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PREVENTIVE EFFECTS OF TOCOTRIENOL-ENRICHED MIXED FRACTION ON PLAQUE FORMATION AND STABILITY IN EARLY AND ESTABLISHED ATHEROSCLEROSIS

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ABSTRACT

Atherosclerosis is the underlying pathology for cardiovascular disease. The preventive effects of tocotrienol-enriched mixed fraction (TEMF) on atherogenesis remain unclear. Objective: To investigate the preventive effects of TEMF supplementation on early and established atherosclerosis. Methods: Twenty New Zealand white rabbits were divided into TEMF (n=10) and placebo (n=10) groups. Treatments were given by oral gavage for 8 weeks followed by a 1% high cholesterol diet (HCD) for another two (to induce early atherosclerosis) or eight weeks (established atherosclerosis). At the end of the study, the aorta was dissected, stained with Sudan IV, and qualitatively analyzed for the atherosclerotic lesion. Immunohistochemistry was performed to detect expression of interleukin-6 (IL-6), CRP, nuclear factor kappa beta, E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein-1 (VCAM-1), smooth muscle actin (SMA), and matrix metalloproteinase-12 (MMP-12). Results: In both early and established atherosclerosis groups, there was a significant reduction of atherosclerotic lesions in TEMF compared to placebo. Atherogenic biomarkers; IL-6, CRP, E-selectin, SMA, and MMP-12 were reduced with TEMF supplementation in established atherosclerosis (p<0.05). Neutral effects were seen in the early atherosclerosis group. Conclusion: TEMF supplementation in the preventive setting decreased atherosclerosis formation and reduces endothelial inflammation in established atherosclerosis lesions while increasing plaque stability.

Key words: Atherosclerosis, inflammation, plaque stability, tocotrienol

INTRODUCTION

Atherosclerosis is the main pathology behind coronary artery disease, the leading cause of premature death worldwide (World Health Organization, 2020). Malaysia has a high burden of the disease (Department of Statistics Malaysia, 2017), resulting in the loss of valuable skilled workers and escalation in healthcare costs.

The pathophysiological mechanism and pathogenesis of atherosclerosis involve endothelial dysfunction caused by a disturbance in laminar blood flow within large and medium-sized arteries (e.g., due to hypertension). This leads to the entry and aggregation of low-density lipoprotein cholesterol (LDL-c) into the intima. The LDL-c becomes oxidized through the action of inflammatory cells and activates CD36 on macrophages, resulting in a cascade of intracellular messenger activation which triggers NF-ƙB and increases chemokine expression (such as CCL-2 and CXCL-4), stimulating more inflammatory cells to enter the intima. Attracted by the chemokines, monocytes adhere to endothelial cells by adhesion molecules (e.g., E-selectin) and migrate through the endothelium into the intima (Bergheanu *et al*., 2017). There, they become macrophages, engulf a substantial amount of LDL-c and transform into foam cells (Kowara & Cudnoch-Jedrzejewska, 2021).

Later, the foam cells undergo apoptosis and necrosis, creating a lipid pool at the core of atherosclerosis. Smooth muscle cells (SMC) proliferate and lose their contractile phenotype to become extracellular matrix synthesizing cells, laying down the plaque scaffold and fibrous cap. The matrix metalloproteinases (MMPs) degrade the extracellular matrix, resulting in increased accumulation of macrophages within an atherosclerotic plaque (Finney *et al*., 2017; Raggi *et al*., 2018). MMP-12 has been shown to lead to more severe atherosclerosis and an increased prevalence of cardiovascular events (Goncalves *et al*., 2015).

This is the most frequently used histological classification for the atherosclerotic plaque is proposed by Stary *et al*. (1995). In this system, atherosclerotic

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plaques are classified into 6 types. Fatty streaks, the earliest form of the lesion, are categorized as Type I. Type VI is the opposite end of the spectrum, composed of a fully formed, large plaque complicated by a rupture and thrombus formation. A fully formed atherosclerotic plaque consists of foam cells, lipid core, and a fibrous cap; this is classified as types III, IV, and V, depending on the size of the lipid core (Stary *et al*., 1995). Early atherosclerosis in this study refers to types I and II lesions while established atherosclerosis is of types III, IV, and V.

As mentioned above, LDL-c is one of the most important risk factors for atherogenesis. Thus, one of the cornerstones of atherosclerosis treatment is LDL-c lowering drugs such as HMG-CoA reductase inhibitors (statins). However, these drugs can exert side effects in patients, the most serious of which are severe rhabdomyolysis, hepatotoxicity, peripheral neuropathy, and impaired myocardial contractility (Bełtowski *et al*., 2009) Thus, investigations to find novel, safer treatment for the condition is a subject of intense research.

Tocotrienol is a component of vitamin E alongside tocopherol, existing in four different isomers: alpha (α)-, beta (β)-, gamma (**γ**)- and delta (**δ**)-tocotrienol (Peh *et al*., 2016). It has antioxidant, anti-proliferative, anti-angiogenic, and anti-inflammatory properties (Malavolta *et al*., 2018). Tocotrienols have been shown to reduce cellular adhesion molecule expressions and monocytic cell adherence *in vitro* studies(Muid *et al*., 2016). Tocotrienol-enriched mixed fraction (TEMF) is derived from naturally occurring tocotrienol-rich oils, such as palm oil (*Elaeis guineensis*), where the tocotrienol component has been concentrated. TEMF has the advantage of being cheaper to produce, as well as having more tocotrienol content than other naturally occurring oils. It has also been reported to reduce atherosclerosis formation in experimentally-induced atherosclerosis in diabetic rats when given concurrently with a high cholesterol diet (Budin *et al*., 2009). A high cholesterol diet is an important independent predictor of cardiovascular disease, apart from diabetes mellitus. Thus, this is an important area to focus on and was the reason for choosing this model for this study.

New Zealand white rabbits fed with a high cholesterol diet are a popular animal model for atherosclerosis (Emini Veseli *et al*., 2017). Rabbits are sensitive to high cholesterol diets leading to the formation of atherosclerosis in topographically similar locations as in humans. Furthermore, the gross and microscopic features of the atherosclerotic lesion are also comparable to humans. They are easy to handle, and once the lesion is induced, the animals can be maintained at a reasonable cost (Baumgartner *et al*., 2016; Emini Veseli *et al*., 2017).

Data on the protective effects of TEMF on inflammatory biomarkers of atherosclerosis and influence on plaque stability *in vivo* are still limited, especially when given before high cholesterol diet feeding. Therefore, this study aims to examine the preventive effects of TEMF supplementation in experimentally induced early and established atherosclerotic rabbits.

MATERIALS AND METHODS

Animal and diets

Twenty adult male New Zealand White rabbits (2.0-3.0 kg) were divided into two groups: TEMF 15 mg/kg (n=10) and placebo (n=10). Before the experiment, the animals were acclimatized for 1 week. The rabbits were given the treatments for eight weeks via oral gavage once daily along with a normal chow diet. Then, each group was fed with a 1% high cholesterol diet (HCD) (Specialty Feeds, Glen Forrest, Australia) for i) two weeks to induce early atherosclerosis and ii) eight weeks to induce established atherosclerosis. The treatments were continued throughout the experiment. Each rabbit was provided with 100 g of diet per day and water ad libitum. They were housed individually in a stainless-steel cage in an air-conditioned room (25 ± 2 °C) with 12 h alternate light and dark period.

TEMF

TEMF (Sime Darby, Malaysia) used in this study consisted of a mixture of both tocotrienol and α-tocopherol (ratio = 70:30%; 40.78% α-tocotrienol, 3.29% β-tocotrienol, 35.67% γ-tocotrienol & 21.54%, δ-tocotrienol and 20.78% α-tocopherol); diluted in palm olein to the desired concentration. Placebo consists of palm super olein (Sime Darby, Malaysia).

Serum collection and analysis

Fasting blood samples of about 8 ml were withdrawn from the marginal ear vein of rabbits following overnight fasting. Serial samples were collected into plain (Becton Dickinson, Malaysia) coated blood tubes at baseline (BO) and post-intervention and post-induction periods. Serum was separated from blood samples by centrifuging (Hettich RotoFix 32A Centrifuge, GMI Inc., USA) at 3500 r.p.m for 10 min and stored at -80 °C (Sanyo, Japan) until analysis.

Serum total cholesterol and LDL-cholesterol (LDL-C) were measured by enzymatic reference methods on an automated analyzer (Hoffman-La Roche Ltd., Switzerland) (LDL-C was measured using the direct method). Serum CRP was measured quantitatively using an enzyme-linked immunoassay (ELISA) (Immunology Consultants Laboratory, Inc, USA) kit. Quality control procedures were performed to ensure the accuracy of all tests.

Tissue examination

At the end of the study, the rabbits were euthanized with an intravenous injection of sodium pentobarbital (15 mg/kg body weight). The aorta was excised longitudinally along the anterior wall and fixed flat on a corkboard overnight in 10% buffered formalin. After fixation, the aortas were washed with 70% ethanol before being stained with Sudan IV for 15 min (Sigma Chemical, USA). The intimal surface was photographed (Canon, UK) to capture the red-stained atherosclerotic areas, then analyzed using image analysis software (analySIS® FIVE, Olympus, Center Valley, USA). The percentage area of atherosclerotic lesion was expressed as a ratio of lesion area to the total area of the intima.

Immunohistochemistry staining

The aorta was sampled as described by Ichikawa *et al*. (2002), fixed in formalin, and embedded in paraffin to form tissue blocks; sectioned at 4 µm thickness, dewaxed, and rehydrated. Tissue antigen was retrieved by microwaving for ten min, followed by incubation in 3% hydrogen peroxide: methanol (1:4) for 15 min for endogenous peroxidase quenching. Sections were then blocked with nonimmune serum and then incubated with the following primary antibodies: interleukin-6 (IL-6), CRP, E-selectin, matrix metalloproteinase-12 (MMP-12) and, smooth muscle actin (SMA); (all antibodies except SMA were purchased from Santa Cruz Biotechnology, USA; SMA was from DAKO, Denmark) for 60 min at room temperature. After washing steps, the sections were incubated with biotinylated secondary antibody for 30 min, followed by horseradish peroxidase-labeled (HRP) streptavidin solutions for 30 min before diaminobenzidine (DAB) treatment for 7 min. They were then counterstained with hematoxylin, dehydrated, and mounted with DPX. For each run, negative and positive controls were included. Positive control was taken from tissue with known expression of the markers. Negative controls were similar to positive control except that the primary antibodies were excluded. Qualitative expressions of respective markers by the endothelium and macrophages within the atherosclerotic lesion were measured using an image analysis software (analysis LS Professionals, Olympus, USA). Pictures of the stained sections were captured by the attached microscope camera (Zeiss, Germany). The positively stained area is represented as the percentage of the whole area of interest in the section.

Statistical analysis

All values in the graphs and tables were expressed as mean ± S.E.M. Data distribution was tested with Kolmogorov Smirnov tests. The statistical significance of the differences between groups was evaluated by using the Mann Whitney-U test from the SPSS software (version 22.0). The *p*-values of 0.05 or less will be considered statistically significant.

RESULTS AND DISCUSSION

Serum lipid and C-reactive protein (sCRP)

We found no significant difference in serum lipid and CRP levels between TEMF and placebo groups. Other studies supplementing vitamin E or tocotrienol-enriched mixed fraction after high cholesterol diet insult, also reported no significant effect of the treatment on serum lipid and oxidative stress markers (Prasad, 2009; Abdul Rahman *et al*., 2016).

However, older studies reported a reduction of serum lipid and HMG-CoA reductase activities with tocotrienol-rich fraction supplementation after HCD feeding. We hypothesize that the studies used different formulations or ratios of tocotrienol and tocopherol; leading to the differences in the results obtained. Our study reported no significant difference in serum lipid level and CRP level even when TEMF was administered before high cholesterol feeding. This is new knowledge to be added on the effects of TEMF on atherosclerosis.

Atherosclerotic lesion and Immunohistochemistry

Atherosclerosis formation

Sudan IV stains fat deposits within the atherosclerotic lesions. In this study, atherosclerosis was seen at the bifurcation of the left subclavian artery of the aortic arch downwards to descending thoracic and abdominal aorta in early (Figure 1a) and established atherosclerosis groups (Figure 1b). When TEMF was administered prior to HCD induction, it reduced atherosclerotic lesions in both early (Mean ± SEM: TEMF vs. placebo: 5.9 ± 2.5% vs. 18.8 ± 1.1%; p=0.01, 68.6% reduction) and established (TEMF vs. placebo: 8.5 ± 2.0% vs. 29.5 ± 4.8 %; p=0.01, 71.2% reduction) atherosclerosis groups (Figure 1c).

Atherosclerotic tissue inflammatory markers

IL-6 and CRP, pro-inflammatory mediators, were found to be expressed by endothelial and foam cells respectively in the atherosclerotic areas of early and established atherosclerosis (Figures 2a & 2b). Supplementation with TEMF in the established atherosclerosis group led to a greater reduction of the markers in TEMF group (p=0.01), Table 1. This was not observed in the early atherosclerosis group (p>0.05). Similar findings were also observed for E-selectin (Table 1).

Viewed as an important inflammatory biomarker in endothelial activation and atherosclerosis, CRP is secreted by the liver in response to IL-6 (Catapano *et al*., 2017; Del Giudice & Gangestad, 2018). The opposite occurs in atherosclerosis where CRP has been suggested to stimulate vascular smooth muscle cells to produce IL-6. This process triggers the vascular inflammatory response involved in the initiation and progression of atherosclerosis (Liu

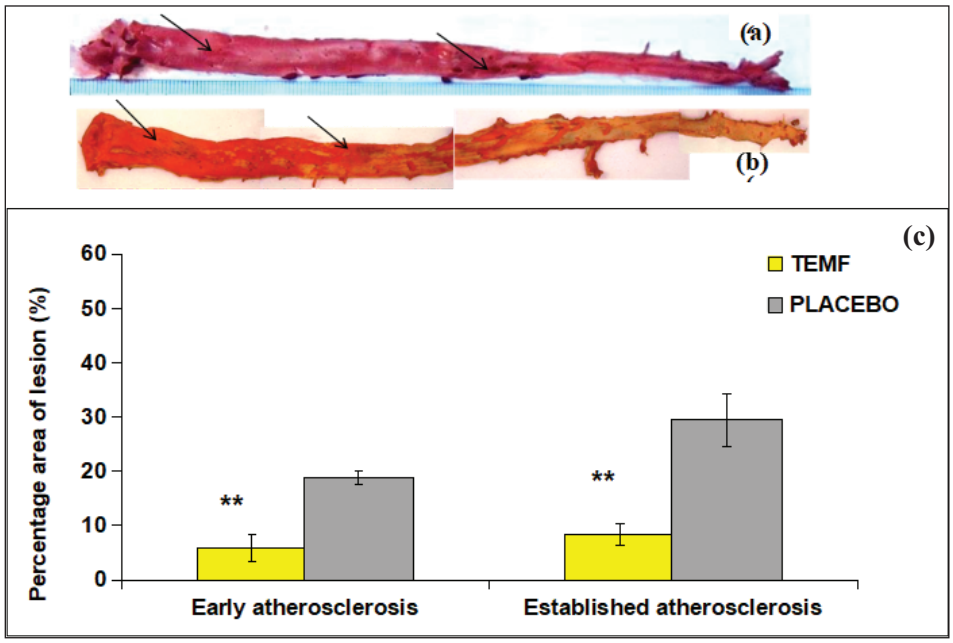

Fig. 1. (a) Early atherosclerotic lesion formation in the aorta (arrows). (b) Established atherosclerosis (arrows). (c) Percentage of atherosclerotic lesions in TEMF and placebo groups in early and established atherosclerosis. (**) indicate significant differences compared between the TEMF and placebo group (p<0.05)

et al., 2010). In this study, both CRP and IL-6 were reduced by TEMF supplementation before prolonged cholesterol feeding.

Data on the effects of TEMF given in a preventive setting on vascular tissue inflammation *in vivo* is limited. Most reports are on the effects of vitamin E supplementation after HCD insult. A study using palm tocopherol supplementation had observed a reduction in atherosclerosis formation in the aorta of (apo) E+/-mice but did not report on the expression of the markers within the atherosclerotic tissue (Black *et al*., 2000). Koga *et al*. (2004) showed a reduction in the number of macrophages in the atherosclerotic lesion with vitamin E supplementation before inducing hypercholesterolemia in rabbits. However, the source and isoform composition of the vitamin E used were not described. Both studies have reported favorable findings on vitamin E and its isoform supplementation on the formation and cellular composition of atherosclerosis. These reports support our findings.

A recent study showed that pure tocotrienol supplementation after high cholesterol diet feeding reduced atherosclerosis formation and vascular inflammation (Rahman *et al*., 2016). The finding in this study supports our results on the mechanism leading to atherosclerosis reduction, which is due to a reduction in endothelial activation and tissue inflammation with TEMF supplementation.

Our study provided great news because TEMF is less expensive to produce compared to pure tocotrienol, thus it is more affordable. This is especially meaningful for the populations most affected by cardiovascular disease: the middle- and lower-income groups (Deans *et al*., 2009).

Markers of plaque stability

We found that smooth muscle actin (SMA) was expressed by cells overlying established atherosclerotic lesions and medial smooth muscle cells (Figure 2d). In this study, TEMF supplement in established atherosclerosis reduced aortic intimal SMA expressions compared to placebo (15.7 ± 2.8 vs. 35.6 ± 3.7 %; p=0.01) (Table 1). No difference was observed in the early atherosclerosis group. Similarly, MMP-12 showed less expressed in TEMF-treated established atherosclerosis group (3.9 ± 2.0 vs. 26.1 ± 2.5%; p=0.01), Table 1. No difference was observed in the early atherosclerosis group.

We postulated that the lack of significant findings in early atherosclerosis is because smooth muscle proliferation and MMP-12 activity are not characteristic of early atherosclerosis (Basatemur *et al*., 2019). The findings in this study suggest that TEMF is potentially beneficial in stabilizing plaque formation in established atherosclerosis.

Lipid-lowering drugs have many side effects that may be challenging for long-term treatment, especially in statin-intolerant subjects. The findings in this study provide a new frontier for the exploration of the potential therapeutic target development apart from the current approach.

One limitation of this study is there was no functional experiment done to study vascular reactivity. Thus, although endothelial reactivity can be implied due to decreased expression of the inflammatory markers, a definitive improvement in endothelial function could not be ascertained.

Table 1. Immunohistochemistry staining of biomarkers expressed as a percentage of the positive area to the entire area of interest

GROUPS	EARLY ATHEROSCLEROSIS			ESTABLISHED ATHEROSCLEROSIS		
BIOMARKERS	TEMF (*n*=5)	Placebo (*n*=5)	*P* value	TEMF (*n*=5)	Placebo (*n*=5)	*P* value
IL-6	7.0 ± 0.7	7.8 ± 1.0	*p*=0.42	7.0 ± 1.9	24.8 ± 3.7	*p*= 0.01*
CRP	4.1 ± 0.9	5.2 ± 1.4	*p*=0.69	5.1 ± 1.7	45.2 ± 3.4	*p*= 0.01*
E-SELECTIN	3.2 ± 0.8	3.7 ± 1.6	*p*=0.69	5.4 ± 1.2	21.2 ± 2.8	*p*=0.04*
SMA	13.8 ± 2.0	17.6 ± 3.8	*p*=0.69	15.7 ± 2.8	35.6 ± 3.7	*p*=0.01*
MMP-12	1.5 ± 0.3	1.9 ± 0.1	*p*=0.31	3.9 ± 2.0	26.1 ± 2.5	*p*=0.01*

Data were expressed as mean ± SEM. Letter (*) indicate significant differences compared between TEMF and placebo of respective biomarkers in both early and established atherosclerosis.

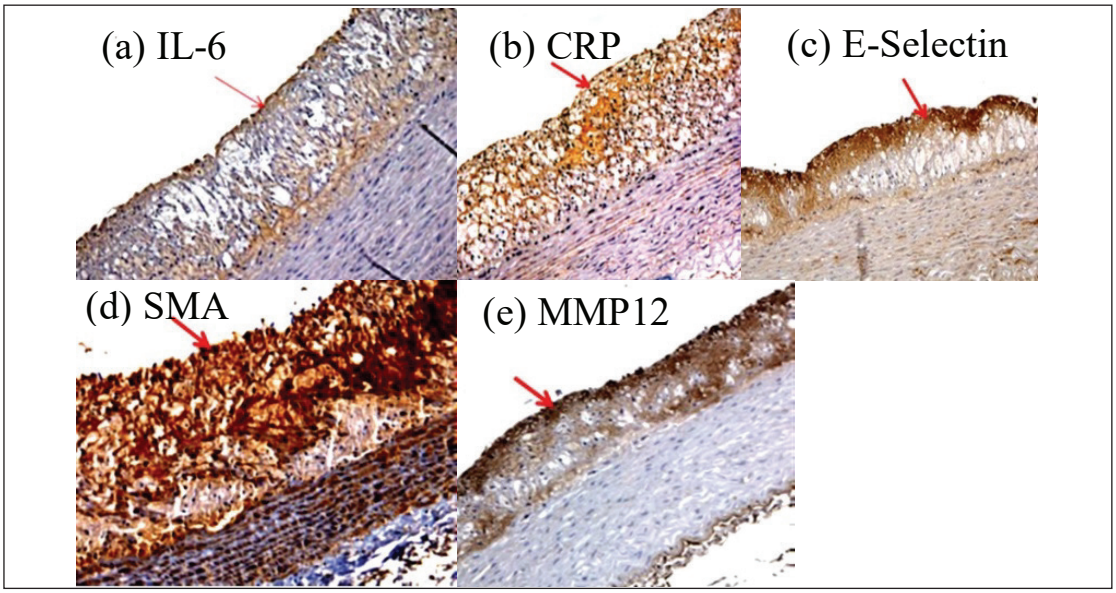

Fig. 2. Photomicrographs of representative immunohistochemistry staining patterns of the markers that were found to be significantly reduced in the TEMF group compared to placebo

CONCLUSION

TEMF supplementation, in the preventive setting, reduces atherosclerotic IL-6, CRP, and e-selectin expression in HCD-induced established atherosclerosis. It also decreased atherosclerosis formation and increase plaque stability by decreasing SMA and MMP-9 formation. This suggests TEMF's potential effect on the prevention of plaque formation and increasing plaque stability.

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ETHICAL STATEMENT

This study was approved by the Institutional Laboratory Animal Care Unit Committee (ACUC/CA/07(01) HAPIZAH).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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