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Low Dose Palm Tocotrienol-Rich Fraction Reduces Aortic Tissue Endothelial Activation in Severely Atherosclerotic Rabbits

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ABSTRACT

Chronic inflammation plays a pivotal role in atherogenesis. Antioxidants have a potential role in the prevention and treatment of atherosclerosis. The effects of palm oil-derived tocotrienol-rich fraction (TRF) supplementation on inflammation are not well established. This study aims to investigate the effects of TRF supplementation on the inflammatory biomarkers and adhesion molecules in severe atherosclerosis. A total of 28 New Zealand white rabbits were given 1% high-cholesterol diet (HCD) for five months and randomised from the second month onwards into one of five intervention groups: Placebo, TRF 15, 30, 60 and 90 mg/kg/day. Treatment was given for three months and the animals were fed HCD throughout the duration. At the end of the study, the aortas were obtained, stained with Sudan IV, fixed in formalin, embedded in paraffin and immunostained for tissue intracellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), E-selectin, smooth muscle actin (SMA), and nuclear factor-κB (NF-κB). The amount of atherosclerotic lesions was not significantly different between the groups and compared to placebo. Qualitative analysis showed lower trend of ICAM-1, IL-6, E-selectin and NF-κB but higher trend of SMA tissue expression in TRF-treated groups especially at low dose of TRF (TRF-15) compared to placebo. Quantitative analysis showed lower ICAM-1 and E-select in positivity in TRF-15 compared to placebo group (25.1 ± 7.4 % vs. 3.8 ± 2.0 %, 23.2 ±6.5 % vs. 4.2± 2.1 %, respectively, $p<0.05$). In conclusion, low dose TRF is potentially beneficial in attenuating vascular endothelial activation in severe atherosclerosis.

Keywords: Aorta, atherosclerosis, inflammation, tocotrienol-rich fraction

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INTRODUCTION

Tocotrienol is a naturally-occurring form of vitamin E other than tocopherol, which exists in four isoforms: α-, β-, γ- and δ-tocotrienol

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(Meganathan, et al., 2014). It differs from tocopherol in the structure of the isoprenoid side chain, where it has an unsaturated geranyl chain compared to the phytyl chain of tocopherol (Dörmann, 2007). This structural difference results in altered membrane distribution and improved interaction with free radicals making tocotrienol a more potent antioxidant than tocopherol (Theriault, Chao, & Gapor, 2002).

Inflammation plays an important role in atherogenesis regardless of the initial cause of endothelial dysfunction (Rajendran et al., 2013). This process is typified by adhesion of circulating monocytes to stimulated endothelial cells, followed by exodus of the monocytes into the sub-endothelial space to form foam cells. The adhesion is aided by expression of endothelial adhesion molecules such as intercellular adhesion molecules-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Muller, 2002). ICAM-1 which is regulated by nuclear factor-κB (NF-κB), serves as a ligand to the integrins found on leucocytes (Muller, 2002).

NF-κB is a transcription factor involved in a number of cellular responses that are involved in various stimuli such as free radicals and oxidised low density lipoprotein (LDL) (Gilmore, 1999). In atherosclerosis, NF-κB is expressed in all stages of disease (Berliner et al., 1996). NF-κB regulates a few other molecules involved in atherosclerosis including ICAM-1. Alpha tocotrienol has been reported to reduce NF-κB activation (Theriault, Chao, & Gapor, 2002). Therefore it has been postulated that alpha tocotrienol decreases expression of adhesion molecules as a result of blockage of NF-κB activation (Theriault, et al., 2002).

Hypercholesterolemia is a major cause of atherosclerosis. Oxidised LDL, a potent inducer of inflammation (Berliner et al., 1996), accumulates in the vascular wall, where it is cytotoxic and chemotactic for monocytes and lymphocytes. It also leads to increase in endothelial expression of adhesion molecules (Berliner et al., 1996). Monocyte activation leads to release of oxygen derived free radicals. These free radicals incur damage to the endothelium which in turn leads to recruitment of more monocytes and other cells to the atherosclerotic area, thus signifying the link between oxidative stress and inflammation in atherogenesis. Therefore, the anti-oxidant properties of Vitamin E are of great interest in this condition.

Although epidemiological studies have reported the reduction of atherosclerosis related cardiovascular risk with administration of vitamin E (Rimm et al., 1993; Stampfer et al., 1993), large scale double blind intervention studies have failed to demonstrate consistent efficacy of vitamin E against atherosclerosis (Tribble, 1999). In addition, majority of the studies used tocopherol as the vitamin E component (Miller et al., 2005). However current research in vitamin E is skewed towards tocopherol, where only 1% of the total literature on vitamin E deals with tocotrienol and the rest concentrates on tocopherol (Sen, Khanna, & Roy, 2007), including large scale intervention studies on efficacy of vitamin E in atherosclerosis. This signifies an important gap in vitamin E research, one which precludes us the full benefits of naturally-occurring vitamin E molecules. Hence, the effects of tocotrienol on plaque stability in both early and severe atherosclerosis and its optimal dose for inflammatory inhibition in atherosclerosis are still not well established. This study aims to investigate the effects of tocotrienol-rich fraction (TRF) supplementation on aortic tissue inflammatory biomarkers in severely atherosclerotic rabbits.

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. A total of 28 New Zealand white rabbits were given 1% high-cholesterol diet (HCD) for two months to induce atherosclerosis 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. All the animals were housed in individual cages, with *ad libitum* access to food and water, maintained at a 12-hour dark/light cycle. The TRF supplements (Gold-Tri E 50® tocotrienol-enriched vitamin E) and placebo E were provided by Sime Darby BioganicSdn. Bhd., Malaysia.

Tissue Collection

At the end of the experiment, rabbits were sacrificed and aortas were obtained. The proximal 5 mm of the aorta was used for immunohistochemistry analysis and the rest was immediately processed for Sudan IV staining.

Sudan IV Staining

For Sudan IV staining, the aortas were cut open longitudinally and fixed overnight with 10% neutral buffered formalin. Subsequently, they were washed with 70% ethanol, then immersed in Sudan IV stain for 15 minutes. After rinsing, the stained aortas were photographed (C-740 Ultra Zoom, Olympus, USA) and analysed for the percentage area of sudanophilia. The area was then calculated by an image analysis software (analySIS® FIVE, Olympus Soft Imaging Solutions, Olympus, USA).

Aortic Immunohistochemical Analysis

Immunohistochemistry to detect ICAM-1, E-selectin, IL-6 and NF-κB, (Santa Cruz Biotechnology Inc., USA) and smooth muscle actin (SMA) (Dako, Denmark) was performed using a conventional streptavidin-biotin-peroxidase method (Santa Cruz Biotechnology Inc., USA). The tissue blocks were sectioned at 5 µm thickness and deparaffinised in xylene. After a few changes of graded concentration of alcohol, the sections underwent antigen retrieval process followed by endogenous peroxidase blocking (in 1% H_2O_2). They were then incubated for 60 minutes in the respective primary antibodies. This was followed by washing steps with phosphate buffer. Then biotinylated secondary antibody was administered, followed by streptavidin horseraddish peroxidase and finally, 3,3'-diaminobenzidine substrate. Hematoxylin was used as counter stain.

The immunostained specimens were scanned by a fully motorised microscope (Olympus BX61, USA). The endothelial lining of the aortic images for all biomarkers except SMA were selected for analysis. For SMA, the intima of the aortic images was chosen. Staining quantification were made based on defined brown endothelial or intimal staining by using the

microscope proprietary software (analySIS® FIVE, Olympus Soft Imaging Solution Olympus, USA) and expressed as percentage area of positivity.

Statistical Analysis

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 22) software. 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.

RESULTS

Sudan Staining

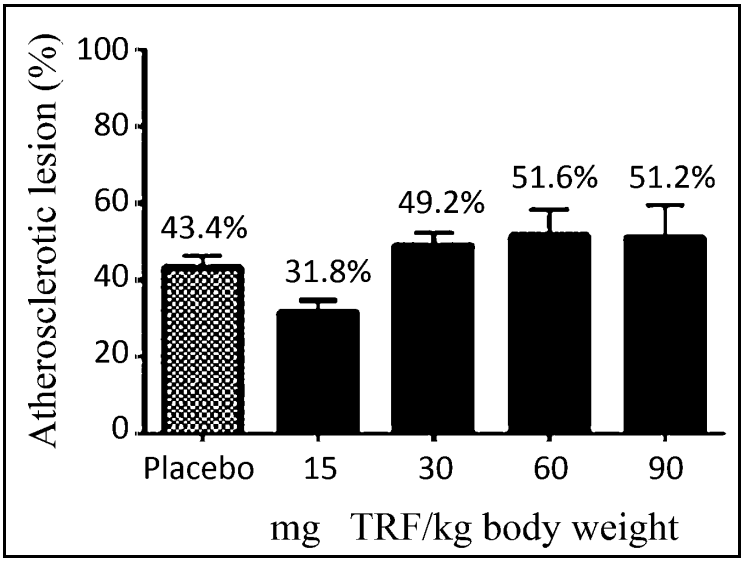

Figure 1. Percentage of atherosclerotic lesions in different treatment groups

All the rabbits developed atherosclerosis, with majority of lesion being of the severe type. The percentage of atherosclerotic lesion in TRF 15 group was the lowest among all the groups (Figure 1).

Immunostaining

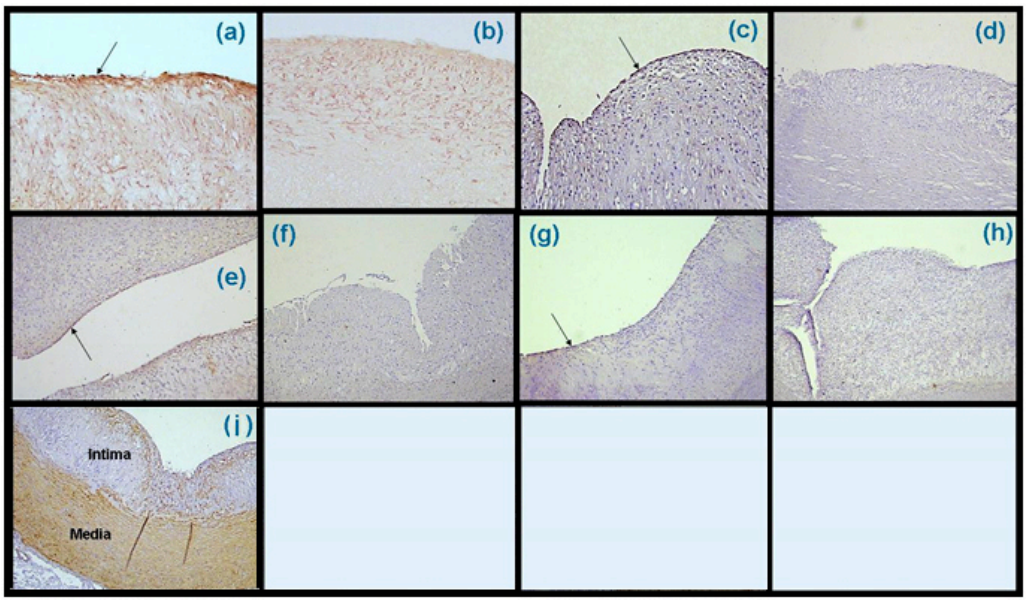

Figure 2. Endothelial and intimal immunohistochemical staining which showed: (a) Positive ICAM-1 (arrow); (b) Negative ICAM-1; (c) Positive NF-κB (arrow); (d) Negative NF-κB; (e) Positive E-selectin (arrow); (f) Negative E-selectin; (g) Positive IL-6 (arrow); (h) Negative IL-6; and (i) Positive intimal and medial SMA

Figure 2 (a – i) shows a typically positive and negative staining of all the immunohistochemical biomarkers.

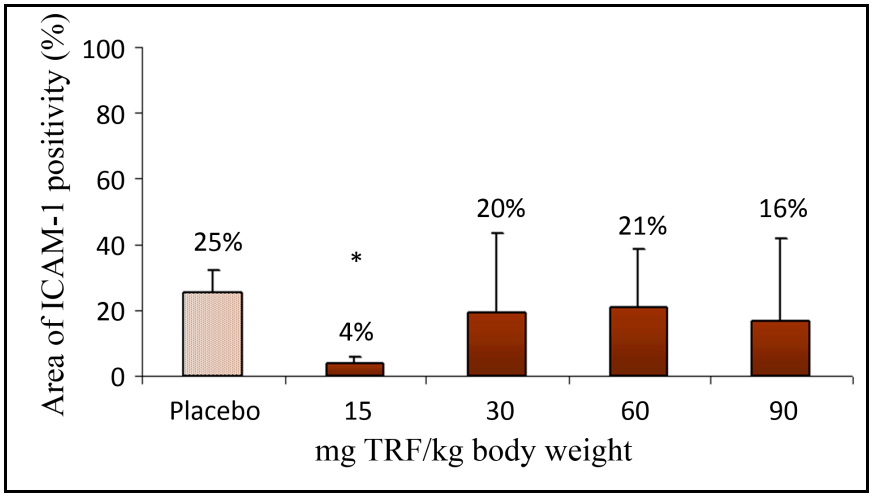

Figure 3. Quantitative Analysis of ICAM-1 immunostaining.* $p<0.05$ compared to placebo; Data expressed as Mean ± SEM

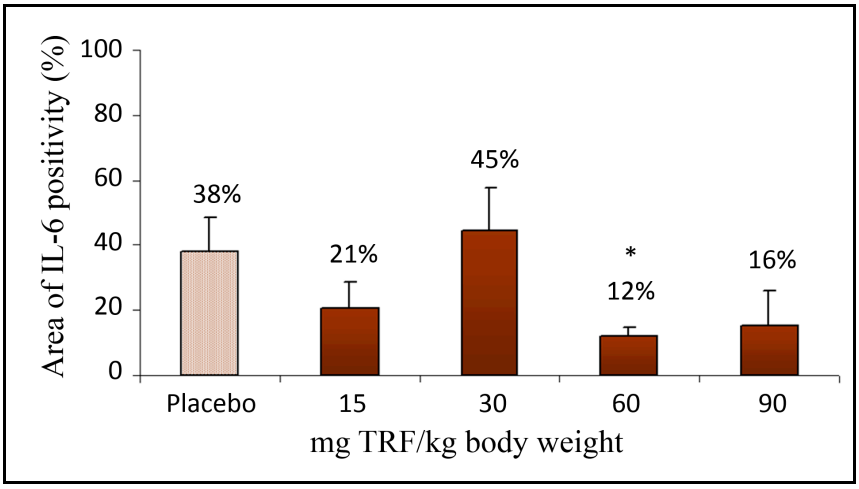

Figure 4. Quantitative analysis of IL-6 immunostaining.* $p<0.05$ compared to placebo; Data expressed as Mean ± SEM

ICAM-1 and IL-6 Expression

There was lower trend of ICAM-1 tissue expression in TRF-treated groups compared to placebo. Quantitative analysis showed lower % of ICAM-1 positivity in TRF-15 compared to placebo group (25 ± 7 % vs. 4 ± 2 % respectively, $p<0.05$) (Figure 3). There was a lower trend of IL-6 tissue expression in TRF-treated groups compared to placebo. The IL-6 expression was lower in the TRF-60 compared to placebo group (12.2 ± 3.1 % vs. 38.4± 11.5% respectively, $p<0.05$) (Figure 4).

E-selectin and NF-kB Expression

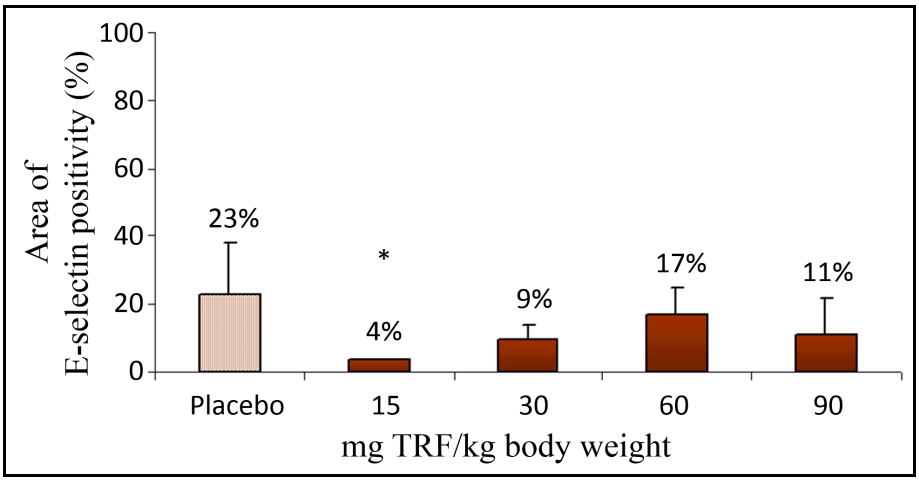

Figure 5. Quantitative analysis of E-selectin immunostaining; Data expressed as Mean ± SEM

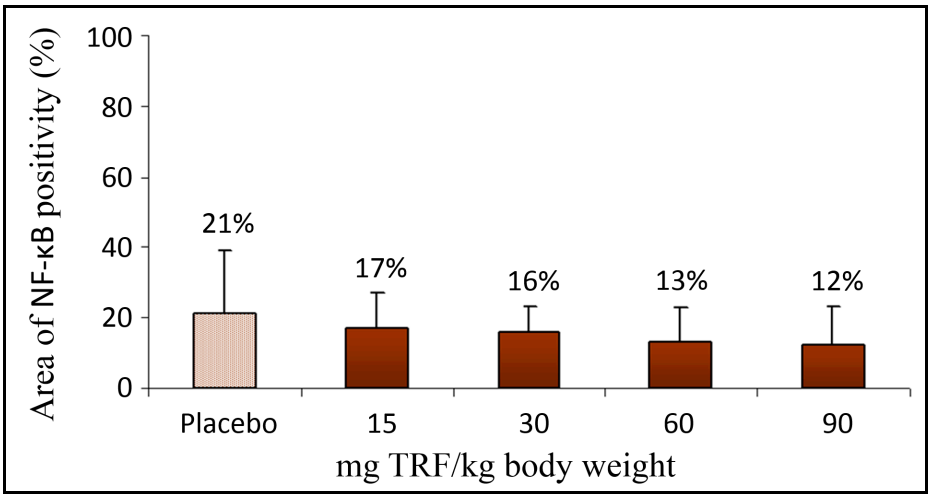

Figure 6. Quantitative analysis of NF-κB immunostaining NF-κB; Data expressed as Mean ± SEM

There was lower trend of E-selectin (Figure 5) and NF-κB tissue expression (Figure 6) in TRF-treated groups compared to placebo. Quantitative analysis showed lower % of E-selectin positivity in TRF-15 compared to placebo group (23.2 ± 6.5 % vs. 4.2 ± 2.1 %, respectively, $p<0.05$).

Smooth Muscle Actin Expression

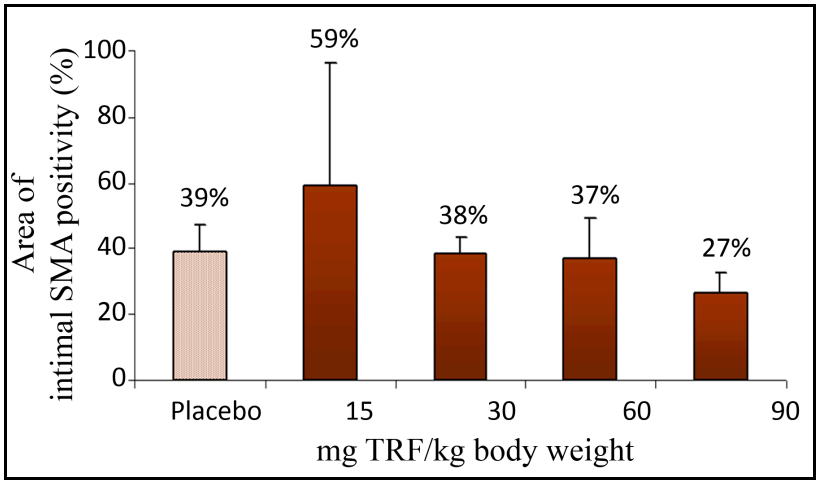

Figure 7. Quantitative analysis of SMA immunostaining; Data expressed as Mean ± SEM

SMA tissue expression (Figure 7) in TRF-treated groups compared to placebo.

DISCUSSION

As tocotrienol is a lipid-soluble antioxidant, the level of circulating biomarkers may not be sufficient to reflect its action in the blood vessels. A tissue culture experiment showed that α-tocotrienol accumulated in human umbilical vein endothelial cells to a level approximately 10-fold greater than that of α-tocopherol (Noguchi, Hanyu, Nonaka, Okimoto, & Kodama, 2003). This result reflects the importance of observing the *in situ* biomarker of atherosclerosis in determining the beneficial effect of tocotrienols. Hence, atherosclerotic lesions and vascular tissue biomarkers were selected to determine if there are anti-atherogenic effects of TRF supplementation, compared to higher doses.

This present study clearly demonstrates that TRF particularly at low dose (15 mg/kg), reduced in vivo aortic tissue endothelial activation and inflammation in HCD induces severe atherosclerosis. In addition, it is clearly shown in the present study that the atherosclerotic lesions were maximally reduced at low dose of TRF (15 mg/kg) rather than at higher doses (30 to 90 mg/kg), compared to placebo. Furthermore, low dose TRF also maximally increased SMA expression, compared to other higher doses. This suggests a great potential of TRF at low dose in attenuating atherogenesis, enhancing plaque stability and subsequently leading to atherosclerotic plaque regression.

ICAM-1, IL-6, E-selectin and NF-κB aortic tissue expression in TRF-treated groups were found to show lower trend compared to placebo. Furthermore, quantitative analysis showed significantly lower ICAM-1, E-selectin and IL-6 positivity in TRF-15 compared to placebo group ($p<0.05$). This suggests that TRF has the capability to inhibit the pro-inflammatory states in severe progressive atherosclerosis. Further quantification analysis revealed TRF supplementation at dosage of 15 and 60 mg/kg body weight shows significant lower endothelial ICAM-1 and IL-6 expression respectively, as compared to placebo. This finding suggests a probable inhibition in the development of atherosclerosis by pro-inflammatory factor mediated by NF-κB activation. This is consistent with the assumption that anti-atherogenic effects of antioxidants may in part be mediated by interference with oxidation-dependent intracellular signaling (Fruebis, Silvestre, Shelton, Napoli, & Palinski, 1999). It is also in agreement with the mechanistic studies on the role of α-tocotrienol in atherogenesis through NF-κB mediated inflammation (Theriault, Chao, & Gapor, 2002). Furthermore, tocotrienols have been found to reduce endothelial expression of adhesion molecules, thus inhibiting the monocytic adherence to HUVEC (Naito et al., 2005).

The lowest dose of TRF (15 mg/kg body weight) which has inhibitory effect on ICAM-1 tissue expression is not supported by TRF intervention at high doses. It has been suggested that tocotrienol at high doses (micromolar concentrations) exert protective effect due to its antioxidant property, while tocotrienol at low doses (nanomolar concentrations) regulates specific neurodegenerative signaling processes (Sen et al., 2007). There is even a study that has reported toxicity of TRF administration at higher tocotrienol dosage (Abd Manan, Mohamed, & Shuid, 2012). Similarly, the low dose used in this experimental atherosclerosis was probably capable of providing sufficient molecule signaling in reducing ICAM-1 tissue expression of the atherosclerotic tissues. A lower expression of NF-κB in TRF-15 group also suggests that low dose tocotrienol may play a role in reducing the expression ICAM-1, E-selectin and IL-6via

the NF-κB pathway. A more recent study on 31 hypercholesterolaemic patients supplemented with low dose delta-tocotrienol reported reduction in plasma inflammatory and oxidative stress biomarkers compared to higher dose supplementation (Qureshi, Khan, Mahjabeen, Trias, Silswal, & Qureshi, 2015). This report is parallel with the findings of this present study.

CONCLUSION

TRF supplementation particularly at low dose decreases *in vivo* ICAM-1, IL-6, E-selectin and NF-κB aortic tissue expression in HCD induced severe atherosclerotic lesions. This suggests that TRF has a potential benefit in attenuating vascular endothelial activation and inflammation, as well as atherosclerotic plaque regression and possibly contributing to enhancing plaque stability. Hence, future studies investigating the therapeutic potential of TRF in reducing atherosclerosis-related complications such as coronary events are warranted.

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