Palm oil tocotrienol fractions restore endothelium dependent relaxation in aortic rings of streptozotocin-induced diabetic and spontaneously hypertensive rats

Dharmani Devi A/P Murugan
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Syed Putra Muharisa, Abdul Gapor Md Topb, Dharmani Murugana, Mohd Rais Mustafa,⁎

aDepartment of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
bMalaysian Palm Oil Board (MPOB), No. 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia

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Abstract

Diabetes and hypertension are closely associated with impaired endothelial function. Studies have demonstrated that regular consumption of edible palm oil may reverse endothelial dysfunction. The present study investigates the effect of palm oil fractions: tocotrienol rich fraction (TRF), α-tocopherol and refined palm olein (vitamin E-free fraction) on the vascular relaxation responses in the aortic rings of streptozotocin-induced diabetic and spontaneously hypertensive rats (SHR). We hypothesize that the TRF and α-tocopherol fractions are able to improve endothelial function in both diabetic and hypertensive rat aortic tissue. A 1,1-diphenyl picryl hydrazyl assay was performed on the various palm oil fractions to evaluate their antioxidant activities. Endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) relaxations were examined on streptozotocin-induced diabetic and SHR rat aorta following preincubation with the different fractions. In 1-diphenyl picryl hydrazyl antioxidant assay, TRF and α-tocopherol fractions exhibited a similar degree of activity while palm olein exhibited poor activity. TRF and α-tocopherol significantly improved acetylcholine-induced relaxations in both diabetic (TRF, 88.5% ± 4.5%; α-tocopherol, 87.4% ± 3.4%; vehicle, 65.0 ± 1.6%) and SHR aorta (TRF, 72.1% ± 7.9%; α-tocopherol, 69.8% ± 4.0%, vehicle, 51.1% ± 4.7%), while palm olein exhibited no observable effect. These results suggest that TRF and α-tocopherol fractions possess potent antioxidant activities and provide further support to the cardiovascular protective effects of palm oil vitamin E. TRF and α-tocopherol may potentially improve vascular endothelial function in diabetes and hypertension by their sparing effect on endothelium derived nitric oxide bioavailability.

Keywords: Rats; Endothelial dysfunction; Hypertension; Palm oil fractions; Nitric oxide; Streptozotocin-induced diabetic

Abbreviations: ANOVA, analysis of variance; ACh, Acetylcholine; DPPH, 1,1-Diphenyl picryl hydrazyl; DMSO, dimethyl sulfoxide; EDNO, endothelium-derived nitric oxide; FRAP, Free radical activity/potential assay; HPLC, High-performance liquid chromatography; NO, Nitric oxide; PE, phenylepherine; RBD, refined, bleached and deodorized; ROS, Reactive oxygen species; SHR, Spontaneously hypertensive rats; SNP, Sodium nitroprusside; STZ, streptozotocin; TRF, Tocotrienol rich fraction; WKY, Wistar Kyoto rats.

1. Introduction

Vascular endothelial cells play a major role in maintaining cardiovascular homeostasis in health. Endothelial dysfunction refers to a condition in which the endothelium loses its physiological properties such as the tendency to promote vasodilatation, fibrinolysis, and antiaggregation [1]. Endothelial dysfunction plays a key role in the pathogenesis of diseases such as hypertension and diabetes [1-3]. The mechanisms of the dysfunction are similar, with oxidative stress exerting complications due to overproduction of reactive oxygen species (ROS), leading...
to decreased bioavailability of endothelium derived nitric oxide (EDNO) [4-6].

Antioxidants are substances that are capable of counteracting the damaging effects of oxidation in tissues, thus preventing autoxidation of biological molecules like lipids, protein and DNA. They are found naturally in the body, in the form of enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, or they can exist as non-enzymatic molecules like vitamin E, vitamin C, and β-carotene [7]. Several antioxidants have been shown to possess beneficial effects in the prevention and treatment of various oxidative-stress-related diseases [8-10]. Dietary supplementation of palm vitamin E has also been demonstrated to improve vascular endothelial dysfunction in streptozotocin (STZ)-induced diabetic rats [11] and lower blood pressure in spontaneously hypertensive rats (SHR) rats [12]. There are 2 chemical forms of vitamin E: tocopherols and tocotrienols.

Palm oil is a major source of vitamin E (having both tocopherol and tocotrienol). During commercial production, crude palm oil is processed to yield vitamin E-lacking refined, bleached and deodorized (RBD) palm olein, along with a co-product of vitamin enriched content known as palm fatty acid distillate. As a result of the refining process, palm olein is composed of a mixture of many compounds (mainly triglycerides) with only a small amount of palm vitamin E. Following a series of purification and concentration performed on palm fatty acid distillate, a highly purified vitamin E is produced. The fraction is rich in tocotrienols, thus termed tocotrienol-rich fraction (TRF) (75% tocotrienols and 25% tocopherol) [13-14]. TRF has been demonstrated to possess antioxidant [15], anticancer [16], anti-inflammatory [17], apoptosis [18] and cardioprotective [19] actions.

Although the effects of tocopherols and tocotrienols on endothelial function have been studied previously, not much work has been conducted to compare the effects of the different palm oil fractions on the endothelial dysfunction in diabetic and hypertensive conditions. Fractions of palm vitamin E with the ability to improve endothelial function may complement medication used in the treatment of hypertension and diabetes. We hypothesize that the TRF and α-tocopherol fractions are able to improve endothelial function in both diabetic and hypertensive aortic tissue. Therefore, the aim of the present study was to evaluate the potential of palm oil vitamin E in improving vascular function in rodent models of diabetes and hypertension. The effects of TRF were compared to an α-tocopherol enriched and vitamin E-free fraction of palm olein.

2. Methods and materials

2.1. Animals

Ten-week-old male Wistar Kyoto rats (WKY) and SHR were obtained from the University of Malaya animal house and kept in a well ventilated room at ambient temperature and provided with normal rat chow and tap water ad libitum. All experiments were reviewed and approved by the University of Malaya Animal Care and Ethics Committee. Diabetes was induced in the WKY rats by administration of STZ 75 mg/kg, intraperitoneally. Blood glucose was determined 3 days later using a glucometer (AccuCheck, Roche Diagnostics) to confirm diabetes. Rats with blood glucose above 17 mmol/L were considered diabetic. The control group (normal WKY) and SHR group received an equal volume of vehicle.

2.2. Preparation of palm oil fractions

Palm oil fractions were obtained from the Malaysian Palm Oil Board, Kuala Lumpur, Malaysia. To standardize the various palm oil fractions, the samples were analyzed using an analytical high-performance liquid chromatography (HPLC) (Hewlett Packard HP 1100, Waldbronn, Germany) equipped with a fluorescence detector system using a YMC 5-μm silica column (150×6.0 mm) and eluting with 0.5% isopropyl alcohol/hexane at 1 mL/min flow rate [13-14]. For vascular studies, extracts of tocopherol (98% purity), TRF and RBD palm olein were prepared as 10 mg/mL by dissolving in 10% dimethyl sulfoxide (DMSO). The extracts were freshly diluted with distilled water to 1 mg/mL concentration before the experiments.

2.3. 1,1-Diphenyl-2-picryl hydrazyl assay

1,1-diphenyl-2-picryl hydrazyl (DPPH) is a stable free radical used to analyze the in vitro free radical scavenging capacity of natural products. Antioxidant activities for the different extracts were measured spectrophotometrically following reaction with DPPH (10−4 mol/L) at 510 nm. In the presence of antioxidants, electrons are donated to the DPPH radicals, changing the purple color (absorbance) of the DPPH radicals. The stock solutions of various extracts were prepared in methanol and diluted to the desired concentration on the day of the experiment with the same solvent. The DPPH activity of the fractions was compared to quercetin, a naturally occurring flavonoid with potent antioxidant activity [20].

2.4. Aortic ring preparation

Eight weeks after induction of diabetes, the STZ-induced or SHR rats (18 weeks olds) were killed by cervical dislocation and the aorta from the thoracic region was excised. The aorta was cleared from any adherent fat and connective tissue with extra care to avoid any damage to the endothelium. It was then cut into small rings (3–5 mm in width) and suspended in a 5-mL organ bath containing Krebs physiological salt solution (pH 7.4) made up of the following composition (mmol/L): NaCl 118, KCl 4.7, CaCl2·2H2O 2.5, KH2PO4 1.2, MgSO4·7H2O 1.2, glucose 11.7, NaHCO3 25.0, and EDTA 0.026. 95% oxygen and 5% carbon dioxide at 37°C were passed continuously through the bathing fluid at 37°C.

Fig. 1. HPLC chromatograms of palm oil vitamin E fractions (A) TRF (B) α-tocopherol and (C) RBD palm olein.
solution. Isometric tension (g) was measured using a force displacement transducer connected to a Mac Lab recording system (ADI Instruments, Australia). The aortic rings were allowed to equilibrate for 20 minutes under resting tension of 1 g before any initiation of experimental protocols. The minimum animal sample required for all of the experiment was calculated using LaMorte’s power calculation (Microsoft Excel spreadsheet, SAMPLESZ.xls).

2.5. Evaluation of aortic rings

After equilibration, the rings were repeatedly stimulated with KCl solution (high K+, 80 mmol/L) for 4 minutes at 10-minute intervals until 2 consecutive equal contractions were reached. Following washout of high K⁺ responses, the aortic rings were incubated for 20 min with indomethacin (10⁻⁵ mol/L) together with the extracts of α-tocopherol (0.01 mg/mL), TRF (0.01 mg/mL), palm olein (0.01 mg/mL), vehicle (0.1% DMSO v/v), or quercetin (10 μmol/L) as positive control. Indomethacin was added to the tissue bath to avoid possible influence of prostaglandins. Vascular relaxation study was performed by doing cumulative concentration–response curves to the endothelium-dependent and endothelium-independent relaxant agonists, acetylcholine (ACh; 10⁻¹⁰ to 10⁻⁵ mol/L) and sodium nitroprusside (SNP; 10⁻¹¹ to 10⁻⁶ mol/L), respectively, on tissues of STZ-induced diabetic, SHR and WKY rats. To test the relaxation responses to ACh and SNP, the aortic rings were pre-contracted with phenylepherine (PE) (1 μmol/L). Aortic rings from 5 to 8 rats were used for each set of experiments [21].

2.6. Statistical analyses

The concentrations indicated in the text or in the figures represent the final tissue-bath concentrations of each respective fraction/drug. The responses were recorded as means ± SEM for the number of rats. Significant differences between means were evaluated using unpaired Student’s t test. When more than 2 groups were compared, data were evaluated by 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (Prism version 4.0, GraphPad software, USA). A value of \( P < .05 \) was considered statistically significant.

3. Results

Fig. 1 shows the chromatograms of the various palm oil fractions. The peaks from HPLC analysis were identified as α-tocopherol and tocotrienol homologues from palm oil vitamin E and corresponded to known tocopherol and tocotrienol standards. The level of vitamin E in tocotrienol rich fraction (TRF) was 72% comprising of tocotrienols and tocopherol at a ratio of 3 to 1; while the α-tocopherol fraction contained 98% α-tocopherol. The total vitamin E content (tocotrienol and tocopherol) detected for palm olein fraction was very low (0.08%).

DPPH antioxidant assay was performed to characterize the antioxidant potency of various palm oil fractions, that is, TRF, α-tocopherol and palm olein. TRF and α-tocopherol exhibited a significant increase in the radical scavenging capability with increasing concentration. Both fractions showed a similar pattern, with maximum inhibition at 85.6% and 86.2%, respectively at 1 mg/mL, and were comparable to quercetin. Palm olein did not exhibit any free radical scavenging capability, having shown no DPPH inhibition in all the doses tested (Fig. 2).

ACh caused concentration-dependent relaxation of PE (10⁻⁶ mol/L)-induced contraction in the aortic rings of WKY, STZ-induced diabetic and SHR rats. Relaxation responses induced by ACh were significantly reduced in STZ-induced diabetic and SHR aorta when compared with WKY (Fig. 3). At 10⁻⁵ mol/L ACh concentration, the maximal relaxation was 65.0% and 51.1% in STZ-induced
diabetic rats and SHR, respectively, whereas the WKY rats demonstrated 80.8% relaxation.

Preincubation with fractions of TRF, α-tocopherol and palm olein did not affect ACh-induced relaxation in WKY compared to its vehicle control (Fig. 4A). For the STZ-induced diabetic group, TRF and α-tocopherol significantly increased the ACh relaxation ($10^{-7} - 10^{-5}$ mol/L) compared to the vehicle control, whereas palm olein exhibited no significant effect. The positive control quercetin also demonstrated a significant improvement in ACh induced relaxation. The maximum relaxation responses at $10^{-5}$ mol/L ACh concentration in aortic rings pre-incubated with TRF was 88.5%, α-tocopherol was 87.4% and palm olein was 70.6% compared to its vehicle at 65.0% (Fig. 4B).

SNP caused concentration-dependent relaxation in the aortic rings of WKY, SHR, and STZ-induced diabetic rats. All the groups exhibited similar preserved relaxation responses. Pre-incubation with different fractions of TRF, α-tocopherol and palm olein as well as quercetin were found to exert no significant effect on the SNP relaxation responses. All relaxation responses were >90% (Fig. 5). The maximal relaxation at the highest concentration used for ACh-induced relaxation and SNP induced relaxation for vehicle, TRF, α-tocopherol and palm olein and quercetin is summarized in Table 1.

At $10^{-5}$ mol/L ACh concentration, SHR aortic rings pre-incubated with TRF, α-tocopherol and quercetin also exhibited increased ACh relaxation while palm olein exhibited no significant effect compared to the vehicle. The maximum relaxation responses observed with TRF was 72.1%, α-tocopherol was 69.8% and palm olein was 58.7% compared to its vehicle at 51.1% (Fig. 4C).

Fig. 4. ACh- induced relaxations in the presence of TRF, α-tocopherol, palm olein and vehicle control in the isolated aortic ring of (A) WKY, (B) STZ-induced diabetic and (C) SHR. Results are expressed as means ± SEM (n = 5–6). Data were analyzed by 1-way ANOVA followed by Bonferroni post hoc test.

Fig. 5. SNP-induced relaxations in the presence of TRF, α-tocopherol, palm olein and vehicle control in the isolated aortic ring of (A) WKY, (B) STZ-induced diabetic and (C) SHR. Results are expressed as means ± SEM (n = 5–6). Data were analyzed by 1-way ANOVA followed by Bonferroni post hoc test.
Maximal relaxation induced by ACh at 10^(-5) mol/L (as well as lower^23^) antioxidant property for lipoprotein. However, there are also studies that reported from the tocotrienol portion of the palm vitamin E. Tocotrienols (75%), the effect of TRF is generally derived from hypertensive rats. As TRF is mostly constituted of α-fractions; particularly, TRF, α-tocopherol and tocotrienol possesses potent antioxidant activity which is related to the phenolic group in the chromanol ring [15]. In 2003, Yoshida et al[22] demonstrated that oral feeding of SHR with a diet containing a small amount of vitamin E which is removed as a result of the refining processes[24]. This would explain the fact that palm olein did not exhibit significant antioxidant activity. The present study demonstrated a similar radical scavenging activity to tocopherols in a homogenous solution. Tocotrienols may have an added advantage in having a higher uniform distribution on the membrane bilayer than α-tocopherol [23]. Palm olein is a mixture of many compounds; however, it is mainly comprised of triglycerides with only a small amount of vitamin E which is removed as a result of the refining processes [24]. This would explain the fact that palm olein did not exhibit significant antioxidant activity. Pre-incubation of the aortic rings with TRF improved ACh induced relaxation in STZ-induced diabetic and SHR without affecting SNP-induced relaxation. This suggests that TRF may be increasing bioavailability of NO via its antioxidant property. A previous study by Abeywardena et al [24] demonstrated that oral feeding of SHR with a diet supplemented with TRF for 12 weeks improved the relaxations in the endothelium dependent in rat aorta but did not alter endothelium independent. The study suggests that pre-incubation with the tocotrienols were without direct effects on the NO signaling pathway in the vascular smooth muscle cells. By scavenging the free radicals, the tocotrienols may have increased the bioavailability of EDNO, allowing normal NO signaling and relaxations. Prevention of any chain breaking effect of oxidized lipid molecules, via inhibition of lipid peroxidation may contribute to the beneficial effects of the tocotrienols [31]. Similarly, α-tocopherol significantly increased ACh-induced relaxation in both STZ-induced diabetic and SHR without altering SNP-induced relaxation. Dietary vitamin E has been documented to restore ACh-induced relaxation in STZ-induced diabetic rat aorta [32] by scavenging ROS (particularly superoxide anions). It has been proposed that the actions of α-tocopherol also increased soluble guanylate cyclase expression leading to an increase in cyclic GMP levels [4]; decreased diacylglycerol levels and activation of protein

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Aortic ring relaxation values from rats</th>
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<tr>
<td></td>
<td>WKY</td>
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<tr>
<td>ACh-induced relaxation (10^-5 mol/L)</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>80.81 ± 1.8</td>
</tr>
<tr>
<td>TRF</td>
<td>79.36 ± 5.5</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>74.55 ± 2.5</td>
</tr>
<tr>
<td>Palm olein</td>
<td>74.43 ± 6.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>87.36 ± 5.5</td>
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<tr>
<td>SNP-induced relaxation (10^-6 mol/L)</td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>94.83 ± 2.5</td>
</tr>
<tr>
<td>TRF</td>
<td>90.74 ± 2.7</td>
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<tr>
<td>α-Tocopherol</td>
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<tr>
<td>Palm olein</td>
<td>96.35 ± 2.6</td>
</tr>
<tr>
<td>Quercetin</td>
<td>101.58 ± 2.8</td>
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</table>

Maximal relaxation induced by ACh at 10^-5 mol/L and SNP at 10^-6 mol/L in the presence of vehicle (0.1% DMSO), TRF (0.01 mg/mL), α-tocopherol (0.01 mg/mL), palm olein (0.01 mg/mL), and quercetin (10 μmol/L) in the isolated rat aortic ring of WKY, SHR and STZ-induced diabetic groups. Results are expressed as means ± SEM (n = 5-6). Data were analyzed by 1-way ANOVA followed by Bonferroni post hoc test. Significant difference from respective vehicle: *p < .05; **p < .01.

4. Discussion

The present study compares the effect of 3 main palm oil fractions; particularly, TRF, α-tocopherol, and palm olein (low vitamin E content), on improving endothelium-dependent relaxation in the isolated aortas of diabetic and hypertensive rats. As TRF is mostly constituted of tocotrienols (75%), the effect of TRF is generally derived from the tocotrienol portion of the palm vitamin E.

The TRF and α-tocopherol palm oil fractions exhibited similar dose-dependent antioxidant activity, while palm olein did not demonstrate significant antioxidant activity, even at high concentrations. It is well documented that vitamin E of both α-tocopherol and tocotrienol possesses potent antioxidant activity which is related to the phenolic group in the chromanol ring [15]. In 2003, Yoshida et al [22] demonstrated that tocotrienols possess a similar free radical scavenging activity to tocopherols in a homogenous solution. Tocotrienols may have an added advantage in having a higher uniform distribution on the membrane bilayer than α-tocopherol [23]. Palm olein is a mixture of many compounds; however, it is mainly comprised of triglycerides with only a small amount of vitamin E which is removed as a result of the refining processes [24]. This would explain the fact that palm olein did not exhibit significant antioxidant activity.

The present study demonstrated a similar radical scavenging capability for TRF and α-tocopherol with DPPH-inhibition assay. Free radical activity/potential assay (FRAP) were previously reported on palm fronds fractions and refined palm oil [25]. TRF possess higher FRAP value while refined palm oil lower FRAP value. TRF has a protective effect on Cu2+-mediated oxidation of low-density lipoprotein. However, there are also studies that reported higher [23] (as well as lower [15]) antioxidant property for tocotrienols when compared to tocopherol. The differences in the palm oil fractions may be due to variation of vitamin E isoforms used to evaluate the antioxidant property as different isoforms provide different antioxidant activity. The nature of experiments, that is, whether they were performed on human or animal models, cell culture or in vitro, may contribute to differences observed for tocotrienol and tocopherol antioxidant activity.

STZ-induced diabetic and SHR aorta exhibited impaired endothelium-dependent relaxations compared to WKY rat aorta. Various studies have also found reduced endothelium dependent relaxations in diabetic [26] and SHR aorta [24]. Oxidative stress is associated with excessive production of ROS like superoxide anions which reduce the bioavailability of nitric oxide (NO) in both diabetic and hypertensive animal models [6,27,28]. Hyperglycemia can directly cause increased ROS generation. High glucose in diabetes can undergo autoxidation and generate hydroxyl radicals [29].

In our study, the endothelium independent relaxation was preserved in the aortic rings of WKY, STZ-induced diabetic and SHR, suggesting that the EDNO signaling pathway in the vascular smooth muscle cells remains intact and that the reduced relaxation responses in the hypertensive and diabetic aorta are likely due to reduced bioavailability of NO. Previous works conducted in our laboratory support this hypothesis [9,21]. Pieper [30] also demonstrated similar relaxations to the NO donor, SNP in aorta of diabetic and euglycemic animals. In the study, the ACh-mediated relaxation was impaired when pre-incubated with L-NAME; the nitric oxide synthase inhibitor, suggesting that EDNO may be involved.

Pre-incubation of the aortic rings with TRF improved ACh induced relaxation in STZ-induced diabetic and SHR without affecting SNP-induced relaxation. This suggests that TRF may be increasing bioavailability of NO via its antioxidant property. A previous study by Abeywardena et al [24] demonstrated that oral feeding of SHR with a diet supplemented with TRF for 12 weeks improved the relaxations in the endothelium dependent in rat aorta but did not alter endothelium independent. The study suggests that pre-incubation with the tocotrienols were without direct effects on the NO signaling pathway in the vascular smooth muscle cells. By scavenging the free radicals, the tocotrienols may have increased the bioavailability of EDNO, allowing normal NO signaling and relaxations. Prevention of any chain breaking effect of oxidized lipid molecules, via inhibition of lipid peroxidation may contribute to the beneficial effects of the tocotrienols [31].

Similarly, α-tocopherol significantly increased ACh-induced relaxation in both STZ-induced diabetic and SHR without altering SNP-induced relaxation. Dietary vitamin E has been documented to restore ACh-induced relaxation in STZ-induced diabetic rat aorta [32] by scavenging ROS (particularly superoxide anions). It has been proposed that the actions of α-tocopherol also increased soluble guanylate cyclase expression leading to an increase in cyclic GMP levels [4]; decreased diacylglycerol levels and activation of protein
kinase C that leads to protection of the vascular wall against lipid peroxidation [33] and increased synthesis of NO synthase [34].

Palm olein or refined palm oil did not improve the relaxation responses induced by either ACh or SNP in either the STZ-induced diabetic or SHR. Palm olein is composed mainly of fatty acids and triglycerides and lacks the antioxidant vitamin E and carotenoids as they are lost during the process of refining [35]. This may explain the lack of antioxidant constituents to scavenge ROS, and thus, improve endothelial function of both STZ-induced diabetic and SHR aorta.

The present study highlighted the potent antioxidant activities of palm oil vitamin E fractions, TRF and α-tocopherol. These fractions are also shown to improve endothelium-dependent relaxation in isolated aorta from SHR and STZ-induced diabetic rats without affecting the endothelium independent relaxation. We accept the hypothesis that TRF and α-tocopherol tocopherol fractions are able to improve endothelial function in both diabetic and hypertensive rat aortic tissue. DPPH assay was used in this study as a measure of total antioxidant capacity. Future research will seek to extend the present study using a battery of antioxidant assays and further investigate their mechanisms. Dietary palm oil has been demonstrated to reduce lipid peroxidation in non-insulin diabetic patient [36]. The in vivo cardiovascular beneficial effect of TRF and α-tocopherol fractions in human subjects needs to be investigated further.

In conclusion, the present results provide evidence of the beneficial vascular protective effect of palm oil in the rat. Incubation of the aortic tissues with TRF and α-tocopherol fractions in vitro suggest that treatment with these fractions may help to restore the normal endothelial function in rodent models of diabetes and hypertension; however, these findings need to be validated in appropriate human studies.

Acknowledgment

We thank the Malaysian Palm Oil Board for providing the palm oil fractions.

References


