

Tocotrienol-Rich Fraction from Palm Oil Prevents Oxidative Damage in Diabetic Rats

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التوكوترينول المشتق من زيت النخيل يمنع ضرر الإجهاد التأكسدي في الجرذان المصابة بداء السكري

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ABSTRACT: Objectives: This study was carried out to determine the effects of tocotrienol-rich fraction (TRF) (200 mg/Kg) on biomarkers of oxidative stress on erythrocyte membranes and leukocyte deoxyribonucleic acid (DNA) damage in streptozotocin (STZ)-induced diabetic rats. **Methods:** Male rats (n = 40) were divided randomly into four groups of 10: a normal group; a normal group with TRF; a diabetic group, and a diabetic group with TRF. Following four weeks of treatment, fasting blood glucose (FBG) levels, oxidative stress markers and the antioxidant status of the erythrocytes were measured. **Results:** FBG levels for the STZ-induced diabetic rats were significantly increased ($P < 0.001$) when compared to the normal group and erythrocyte malondialdehyde levels were also significantly higher ($P < 0.0001$) in this group. Decreased levels of reduced glutathione and increased levels of oxidised glutathione ($P < 0.001$) were observed in STZ-induced diabetic rats when compared to the control group and diabetic group with TRF. The results of the superoxide dismutase and glutathione peroxidase activities were significantly lower in the STZ-induced diabetic rats than in the normal group ($P < 0.001$). The levels of DNA damage, measured by the tail length and tail moment of the leukocyte, were significantly higher in STZ-induced diabetic ($P < 0.0001$). TRF supplementation managed to normalise the level of DNA damage in diabetic rats treated with TRF. **Conclusion:** Daily supplementation with 200 mg/Kg of TRF for four weeks was found to reduce levels of oxidative stress markers by inhibiting lipid peroxidation and increasing the levels of antioxidant status in a prevention trial for STZ-induced diabetic rats.

Keywords: Antioxidants; Diabetes Mellitus; Oxidative Stress; Tocotrienols.

المخلص: الهدف: تهدف هذه الدراسة الي تحديد تأثير فعالية التوكوترينول (200 ملجم/كجم) على المؤشرات الحيوية للإجهاد التأكسدي في أغشية كريات الدم الحمراء وجزئية الحمض النووي الريبي منقوص الأكسجين (DNA) في الجرذان المصابة بداء السكري المحدث بواسطة الستريبتوزوتوسين. الطريقة: تم تقسيم 40 جرذ ذكر عشوائيا الي أربع مجموعات متساوية كالتالي: مجموعة طبيعية ومجموعة طبيعية أعطت توكوترينول ومجموعة مصابة بداء السكري ومجموعة مصابة بداء السكري أعطت توكوترينول. بعد فترة أربع اسابيع من العلاج تم قياس مستوى الجلوكوز في الدم ومستويات المؤشرات الحيوية للإجهاد التأكسدي وحالة مضادات الأوكسدة لكريات الدم الحمراء. النتائج: أظهرت مجموعة الجرذان المصابة بداء السكري ازديادا معنويا احصائيا في مستوى الجلوكوز ومستوى المالونديليهايد مقارنة بالمجموعة الطبيعية. كما أظهرت النتائج بأن هناك انخفاضا في مستوى الحالة المختزلة للجلوتاثيون وبالمقابل ازدياد في مستوى الحالة المؤكسدة للجلوتاثيون في المجموعة المصابة بداء السكري مقارنة بالمجموعة الطبيعية. من جهة أخرى كان هناك انخفاض معنوي احصائي في مستوى نشاط انزيمي السوبر اكسيد ديسميوتيز و الجلوتاثيون بيروكسيديز في المجموعة المصابة بداء السكري. أيضا كان هناك ارتفاع معنوي احصائي في مستوى الضرر في جزئية (DNA) لكريات الدم البيضاء في المجموعة المصابة بداء السكري مقارنة بالمجموعة الطبيعية. لقد بينت النتائج بأن العلاج بالتوكوترينول كان فعالا في معادلة سمية وضرر جزئية (DNA) في مجموعة الجرذان المصابة بداء السكري. الخلاصة: وجد أن تناول 200 ملجم / كجم يوميا لمدة أربعة اسابيع فعال في تخفيض مستويات المؤشرات الحيوية للإجهاد التأكسدي وذلك عن طريق تثبيط تأكسد الدهون وزيادة في مستويات مضادات الأوكسدة في الجرذان المصابة بداء السكري.

مفتاح الكلمات: مضادات الأوكسدة؛ مرض السكري؛ الإجهاد التأكسدي؛ التوكوترينول.

ADVANCES IN KNOWLEDGE

- The antioxidant potential of tocotrienols has been a focus of scientific interest in recent years. Recent findings have shown that tocotrienols are responsible for the majority of the activity of vitamin E. This study shows that a tocotrienol-rich fraction (TRF) from palm oil at 200 mg/Kg of body weight for four weeks provides sufficient antioxidant activity to reduce oxidative stress, by inhibiting lipid peroxidation and reducing levels of deribonucleic acid (DNA) damage, as well as increasing the status of the antioxidant system in a prevention trial for diabetic rats.

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APPLICATION TO PATIENT CARE

- In terms of using tocotrienols for antioxidant agent activity in diabetic humans, the finding of this study suggests that TRF at 200 mg/Kg for four weeks may play an important role in reducing the complications of oxidative stress-mediated diabetes mellitus.

ACCORDING TO THE WORLD HEALTH Organization (WHO), a total of 171 million people in all age groups worldwide (2.8% of the world's population) have diabetes; this number is expected to rise to 366 million (4.4% of the world's population) by 2030.¹ A balance between the rate of free radical generation and the rate of radical elimination is important so that cellular radical generation is not harmful.² However, if there is a significant increase in radical generation or a decrease in radical elimination of the cells, this balance is disrupted, resulting in the formation of oxidative stress.³ Free radicals and oxidative stress are among the factors involved in the pathogenesis of diabetes; in particular, they appear to play a major role in the development of chronic complications from diabetes.⁴

Vitamin E is widely accepted as the first line of defense against lipid peroxidation, protecting the polyunsaturated fatty acids in the cell membranes through its free radical-quenching activity in the biomembranes in the early stages of the free radical attack.⁵ Palm oil contains a larger concentration of tocotrienols (70%) than any other natural source, except annatto (100%).⁶ Tocotrienols and tocopherols act as potent antioxidants, serving to protect the cellular membranes from free radical-catalysed lipid peroxidation.⁷ A number of health-related biological properties of tocotrienols have been identified, including anti-cancer, anti-cholesterolemic, antihypertensive, antioxidant, immunomodulatory and neuro-protective properties.⁶

Tocotrienol-rich fraction (TRF) has unique biological properties that make it a potential neuroprotective dietary factor. In addition to its antioxidant activity, TRF at micromolar concentrations displays cholesterol-lowering activities in cells, animal models and some, but not all, human subjects by means of the inhibition of the activity of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase.⁸ Furthermore, TRF at lower concentrations (~10 nmol/L) modulates signalling pathways involved in neuronal cell

death in cell culture experiments.⁸ Currently, TRF is addressed as an anti-apoptotic and anti-inflammatory agent, and emerging findings suggest a role for nuclear factor kappa-B cells (NF- κ B) as a mediator of the effects of dietary factors on neuronal plasticity.⁹

Our previous study showed that the TRF of palm oil (200 mg/Kg of body weight) has some protective effects as it reduces lipid peroxidation and protein oxidation, and prevents the physical and morphological alterations of erythrocytes after four weeks.¹⁰ Another study reported that the supplementation of TRF reduced oxidative stress and blood glucose levels and had protective effects on diabetes-induced pancreatic damage in diabetic rats.¹¹ The purpose of this study was to investigate the effects of TRF on oxidative stress and antioxidant status on erythrocytes in streptozotocin (STZ)-induced diabetic rats.

Methods

For this study, male Sprague-Dawley rats were provided by the Laboratory Animal Resources Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur. The animals were 8–10 weeks old and weighed 200–250 g. During the study period, the rats had unrestricted access to food and tap water with a standard diet containing 22% crude protein, 5% crude fibre, 3% fat, 13% moisture, 8% ash, 0.85–1.2% calcium, 0.6–1% phosphorus and 49% nitrogen-free extract in the form of mouse pellet 702-P (Gold Coin Holdings, Kuala Lumpur, Malaysia). They were housed in pairs in plastic cages with wood chips for bedding (changed weekly for the normal groups and daily for the diabetic groups). The animals were acclimatised to standard laboratory conditions, with a temperature of 25 °C with standard light-darkness cycles of 12 hours for one week before experimentation. The study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee.

The experiment was designed with 40 rats, divided randomly into four groups of 10 rats: a normal group; a normal group supplemented

with TRF; a diabetic group, and a diabetic group supplemented with TRF. Initially, all experimental groups had similar body weights (200–250 g). At the end of the study period, both diabetic groups (STZ-induced diabetic rats and diabetic rats with TRF) showed a significant loss of body weight (average weight 190 g) when compared to the normal groups (average weight 319 g). Diabetes was induced after an overnight fast by a single intravenous injection of STZ via the tail vein (45 mg/Kg of body weight), which was freshly dissolved in a normal saline solution (9 g/L of sodium chloride). Three days later, blood was collected via the tail vein and the glucose concentration was measured by a strip-operated blood glucose sensor (Companion 2, Medisense Ltd., Birmingham, UK). Rats with blood glucose levels of >7.0 mmol/L were included in the study.

TRF was obtained from the Sime Darby Group (Kuala Lumpur, Malaysia) as follows: α -tocopherol at 171.1 mg/g, α -tocotrienol at 190.4 mg/g, β -tocotrienol at 36.0 mg/g, γ -tocotrienol at 211.2 mg/g and δ -tocotrienol at 150 mg/g. It was administered orally at a dose of 200 mg/Kg of body weight per day throughout the four-week feeding period, and supplementation was begun the same day. After four weeks of supplementation, all the rats underwent overnight fasting and were then euthanised by a cardiac puncture while under deep anaesthesia with diethyl ether.

The biochemical investigation commenced while the rats were under deep anaesthesia. A blood sample was taken from each of the rats via a cardiac puncture using a 21 gauge needle with a 10 ml syringe. Samples were then transferred into tubes containing a sodium fluoride plastic tube (fluoride oxalate tube, #X2230, Teklab Ltd., UK) for fasting blood glucose (FBG) analysis and ethylene diamine tetra acetic acid (EDTA) tubes (BD Vacutainer® Plus Plastic K2EDTA Tube #368589, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) and kept on ice. Plasma glucose levels were analysed on the same day using enzymatic glucose-oxidase kits (Glucose Oxidase Reagent, # TR15103, Fisher Scientific Ltd., Loughborough, UK). Erythrocyte pellets were separated from the plasma and the leukocyte layer by centrifugation at 4,000 rpm for 10 mins at 4 °C. The supernatant plasma was pipetted into 1.5 ml Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and frozen

at -80 °C until use. After the removal of the buffy coat, the erythrocyte pellets were prepared by being washed three times with a cold (4 °C) normal saline (9%) solution. These pellets were then stored at -80 °C for biochemical analyses. The amount of blood taken from each rat varied from 6–10 ml.

Erythrocyte malondialdehyde (MDA) samples were measured using the high performance liquid chromatography (HPLC) method with some modifications as described by Pilz *et al.*, based on the derivation with 2, 4-dinitrophenylhydrazine (DNPH).¹² The total MDA was prepared from the erythrocytes by perchloric acid deproteinisation whereas an alkaline hydrolysis step for 30 mins at 60 °C was introduced prior to protein precipitation for the determination of the total (free and bound) MDA. The standard and samples were processed under the same conditions as described in this section.

The MDA standard was prepared by dissolving 25 μ l of 1,1, 3,3-tetraethoxypropane (TEP) in 100 ml of 1% sulfuric acid to give a 1 mM stock solution and was kept at 4 °C overnight. The working standard was prepared by the hydrolysis of the 1 ml of TEP stock solution in 50 ml of distilled water (dH₂O) and incubated for two hours at room temperature.¹³ The concentration of 1, 2, 4, 6, 8, and 10 μ mol was prepared from the working standard to get the standard curve for the estimation of total MDA.

A solution of 200 μ l of sodium hydroxide (NaOH) of 1.3 M was added to the 50 μ l standard or sample. For the protein-bound alkaline hydrolysis step, the mixture was incubated in a 60 °C water bath for 30 mins and then cooled in ice for five mins. The hydrolysis samples were acidified with 100 μ l of 35% (volume/volume [v/v]) perchloric acid. After centrifugation at 1,000 g for 10 mins, 300 μ l of the supernatant were mixed with 12.5 μ l of the DNPH solution and incubated in the dark at room temperature for 30 mins. Finally, 40 μ l was injected into the HPLC system.

Analytical HPLC separations were performed with a 655A-12 liquid chromatography instrument (SCL-10AVP system controller, Shimadzu Scientific Instruments, Kyoto, Japan) equipped with an auto injector (655A-40) and a variable-wavelength ultraviolet (UV) detector (655A) operated at 310 nm on a 125 \times 3 mm Nucleosil C18 column of 5 μ m particle size on reversed-phase with an integrated precolumn (SepServ HPCL, Separation Service

Berlin Ohg, Berlin, Germany). The column was kept at 30 °C in a column oven with a mobile phase. The HPLC mobile phase was prepared by mixing 380 ml acetonitrile with 620 ml of dH₂O acidified with 0.2% (v/v) acetic acid and degassed under reduced pressure with a flow-rate of 1 ml/min.

Superoxide dismutase (SOD) activity was determined according to Fridovich and Beyer's method, based on the inhibition or reduction of nitroblue tetrazolium (NBT).¹⁴ A reaction mixture of a substrate solution containing L-methionine (30 mg/ml), tritone X-100 (1%) and NBT (1.14 mg/ml) was added in a phosphate buffer saline (PBS) solution (pH 7.8). Lyses erythrocytes (20 µl) were added to 0.1 ml of cold dH₂O and were kept at 4 °C for 15 mins. The mixture (1 ml) was added to 20 µl of lyses erythrocyte or a PBS solution (8 g of sodium chloride [NaCl], 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of monopotassium phosphate) (control), and 10 µl of riboflavin was then added. The samples were incubated for seven mins. The absorbance of the mixture at 560 nm was determined using a spectrophotometer UV-160A (Shimadzu Scientific Instruments). The enzyme activities were calculated in U/g haemoglobin (Hb).

Glutathione peroxidase (GPx) activity was determined according to the Lawrence method, based on the principle that GPx catalyses the oxidation of glutaredoxin (GRx) and nicotinamide adenine dinucleotide phosphate (NADPH).¹⁵ The oxidised glutathione was immediately converted to the reduced form with a concomitant oxidation, NADPH to NADP⁺. A 100 µl sample of lysed erythrocyte and 0.8 ml of a buffered solution (1 mM of sodium azide, 1 mM of EDTA, 0.2 mM of NADPH, 50 units of GRx, 1 mM of GSH and 50 mM of PBS; pH 7) were incubated at room temperature for 5 mins, then 100 µl (0.25 mM) of hydrogen peroxide was added. The absorbance of the mixture at 340 nm was determined using the Shimadzu Scientific Instruments spectrophotometer. Activities of enzymes were calculated in U/g Hb. A commercial glutathione detection kit (BioVision, Inc., Milpitas, California, USA) was used to measure GSH, GSSG and total glutathione. The readings for samples and the standard were determined using a fluorescence plate reader equipped with an excitation wavelength/emission wavelength (Ex/Em) of 340/420 nm (SkanIt

Software, Thermo Fisher Scientific Inc.).

The concentration of vitamin E in the rat erythrocyte samples was measured by the HPLC method with some modification of the procedures described by Brandt *et al.*¹⁶ Briefly, α-tocopherol and α-tocopheryl-acetate (the internal standard) in the samples were extracted into hexane. The hexane extract was dried under nitrogen and dissolved in the mobile phase for injection into the HPLC for separation with detection by UV. The mobile phase used was composed of methanol and toluene of HPLC grade (BDH Merck Ltd., Poole, UK) in the ratio of 80:20.

A stock standard of 10 mmol/L of α-tocopherol (BDH Merck Ltd.) was prepared by dissolving 0.0216 g of vitamin E in 5 ml of absolute ethanol. Different standards concentrations of α-tocopherol (10, 20, 30, 40 and 50 µmol/L) were prepared. The internal standard, α-tocopheryl acetate, was prepared by dissolving 0.0236 g of α-tocopheryl acetate in 5 ml of absolute ethanol and then pipetting 0.75 ml of this into 50 ml of absolute ethanol. The final concentration of α-tocopheryl acetate in every standard and sample was 150 µmol/L.

Lyphochek 1 and Lyphochek 2 (Bio-Rad Laboratories, Hercules, California, USA) were used as the low and high precision controls, respectively, and treated as plasma samples. A 100 µl of sample or standard was added to 100 µl of ethanol together with 100 µl of α-tocopheryl acetate. Hexane (200 µl) was added and centrifuged at 2,500 g (for 10 mins at 4 °C). Then 120 µl of the upper layer (hexane extract) was evaporated. A total of 60 µl of the mobile phase were added to dissolve the extract, and this was immediately loaded for injection into the HPLC system.

Analytical HPLC separations were performed as previously described in this section. The running condition of HPLC was seven mins per sample. The flow-rate was 1 ml/min. The injection volume was 20 µl and the detection wavelength was at 292 nm. The retention time was ~4.4 mins for vitamin E and ~5.3 mins for α-tocopheryl acetate.

A calibration curve was constructed by plotting the peak area ratio of vitamin E and α-tocopheryl-acetate against the vitamin E standard values. The slope obtained from the calibration curve was used to determine the α-tocopherol concentration in the sample by the following calculation:

The deoxyribonucleic acid (DNA) oxida-tive

Table 1: Effects of treatment with tocotrienol-rich fraction on antioxidant status and oxidative stress

Group	SOD in U/gHb	GPx in U/gHb	GSH in $\mu\text{mol/gHb}$	GSSG in nmol/gHb	MDA in nmol/gHb
Normal	3673 \pm 182	71.6 \pm 1.8	8.32 \pm 0.66	73.34 \pm 2.1	1.9 \pm 0.13
Normal with TRF	4028 \pm 161 [†]	65.3 \pm 4.7 [†]	18.70 \pm 0.7 [°]	79.24 \pm 1.9 [†]	7.5 \pm 0.6
Diabetic	2916 \pm 126 [°]	39.3 \pm 2.6 [°]	4.91 \pm 0.3 [°]	92.75 \pm 4.6 [°]	76.6 \pm 6.8 [°]
Diabetic with TRF	3364 \pm 137 [†]	46.5 \pm 3.4	8.84 \pm 0.4 [†]	77.76 \pm 1.4 [†]	13.1 \pm 0.3 ^{††}

SOD = superoxide dismutase; GPx = glutathione peroxidase; GSH = reduced glutathione; GSSG = oxidised glutathione; MDA = malondialdehyde; TRF = tocotrienol-rich fraction. Results are expressed as mean \pm standard error of mean; [°]Significantly different as compared to the control group ($P < 0.05$); [†]Significantly different as compared to the diabetic group ($P < 0.05$).

damage was then measured as follows. The alkaline single-cell gel electrophoresis assay procedure was performed according to the method described by Singh *et al.*¹⁷ For slide preparation, 100 μl (0.5%) of normal melting agarose at about 121 $^{\circ}\text{C}$ in calcium ion- and magnesium ion-free butylphthalate plasticised styrene, was immediately covered with a cover slip and kept on ice for 5 mins to allow the

$$\frac{\text{Peak area ratio of the sample}}{\text{Slope of the calibration curve}} = \text{Vitamin E concentration} \left(\text{in } \frac{\mu\text{mol}}{\text{L}} \right)$$

agarose to solidify. Around 10 μl of whole blood was suspended in 80 μl (0.5%) of low melting agarose. The cell suspension was rapidly pipetted onto the first agarose layer and spread using a cover slip, and the slides were immersed in a lysis buffer solution (2.5 M of NaCl, 100 mM of sodium EDTA, 10 mM of trisaminomethane hydrochloride (Tris-HCl) and 1% of Triton X-100), and 10% dimethyl sulfoxide was added just before use at 4 $^{\circ}\text{C}$ for a minimum of one hour. The slides were placed in a horizontal gel electrophoresis tank and filled with fresh cold electrophoresis solution (10 N of sodium hydroxide and 200 mM of sodium (in the form of Na_2) EDTA) and were left in the solution for 20 mins to allow the DNA to unwind. Electrophoresis was conducted at 4 $^{\circ}\text{C}$ for 20 mins. The slides were neutralised with a Tris-HCl buffer (pH 7.5) three times for five mins and stained with ethidium-bromide (100 $\mu\text{g/ml}$) for 10 mins. Slides were examined at x 20 magnification using a fluorescence microscope with 100 cells per slide analysed using image analysing software (Comet Assay Software Project (CaspLab), Version 1.2.2XCAP[™] Image Processing Software (Version 1.1.1), CaspLabEPIX, Inc., Swietokrzyska Academy, Kielce, Poland).

All results were expressed as mean \pm standard error of mean (SEM). The data were analysed

using the Statistical Package for the Social Sciences (SPSS), Version 20.0 (IBM, Corp., Chicago, Illinois, USA), and Microsoft Office Excel 2007 (Microsoft, Inc., Redmond, Washington, USA) was used to draw the graphs. The Shapiro-Wilk test was used to check the normality of the variables. Accordingly, a one-way analysis of variance (ANOVA) test followed by a *post hoc* least significant difference (LSD) multiple comparison test to estimate the significance of difference between groups, and the Kruskal-Wallis one-way ANOVA test was used to analyse the data that followed non-normal behaviour of distribution patterns, respectively. The difference between groups was considered significant when $P < 0.05$.

Results

As far as plasma glucose levels are concerned, Figure 1 shows that the levels of FBG were significantly increased ($P < 0.0001$) in the STZ-induced diabetic rats compared to the normal group. On the other hand, FBG levels were significantly lower in the diabetic group ($P < 0.001$), which had been supplemented with TRF, in comparison with the STZ-induced diabetic rats; however, this improvement was not sufficient to have restored the blood glucose levels to their normal state.

As to the MDA erythrocyte membrane, the erythrocyte MDA levels were statistically significantly higher in the STZ-induced diabetic rats (76.69 \pm 6.8) compared with the normal rats (1.9 \pm 0.1; $P < 0.0001$) [Table 1]. On the other hand, the diabetic rats treated with TRF showed significantly decreased (13.11 \pm 0.3; $P < 0.001$) MDA levels compared with STZ-induced diabetic rats.

The results for the erythrocyte antioxidants status were as follows. In the diabetic group there was significantly decreased activity of SOD, GPx

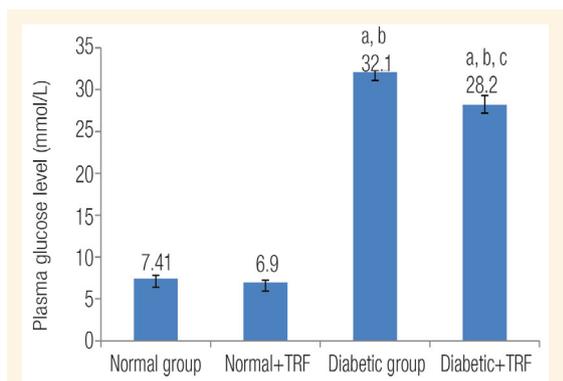


Figure 1: The effect of treatment with tocotrienol-rich fraction (TRF) on blood glucose in the experimental groups of rats.

a = Significantly different compared to the normal group ($P < 0.05$); *b* = Significantly different from the normal with TRF group ($P < 0.05$); *c* = Significantly different from the diabetic group ($P < 0.05$).

and levels of GSH (P values were 0.006, 0.0001 and 0.001, respectively). On the other hand, levels of GSSG were significantly increased ($P = 0.002$) compared with the normal group. However, TRF supplementation produced a significant increase in SOD activity and levels of GSH, as well as a decreased level of GSSG in the diabetic with TRF group when compared to the STZ-induced diabetic rats group [Table 1 and Figure 1].

As far as the vitamin E erythrocyte membrane was concerned, the concentration of vitamin E was significantly lower in the diabetic groups (STZ-induced diabetic rats and diabetic rats with TRF)

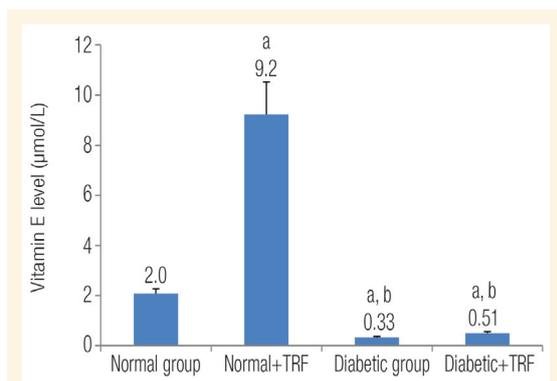


Figure 2: The effect of treatment with tocotrienol-rich fraction (TRF) on the concentration of vitamin E in the experimental groups of rats.

a = Significantly different from the normal group ($P < 0.05$); *b* = Significantly different from the normal with TRF group ($P < 0.05$).

compared with the normal group (0.33 ± 0.03 and 0.55 ± 0.05 versus 2.07 ± 0.29). P values were 0.01 and 0.04, respectively. On the other hand, vitamin E levels in the normal group supplemented with TRF were significantly increased when compared to other groups ($P = 0.0001$) [Figure 2].

The levels of DNA damage were significantly higher in the STZ-induced diabetic rats compared to normal rats [Table 2]. TRF supplementation was found to minimise the DNA damage parameters in diabetic rats with TRF. Figure 3 shows the normal and damaged cells with long tails of DNA migration.

Table 2: Effect of treatment with tocotrienol-rich fraction on the level of deoxyribonucleic acid (DNA) damage (determined by tail length, tail moment and percentage of tail DNA) of the leukocytes

	TL in μm	TM in arbitrary units	Tail DNA in %
Normal group	4.57 ± 0.23	0.015 ± 0.002	0.114 ± 0.016
Normal with TRF group	4.03 ± 0.15	0.0086 ± 0.001	0.084 ± 0.012
Diabetic group	$127.38 \pm 3.17^{\circ}$	$39.009 \pm 1.45^{\circ}$	$24.93 \pm 0.66^{\circ}$
Diabetic with TRF group	$45.28 \pm 0.75^{*\dagger}$	$3.93 \pm 0.12^{\dagger}$	$7.93 \pm 0.16^{*\dagger}$

TL = tail length; TM = tail moment; DNA = deoxyribonucleic acid; TRF = tocotrienol rich fraction.

Results are expressed as mean \pm standard error of mean; $^{\circ}$ Significantly different as compared to the control group ($P < 0.05$); † Significantly different as compared to the diabetic group ($P < 0.05$).

Discussion

This study shows that a daily supplementation of TRF (200 mg/Kg) reduced the levels of oxidative stress markers in STZ-induced diabetic rats. The results of the FBG tests indicated increased plasma glucose levels in STZ-induced diabetic rats, which could be due to the inhibition of insulin secretion resulting from the injection of STZ. Previous studies have demonstrated that injections of STZ in experimental diabetic rats led to the degeneration of beta cells of the islets of Langerhans, thereby increasing FBG levels.¹⁸ In this study, the supplementation of TRF at 200 mg/Kg for four weeks improved blood glucose levels. However, the improvement was not enough to restore blood glucose levels to a normal state.

The mechanism through which tocotrienols reduce blood glucose levels in patients and in pre-clinical animal models was investigated by Fang

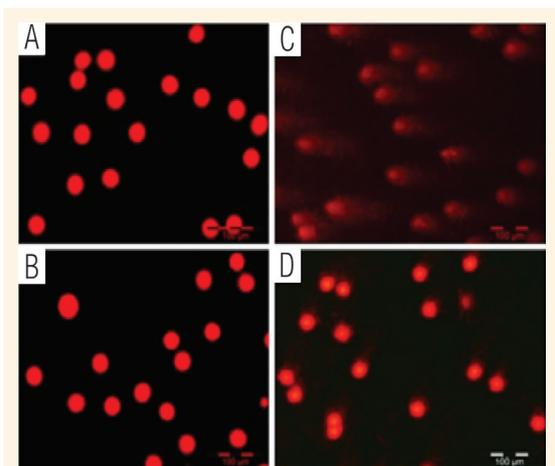


Figure 3: Single-cell gel electrophoresis assay image of the deoxyribonucleic acid (DNA) using rat whole blood (10 µl) directly for the assay. Single-cell gel electrophoresis image of the DNA in the (A) normal group; (B) normal with tocotrienol-rich fraction (TRF) group; (C) diabetic group, and (D) diabetic with TRF group.

*et al.*¹⁹ They proposed that tocotrienols function as peroxisome proliferator-activated receptor (PPAR) modulators. PPARs are ligand-regulated transcription factors that play essential roles in energy metabolism. Synthetic PPAR α and PPAR γ ligands have been used recently in the treatment of hyperlipidaemia and diabetes. Both α - and γ -tocotrienol activated PPAR α , while δ -tocotrienol alone activated PPAR α , PPAR γ and PPAR δ in reporter-based assays.

Several studies have shown an increased lipid peroxidation in clinical and experimental diabetes.²⁰ In the first part of this study, we confirmed that oxidative stress is enhanced in STZ-induced diabetic rats as demonstrated by the significant augmentation in diabetic erythrocyte MDA. MDA is a late-stage lipid oxidation by-product that can be formed non-enzymatically, or is a by-product of cyclooxygenase activity.²¹

Previous studies have reported elevated levels of lipid peroxidation products in the erythrocytes, plasma and retinas of diabetic patients and animals.²² The findings of the present study showed elevated levels of lipid peroxidation in erythrocyte STZ-induced diabetic rats, and this finding was in agreement with the reports documented in the literature.^{22–24} Increased levels of lipid peroxide may cause oxidative injury to the erythrocytes and cross-linking in the membrane proteins and lipids.²⁵ Moreover, the levels of MDA were slightly increased

in the normal group treated with TRF compared to the control group, although the difference was not statistically significant. This might be related to having exposed the rats to stressors during the force-feeding of the TRF supplementation.

In this study, four weeks of TRF supplementation showed significantly lower erythrocyte lipid peroxidation in the diabetic with TRF group compared to the STZ-induced diabetic group. Moreover, the normalisation of lipid peroxides in the STZ-induced diabetic rats treated with TRF was in agreement with Sung *et al.*, who found that vitamin E inhibited the thiobarbituric acid reactive substances in the erythrocyte membrane.²⁶ Also, the formation of lipid peroxidation products such as MDA was prevented by vitamin E treatment. Evidence from the present study suggests that TRF supplementation may help prevent and protect against free radical production in diabetes.

Increased oxidative damage and changes in the antioxidant defense system have been reported in experimental diabetes and have been associated with the development of diabetic complications.²⁷ Previous studies have shown that the presence of oxidative stress in diabetes mellitus, and antioxidant enzymes such as SOD, catalase (CAT) and GPx as well as molecules such as GSH, vitamins E and C, and beta carotene, increase the response of the antioxidant defense system to oxidative stress in the body.^{28,29} In the present study, the levels of erythrocyte antioxidant enzymes, SOD and GPx, were decreased in both the STZ-induced diabetic rats and the diabetic with TRF group in comparison to the normal group. These findings agree with other research showing that the presence of oxidative stress in rat erythrocytes was caused by STZ-induced diabetes.^{28,30} In this study, supplementation with TRF was found to augment the antioxidative system and increase the levels of antioxidant enzymes, such as SOD, which catalytically converts superoxide anion radicals to hydrogen peroxide; this was especially remarkable in the diabetic with TRF group. While GPx activity was not significantly different in the diabetic with TRF group compared with the STZ-induced diabetic group, these results agree with some previous studies which showed that GPx appears to be a relatively stable enzyme;^{31,32} thus, it may only be inactivated under conditions of severe oxidative stress.³³ In addition, other previous

experimental animal studies have reported that high levels of glucose have no effect on GPx activity and its gene expression.³⁴

Decreased erythrocyte GSH together with significantly increased GSSG was observed in STZ-induced diabetic rats when compared with the normal rats. These findings are consistent with other studies.³² The current study found that the supplementation of TRF significantly increased the levels of GSH and decreased GSSG in the diabetic group treated with TRF, and may indicate that high concentrations of TRF can significantly reduce the levels of lipid peroxidation and the reduction of oxidative agents, thereby increasing levels of antioxidant GSH.

The increased production of reactive oxygen species (ROS) under diabetic conditions underlines the increased amount of oxidatively-damaged DNA in different tissues. The presence of ROS may lead to increased DNA damage in peripheral blood lymphocytes that may be revealed by the single-cell gel electrophoresis assay. Significant differences were detected between the control and STZ-induced diabetic rats in terms of the frequency of damaged cells. The tail moment (TM) and tail length (TL) parameters for DNA damage were significantly higher in the STZ-induced diabetic rats than in the control rats, which might indicate that these cells are handling more oxidative damage on a regular basis. Also, clear differences between the STZ-induced diabetic rats and diabetic rats with four weeks of TRF supplementation were observed on the basis of the extent of DNA migration during single-cell gel electrophoresis, supporting the hypothesis that antioxidants protect cells from DNA damage. A previous study showed that antioxidants such as vitamin E exhibited a protective effect against oxidative DNA damage.³⁵

Conclusion

This study showed that a daily oral supplementation of 200 mg/Kg of TRF of palm oil had the beneficial effect of reducing levels of oxidative stress markers by an inhibition of lipid peroxidation and an increase in the levels of antioxidant defense system. This was demonstrated through a significant increase in the SOD activity, and the GSH levels in diabetic rats supplemented with TRF. In contrast, GPx activity and vitamin E levels were not

significantly affected following four weeks of TRF supplementation. Furthermore, the antioxidant properties of TRF showed some protective effects in the reduction of the percentage of damaged DNA in the tail. The DNA TL and TM of the leukocyte single-cell gel electrophoresis assay were used as indicators of oxidative DNA damage. Additionally, TRF was also found to have a beneficial effect in improving hyperglycaemic status, which resulted in a lowering of the plasma glucose levels after four weeks of supplementation. This study suggests that TRF supplementation plays an important role in reducing oxidative stress-induced diabetes mellitus.

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