INTRODUCTION

Inflammation plays an important role in the pathogenesis of atherosclerosis, regardless of the initial cause of endothelial dysfunction, leading to coronary artery disease (CAD) and stroke (Linton et al., 2019). Endothelial leukocyte adhesion molecule-1 (e-selectin), intercellular adhesion molecules-1 (ICAM-1), vascular cell adhesion molecules-1 (VCAM-1) and proinflammatory cytokines such as interleukin-6 (IL-6) are variably involved in atherogenesis (El-Solh et al., 2002). Immunocytochemical studies have shown that adhesion molecules are expressed in human atherosclerotic plaques (Mingyou et al., 2015). Clinical studies have also suggested that adhesion molecules have important roles in atherogenesis and are predictive of future myocardial infarction (Libby et al., 2019).

The nuclear factor kappa B (NFκB) is a transcription factor which plays a role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules and chemokines (Mussbacher et al., 2019). Targeting proteins that control the NF-κB signalling pathway may be useful for the treatment of inflammatory diseases (Bienke & Ley, 2004). Endothelial nitric oxide synthase (eNOS) is expressed in vascular ECs, especially at the endothelial layer of medium to large-sized blood vessels (Fish et al., 2005). eNOS play an important role in nitric oxide (NO) production by ECs (Hickey, 2001). NO modulates leukocyte-endothelial cell activation through the direct effect of NO on the regulation of cytokines and adhesion molecule expression by the transcription factor NFκB (Liu et al., 2019).
VITAMIN E ISOMERS AND BIOMARKERS OF EARLY ATHEROGENESIS

2017). NO induces transcription of IkBa, an inhibitor of NFkB thus stabilizing the inhibitory NFkB/ IkBa complex in the cytosol (Peng et al., 1995).

The inflammatory and endothelial activation biomarkers have been suggested as a potential guide for monitoring therapeutic intervention among patients with an increased risk of CAD and stroke (Upadhyay et al., 2015). They can be considered early biomarkers of atherogenesis. Therefore, these studies have provided the basis to consider anti-inflammatory therapy as a way of inhibiting endothelial cell adhesion and hence reducing the risk of developing CAD. Several studies in vitro have indicated that antioxidants such as a statin, ascorbic acid, flavonoids and vitamin E are potential indicators of adhesion molecule expression (Aquila et al., 2019).

Natural vitamin E, as opposed to the synthesized one, is a mixture of eight different isomers, namely α-, β-, γ- and δ- tocopherols (TOCs) and α-, β-, γ- and δ- tocotrienols (TCTs) which all have vitamin activities and therefore are called vitamers (Szewczyk et al., 2021). All vitamers of vitamin E are lipid soluble antioxidants and can be found incorporated in cell membranes (Linton et al., 2019). Vitamin E exerts its antioxidant activity through its ability to break radical-propagated chain reactions and protect cell membranes from lipid peroxidation by trapping peroxyl radicals (Dutta & Dutta et al., 2003). Out of these four TCT isomers, α and γ are abundant, especially in the palm-oil-derived TRF. Structurally, TCTs and TOCs compounds are similar, except that TCTs have an unsaturated side chain with three trans double bonds at 3′, 7′, and 11′ positions, whereas TOCs have a fully saturated aliphatic side chain (Niki & Abe, 2019). Tocopherols and tocotrienols shared the same vitamin E family, however, emergent literature has identified unique biological functions of tocotrienols that are not shared by tocopherols (Niki & Abe, 2019).

Epidemiological studies have indicated the beneficial effects of Vitamin E in the reduction of cardiovascular events but in various clinical trials, the results were contradictory (Rim et al., 2003, Ziegler et al., 2020). A meta-analysis revealed that vitamin E supplementation appears to have neutral effects but has no effect on all-cause mortality from chronic diseases at doses up to 5,500 IU/d (Abner et al., 2011). In that study, it is also suggested that supplementation with vitamin E as alpha-tocopherol cannot be endorsed as a means of reducing mortality for the specified populations. Vitamin E consists of both tocopherols and tocotrienols. Currently, the biological relevance of other vitamin E isomers other than α-TOC fetches the focus of cardiovascular prevention research, due to its distinct properties compared to α-TOC (Ziegler et al., 2020). TCTs have been described to have greater antioxidant activity than α-TOC (Muid et al., 2013, Miyazawa et al., 2019). Although TCTs’ activity is superior to TOCs, the potential role of TCTs in the prevention of atherosclerosis and cardiovascular diseases is yet to be established. Furthermore, the comparison of tocotrienols with α-TOC as an anti-atherogenic agent in vitro is yet to be investigated. Therefore, this study aimed to compare the effectiveness of TRF, α- TOC and pure TCT isomers (α-, γ- and δ-TCT) in reducing inflammation, endothelial activation, monocytes binding activity (MBA) and increasing eNOS in human endothelial cells based on area under the curve analysis.

MATERIALS AND METHODS

Materials

Tocotrienol-rich fraction (TRF) derived from palm oil was provided by Sime Darby Plantations, Malaysia. Pure isomers of α-, γ- and δ-TCT and α-TOC (>97%) isolated from palm oil were provided by Davos Lifesciences, Singapore. Cell culture medium and supplements are manufactured by Cascade Biologics, USA. Antibiotics (Penicillin/ streptomycin) were purchased from PAA laboratories, Austria. [3-(4, 5-Dimethylthiazol-2-yl)]-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Fluka, Germany. Accutase was purchased from ICN Biomedical, USA. Phosphate buffer saline (PBS) was obtained from MP Biomedicals, France. ELISA test kits for IL-6, TNF-α, sICAM-1, sVCAM-1 and e-selectin were purchased from eBioscience, Austria. NFkB binding assay kit was obtained from Cayman Chemicals, USA. Quantikine eNOS immunoassay kit was manufactured by R&D BioSystems (USA). All chemicals used in this assay were of tissue culture grade.

Cells Viability

TRF was subjected to cell viability analysis using an MTT assay. Firstly, 100 µL of 1 × 10⁶ cells/mL were incubated with TRF (0.3 – 100 µM) for 24 h. Untreated cell populations were served as a control. MTT solution (20 µL) was added to each well, followed by 4 h of incubation at 37 °C. Then, 100 µL of DMSO was added before room temperature incubation for 30-50 min. The absorbance of each well was read at 550 nm wavelength in a microplate reader (Micro Quant, Biotek Instruments Inc, USA).

Incubation of TRF and Vitamin E Isomers in LPS stimulated HUVECs

In this study, HUVECs in 25 cm² culture flasks were treated with different concentrations of TRF or α-, γ-, δ-TCT isomers or α-TOC (0.3 – 10 µM) together with LPS (1 µg/mL) for 16 h in CO₂ incubator set at 37 °C.
**Protein expression**

Following incubation, culture medium and cells were collected for protein expression analysis of inflammation, endothelial activation biomarkers, eNOS as well as NFκB activation by Enzyme link of immunosorbent assay (ELISA) according to manufacturer’s instructions. The absorbance of the antigen-antibody complex was measured spectrophotometrically at 405 nm using the Micro Quant spectrophotometer (Biotek Instruments, USA).

**Monocytes binding activity assay**

Monocyte binding activity was performed by Rose Bengal Staining (Ariff et al., 2020). After incubation, the absorbance in each well was read at 570 nm wavelength with a microplate reader (Tecan Safire, Switzerland).

**The area under the curve and statistical analysis**

Analysis of the area under the curve (AUC) for each TRF, α-, χ-, δ-TCT isomer and α-TOC for all concentrations combined (0.3 – 10 μM) were performed using the Graph pad Version 4.3 software. The area under the curve analysis was chosen for this study. This analysis was chosen due to the various concentrations of vitamin E tested in this study. All data were analysed by a statistical package programme, SPSS version 21.0. The level of significance was set at p<0.05. Percentage of inhibition for each vitamin E isomer is accounted as:

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\% \text{Inhibition} = \frac{\text{Area under the curve LPS stimulated alone} - \text{Area under the curve LPS stimulated alone with TRF or-α or-χ- or δ-TCT or α-TOC}}{\text{Area under the curve LPS stimulated alone}} \times 100
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**RESULTS AND DISCUSSION**

**Cells Viability**

Figure 1 indicated TRF starting at 10 μM and higher concentrations had reduced cell viability when compared to untreated controls. Therefore, TRF starting from 10 μM and below was used for this experiment. Similarly, Sue-Mian et al. (2010) showed similar results where, γ-TCT and α-TOC at 10 μM had cell viability of more than 85% but with different types of cells (neuronal cells). A study by Selvaduray et al. (2011) also used a similar dosage of TRF, γ-TCT and δ-TCT and α-TOC as reported in this study.

**Protein expression**

Figure 2 shows, among TRF and vitamin E isomers, only TRF, γ-TCT and δ-TCT showed inhibition of IL-6 protein expression. δ-TCT showed the highest inhibition of IL-6 (25.6%) compared to TRF (9.9%) and γ-TCT (12.0%). Only a slight inhibition of TNF-α was shown by δ-TCT (6.9%) and α-TOC (3.2%). Interestingly, TRF and vitamin E isomers showed inhibition of NFκB activation with δ-TCT (22.4%) exhibited as the highest. In this study, LPS stimulated controls were considered as 0% of inhibition. Therefore, the higher % inhibition of TRF and vitamin E isomers used in this experiment is better. A negative % inhibition value indicated no inhibitory effects.

TRF indicated the highest e-selectin % of inhibition. Previous studies reported that TCT reduced IL-6 in other cell lines such as macrophages and monocytes; this study, however, is one of a few that report on human endothelial cells (Wu et al., 2008; Qureshi et al., 2011). In this study, AUC analysis revealed that pure TCT, particularly δ-TCT, had greater inhibitory effects on IL-6 and TNF-α. Interestingly, this inhibitory effect is conquered by the inhibition of NFκB activation by δ-isomers. NFκB is the transcription factor for the expression of IL-6 and TNF-α. These results agreed with the other investigators (Theriault et al., 2002; Naito et al., 2005). In this present study, pure TCT isomers have shown a better reduction of NFκB activation than TRF, while α-TOC showed a lack of beneficial effects. It has been suggested that targeting proteins that control the NFκB signalling pathway regulating the proteolysis of p105 may be useful for the treatment of inflammatory diseases (Bienke & Ley 2004). It has been suggested that any supplement or treatment that can deactivate the transcriptional factor NFκB can thus inhibit these pro-inflammatory cytokines and adhesion molecules which in turn will be capable of slowing down the progression of atherosclerosis (Theriault et al., 2002).

Figure 3 indicated TRF and TCT isomers but not α-TOC showed inhibition of ICAM-1 protein expression. Among all, δ-TCT indicated the highest % of inhibition. For VCAM-1 protein expression, only α-TCT did not inhibit ICAM-1 protein expression. Similarly, δ-TCT exhibited the highest % inhibition of VCAM-1 protein expression. For e-selectin, only TRF, γ-TCT and δ-TCT showed inhibition of its protein expression from endothelial cells incubated with LPS alone.

Theriault et al. (2002) has reported the ability of TCT in the inhibition of ICAM-1, VCAM-1 and e-selectin by blocking the activation of NFκB, however, it was only limited to α-TCT (Theriault et al., 2002). A similar study, also suggested that α-TCT has greater inhibitory effects in reducing endothelial activation biomarkers than α-TOC in HUVECs (Theriault et al., 2002). Naito et al. (2005) on the other hand only reported the inhibitory effects on VCAM-1 by various TCT isomers (Naito et al., 2005). Adhesion of leukocytes with vascular endothelial cells is an important process during an inflammatory response. The first event involves the rolling of leukocytes along the endothelial membrane, a process which is mediated by e-selectin. E-selectin, which is expressed
Fig. 1. Effects of TRF on cell viability. HUVECs were grown in 96 well microplates until confluent. Cells were pre-treated with TRF (0.3 – 100 µM) for 24 h. Data are expressed as Mean ± SEM = Mean values ± Standard error of means from 3 biological replicates (n=3).

Fig. 2. IL-6, TNF-α protein expression and NFκB activation of TRF, α-, γ-, δ-TCT and α-TOC in LPS stimulated HUVECs based on the area under the curve analysis. ANOVA, p<0.05 (each markers). Data are expressed as Mean ± SEM = Mean values ± Standard error of means from 3 biological replicates (n=3).

Fig. 3. ICAM-1, VCAM-1 and e-selectin protein expression of TRF, α-, γ-, δ-TCT and α-TOC in LPS stimulated HUVECs based on the area under the curve analysis. ANOVA, p<0.05 (each markers). Data are expressed as Mean ± SEM = Mean values ± Standard error of means from 3 biological replicates (n=3).
by endothelial cells, mediates the interaction between endothelial cells and monocytes (Čejková-Líva et al., 2016). After rolling, firm adhesion is accomplished by endothelial adhesion molecules (VCAM-1 & ICAM-1). Therefore, the monocyte binding activity is suggested to be correlated with adhesion molecule expression.

**Monocytes binding activity assay**

Figure 4 indicated that similar to ICAM-1 expression, TRF and pure TCT isomers, but not α-TOC, have inhibitory effects on monocyte binding activity in which δ-TCT exhibits the highest inhibition. This finding concurs with Naito et al. (2005). Our group previously reported a positive correlation between monocyte binding activity with adhesion molecules (Muid et al., 2017). Naito et al. reported that the potency of α-TCT in inhibiting adhesion of monocytes to endothelial cells was contributed by the increase in intracellular accumulation compared to α-TOC (Naito et al., 2005). Therefore, this could explain why α-TOC did not cause inhibitory effects on monocyte binding activity in this study.

**Protein expression of eNOS**

Thus far, there have been no previous studies addressing the comparison in effects between TRF, pure TCT isomers and α-TOC on eNOS expression in stimulated endothelial cells. Figure 5 showed co-cultivation of TRF, γ- and δ-TCT with LPS for 16 hr exhibited increment of eNOS protein expression from LPS controls. Both α-TCT and α-TOC did not lead

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**Fig. 4.** Monocytes binding activity of TRF, α-, γ-, δ-TCT and α-TOC in LPS stimulated HUVEC based on the area under the curve analysis. ANOVA, *p*<0.05 (each markers). Data are expressed as Mean ± SEM = Mean values ± Standard error of means from 3 biological replicates (*n*=3).

**Fig. 5.** eNOS protein expression of TRF, α-, γ-, δ-TCT and α-TOC in LPS stimulated HUVEC based on the area under the curve analysis. ANOVA, *p*<0.05 (each markers). Data are expressed as Mean ± SEM = Mean values ± Standard error of means from 3 biological replicates (*n*=3).
VITAMIN E ISOMERS AND BIOMARKERS OF EARLY ATHEROGENESIS


In this study, AUC analysis revealed that pure TCT, particularly γ- and δ- isomers had a greater reduction in inflammation and endothelial activation and greater eNOS increment than TRF. Therefore, this study suggests that pure TCT isomers have beneficial effects in terms of reducing inflammation and endothelial activation, unlike α-TOC which exhibits detrimental effects on TCT benefits. Despite the combination, TCTs and α-TOC mixtures still have atheroprotective properties, they are still inferior to that of pure TCT, particularly γ- and δ- isomers. α-TOC has been shown to interfere with the functions and potential benefits of TCTs such as its role in lipid lowering activity (Qureshi et al., 1996). Qureshi et al (2010) also reported that α-TOC does not play any role in inhibiting LPS-induced inflammation. Studies comparing varying amounts of α-TOC in TCT mixtures found that lower α-TOC content yields better results (Qureshi et al., 1996; Qureshi et al., 2011).

It has been suggested that for cardiovascular disease, the rule-of-thumb for an effective composition is greater than 60% of γ- and δ-TCT isomers and 0 - 15 % α-TOC. α-TOC content in TRF preparation that has been used in this study is at 30%. This could be the reason TRF exhibits weaker effects compared to pure TCT isomers. Furthermore, the presence of an isoprenoid side chain in TCT is accounted for by the superior activity of TCT over TOC. Structurally, TCTs and TOCs can be distinguished by their side chains, and it has been reported that the unsaturated side chain of TCT allows it to pass through cell membranes more efficiently and at a faster rate than the saturated phytol side chain of TOCs (Miyazawaa et al., 2008). For this reason, the greater anti-inflammatory, anti-endothelial activation may be due in part to their effective incorporation into endothelial cells (Miyazawaa et al., 2008). In addition, it is suggested to use the correct ratio of TCT: α-TOC concentration (>60% TCT: <15% α-TOC) in TRF preparation.

This present study indicates that pure TCT particularly γ- and δ- isomers exhibited greater potency than TRF (TCTs:α-TOC ratio = 70:30 %) by showing greater reduction of IL-6, ICAM-1, VCAM-1, NFκB and monocyte binding activity. In addition, γ- and δ-TCT isomers have greater potency than TRF in inducing eNOS expression in LPS-stimulated ECs. TCTs in combination with α-TOC in TRF still have the atheroprotective properties though to a lesser degree when compared with that of TCT particularly γ- and δ- isomers.

CONCLUSIONS

In this study, TRF and TCT isomers, especially γ- and δ-TCT inhibit inflammation and endothelial activation in human endothelial cells, in contrast to α-TOC. Tocotrienol (TCT) isomer on its exhibits a greater reduction of inflammation and endothelial activation than TRF. It is hence timely to investigate the in-vivo anti-atherosclerotic property of TRF and TCT in animal studies and further human clinical trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


