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Neutral Effects of Tocotrienol-rich Fraction Supplementation on Serum Lipids, C-reactive protein and Plasma Lipid Peroxidation in Rabbits with Severe Hypercholesterolaemia and Atherosclerosis

Azlina A. Razak[1], Effat Omar[1,2], Suhaila Muid[1,2] and Hapizah Nawawi[1,2]*

[1]Institute of Pathology, Laboratory and Forensic Medicine (I-PPerForM), Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia
[2]Faculty of Medicine, Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, 47000 Sungai Buloh, Selangor, Malaysia

ABSTRACT

Tocotrienols have been reported to possess potent cholesterol lowering, anti-hypertensive, anti-inflammatory and anti-oxidative properties which are superior to tocopherols. Emerging evidence suggests pure tocotrienols have anti-atherogenic properties. However, optimal doses of oftocotrienol-rich fraction (TRF) in progressive atherogenesis remain unclear. This animal model experiment was designed to investigate the effects of a range concentration of TRF supplementation on the extent of atherosclerosis and soluble lipids, inflammatory and oxidative stress biomarkers in high-cholesterol diet (HCD) induced hypercholesterolaemic (HC) rabbits with atherosclerosis. A total of 28 New Zealand white rabbits were given 1% high-cholesterol diet (HCD) for two months and then randomised into five groups: Placebo (n=7), TRF 15 mg/kg (n=5), TRF 30 mg/kg (n=6), TRF 60 mg/kg (n=5) and TRF 90 mg/kg (n=5) daily. The treatment was given for three months and the animals were fed HCD throughout the duration. Aortic vessels were obtained to assess the extent of atherosclerotic lesions at the end of the study. Fasting serum lipids (FSL), C-reactive protein (CRP), malondialdehyde (MDA) and 8-isoprostane levels were measured at baseline, one and two months post-HCD, one, two, and three months post-intervention. There were no differences in the extent of the atherosclerotic lesions, percentage changes of FSL, MDA, 8-isoprostane and CRP levels between the placebo and TRF groups. In conclusion, TRF across all doses studied have neutral effects on atherosclerotic lesions, soluble lipids, biomarkers of oxidative stress, coronary risk and inflammation in severely atherosclerotic rabbits with progressive and continuous insult by high cholesterol feeding.

Keywords: Atherosclerosis, inflammation, lipids, oxidative stress, soluble biomarkers, tocotrienol-rich fraction

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E-mail addresses:
azurinas@gmail.com (Azlina A. Razak),
effat2011.eo@gmail.com (Effat Omar),
suhaila777@gmail.com (Suhaila Muid),
hapizah.nawawi@gmail.com (Hapizah Nawawi)
*Corresponding Author

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INTRODUCTION

Atherosclerosis is the main underlying pathology in coronary heart disease (CHD), leading to clinical events such as acute myocardial infarction and unstable angina. Therefore, most clinicians target to reduce atherosclerosis by treating cardiovascular risk factors (Roger et al., 2012). Amongst the cardiovascular risk factors, hypercholesterolaemia was recognised to play a central role in the initiation of atherosclerosis or fatty streak development. It is characterised by elevated low-density lipoprotein (LDL) levels in the blood. Since LDL is the major cholesterol-carrying lipoprotein in plasma, there is a higher chance for it to undergo modification such as oxidation, proteolysis and aggregation and subsequently get trapped in the vascular walls (Berliner et al., 1996; Stocker & Keaney, 2004).

Following initiation, atherosclerosis is then manifested in stages of early, developing and mature lesions. In general, atherosclerotic lesions are characterised by lipid deposition, fibrosis, and calcification of lesions (Heistad, 2006). Lipid component of these lesions can be visualised using special staining such as Sudan IV (Tangirala, Rubin, & Palinski, 1995; Zhang et al., 2010). In hypercholesterolaemic rabbits induced severe atherosclerosis, a time-dependent increase in plasma CRP has been shown (Yu et al., 2012). This suggests a predominant systemic inflammatory state in response to continuous high plasma cholesterol level. Cholesterol feeding in animal model of atherosclerosis also produced increased lipid peroxidation (Mahfouz & Kummerow, 2000). Therefore, measuring soluble biomarkers in blood such as circulating lipids, serum CRP and products of lipid peroxidation is useful to monitor the on-going process of atherosclerosis. Two relevant products of lipid peroxidation in experimental atherosclerosis are 8-iso-Prostaglandin F2α (or 8-Isoprostane) and malondialdehyde (MDA). 8-Isoprostane is a stable end-product from the peroxidation of arachidonic acid (Morrow et al., 1990), while MDA is a short chain aldehyde which arises from ROOH fragmentation of free radical generation.

Vitamin E in the form of tocotrienols are naturally synthesised only by plants and found concentrated in rice bran and palm oil (Ahsan, Ahad, Iqbal, & Siddiqui, 2014). The palm tocotrienol can be produced in the form of tocotrienol-rich fraction (TRF), which comprises 30% tocopherol and 70% tocotrienol (Sundram, Sambanthamurthi, & Tan, 2003). TRF is currently available in the market for human consumption. Tocotrienols differ from tocopherols by possessing three double bonds in the phytyl side chain. In general, the term vitamin E covers four isomers of tocopherols (α, β, γ and δ) and four isomers of tocotrienols (α, β, γ and δ), all of which are lipid-soluble and chain-breaking antioxidants (Brigelius-Flohe & Traber, 1999). Previously, the effectiveness of pure tocotrienols in reducing inflammation and endothelial activation in human endothelial cells *in vitro* was reported (Muid, Froemming, Rahman, Ali, & Nawawi, 2016). Rahman et al. (2016) also reported that pure tocotrienols reduced aortic inflammation, endothelial activation and proteolytic enzymes. Similarly, TRF was also found to exhibit anti-atherogenic properties in experimental atherosclerosis (Mohd. Ismail, Abdul Ghafar, Jaarin, Hla Khine, & Md. Top, 2000; Norsidah, Asmadi, Azizi, Faizah, & Kamisah, 2013) and patients with carotid stenosis (Tomeo, Geller, Watkins, Gapor, & Bierenbaum, 1995). However, the effect of TRF to modulate circulating lipids and systemic inflammation and lipid peroxidation in progressive HCD-induced severe atherosclerosis is not known. Therefore, this experimental atherosclerosis study aimed to further examine the effects of TRF supplementation

on progressive atherosclerotic lesions and soluble biomarkers in five-month cholesterol-fed rabbits. The doses of TRF supplement used were based on previous literature reports (Ahmad, Khalid, Luke, & Ima Nirwana, 2005; Rahman et al., 2016).

MATERIALS AND METHODS

Rabbits and High-cholesterol Diet

This study had been approved by the Institutional Animal Ethics Committee and conformed to the institutional and national guidelines on use of animals in research. The researchers used 28 male New Zealand white rabbits weighing (2.0 - 3.0 kg) which given 1% high-cholesterol diet (HCD) for two months to induce hypercholesterolaemia and establish atherosclerosis, following which, they were randomised into one of five groups: Placebo (n=7), TRF 15 mg/kg (n=5), TRF 30 mg/kg (n=6), TRF 60 mg/kg (n=5) and TRF 90 mg/kg body weight (n=5) daily. The treatment was given for three months and the animals were fed HCD throughout the duration. All animals were housed in individual cages, with *ad libitum* access to food and water, maintained at a 12-hour dark/light cycle.

Diet and TRF Supplement

HCD was purchased from Bio-Serv, USA. The TRF supplement (Gold-Tri E 50®, Sime Darby Bioganic Sdn. Bhd., Malaysia) consisted of γ-tocotrienol: 174.2 mg/g, α-tocotrienol: 145.6 mg/g, δ-tocotrienol: 128.2 mg/g, β-tocotrienol: 11.4 mg/g and α-tocopherol: 95.8 mg/g. The placebo group was treated with distilled water.

Blood and Tissue Collection

Blood was taken for serum lipids, C-reactive protein (CRP), malondialdehyde (MDA) and 8-isoprostane levels at baseline (BL), one and two months intra-HCD, one, two, and three months post-treatment. About 10 ml of fasting blood samples were collected into plain and ethylene diamine tetra acetic acid (EDTA) coated blood tubes as anticoagulant from middle arterial veins at baseline (BL), one and two post-HCD, one, two, and three months post-TRF treatment. At three months post-TRF treatment the rabbits were sacrificed and aortic vessels were obtained. The blood tubes were centrifuged at 1400 × g for 10 minutes. Serum and plasma EDTA were isolated and kept in -80°C until analysis. The aorta, from the bifurcation point of the left subclavian artery to the iliac bifurcation, were removed from each rabbit.

Sudan IV Staining

These aortic vessels were processed immediately for macroscopic Sudan IV staining, which was performed according to the method by Hamm, Kaplan, Clarkson and Bullock (1983). The aortas were gently rinsed with normal saline, then cut open longitudinally to expose the lumen and pinned flat on a wooden board in a stainless-steel tray (8 × 16 cm). The opened aortic vessels were then fixed with 10% neutral buffered formalin overnight. After the process, the aortic vessels were taken out from the immersion and washed with 70% ethanol, followed

by immersion in Herskhemers solution for 15 minutes at room temperature and destained under running tap water for one hour. The stained aortic vessels were put on smooth contrast background for imaging.

The areas of the entire aortic vessels were captured at ultra zoom by a digital camera (C-740 Ultra Zoom, Olympus, USA). The percentage area of dark red regions (the area of atherosclerotic lesions) was calculated within the total surface area of the aortic vessel by using soft imaging solution (analySIS® FIVE, Olympus, USA).

Fasting Serum Lipid Profiles

Fasting lipid profiles [total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL-c) and low-density lipoprotein (LDL-c)] were measured by Cobas Integra 400 Plus automated chemistry analyser (Hoffman-La Roche Ltd., Switzerland), according to the manufacturer's protocol and using Roche's calibrator for automated system (cfas), a lyophilised calibrator based on human serum. The use of this method for measuring lipids in rabbit serum has also been reported elsewhere (Idris et al., 2014; Rahman et al., 2016). For TC, the within and between batch coefficient of variations (CVs) at 5.3 mmol/L were 1.3% and 2.2%, respectively while that for TG at 1.6 mmol/L were 1.6% and 1.9%, respectively. For HDL-c, the within and between batch CVs at 1.2 mmol/L were 0.9% and 1.7%, respectively; while the CVs for LDL-c at 4.3 mmol/L were 1.1% and 1.8%, respectively (Method Manual Cobas Integra 03/2002, 2002).

C-reactive Protein (CRP)

Quantitative determinations of rabbit serum CRP were based on enzyme-linked immunosorbent assay (ELISA) using rabbit CRP E-15CRP-8 ELISA kits (ICL Inc., USA). A standard curve for each microplate assay was prepared. The tests were run according to manufacturer's manual by automated EIA analyser (CODA, Bio-Rad Laboratories, USA). The calculated limit of detection in this assay was 0.3 ng/mL. The within and between batch CVs of rabbit CRP at 3.2 μg/mL were 5.3% and 9.1%, respectively (Rabbit CRP Package Insert, 2006).

Oxidative Stress Biomarkers

The MDA content in serum was determined as thiobarbituric acid-reactive substances (TBARS) as described by Ledwoż, Michalak, Stępień and Kądziołka (1986), with slight modification. The within and between batch CVs of MDA at 7.2 nmol/g were variation that ranged from 1-6% and 5.2%, respectively (Ledwoż et al., 1986). Results were expressed as TBARS nmol per gram serum protein (3.1), using 1,1,3,3,tetramethoxypropane (Sigma) as standard.

The measurement of 8-Isoprostane was performed by a validated method adapted from Bohnstedt and colleagues (2003) using liquid chromatography (Agilent 1100 Series, Agilent Technologies, USA) coupled to tandem mass spectrometry system with turbo ion spray (API 4000™ LC/MS/MS System, Applied Biosystems, USA). Bohnstedt et al.(2003) reported that the within and between batch CVs of 8-Isoprostane at 0.28 ng/mL were 7.0% and 7.8%,

respectively. In our laboratory, the within and between batch CVs of 8-Isoprostane at 0.5 ng/mL during the method development were 1.6% and 4.8%, respectively.

Statistical Analysis

For between group differences, student's t-test or Mann-Whitney test was used for variables with normal or non-normal distribution respectively. Within group pre- and post-treatment differences for each variable were analysed by paired t-test or Wilcoxon matched-pair test for those variables with normal distribution and non-normal distribution respectively. Normality was tested with Kolmogrov-Smirnov test. Probability value of $p<0.05$ was taken as significant. The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 22.

RESULTS

Percentage of Atherosclerotic Lesions

At the end of the experiment, atherosclerotic lesions were analysed in all the rabbits. There were no differences observed in the extent of atherosclerosis, as represented by the Sudan IV positive areas, between the placebo and treatment groups (Figure 1).

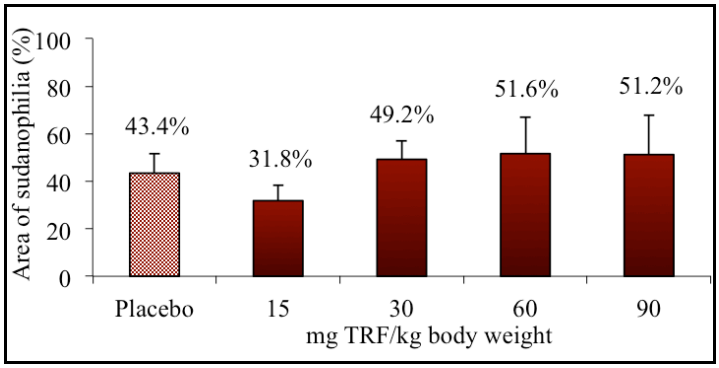


Figure 1. Extent of atherosclerosis defined by percentage of sudanophilia area in all groups at 3 months post-intervention

Lipid Profiles

Total cholesterol, triglyceride, LDL-c and HDL-c levels significantly increased in all the groups at one and two months post HCD administration compared to baseline. The levels and percentage changes of the lipid profiles and baseline, one and two months post HCD are summarised in Table 1. Levels of all lipid parameters remained unaltered post-supplementation at 3 to 5 months compared to post HCD baseline (that is, at two months post HCD prior to randomisation) (Figure 2).

Table 1
Level of serum lipids in placebo and TEMF groups at baseline (BL), 1 month and 2 months post HCD (High cholesterol diet)

Groups	Time points	TC (mmol/L)	TG (mmol/L)	HDL-c (mmol/L)	LDL-c (mmol/L)
Placebo	BL	1.6 ± 0.2	0.6 ± 0.1	1.0 ± 0.2	0.4 ± 0.1
	1 month	12.9 ± 2.4*	0.6 ± 0.2	3.2 ± 0.4*	12.5 ± 2.8*
	2 months	16.7 ± 3.4*	0.6 ± 0.1	3.7 ± 0.7*	15.9 ± 2.8*
TEMF 15	BL	1.1 ± 0.1	0.6 ± 0.0	0.8 ± 0.1	0.2 ± 0.1
	1 month	23.0 ± 6.1*	1.6 ± 1.0	5.2 ± 0.5*	20.7 ± 5.3*
	2 months	25.5 ± 6.6*	1.6 ± 0.6	5.4 ± 1.0*	27.5 ± 7.3*
TEMF 30	BL	1.3 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
	1 month	19.6 ± 5.8*	1.5 ± 1.0	4.1 ± 0.7*	19.1 ± 6.4*
	2 months	15.5 ± 3.2*	1.4 ± 0.8	3.6 ± 0.7*	13.8 ± 2.7*
TEMF 60	BL	1.4 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	0.2 ± 0.1
	1 month	20.4 ± 4.4*	0.7 ± 0.1	4.3 ± 1.0*	17.6 ± 5.4*
	2 months	18.9 ± 4.6*	0.9 ± 0.3	4.1 ± 1.0*	17.2 ± 4.4*
TEMF 90	BL	1.3 ± 0.2	0.9 ± 0.3	0.8 ± 0.2	0.4 ± 0.2
	1 month	22.4 ± 4.0*	1.1 ± 0.5	6.1 ± 0.4*	19.2 ± 4.2*
	2 months	12.4 ± 3.1*	1.7 ± 1.1	2.8 ± 0.5*	10.3 ± 2.5*

Note: Data are expressed as Mean ± SEM. * p<0.05 when compared to BL in respective groups

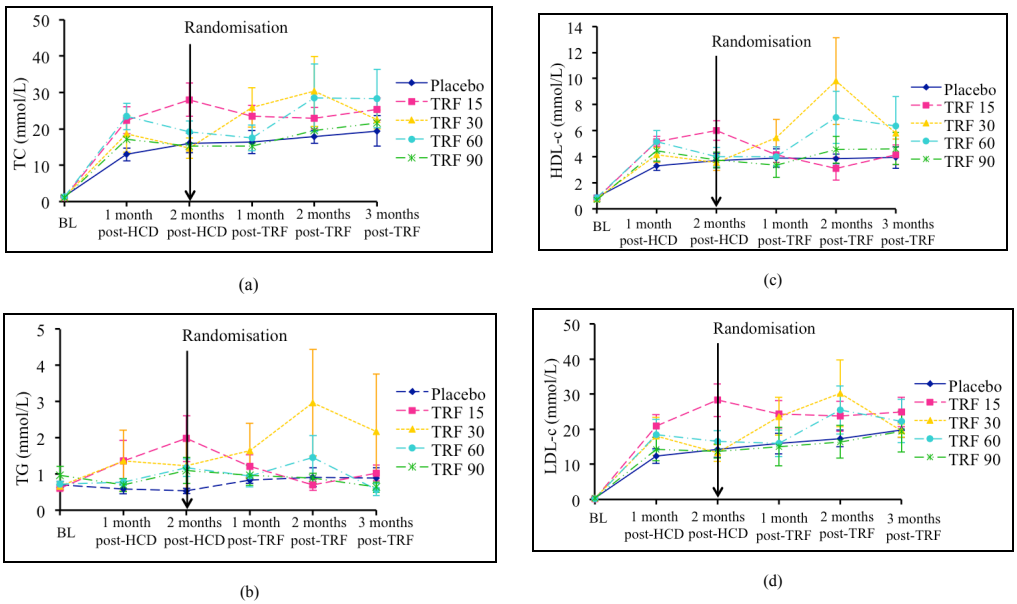
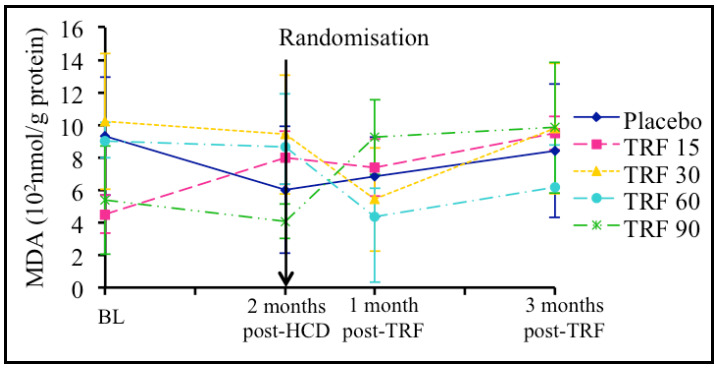


Figure 2. . Fasting lipid profiles: (a) TC; (b) TG; (c) HDL-c; and (d) LDL-c in all groups at BL, 1 and 2 Months Post-HCD, 1, 2 and 3 months post-treatment

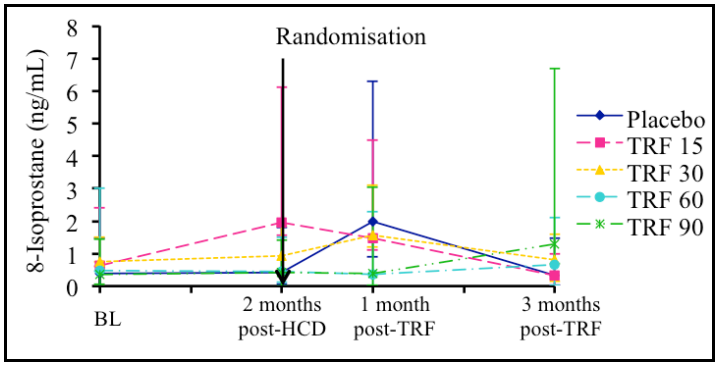
Data are expressed as Mean ± SEM. No significant differences ($p>0.05$) in lipid profiles of TRF groups compared to placebo were observed. Different letters indicate significant differences compared to BL levels ([a] Placebo, [b] TRF 15, [c] TRF 30, [d] TRF 60, [e] TRF 90, $p<0.05$).

CRP, MDA and 8-isoprostane Levels

TRF treated groups, across all concentrations showed no significant changes in serum MDA, 8-isoprostane and CRP levels compared to the placebo groups (Figures 3 and 4).



(a)



(b)

Figure 3. . Levels of: (a) Plasma MDA; and (b) 8-isoprostane, in all Groups at BL, 2 months post-HCD, 1 and 3 months post-treatment

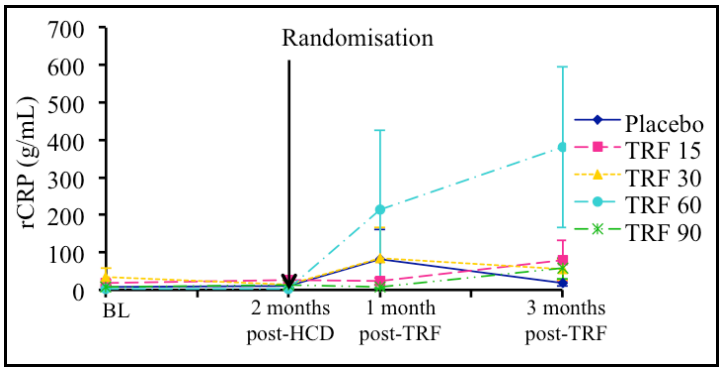


Figure 4. Serum CRP levels in all groups at BL, 2 months post-HCD, 1 and 3 months post-treatment

DISCUSSION

This study used 1% high cholesterol diet to induce hypercholesterolaemic state and severe atherosclerosis in a rabbit model experiment. Cholesterol-fed rabbits were used in investigating the effects of TRF because they are the established experimental model for research in hypercholesterolaemia-induced atherosclerosis (Finking & Hanke, 1997). Blood soluble biomarkers were used to determine the cholesterol lowering, anti-inflammatory and anti-oxidative properties. Furthermore, inducement of hypercholesterolaemic state and severe atherosclerosis enable the investigation on the systemic hypolipidaemic action, antioxidant and anti-inflammatory activities in active progression of severe atherosclerosis.

A previous study showed that hypercholesterolaemic-induced rabbits exhibited an increase in oxygen free radical production from one month of cholesterol feeding onward (Ohara, Peterson, & Harrison, 1993). Various percentages of dietary cholesterol were used to induce the hypocholesterolaemic status in other experimental atherosclerosis and vitamin E interventions (Mohd. Ismail et al., 2000; Norsidah et al., 2013; Prasad & Kalra, 1993). In this present study, 1% high cholesterol diet was given for two months prior to randomisation, followed by another three months of HC diet together with the treatment. Serum TC and LDL-c levels increased significantly before randomisation and remained high with or without tocotrienol-rich supplementation across all doses studied (15-90 mg/kg). This finding suggests that the oral administration of TRF supplement does not suppress severe hypercholesterolaemia which may in turn indicate its lack of benefit in terms of cholesterol lowering in severe hypercholesterolaemic conditions such as familial hypercholesterolaemia.

Overall, this present study showed that the percentage of atheromatous lesion was not significantly reduced in TRF treated groups as compared to placebo group. However, low dose TRF at 15 mg/kg, had a trend of atherosclerotic regression but this did not indicate any statistical significance ($p=0.06$). It is also worth noting, however, that this study attempted to use TRF supplementation to inhibit the progressive atherogenesis in severe atherosclerosis, with a background of continuous insult by high cholesterol diet for a total period of five months. The design mirrored clinical trial on supplementation in patients with established atherosclerotic plaque progression such as tocotrienol trial in patients with carotid stenosis

(Tomeo et al., 1995). Investigation on this aspect is important, as it provides possible mechanistic value of TRF intervention in plaque formation and potentially its stability. Most anti-atherogenic properties of other investigated compounds are designed in the manner where there is regression of atherosclerosis. To study regression of lesions, it is suggested to induce the rabbits with 1.0 - 1.5% cholesterol or saturated fat and switch back to normal/basal diet at the point of intervention (Stein & Stein, 2001). Another experimental atherosclerosis design with positive effect of antioxidants is concurrent supplemental antioxidants and atherogenic diet. The antioxidants may be fed through oral gavage or incorporated with the atherogenic diet (Black, Wang, Maeda, & Coleman, 2000; Norsidah et al., 2013).

Although palm vitamin E enriched tocotrienols are found to have lipid lowering effects (Palau, Lightner, Brannon, & Krishnan, 2013; Parker, Pearce, Clark, Gordon, & Wright, 1993; Theriault, Chao, Wang, Gapor, & Adeli, 1999), the result in this experimental atherosclerosis utilising rabbits does not support the previous positive finding. The previous rabbit model in investigating the effects of tocotrienols on cholesterol and atherosclerosis by Hasselwander et al. (2002) is in agreement with the outcome of this present study. However, the previous study employed dietary mixed tocotrienols supplementation rate of 500mg/kg diet which is equal to approximately 20 mg/kg body weight, while the current study uses slightly lower and higher doses (15-90 mg/kg).

In the present study, serum CRP levels are measured as a soluble biomarker as an indicator of systemic inflammatory status. A time-course measurement of rabbit CRP sera indicated non-significant changes throughout the study in all groups. Limited original contributions have been found to indicate direct systemic anti-inflammatory effect of TRF in experimental atherosclerosis, although tocotrienols have been found to reduce expression of aortic inflammatory markers and increase plaque stability via reduction of vascular matrix metalloproteinases (Rahman et al., 2016).

The selected lipid peroxidation indices, MDA and 8-isoprostane, revealed non-significant changes throughout the study. Comparison among potent antioxidants on anti-atherosclerotic effect suggests that vitamin E may provide protective properties against hypercholesterolaemic atherosclerosis not linked to its antioxidant property (Özer & Azzi, 2000). For example, one of the probucol analogue, bis(3,5-di-*tert*-butyl-4-hydroxyphenylether) propane, failed to prevent atherosclerosis in rabbit despite its strong inhibition on LDL oxidation *in vitro* (Fruebis, Silvestre, Shelton, Napoli, & Palinski, 1999). It is also speculated that the undetectable anti-inflammatory action by TRF may be due to its characteristic as a mixture of tocotrienols and tocopherols, as it is reported that tocopherol is capable in attenuating the hypocholesterolaemic activity of tocotrienols (Qureshi, Pearce, Nor, & Gapor, 1996). However, further study is necessary to clarify this possibility.

CONCLUSION

TRF has no *in vivo* effect on soluble biomarkers of coronary risk in severe HC and experimental atherosclerosis.

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