UNIVERSITI PUTRA MALAYSIA

EFFECTS OF TOCOTRIENOL SUPPLEMENTATION ON PLATELET AGGREGATION IN SUBJECTS WITH METABOLIC SYNDROME

GAN YEE LIN

FBSB 2014 16

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By

GAN YEE LIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2014

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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GAN YEE LIN

April 2014

Chair : Prof. Lai Oi Ming, PhD

Faculty : Biotechnology and Biomolecular Sciences

An occlusive thrombus in vasculature either in arterial or venous, is a pathological condition that predisposes to most cases of cardiovascular diseases (CVDs). As reported by World Health Organisation (WHO), CVDs were among the major cause of deaths for noncommunicable diseases. Prescription of antithrombotic agents (e.g. aspirin, clopidogrel and warfarin) is a standard treatment given to patients to reduce the risk of death from cardiovascular events. However, presence of side effects and inter-individual variability in response towards these antithrombotic agents may limit the prevention of cardiovascular events throughout the world including Malaysia. Tocotrienols are a group of vitamin E besides tocopherols. Previous animal studies showed significant inhibition of platelet aggregation and antithrombotic effect after tocotrienol administration, but results in human trials are controversial. Therefore, this study was designed to ascertain the effect of tocotrienol supplementation on platelet reactivity in subjects with metabolic syndrome (MetS), by taking into account that MetS subjects are associated with prothrombotic state due to higher platelet reactivity and blood coagulability. Thus, an initiation of antithrombotic therapy is important for them to prevent thrombotic events. In this connection, a randomised, double-blinded, crossover, and placebo-controlled human trial was conducted with a total of 31 MetS subjects (15 males and 16 females) completed the study. Subjects recruited received two interventions in a random order, separated by a 15-day washout period in between the interventions. During the intervention, subjects consumed palm mixed tocotrienols 200 mg or placebo twice daily for 14 days followed by a postprandial study day. Post-intervention results demonstrated that reactivity of arachidonic acid and adenosine 5’-diphosphate (ADP) signalling platelet aggregation was not significantly different ($p > 0.05$) between tocotrienol and placebo interventions. For the results in terms of postprandial change, tocotrienols also did not exert inhibitory effect ($p > 0.05$) on these platelet aggregation measurements compared to placebo. No significant differences ($p > 0.05$) were found on the degree of platelet activation induced by thrombin mimic peptide and haemostatic measures (plasma D-dimer, plasminogen activator inhibitor type 1 (PAI-1), fibrinogen and undercaroxylated osteocalcin (ucOC)) between tocotrienol and placebo interventions, both post-intervention and postprandial. Among the inflammatory measures including plasma soluble P-selectin (sP-selectin), plasma

soluble E-selectin (sE-selectin), plasma soluble intracellular adhesion molecules 1 (sICAM-1), plasma soluble vascular cell adhesion molecules 1 (sVCAM-1) and serum high sensitivity C-reactive protein (hsCRP), the results showed that there were no significant differences ($p > 0.05$) between tocotrienol and placebo interventions, both post-intervention and postprandial except for plasma sICAM-1, in which tocotrienols significantly lowered ($p < 0.05$) the plasma sICAM-1 during postprandial. Post-intervention results also showed that brachial systolic blood pressure (SBP) and aortic pulse pressure were significantly lower ($p < 0.05$) in tocotrienol intervention compared to placebo intervention. In conclusion, this study demonstrated that 14 days of 400 mg tocotrienol supplementation did not exert antithrombotic effects on platelet aggregation induced by ADP and arachidonic acid, thrombin mimic peptide induced platelet activation, and haemostatic and inflammatory measures.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN PENGAMBILAN TOKOTRIENOL TERHADAP PENGAGREGATAN PLATELET DALAM SUBJEK DENGAN SINDROM METABOLIK

By

GAN YEE LIN

April 2014

Pengerusi : Prof. Lai Oi Ming, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Pembentukan trombus dalam saluran darah, sama ada dalam arteri atau vena, adalah suatu keadaaan patologi yang lazimnya punca kepada kes-kes kejadian penyakit kardiovaskular. Menurut laporan WHO, penyakit kardiovaskular merupakan punca utama kematian bagi kategori penyakit tidak berjangkit. Preskripsi agen antitrombotik (contohnya aspirin, clopidogrel and warfarin) merupakan rawatan yang sering diberi kepada pesakit untuk mengurangkan risiko kematian akibat penyakit kardiovascular. Namun demikian, kewujudan kesan-kesan sampingan agen antitrombotik serta keberkesanannya terhadap setiap individu yang berbeza mungkin menghadkan keupayaan agen tersebut dalam pencegahan kejadian penyakit kardiovaskular di seluruh dunia termasuk negara Malaysia. Selain tokoferol, tokotrienol merupakan salah satu kumpulan vitamin E. Kajian praklinikal haiwan telah membuktikan bahawa rawatan tokotrienol dapat menghambat platelet agregasi dan menunjukkan kesan antitrombotik yang signifikan, akan tetapi, hasil kajian dalam kajian klinikal manusia adalah kontroversial. Oleh demikian, matlamat kajian ini adalah untuk menentukan kesan pengambilan tokotrienol terhadap kereaktifan platelet dalam subjek sindrom metabolik, dengan mengambil kira bahawa subjek sindrome metabolik adalah berhubung kiat dengan keadaan pratrombotik akibat peningkatan kereaktifan platelet dan koagulabiliti darah. Justeru, langkah permulaan terapi antitrombotik adalah penting bagi mencegah kes kejadian trombotik. Sehubungan ini, satu kajian klinikal secara rawak, rabun dua pihak, bersilang dan kawalan-plasebo telah dilaksanakan dengan sejumlah 31 subjek sindrom metabolik telah mengambil bahagian dalam kajian ini sehingga akhir. Subject dipilih secara rawak untuk pengambilan dua jenis kapsul yang berlainan secara turutan, di mana satu tempoh rehat sekurang-kurangnya 15 hari telah diberikan selepas rawatan pertama dan sebelum sukarelawan mengambil suplemen jenis kedua. Semasa rawatan, subject mengambil satu kapsul tokotrienol (200 mg) atau plasebo sebanyak dua kali sehari selama 14 hari diikuti dengan kajian pasca prandial. Selepas 14 hari rawatan, hasil kajian menunjukkan tiada perbezaan yang signifikan ($p > 0.05$) dalam kereaktifan pengagregatan platelet dengan ransangan dari asid arakidonik atau ADP antara kumpulan tokotrienol dan plasebo. Penemuan pasca prandial turut menunjukkan bahawa pengambilan tokotrienol tidak memberi apa-apa kesan yang

signifikan ($p > 0.05$) terhadap pengagregatan platelet berbanding dengan plasebo. Didapati tiada perbezaan yang signifikan ($p > 0.05$) antara kumpulan tokotrienol dan plasebo dari segi tahap pengaktifan platelet (diransang dengan thrombin tiruan peptida) dan paras biopenanda hemostatik (plasma D-dimer, PAI-1, fibrinogen dan ucOC) selepas pengambilan tokotrienol selama 14 hari dan pasca prandial berbanding dengan plasebo. Selain itu, paras biopenanda keradangan termasuk plasma sP-selectin, sE-selectin, sICAM-1 dan sVCAM-1 dan serum hsCRP tidak berbeza signifikan ($p > 0.05$) selepas 14 hari suplementasi dan pasca prandial antara dua kumpulan kecuali biopenanda plasma sICAM-1 yang dilaporkan parasnya agak rendah semasa pasca prandial secara signifikan ($p < 0.05$) selepas pengambilan tokotrienol berbanding dengan plasebo. Tokotrienol juga didapati menurunkan tekanan darah sistolik brakial dan tekanan nadi aorta secara signifikan ($p < 0.05$) berbanding dengan plasebo selepas pengambilan selama 14 hari. Kesimpulannya, kajian ini menunjukkan bahawa dalam subjek sindrom metabolik, pengambilan 400 mg tokotrienol selama 14 hari tidak mempamerkan kesan antitrombotik terhadap pengagregatan platelet yang dirangsang dengan ADP dan asid arakidonik, pengaktifan platelet yang dirangsang dengan thrombin, dan biopenanda-biopenanda hemostatik dan keradangan.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation to Malaysian Palm Oil Board (MPOB) for giving me the chance to carry out this trial and the facilities and grant support. I would also like to extend my greatest gratitude to my supervisor, Prof. Dr. Lai Oi Ming, and co-supervisors Dr. Chew Boon How, Dr. Kalanithi Nesaretnam and Dr. Fu Ju Yen for your valuable guidance, assistance and encouragement in bringing this project to a successful end. A big thank you to my supervisory committee members for their valuable time spent in aiding my thesis writing. It is also my duty to record my thankfulness to Prof. Dr. Yuen Kah Hay, Dr. Teng Kim Tiu, Dr. Kanga Rani Selvaduray, Dr. Maria Madon, Ms. Puvaneswari a/p Meganathan, staffs in Nutrition Unit and Genomic Unit for their kind assistance and valuable opinions along the project. A special note of thanks to the subjects who had participated in this trial and Hovid Bhd. for the dedication of Tocovid™ *SupraBio*™ 200 mg and placebo capsules. I also wanted to thank my labmates: Mo Shuen Yeing, Chang Lin Faun, Doryn Tan, Nur Afiqah bt Kamaruzzaman and the intern students who had lend their helping hands during screening and trial run of this project. Last but not least, I would like to express my heartfelt appreciation to my family members for the endless support, encouragement and understanding showered along this project.

I certify that a Thesis Examination Committee has met on 14 April 2014 to conduct the final examination of Gan Yee Lin on her thesis entitled "Effects of Tocotrienol Supplementation on Platelet Aggregation in Subjects with Metabolic Syndrome" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Noorjahan Banu binti Mohammed Alitheen, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Amin bin Ismail, PhD
Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Internal Examiner)

Loh Su Peng, PhD
Associate Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Internal Examiner)

Nur Aishah binti Mohd Taib, PhD
Professor
University of Malaya
Malaysia
(External Examiner)

_____

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 23 June 2014

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Lai Oi Ming, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Chew Boon How, MMed.
Senior Medical Lecturer
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Member)

Kalanithi Nesaretnam, PhD
Regional Manager
Product Development and Advisory Services Division
Malaysian Palm Oil Board
(Member)

Fu Ju Yen, PhD
Research Officer
Product Development and Advisory Services Division
Malaysian Palm Oil Board
(Member)

BUJANG KIM HUAT, PhD
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Universiti Putra Malaysia

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Signature:	Signature:
Name of Member of Supervisory Committee: **Dr. Kalanithi Nesaretnam**	Name of Member of Supervisory Committee: **Dr. Fu Ju Yen**

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AIx@75	Augmentation index adjusted to heart rate at 75
ALP	Alkaline phosphatase
Alx	Augmentation index
AMP	Adenosine monophosphate
Apo A1	Apolipoprotein A1
Apo B100	Apolipoprotein B100
ARU	Aspirin reactivity unit
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CRP	C-reactive protein
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DP	Diastolic pressure
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty acid methyl ester
FITC	Fluorescein isothiocyanate
GGT	Gamma-glutanyl transpeptidase
GP IIb/IIIa	Glycoprotein IIb/IIIa
HAECs	Human aortic endothelial cells
Hb	Haemoglobin
HDL-C	High density lipoprotein cholesterol

HMG-CoA	β-hydroxy-β-methylglutaryl-coenzyme A
HPLC	High performance liquid chormatography
hsCRP	High sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule 1
IDF	International Diabetes Federation
IOM	Institute of Medicine
iso-TRAP	(DL-Isoser[1])-thrombin receptor activating peptide-6 trifluoroacetate salt
IV	Intravenous
LDL-C	Low density lipoprotein cholesterol
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MetS	Metabolic syndrome
MFI	Mean fluorescence intensity
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor type 1
PAR	Protease-activated receptor
PAU	Platelet aggregation unit
PerCP	Peridinin chlorophyll protein complex
PIVKA-II	Protein induced by vitamin K absence-factor II

PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PMC	2,2,5,7,8-pentamethyl-6-chromanol
PRP	Platelet rich plasma
PRU	P2Y12 reactivity unit
RBC	Red blood cell
SBP	Systolic blood pressure
SD	Standard deviation
sE-selectin	Soluble E-selectin
SEVR%	Sub-endocardial viability ratio index
SGOT	Serum glutamic oxaloacetic transminase
SGPT	Serum glutamic pyruvic transaminase
sICAM-1	Soluble intracellular adhesion molecules 1
SP	Systolic pressure
sP-selectin	Soluble P-selectin
sVCAM-1	Soluble vascular cell adhesion molecules 1
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TC	Total cholesterol
TNF-α	Tumour necrosis factor-α
TRF	Tocotrienol-rich fraction
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2

ucOC	Undercaroxylated osteocalcin
ULOQ	Upper limit of quantification
US FDA	United States Food and Drug Administration
VCAM-1	Vascular cell adhesion molecule 1
VLDL-C	Very low density lipoprotein cholesterol
vWF	von Willebrand factor
WBC	White blood cell
WHO	World Health Organisation
α	Alpha
α-TTP	α-tocopherol transfer protein
β	Beta
δ	Delta
γ	Gamma

CHAPTER 1

INTRODUCTION

Thrombosis is one of the major causes of morbidity, mortality and disability. It is a pathological condition which involves the formation of thrombus in vasculature and thus alters or occludes the normal blood flow. It can take place either in arterial or venous circulation. With respect to arterial thrombosis, thrombus is formed in an area with high shear stress, especially where atherosclerotic plaques are disrupted (Lijfering *et al.*, 2011; Turpie and Esmon, 2011). Several high mortality rate of cardiovascular diseases (CVDs) such as myocardial infarction, cerebrovascular disease and peripheral arterial disease are triggered by arterial thrombosis (Freedman, 2005). On the other hand, venous thrombosis occurs in an intact vein, an area with low blood flow and shear stress. Venous thrombosis is associated with an increased risk of developing deep vein thrombosis or pulmonary embolism (Franchini and Mannucci, 2012; Lijfering *et al.*, 2011). Arterial thrombi comprise predominantly platelet-rich "white thrombi", whilst venous thrombi consist mainly of red cell-rich "red thrombi". Nonetheless, both thromboembolic disorders' thrombi are composed of platelets, fibrin, leukocytes and erythrocytes. Some common pathogenesis biological mechanisms shared between thrombosis of artery and vein included the activation of endothelium, platelets, and coagulation (Franchini and Mannucci, 2012). Recognition of the types of cells, agonists, receptors, enzymes and cellular molecules involved in pathogenesis of thrombosis leads to the major improvement in the development of antithrombotic drugs for the clinical management of patients with thromboembolic disorders.

At present, antithrombotic drugs such as antiplatelets, anticoagulants and fibrinolytic are the standard therapies being applied in the clinical setting for the thrombosis management. They function by targeting the key pathways of coagulation, platelet activation and aggregation including the adenosine 5'-diphosphate (ADP) signalling pathway, thromboxane A_2 (TxA_2) synthesis, activation of glycoprotein IIb/IIIa (GP IIb/IIIa), activity of thrombin and activated factor X, γ-carboxylation of vitamin K and lysis of formed thrombus (Joint Formulary Committee, 2013; Ryan *et al.*, 2013). Some of the antithrombotic drugs have been medicinally available for several decades for the prevention and treatment of arterial and venous thrombosis. However, these antithrombotic drugs possess several drawbacks such as side effects and inter-individual variability in response towards drug's therapeutic effects, may limit their usage or effectiveness (Gross and Weitz, 2009; Sikka and Bindra, 2010; Turpie and Esmon, 2011).

The observation of high residual platelet reactivity after antithrombotic therapies reported in published evidences has indicated that these drugs are insufficient to combat the thromboembolic disorders (Michelson, 2010; Musallam *et al.*, 2011; Sweeny *et al.*, 2009). This may be due to the inter-individual response towards the therapeutic effects of antithrombotic drugs. Growing body of evidences have shown

that some patients have low response towards antiplatelet or anticoagulant therapies of aspirin, clopidogrel, warfarin or heparin (Ancrenaz *et al.*, 2010; Cambria-Kiely and Gandhi, 2002; Hirsh and Raschke, 2004; Musallam *et al.*, 2011; Sinxadi and Blockman, 2008; Sweeny *et al.*, 2009). Similar situation also observed in Malaysia. A study conducted by Ibrahim *et al.* (2013) reported that there were 4.69% low responders for aspirin therapy and 21.9% low responders for clopidogrel therapy in acute coronary syndrome patients. Yet, the mechanisms causing this low response towards antithrombotic drugs have not been fully elucidated. Several potential explanations have been recognised. These included the genetic polymorphisms, pharmacodynamic and pharmacokinetic of drugs interaction and high platelet reactivity (Acikel and Akdemir, 2010; Ancrenaz *et al.*, 2010; Sikka and Bindra, 2010). By taking into consideration the risk-benefit ratio assessment, the presence of side effects of each antithrombotic drug has also limits its usage for the prevention and treatment of thrombosis (Gross and Weitz, 2009; Joint Formulary Committee, 2013; Michelson, 2010; Ryan *et al.*, 2013). Thus, development of new safe and effective antithrombotic agents to strengthen the health care system is an ongoing effort.

Vitamin E, a plant-derived tocols vitamin with antioxidant characteristic, is found to be able to improve cardiovascular outcome (Stephens *et al.*, 1996). More interestingly, tocotrienols, a group of vitamin E beyond the commonly known tocopherols, are previously found to possess antithrombotic properties. Previous animal studies have shown that tocotrienols were able to inhibit collagen and ADP induced platelet aggregation and its effects were significantly better than α-tocopherol (Qureshi *et al.*, 2011). Further, in human study, supplementation of tocotrienols has been reported to decrease the collagen and ADP induced platelet aggregation significantly (Qureshi *et al.*, 1991a). In addition, several animal studies also have demonstrated that tocotrienols were able to reduce the platelet activation biomarkers such as plasma thromboxane B_2 (TxB_2) and platelet factor 4 (Qureshi *et al.*, 1991b; 2001a; Qureshi and Peterson, 2001). Similar results were shown by Qureshi *et al.* (1995; 1997) in several other human studies where four weeks of tocotrienol supplementation have significantly decreased the levels of TxB_2 and platelet factor 4 in hypercholesterolemic subjects.

Meanwhile, contradictory findings have been published as well. Different groups of researchers have demonstrated that tocotrienols were not able to inhibit the collagen, adrenaline or ADP induced platelet aggregation in animal and human studies (Koba *et al.*, 1992; Mensink *et al.*, 1999; Tomeo *et al.*, 1995; Wahlqvist *et al.*, 1992; Watkins *et al.*, 1993). Additionally, measurement of platelet activation biomarkers such as plasma and urinary TxB_2 and platelet adenosine triphosphate (ATP) release has shown no difference after tocotrienol supplementation (Mensink *et al.*, 1999; Tomeo *et al.*, 1995; Wahlqvist *et al.*, 1992; Watkins *et al.*, 1993). Hence, the antithrombotic effect of tocotrienols remains uncertain with these inconclusive findings.

Based on the published tocotrienols human studies, it was found that dosages of tocotrienols between the previous human studies were different. Significant suppressive effect on platelet aggregation was observed after four weeks of palm tocotrienol-rich fraction (TRF) supplementation at a daily dose of 200 mg (Qureshi *et al.*, 1991a). Conversely, administration of palm TRF at a daily dose ranged from 160 mg up to 336 mg had showed no significant inhibitory effect on platelet aggregation (Mensink *et al.*, 1999; Tomeo *et al.*, 1995; Wahlqvist *et al.*, 1992). Based on the study conducted by Qureshi *et al.* (2011) in dogs, dosage used in this study was 10 mg TRF/kg of body weight. When the dosage was translated to human equivalent dose with an assumption of 75 kg for human's body weight, it was found that the tocotrienol human equivalent dose was 405 mg. Thus, summarizing from the above studies, in order to ascertain the antithrombotic effect of tocotrienols in current study, a higher dose of 400 mg tocotrienols could be supplemented in humans.

Apart from the dosage of tocotrienols, platelet aggregation methods were also varied between studies. The studies which used whole blood impedance platelet aggregometry for platelet aggregation analyses had demonstrated that tocotrienols had no significant inhibition effect on platelet aggregation. In constrast, previous studies which used light transmission turbidimetric platelet aggregometry showed that tocotrienols significantly reduced ADP and collagen induced platelet aggregation. Light transmission turbidimetric platelet aggregometry is a method that requires a complex sample preparation which may alter platelet behaviour, and a trained operator to perform the test (Harrison, 2005; Harrison *et al.*, 2007). Besides that, results obtained were not reproducible even in the same laboratory (Musallam *et al.*, 2011). On the other hand, whole blood impedance platelet aggregometry also was reported to be poor in standardised method and thus it was difficult to compare findings between the studies (Harrison, 2005). In order to address the drawbacks of these methods, it is crucial to adopt a universal standardised method that requires no sample processing and skillful operator in addition to its ability to produce reproducible results. VerifyNow instrument is a fully automated cartridge-based instrument that fulfills these desirable attributes (Harrison *et al.*, 2007; Harrision and Keeling, 2007).

In addition, previous studies had been focused only on the investigation of the long term tocotrienol supplementation effect on the fasting measurement of platelet aggregation. There is lack of study looking into the postprandial effect of tocotrienols on platelet aggregation and thrombotic markers. In fact, humans spend most of their waking hours in postprandial state which triggers a series of biochemical events including platelet activation, hypercoagulable state and endothelial dysfunction (O'Keefe and Bell, 2007).

In view of these gaps in current research, further exploration which includes a daily dose of 400 mg tocotrienols and use of VerifyNow instrument for platelet aggregation measurement is thus needed to fully understand the potential of tocotrienols being the antithrombotic agent and sort out the pathway that tocotrienols are targeting in reducing the thrombotic events. This needs to consider not only the

fasting measurement after a period of supplementation but also postprandially. Seeing that tocotrienol is a natural product extracted from plant, it may be beneficial as a potential supplement to enhance the cardiovascular health and simultaneously circumventing the side effects associated with the current antithrombotic drugs.

As such, this randomised double-blind, crossover and placebo-controlled human clinical trial was designed to determine the antithrombotic effect of tocotrienols in subjects with metabolic syndrome (MetS) at a daily dose of 400 mg. MetS subjects are associated with abdominal obesity, hypertension, atherogenic dyslipidemia, insulin resistance, prothrombotic state and proinflammatory state. This series of conditions alter the haemostasis balance by increasing the platelet reactivity and inducing hypercoagulability and hypofibrinolytic states. Hence, an increased risk of cardiovascular diseases in MetS subjects may be attributed by its high tendency of thrombus formation. Prevention of thrombotic event is thus important for this group of people.

The specific objectives of this study are:

i. To ascertain the effect of tocotrienol supplementation (400 mg) on platelet aggregation in subjects with MetS.
ii. To find out the antithrombotic mechanism of tocotrienols via the platelet activation pathway, coagulation pathway, inflammatory pathway, heamodynamic improvement or changes in lipid profile in subjects with MetS.
iii. To determine the postprandial effect of tocotrienol supplementation on the platelet aggregation and activation, coagulation, inflammatory and haemodynamic markers in subjects with MetS.
iv. To determine the safety and tolerance of tocotrienol supplementation in subjects with MetS.

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