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Palm vitamin E reduces oxidative stress, and physical and morphological alterations of erythrocyte membranes in streptozotocin-induced diabetic rats

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Key Words

Diabetes mellitus; Erythrocyte membrane;
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Palm vitamin E

Abstract

Hyperglycemia-induced oxidative stress in diabetic conditions promotes alterations of function and other properties of the erythrocyte membrane. In this study, we examined the effects of palm vitamin E (200 mg/kg) on oxidative stress and the physical and morphological characteristics of erythrocyte membranes in streptozotocin-induced diabetic rats. Forty male rats were randomly divided into 4 groups as follows: Group 1, control group; Group 2, normal plus palm vitamin E treatment; Group 3, untreated diabetic group; and Group 4, diabetic plus palm vitamin E treatment. After 4 weeks of treatments, we determined complete blood count, erythrocyte osmotic fragility, erythrocyte morphology, levels of erythrocyte malondialdehyde and protein carbonyl, levels of erythrocyte glutathione and oxidized glutathione, and total plasma antioxidant capacity. The results of osmotic fragility were significantly higher in the diabetic group compared to the control and diabetic with palm vitamin E. There were significant morphological changes in the erythrocytes of the diabetic group compared to the control group. We also found a significant decrease in the hemoglobin concentration, hematocrit percentage, and erythrocyte distribution width in the diabetic group. Erythrocyte malondialdehyde and protein carbonyl levels were significantly higher in the diabetic group compared to the control group. Decreased levels of reduced glutathione and increased level of oxidized glutathione were observed in the diabetic group compared to the control and diabetic with palm vitamin E groups. Plasma total antioxidant capacity was significantly lower in the diabetic group than in the control group, whereas plasma total antioxidants in the diabetic with palm vitamin E group were significantly increased compared to the untreated diabetic group. In conclusion, 4 weeks of daily supplementation with 200 mg/kg palm vitamin E could reduce the levels of oxidative stress markers by inhibiting lipid peroxidation and protein oxidation in streptozotocin-induced diabetic rats.

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INTRODUCTION

Diabetes is one of the leading causes of death and disability worldwide [1]. In 2000, the World Health Organization recorded a total of 171 million people (2.8% of the population) with diabetes for all age groups worldwide, and these numbers are expected to rise to 366 million (4.4% of the population) by 2030 [2]. To date, many studies have suggested that high

levels of glucose in the blood can cause cellular membrane damage and cell death of a number of cell types, including cultured pericytes, endothelial cells, kidney cells, retinal cells, and red blood cells (RBCs) [3].

Diabetes is a group of metabolic diseases characterised by high levels of blood sugar (hyperglycemia). It results from defects in insulin production, insulin

action, or both, as well as impaired functions in the metabolism of carbohydrate, lipid, and protein that lead to long-term complications [1, 4].

Human erythrocytes are among the cells that are highly exposed to peroxidation damage caused by free radicals [3]. As the RBC emerges from the bone marrow, it loses its nucleus, ribosomes, and mitochondria, as well as the capacity for protein synthesis. The human erythrocyte, because of its role as O₂ and CO₂ transporter, is under constant exposure to reactive oxygen species (ROS) and oxidative stress. Oxidative stress occurs in the cells or tissues when the ROS concentration exceeds the level of antioxidant protection [5].

In the past few decades, there has been a growing interest in characterising erythrocyte membrane defects in several diseases [6], as changes in the membrane structure can contribute to disease progression [7]. Hyperglycemia-induced oxidative stress in diabetic conditions promotes alterations of the erythrocyte membrane function and other properties, which leads to adverse diabetic complications. Therefore, the search for factors that can reduce the risk associated with oxidative stress-induced erythrocyte damage in diabetes is fundamental [7]. The presence of microvascular and atherosclerotic disorders is a well-known complication in diabetes mellitus, and these complications are highly associated with the increased oxidative stress and abnormalities in erythrocyte composition [3, 8]. The oxidative damage to erythrocytes in diabetic conditions is apparent as disturbed expression or activity of the free radical-scavenger systems [9] or by free radical-associated modifications in the structure and/or components of the erythrocyte membranes [10, 11]. The increased levels of ROS in the diabetic erythrocytes affect their function, leading to diminished lifespan, reduced deformability [12], and increased microviscosity [13], aggregation, and adhesiveness to the endothelial layer, which enhances thrombotic events [14].

Vitamins E has been shown to have beneficial effects in diabetics via its antioxidant properties, which enable it to reduce cell membrane damage, improve glucose metabolism, increase the effectiveness of insulin, and reduce the attachment of glucose to proteins [15]. Vitamin E is a well-known lipophilic free radical scavenger that is mainly localised in the biological membranes. Thus, this study aimed to examine the effect of palm vitamin E (PVE) treatment on oxidative stress and physical and morphological alterations of erythrocyte membranes in streptozotocin (STZ)-induced diabetic rats. To achieve this, the hematological parameters, morphological analysis, osmotic fragility, and erythrocyte membrane stability were examined.

MATERIALS AND METHODS

Experimental design

Forty male Sprague–Dawley rats aged 6–8 weeks and weighing 200–250 g were provided by Laboratory Animal Resources Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The rats were handled according to the approval guidelines issued by the University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC no: FSKB/BIOMED/2010/JAMALUDIN/20-MAY/304-MAY-2010-MAY-2013). The rats were housed in plastic cages (2 rats/cage) and fed with mouse pellet 702 P (Gold Coin Sdn. Bhd.) and tap water *ad libitum*. The animals were acclimatised to standard laboratory conditions at 25°C with 12-h light-dark cycles for 1 week. Prior to the experiments, the rats were divided into 4 groups containing 10 rats each, as follows: normal rats supplemented with distilled water served as control group (Group 1), normal rats supplemented with 200 mg/kg body weight (BW) PVE (Group 2), untreated diabetic rats (Group 3), and diabetic rats supplemented with 200 mg/kg PVE (Group 4). Induction of diabetes was achieved following an overnight fasting by a single intravenous injection (via tail vein) of STZ (Sigma, St. Louis, MO, USA) at 45 mg/kg BW. After 3 days, blood samples were collected via the tail vein, and glucose concentration was measured using a strip-operated blood glucose sensor (Companion 2; Medisense Ltd., Birmingham, UK). Rats with blood glucose levels of >15.0 mM were selected for the study. PVE (provided by Malaysian Palm Oil Board) was orally administered at 200 mg/kg BW daily for 4 weeks. Following 4 weeks of PVE supplementation, the rats were fasted overnight, and blood samples were collected by cardiac puncture under deep anaesthesia with diethyl ether. Blood was collected into tubes containing sodium fluoride and EDTA for fasting blood glucose and erythrocyte analyses, respectively.

Erythrocyte isolation

Erythrocyte isolation was achieved through centrifugation of blood at 4,000 rpm at 4°C for 10 min. After the removal of plasma and buffy coat, erythrocyte pellets were washed 3 times with cold 9% normal saline. Erythrocytes were stored at –80°C until further biochemical analyses.

Measurement of erythrocyte fragility

Erythrocyte fragility was determined according to the method previously described by Godal *et al* [16]. Blood (50 µl) was added to 5 ml of buffered sodium saline containing sodium chloride solution ranging from 0.1 to 0.9%. Samples were carefully mixed and incubated at room temperature for 30 min, followed by centrifugation at 2000 rpm for 5 min. The absorbance

of the supernatant was measured using a spectrometer at 540 nm. The hemolysis of RBCs in each tube was expressed as a percentage against the maximum value of absorbance of distilled water.

Scanning electron microscopy

Morphological observations of the erythrocytes by scanning electron microscopy (SEM) were performed according to the method described by Przybylska *et al* [17]. Erythrocytes were washed 3 times by adding 5 portions of normal saline buffer (pH 7.4) to 1 portion of whole blood, and the mixture was centrifuged at 3500 rpm for 20 min. The washed RBCs were fixed with 2.5% (w/v) glutaraldehyde in the same buffer to achieve about 50% of final hematocrit (Hct) for 1 h, and then allowed to settle on standard microscopic cover glasses for 1 h. Samples were post fixed with 1% (w/v) osmium tetroxide for 1 h, and dehydrated with ascending ethanol series (30, 50, 70, 85, and 95% v/v) in 100% acetone. Subsequently, all samples were dried with CO₂, coated with gold-palladium, and examined by SEM (Tecnai G2, FEI, Hillsboro, OR, USA). Altered erythrocyte shape was evaluated by counting at least 500 cells (50 RBCs for each SEM field at 3000x magnification), and the mean morphological index was calculated.

Hematological study

Freshly collected blood samples were analysed using an automatic hematology analyser: Veterinary package software enables the cell-DYN-3700 hematology system to analyse animal samples. Total RBC counts, total white blood cell counts (WBCs), hemoglobin (Hb) concentration, Hct, mean cell hemoglobin (MCH), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), RBC distribution width (RDW), and platelets (Plt) were analysed.

Measurement of malondialdehyde in erythrocytes by HPLC

Malondialdehyde (MDA) levels in rat erythrocyte samples were measured by HPLC with slightly modifying the procedures described by Pilz *et al* [18], based on the derivation with 2,4-dinitrophenylhydrazine (DNPH). Free MDA was prepared from the erythrocytes by perchloric acid deproteinisation, while an alkaline hydrolysis step for 30 min at 60°C was introduced prior to protein precipitation for the determination of total (free and bound) MDA. Standards and samples were processed under the same conditions as described.

MDA standard was prepared by dissolving 25 µl of 1,1,3,3-tetraethoxypropane (TEP) in 100 ml of 1% sulphuric acid to yield 1 mM stock solution, which was kept at 4°C overnight. Working standards were prepared by the hydrolysis of 1 ml of TEP stock

solution in 50 ml of distilled water (dH₂O) and incubated for 2 h at room temperature. The TEP stock solution was diluted to working standards of 1, 2, 4, 6, 8, and 10 µmol to generate the standard curve for the estimation of total MDA.

Sample preparation

To 50 µl of the standard or sample, 200 µl of 1.3 M NaOH was added, and the mixture was vortexed for alkaline hydrolysis of bound proteins. The mixture was incubated in a water bath at 60°C for 30 min, and then cooled on ice for 5 min. The hydrolysis samples were acidified with 100 µl of 35% (v/v) perchloric acid. After centrifugation at 1000g for 10 min, 300 µl of supernatant was transferred to a 2-ml HPLC vial, mixed with 12.5 µl of DNPH solution, incubated at room temperature for 30 min in the dark, and then 40 µl was injected onto the HPLC system.

HPLC analysis

Analytical HPLC separations were performed with a Model 655A-12 liquid chromatography (Shimadzu SCL-10AVP) PDA (M10AVP) equipped with an auto injector (655A-40) and a variable-wavelength UV detector (655A) operated at 310 nm (0.0025 AUFS) on a 125 x 3 mm Nucleosil C₁₈ column of a particle size of 5 µm for reverse-phase chromatography with integrated precolumn (SepServ, Berlin, Germany). The column was kept at 30°C in a column oven with a mobile phase flow rate of 1 ml/min.

Measurement of protein carbonyl

The determination of protein carbonyl from erythrocyte membranes was performed, according to a previously described method [19, 20]. Erythrocyte membrane protein extract (50 µl) was precipitated with 20% cold trichloroacetic acid (TCA) and incubated on ice for 15 min, and then collected by centrifugation for 5 min at 15000g. DNPH (200 µl) was added to the protein pellet of each sample to yield a final protein concentration of 1-2 mg/ml. Samples were allowed to stand in the dark at room temperature for 1 h with vortexing every 10 min, and then, the samples were precipitated with 10% TCA (final concentration) and centrifuged for 5 min. The supernatants were discarded, the protein pellets were washed once again with 10% TCA, and then washed 3 times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove any free DNPH. Samples were then resuspended in 1 ml of 5 M urea, vortexed, and incubated in a water bath for 15 min at 37°C. Carbonyl content was determined from the absorbance at 380 nm. The carbonyl content was calculated using an absorption coefficient (e) of 22,000 M⁻¹·cm⁻¹, and the data are expressed as nmol/mg-protein.

Measurement of reduced, oxidized, and total glutathione levels

A commercial glutathione (GSH) detection kit (Biovision, Livingston, NJ, USA) was used to measure GSH, oxidized GSH (GSSG), and total GSH. The values for samples and standards were determined using a fluorescence plate reader equipped with Ex/Em 340/420 nm (SkanIt software, Thermo Fisher Scientific).

Ferric reducing antioxidant power assay

Total antioxidant capacity was determined based on the method described by Benzie and Strain [21], and the samples were analysed using a microplate reader (Bio-Rad microplate, Manager 6.1 Mark software). The ferric reducing antioxidant power (FRAP) value was directly proportional to the change in absorbance at 593 nm caused by the reduction of a ferric tripyridyltriazine (TPTZ) complex to a ferrous TPTZ complex at pH 3.6, during which an intense blue colour was formed. Standard FRAP solution (1000 $\mu\text{mol/l}$) was prepared by adding 27.8 mg of $\text{Fe SO}_4(\text{H}_2\text{O})_7$ to MilliQ water and making up to 100 ml. The standard was used as FRAP calibrator. The sample (10 μl) was added to 300 μl of FRAP reagent, which was freshly prepared each time by mixing 300 mmol/l acetate, 20 mmol/l ferric chloride, and 10 mmol/l TPTZ. Samples were incubated for 4 min at 37°C, and the absorbance change at 593 nm in the first 4 min of the reaction time of each sample was compared to that of the 1000 $\mu\text{mol/l}$ ferrous standard. The FRAP values were calculated as $\mu\text{mol/l}$.

Statistical analysis

All results are expressed as the mean $\pm$ standard error of the mean (S.E.M.) values. The data were analysed by one-way analysis of variance (ANOVA), followed by post-hoc LSD multiple comparison test to estimate the significant difference between groups. The difference between groups was considered significant when $p < 0.05$.

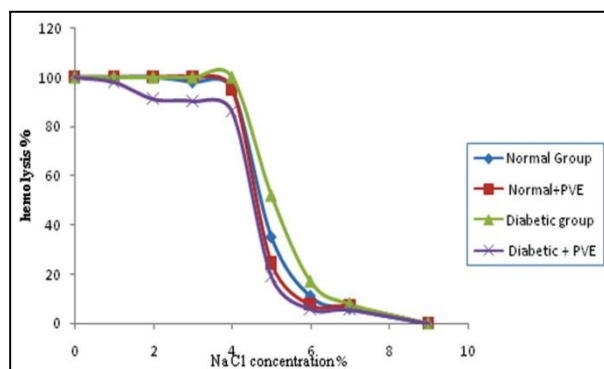


Figure 1. Percentage of osmotic fragility of erythrocytes against sodium chloride at different concentrations.

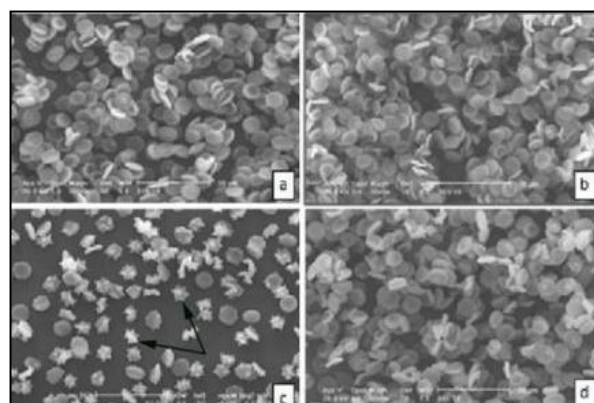


Figure 2. Effects of PVE on the morphology of rat erythrocytes. SEM images of the erythrocytes from the normal group served as a positive control (a); normal + PVE treated (b); diabetic group not treated with PVE as the negative control (c); and diabetic + PVE treated groups (d). The echinocytes can be observed in (c) and (d). [SEM, scanning electron microscopy; PVE, palm vitamin E]

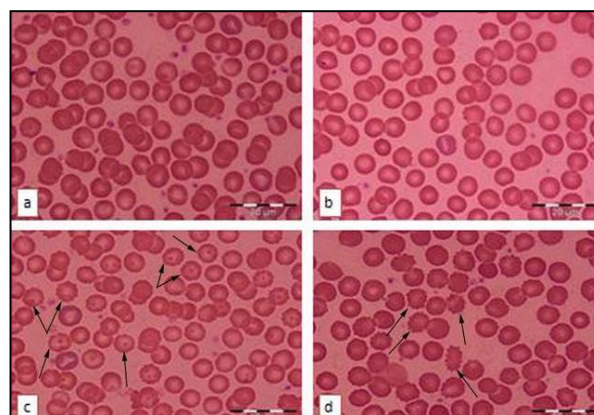


Figure 3. Effects of PVE on the morphology of rat erythrocytes. Light microscopy images of the erythrocytes from the normal group as the positive control (a); normal + PVE (b); diabetic group not treated with PVE as the negative control (c); and diabetic + PVE treated groups (d). The echinocytes and target cells can be observed in (c) and (d).

RESULTS

Erythrocyte fragility

The effects of PVE on erythrocyte osmotic fragility curves of the normal and diabetic groups are shown in Fig.1, where the percentage of hemolysed cells has been plotted as a function of the percentage concentration of NaCl. It is clear from Fig.1 that the fragility curve of diabetic rats was shifted to the right when PVE treatment was administered.

Erythrocyte morphology

The morphological changes of rat erythrocytes were observed under an SEM (Fig.2) and a light microscope

(Fig.3). The diabetic group showed morphological changes in the form of echinocytes and target cells. We found a higher percentage of echinocytes among the diabetic groups compared to the control group (70 ± 1.5 vs 3 ± 0.7 , $p = 0.001$). In contrast, the percentage of echinocytes was significantly lower in the diabetes with PVE group (20 ± 1.6 , $p = 0.005$; Fig.4).

Hematological parameters

Table 1 shows the complete blood count of normal and STZ-induced diabetic rats. The diabetic group had a significantly lower Hb concentration and Hct percentage compared to the control group, and a significantly increased percentage of Plt and WBC values (137 ± 2 , 40.2 ± 1.2 , 19.2 ± 0.9 , 1186.4 ± 34 , respectively) in diabetic group when compared with the normal group (143.9 ± 1 , 46.2 ± 0.7 , 17.0 ± 1.1 , 1070.5 ± 63 , respectively) and p values were 0.007, <0.001 , <0.001 and 0.008, respectively. In contrast, there were no differences in RBCs or MCH, MCV, and MCHC values between the diabetic and control groups.

Malondialdehyde and protein carbonyl of erythrocyte membrane

Erythrocyte MDA was significantly higher in STZ-induced diabetic rats compared to the control rats (76.69 ± 6.897 vs 1.9 ± 0.042 nmol/gHb, $p < 0.001$; Fig.5). On the other hand, diabetic rats treated with PVE showed significantly lower erythrocyte MDA compared to the untreated diabetic rats (13.11 ± 0.348 , $p < 0.001$; Fig.5). In addition, the findings of the present study showed that the levels of erythrocyte membrane protein carbonyl were significantly increased in STZ-induced diabetic rats compared to the control rats (0.202 ± 0.013 vs 0.115 ± 0.014 nmol/mg-protein, $p < 0.05$; Fig.6), whereas diabetic rats treated with PVE showed significantly lower erythrocyte protein carbonyl compared to the untreated diabetic rats (0.09 ± 0.012 , $p < 0.05$; Fig.6).

Erythrocyte antioxidant status

Decreased levels of GSH and increased level of GSSG were observed in the diabetic group (4.91 ± 0.3 and 92.75 ± 4 , respectively) compared to the control group (8.32 ± 0.66 and 73.34 ± 2 , respectively; $p < 0.001$) but PVE supplementation significantly increased these levels of GSH (8.8 ± 0.44 , $p < 0.001$; Table 2). We found a similar trend for plasma total antioxidant capacity as to be significantly lower in diabetic group than that in controls (342.79 ± 45.31 vs 552.06 ± 41.04 , $p < 0.001$), whereas plasma total antioxidants in diabetic rats treated with PVE showed significantly increased levels when compared to untreated diabetic group (632.01 ± 15.37 , $p < 0.005$; Table 2).

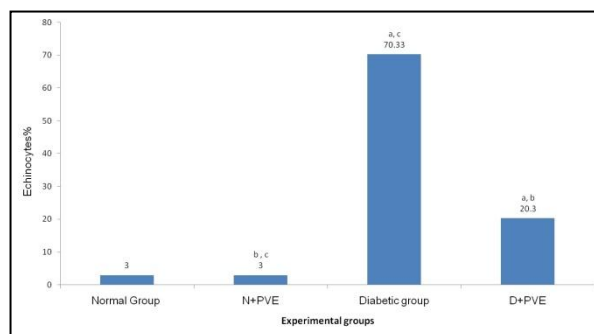


Figure 4. Effects of PVE on the morphology of the RBCs from normal and STZ-induced diabetic rats. Results are expressed as the mean $\pm$ S.E.M.; (a) significantly different from the normal group ($p < 0.001$), (b) significantly different from the diabetic group ($p < 0.001$), (c) significantly different from the diabetic treated with PVE group ($p < 0.05$).

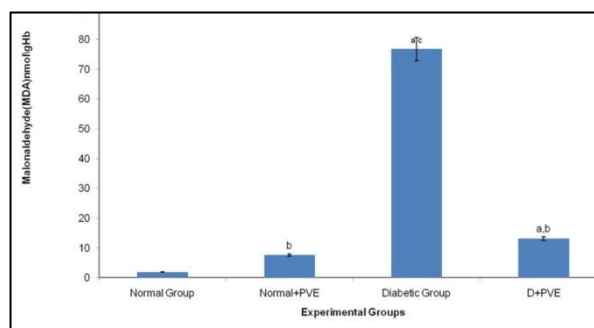


Figure 5. Effects of PVE treatment on MDA concentration in the normal and STZ-induced diabetic rats. Results are expressed as the mean $\pm$ SEM; (a) significantly different from the normal group ($p < 0.001$), (b) significantly different from the diabetic group ($p < 0.001$), (c) significantly different from the diabetic treated with PVE group ($p < 0.001$). [MDA, malondialdehyde; PVE, palm vitamin E]

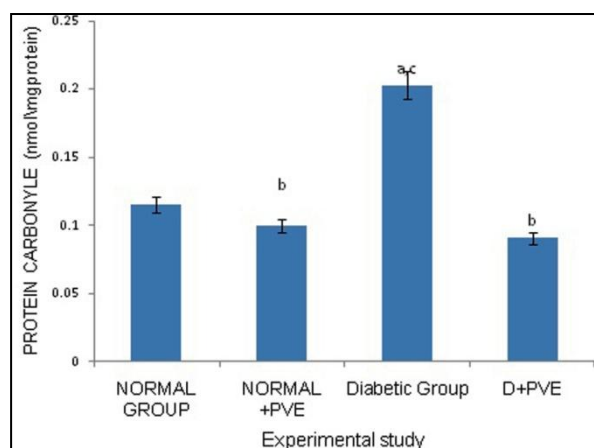


Figure 6. Effects of PVE treatment on the level of protein carbonyl in the normal and STZ-induced diabetic rats. Results are expressed as the mean $\pm$ S.E.M.; (a) significantly different from the normal group ($p < 0.05$), (b) significantly different from the diabetic group ($p < 0.05$), (c) significantly different from the diabetic treated with PVE group ($p < 0.05$).

Table 1. Effects of 4-week treatment with PVE on hematological parameters

Groups	RBC ($\times 10^{12}/L$)	Hb (g/L)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (fl)	WBC ($\times 10^9/L$)	Plt ($\times 10^9/L$)
Normal group	8.1 $\pm$ 0.1	143.9 $\pm$ 1	46.2 $\pm$ 0.77	80.0 $\pm$ 0.7	17.5 $\pm$ 0.15	20.03 $\pm$ 2	19.8 $\pm$ 0.6	17 $\pm$ 1.1	1070.5 $\pm$ 36
Normal + PVE group	8.1 $\pm$ 0.1	146.9 $\pm$ 3	44.3 $\pm$ 0.74	81.4 $\pm$ 0.8	17.9 $\pm$ 0.2	22 $\pm$ 0.06	17.0 $\pm$ 0.3	17.6 $\pm$ 1.1	937.4 $\pm$ 97
Diabetic group	7.9 $\pm$ 0.1	137.7 $\pm$ 2*	40.2 $\pm$ 1.2*	79.9 $\pm$ 0.9	17.3 $\pm$ 0.28	21.7 $\pm$ 0.1	17.8 $\pm$ 0.5*	19.2 $\pm$ 0.9*	1186.4 $\pm$ 43*
Diabetic + PVE group	8.2 $\pm$ 0.1	151.4 $\pm$ 3	47.9 $\pm$ 0.72	81.5 $\pm$ 0.9	18.2 $\pm$ 0.23	22.7 $\pm$ 0.2	16.2 $\pm$ 0.39	17.7 $\pm$ 1.6	924.3 $\pm$ 66

Results are expressed as the mean $\pm$ S.E.M.; * p < 0.05 compared to the normal group.

RBC, red blood cells; WBC, white blood cells; Hb, hemoglobin concentration; Hct, hematocrit; MCH, mean cell hemoglobin; MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; RDW, red blood cell distribution width; Plt, platelets.

Table 2. Effect of 4-week treatment with PVE on antioxidant status in normal and STZ-induced diabetic rats

Experimental groups	GSH (μ mol/gHb)	GSSG (nmol/gHb)	FRAP (μ mol/l)
Normal group	8.32 $\pm$ 0.66	73.34 $\pm$ 2.1	552 $\pm$ 41
Normal + PVE group	18.7 $\pm$ 0.7***	79.24 $\pm$ 1###	539.6 $\pm$ 3
Diabetic group	4.91 $\pm$ 0.3***	92.75 $\pm$ 4***	342.7 $\pm$ 45***
Diabetic + PVE group	8.84 $\pm$ 0.4###	77.76 $\pm$ 1###	632 $\pm$ 15###

Results are expressed as the mean $\pm$ S.E.M.; *** p < 0.001 compared to normal, and ### p < 0.001 compared to the diabetic group.

GSH, reduced glutathione; GSSG, oxidized glutathione; FRAP, ferric reducing antioxidant power.

DISCUSSION

In the current *in vivo* cross-sectional study, we investigated the effect of PVE supplementation on the physical and morphological changes to erythrocytes. A number of studies have been carried out to investigate the antioxidant activity of vitamin E at different doses, but this study was carried out to see whether 200 mg/kg BW PVE provided sufficient antioxidant activity to reduce oxidative stress. Recent studies showed that the tocotrienol-rich fraction (TRF) of palm oil (200 mg/kg BW) was able to prevent the progression of changes to the vascular wall occurring in diabetes mellitus, lower the blood glucose level, and improve dyslipidemia [22, 23]. Another study reported that supplementation of TRF at 200 mg/kg was able to improve wound healing in type 1 diabetes-induced rats [24].

A similar study showed that administration of palm oil extract (containing 80% tocotrienols) at a dose of 200 mg/kg over a period of 4-6 weeks was associated with a significant reduction in serum total cholesterol, LDL cholesterol, apolipoprotein B, thromboxane B₂, and platelet factor, and no adverse effects were reported [25]. These findings were in agreement with a previous study conducted on 36 hypercholesterolemic subjects who were maintained on the American Heart Association step 1 diet and received either a TRF from palm oil (equivalent to 220 mg/day of tocotrienols) or 200 mg/day of pure gamma tocotrienol over a 4-week period [26]. Significant reductions in serum total

cholesterol and apolipoprotein levels were observed with both preparations and no adverse side effects due to tocotrienol administration were reported.

Several studies have shown increased lipid peroxidation in clinical and experimental diabetes [27, 28]. The severity of protein and lipid damage is related to the concentration of oxidants in the cells, and hence, to the efficiency of the lipid repair mechanisms. Hydrogen peroxide and superoxide levels in the erythrocytes are involved in the oxidative degradation of hemoglobin and oxidation of lipid peroxidation [29].

In the first part of this study, oxidative stress was enhanced in STZ-induced diabetic rats, as demonstrated by the significant augmentation in diabetic erythrocyte MDA. A major protective mechanism against oxidative damage is membrane integrity. Erythrocytes with intrinsic membrane defects are more susceptible to lipid peroxidation than normal erythrocytes.

The results presented here indicate increased fragility of erythrocytes in untreated diabetic rats, which can be correlated to increased lipid peroxidation of erythrocytes. Lipid peroxidation has been implicated in the alterations of membrane structure and function. Increased lipid peroxidation has been reported to cause an increase in osmotic fragility and a decrease in cell fluidity [30]. These results agree with other previous studies that reported a significant positive correlation between the glucose-induced membrane lipid

peroxidation and the increased osmotic fragility of the erythrocyte membrane, which can cause changes in the properties of the RBC membrane and erythrocytes of STZ-induced diabetic rats [31]. In addition, the findings of this study demonstrated a significant decrease in erythrocyte osmotic fragility in the diabetic rats supplemented with PVE, which may be due to the protective effects of vitamin E present in the erythrocyte membranes. A previous study showed that vitamin E was a universal participant in antioxidant defence reactions in biological membranes, since it acts at all steps of membrane oxidative damage [32] and vitamin E acts as a first line of defence against peroxidation of polyunsaturated fatty acid [33].

The morphological changes in erythrocytes during the course of the disease reflect the effect of the altered environment, which is measured from the microscopic images by vibration in shape descriptors and application of wavelet transforms [34]. The normal mammalian RBC is a flexible biconcave disk. The present study shows that hyperglycemia induced a morphological change in the erythrocytes from the normal discoid shape to an echinocytic form. The diabetic groups had a lower percentage of discocytes and a significant increase in the altered erythrocyte forms (echinocytes) compared to the normal group. These results are in agreement with a previous study showing that oxidative stress and increased insulin production may further induce changes in the shape of the erythrocytes, as observed under *in vitro* conditions by the incubation of erythrocyte with H₂O₂, which transforms the discocytes to echinocytes because of the involvement of membrane proteins [35].

The elevated concentrations of glucose result in a decrease in membrane deformability, causing the cells to become more rigid [7, 36, 37]. The present study showed that 4 weeks of PVE supplementation resulted in reduced damage to erythrocyte morphology in diabetic rats. This finding suggests that PVE might be a potent inhibitor of protein oxidation and lipid peroxidation in rat erythrocytes. It was also suggested that improved glycemic status could be related to an improvement in glucose metabolism.

According to hematological parameters, there was a statistically significant decrease in Hb concentration, Hct percentage, and RDW. At the same time, Plt and WBC counts were significantly increased in the diabetic group compared to the normal group. Elevated WBC count has been proposed as a diagnostic feature of metabolic syndrome [38, 39]. Several epidemiologic studies have indicated a close relationship between WBC count and components of metabolic syndrome [40, 41]. Although a number of studies have confirmed the associations of hematologic parameters with insulin resistance, the finding of this study showed no

significant difference in RBC, MCV, MCH, and MCHC between the diabetic and control groups.

MDA is a late-stage lipid oxidation by-product that can be formed non-enzymatically or as a by-product of cyclooxygenase activity [42]. Previous studies showed elevated levels of lipid peroxidation products in the erythrocytes, plasma, and retinas of diabetic patients and animals [3, 43]. Lipid peroxidation causes polymerisation of membrane components and decreases cell deformability [29]. Products of lipid oxidation, such as oxidized cholesterol and oxidized unsaturated fatty acyl groups of phospholipids, may affect the structure and function of the membrane bilayer.

The present study showed elevated lipid peroxidation in the erythrocytes of diabetic rats, in agreement with previous reports [44-46]. Increased levels of lipid peroxide may cause oxidative injury to the erythrocytes, cross-linking membrane proteins and lipids [47]. In this study, diabetic rats treated with vitamin E, a potent free radical scavenger, for 4 weeks showed significantly lower erythrocyte lipid peroxidation than the untreated diabetic rats. Moreover, the normalisation of lipid peroxides in the vitamin E-treated diabetic rats was in agreement with the data obtained by Sung [48], who found that vitamin E inhibited the thiobarbituric-reactive substances in erythrocyte membranes. Moreover, lipid peroxidation as measured by MDA concentration was prevented by treatment with vitamin E. It is evident from the present study that palm vitamin E supplementation may help in the prevention of and/or protection against production of free radicals in diabetes.

Carbonyl content is a general indicator and the most commonly used marker of protein oxidation in diabetes [49, 50]. Carbonylation of proteins often leads to loss of protein function, which is considered a widespread marker of severe oxidative stress, damage, and disease-derived protein dysfunction [51, 52]. Our results indicate that erythrocyte membrane proteins were more affected by carbonyl stress in type 1 diabetic rats compared to the control rats. This observation indirectly suggests an increase in free radical-mediated damage of the cell membrane. The levels of MDA and protein carbonyl, as markers of lipid and protein oxidation, did not increase in the diabetic rats treated with PVE. These findings may indicate that the mechanism of lipid and protein metabolism is impaired in the erythrocytes of the STZ-induced diabetic rats. Increased blood levels of MDA and carbonyl in subjects with diabetes may lead to impairment of the balance between oxidative stress and antioxidant defence mechanisms by depletion of enzymatic antioxidants. The findings of this study are in agreement with previously published data [53, 54].

Previous studies on type 2 diabetes mellitus showed an association between impaired glycemic control and high carbonyl content in the RBC membranes [55] and plasma proteins [56].

Increased oxidative damage and changes in the antioxidant defence system have been reported in experimental diabetes, and have been associated with the development of diabetic complication [57]. Recent studies on the effects of various antioxidants on glutathione concentrations found that glutathione was decreased in the liver [58], kidney [59], pancreas [60], plasma, red blood cells [61], nerves, and precataractous lens [62] of chemically induced diabetic animals.

Erythrocyte-reduced GSH were significantly reduced in the untreated diabetic group compared to the control group, and on contrary GSSG were significantly increased in untreated diabetic group compared to control group; these findings are consistent with the literature supplied by other investigators for patients with type 1 diabetes mellitus [9, 63-66]. In addition, these results suggest that supplementation with PVE significantly increases the levels of GSH in the erythrocytes of diabetic rats treated with PVE and these results may indicate that high concentrations of PVE can significantly reduce the levels of lipid peroxidation and oxidative agents, leading to the increase in GSH level [58, 60].

Ferric-reducing antioxidant power, a non-specific test for the total antioxidant status, was lower in the untreated diabetic group compared to the control group, indicating their decreased defence capacity against oxidative stress. Lower total antioxidant capacity in the diabetic group may be due to higher production of oxidative agents and thereby, higher consumption of antioxidants [66].

In conclusion, the findings of this study may indirectly suggest an increase in the free radical-mediated damage to the erythrocyte cell membrane. Furthermore, a prevention trial involving diabetic rats showed that 4 weeks of daily supplementation with 200 mg/kg of PVE was able to reduce the levels of oxidative stress markers by inhibiting lipid peroxidation and protein oxidation and enhancing the antioxidant defence system. These results suggest that PVE may have significant protective effects against the adverse effects of oxidative stress in STZ-induced diabetic rats.

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CONFLICTS OF INTEREST

None were declared.

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