Therefore, in this present study the mechanism on how PV could improve glycaemic status is uncertain, but it could be due to the stimulation of glucose metabolism and also through its antioxidant effects, which protect the pancreas from oxidative damage.

DNA damage in mononuclear cells from diabetic subjects has been reported recently [24]. The levels of DNA damage in mononuclear cells from diabetic patients with poor glycemic control were higher as compared with patients with good glycemic status. However, no significant changes could be seen in the levels of DNA damage in subjects with good glycemic control compared to non-diabetic patients [25].

In the present study, we used the Alkaline Comet Assay to measure basal levels of DNA damage in freshly isolated lymphocyte. There were significant increases in the levels of DNA damage in the lymphocyte from No Suppl diabetic rats. Our results also indicated that MDA levels were significantly higher in Non Suppl diabetic rats. This finding shows that DNA damage is associated with the production of free radicals. Free radicals including hydroxyl radicals, singlet oxygen, peroxyl radicals and peroxynitrite are able to produce other modification to the DNA bases as well as strand breakage and various other DNA damage [26].

It has been suggested that administration of antioxidant may ameliorate pathogenesis of diabetes complication due to free radical toxicity [27, 28]. The presence of antioxidants can influence the reaction of the cells to external mutagens and also the ability to detoxify of the mutagens [29]. Study showed that fluvastatin, and its metabolites have been reported to have antioxidative effect against oxidative DNA damage either in vitro [30] or in vivo [26]. Cumulative evidence had demonstrated that vitamin E significantly reduces free radical induced chromosomal damages. The protective effect of vitamin E on oxidative stress induced DNA damage may be mediated through inhibition of free radical formation and also as a free radical scavenger [31]. Vitamin E has been shown to reduce H2O2 -induced HO • generation and subsequent DNA pair base modification in human oral epithelial cells [32] and H2O2 induced DNA strand break in human skin cell [33]. In addition to inhibiting free radical formation, vitamin E has been suggested to enhance the repair of DNA damage by increasing the rate of removal of damage DNA [34]. Vitamin E supplementation also had resulted significant decrease in DNA damage in diabetic patients and smokers [35].

In the present study PV supplementation for eight weeks managed to prevent the increase in MDA levels and inhibit DNA damage in diabetic rats. This finding indicates that PV has the potential in reducing oxidative damage. The percentage of comet tail in PV group is significantly low when compared with untreated diabetic rats, although markedly higher when compared with the NDM group. However, the levels of DNA damage as measured by tail moment, showed no significant different between NDM and PV groups. This indicated that the DNA damage that occurs in PV supplementation rats had been repaired.

The exact mechanism how palm vitamin E reduced DNA damage in this study we does not fully understood. The most probable mechanism is through its antioxidative effects. Tocotrienol, which is the main isomer of vitamin E in PV is an excellent antioxidant [8] [9]. A recent study has shown that PV has the potential in improving oxidative stress in diabetic animal [10]. Tocotrienol also possess greater antioxidant effect than tocopherol [36]. Tocotrienol may be more effective than tocopherol in lowering the concentration of peroxides in the plasma and possessed greater antioxidative activity toward peroxyl/alkoxyl radicals in vitro [36].

Study has demonstrated a relationship between chronic hyperglycaemia and the development of secondary complications in diabetes patients. It has been postulated that oxidative stress due to chronic hyperglycaemia may play an important role in etiology of diabetic complications [14, 15]. In the present study the antioxidant properties of PV seem to be major contributing factors to the improvement in oxidative damage. As glycemic control plays an important role in the progression of diabetes mellitus and the development of secondary complication, the reduction of oxidative stress in PV supplementation diabetic rats may be associated with the improvement in glycemic index. In conclusion PV may has the potential to reduce oxidative stress and inhibiting DNA damage in diabetic rats. This is possibly through the improvement of glycaemic status and its antioxidant properties.

Acknowledgments

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Refences


9. Abdul Mutalib MS, Khaza’ai H, Wahle KWJ. Palm-tocotrienol rich fraction (TRF) is a more effective inhibitor of LDL oxidation and endothelial cell lipid peroxidation than (-tocopherol in vitro. Food Research International. 2003; 36(5): 405-413.


