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Actions of annatto-extracted tocotrienol supplementation on obese postmenopausal women: study protocol for a double-blinded, placebo-controlled, randomised trial

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ABSTRACT

Introduction Obesity is a major health concern in postmenopausal women, and chronic low-grade inflammation contributes to the development of obesity. Cellular studies and high-fat-diet-induced obese mouse model mimicking obesity show the antiobesity effect of annatto-extracted tocotrienols (TT) with antioxidant capability. We aim to assess the safety and efficacy of TT consumption for lipid-related parameters in obese postmenopausal women.

Methods and analysis Eligible obese postmenopausal women will be randomly assigned to placebo group (430 mg olive oil) and TT group (DeltaGold Tocotrienol 70%) for 24 weeks. In the present study, the primary outcome is total/regional fat mass and visceral adipose tissue. The secondary outcomes include lipid profile in serum, mRNA expression of fatty acid synthase and carnitine palmitoyltransferase 1A in fat tissue, oxylipins and endocannabinoids in plasma and adipose tissue, abundance and composition of intestinal microbiome in faeces, high-sensitivity C-reactive protein (hs-CRP) in serum and leptin in serum. Every participant will be evaluated at 0 (prior to starting intervention) and 24 weeks of intervention, except for serum lipid profile and hs-CRP at 0, 12 and 24 weeks. '*Intent-to-treat*' principle is employed for data analysis. Hierarchical linear modelling is used to estimate the effects of dietary TT supplementation while properly accounting for dependency of data and identified covariates. To our knowledge, this is the first randomised, placebo-controlled, double-blinded study to determine dietary TT supplementation on an obese population. If successful, this study will guide the future efficacy TT interventions and TT can be implemented as an alternative for obese population in antiobesity management.

Ethics and dissemination This study has been approved by the Bioethics Committee of the Texas Tech University Health Sciences Center, Lubbock. An informed consent form will be signed by a participant before enrolling in the study. The results from this trial will be actively disseminated through academic conference presentation and peer-reviewed journals.

Trial registration number NCT03705845.

Strengths and limitations of this study

- This is the first randomised, double-blinded, placebo-controlled trial to investigate the effects of dietary annatto-extracted tocotrienol supplementation on obesity-related outcomes in obese postmenopausal women.
- This study will be performed at a single research centre with experience in conducting independent, investigator-initiated, randomised controlled trials in nutrition research.
- Annatto-extracted tocotrienol supplement is not available over the counter worldwide.
- There is no long-term follow-up.

INTRODUCTION

Obesity is now recognised as a worldwide epidemic disease, which leads to the development of several comorbidities such as insulin resistance, dyslipidaemia and metabolic syndrome (WHO 2011).[1] With advances in basic redox biology, emerging evidence indicates that chronic low-grade inflammation paves the way for the development of comorbidities in obesity.[2] Obesity-induced chronic low-grade inflammation, called metaflammation, is initiated by excess nutrients in metabolic cells.[2] The inflammatory signalling conducted by these metabolic cells eventually causes activation of specialised immune cells and leads to an unresolved inflammatory response within the adipose tissue.[2] Therapeutic interventions to inhibit inflammatory pathways in obesity have shown beneficial effects on insulin sensitivity in mouse models and human trials.[2]

Oxylipins (OxL) are oxygenated fatty acid metabolites derived from n-6 polyunsaturated fatty acids (PUFA) (ie, linoleic acid, dihomo-γ-linolenic acid and arachidonic

acid (AA)) or n-3 PUFAs (ie, α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) through the actions of cyclo-oxygenases, lipoxygenase (LOX) and cytochrome P450 enzymes.[3] In postmenopausal women (PMW) at risk of metabolic syndrome, the amounts of OxLs were modified by dietary n-3 PUFA such that the levels of proinflammatory were reduced and anti-inflammatory enhanced.[4] Particularly, DHA feeding reduced plasma levels of various inflammatory cytokines and OxLs (5-LOX-dependent inflammatory mediators and vasoconstrictive 20-hydroxy-eicosatetraenoic acid (HETE) in a rodent model of obesity).[5] Emerging evidence suggests that AA-derived OxLs contribute to obesity-associated inflammation.[6 7] For example, 5-LOX products of AA (eg, 5- HETE or 5-oxo-eicosatetraenoic acid) exert proinflammatory effects by increasing the production of proinflammatory cytokines and inducing chemotaxis to attract inflammatory cells in the blood vessels in adipose tissue from obese mice.[8 9] 12-LOX products of AA (eg, 12-HETE, 12-hydroperoxyeicosatetraenoic acid) have also been shown to play a direct role in obesity-induced inflammation[10] and insulin resistance.[11–13] Obese subjects with low-grade inflammation had elevated levels of several AA-derived OxLs (eg, 5, 8, 11, 12, 15 and 20-HETE) compared with those with no inflammation.[7] On the other hand, OxLs that originate from n-3 PUFA (eg, EPA and DHA) have demonstrated their ability to suppress obesity-associated inflammation as seen in inhibiting macrophage recruitment and increasing adiponectin secretion in adipose explants isolated from obese mice.[14] Supplementation of n-3 PUFA for 8 weeks significantly decreased AA-derived OxLs and increased EPA and DHA-derived OxLs in hypertriglyceridaemic subjects along with mitigated inflammatory and vascular status.[15] Since chronic low-grade inflammation in obesity is a systemic problem, administration of dietary supplements with potent anti-inflammatory function can be a good strategy to combat inflammation in obesity.

Tocotrienols (TT) and tocopherol are two subclasses of vitamin E, which can be further classified into four isomers (α, β, δ and γ). The unsaturated side chain of TT allows for more efficient penetration into tissues compared with the completely saturated side chain of tocopherol. TT is lipophilic in nature and it is found in association with lipoproteins, fat deposits and cellular membranes.[16 17] TT has been shown to protect the PUFAs from peroxidation.[16 17] In animal studies, dietary TT (palm TT-rich fraction, δ-TT and γ-TT) decreased body weight[18] and fat mass[19 20] reduced plasma concentrations of free fatty acids, triglycerides and cholesterol[21–25] and improved glucose and insulin tolerance.[18–20 25 26] Many epidemiology studies showed that dietary TT intake is associated with decreased serum concentrations of total cholesterol and lipoproteins of study subjects.[23 27–31] In humans, TT supplementation (34.6% α-TT+43.5% γ-TT) reduced microalbuminuria and high-sensitivity C-reactive protein (hs-CRP), indicators of acute inflammation, in patients with type 2 diabetes.[32] Intake of grape seed oil with high levels of TT was shown to attenuate serum levels of hs-CRP and tumour necrosis factor-α and improve insulin resistance in overweight/obese women.[33] The review of the supporting evidence strongly suggests that TT can offer benefits to control obesity due to its potent anti-inflammatory-dependent biological activities. In addition, although δ-TT and γ-TT are considered as the most potent isomers among eight vitamin E isomers,[34] their clinical relevance to combat obesity along with mechanisms has never been investigated in humans. Furthermore, our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen *et al*, unpublished findings). Yet, it is not known whether supplementation with TT would benefit total fat mass reduction, improve lipid metabolism and modify OxL in plasma and adipose tissue of humans. Additionally, there is not a single study yet to evaluate how TT supplementation affects gut microbiome in humans, particularly obese PMW. We previously reported that 12-week annatto-extracted TT supplementation suppressed bone resorption and oxidative stress in PMW with low bone mass.[35] However, there is no study to evaluate how TT supplementation would affect obese PMW. Therefore, the objective of this pilot study is to test a mixture of 90% δ-TT+10% γ-TT dietary supplementation for feasibility, and to quantitatively assess its potential antiobesity effects on obese PMW.

In this paper, we present the design of a 24-week double-blinded, placebo-controlled and randomised trial as well as a discussion of the overall challenges when conducting this trial. The results from this trial will be reported at the completion of the study in accordance with the Consolidation of Standards for Reporting Trials guidelines.

METHODS

Study design

This is a 24-week double-blinded, placebo-controlled and randomised intervention trial with allocation 1:1 for two treatment arms. Women with at least 1 year after menopause will be recruited for this study. After screening, qualified participants will be randomly assigned to one of the two treatment groups: placebo and TT. The participants in the placebo group will receive one 430 mg olive oil softgel per day for 24 weeks. The TT participants will receive one 430 mg 70% pure TT softgel (representing 300 mg TT) for 24 weeks. The primary outcome measure is total fat mass and the secondary outcome measures are serum lipid profile, leptin, adiponectin, lipid metabolism-related gene expression in adipose tissue, inflammation-associated biomarkers (serum CRP, plasma and adipose tissue OxL) and gut microbiome analysis in faeces. Both primary and secondary outcome measures will be taken from participants at baseline and 24 weeks, except for serum lipid profiles and CRP, which will be assessed at baseline, 12 and 24 weeks. Liver function will also be monitored by assessing the activity of aspartate

Table 1 Timetable of activities planned during the course of the study directly related to participants

	Weeks										
Activity	−4	−2	0	3	6	9	12	15	18	21	24
Enrolment	x										
Screening (liver function, TSH)		x									
Randomisation			x								
Intervention			x	x	x	x	x	x	x	x	x
Body composition			x								x
Fat biopsy (optional)			x								x
Blood for outcomes			x				x				x
Liver function assessment			x				x				x
Pill counts			x				x				x
Food intake assessment			x								x
Physical activity survey			x								x
Self-report adverse event			x	x	x	x	x	x	x	x	x

TSH, thyroid-stimulating hormone.

aminotransferase (AST) and alanine aminotransferase (ALT) at baseline, 12 and 24 weeks. Physical activity and food frequency questionnaires will be assessed at baseline and 24 weeks. Investigators and outcome assessors evaluating the endpoints will be blinded to intervention assignment. The time points of all participant-related actions to be taken during the study period are presented in table 1.

Study setting, study population and recruitment

The subjects, ambulatory women with at least 1-year postmenopausal history, for this present study will be recruited from Lubbock and surrounding areas in Texas. Ethnicity or race is not a factor in the inclusion of subjects. Direct person-to-person solicitation in the obstetrician-gynaecologist clinic and health fairs, flyers, non-solicited email system, campus announcements, local radio, newspapers, senior newsletters and TV scripts will be used to recruit potential subjects. In addition, we plan to recruit the minority participants who have limited access to such intervention due to social and cultural factors. Advertisement in minority newspaper and radio will also be implemented. According to our past experience in subject recruitment, it is not difficult to recruit qualified participants within a reasonable time frame from our existing subject pool with the methods described above. If a longer period is needed to recruit the subjects, the study supplement intervention may take place in a staggered fashion, that is, a block randomisation strategy will place participants into subgroups for treatments on a first-come, first-served basis.

Screening

Prescreening will be conducted through a phone interview and will cover age, body weight, height, menstrual history, status of any serious chronic diseases and availability for the study period. Subjects who pass the prescreening will attend an informed consent session and sign consents and Health Insurance Portability and Accountability Act (HIPAA) forms before completing a detailed questionnaire with demographic, health and dietary information. Visits for fasting blood screening will follow afterwards.

Inclusion criteria

1. PMW with body mass index (BMI) ≥30 kg/m^2.
2. Normal function of thyroid, liver and kidney.
3. Sedentary using International Physical Activity Questionnaire-short form.[36]

Exclusion criteria

1. Unstable body weight (more than 5% change in body weight) within 3 months before intervention begins.
2. Changes to medications or supplements (ie, steroids, statins) within 3 months of the baseline study visit that could affect lipid metabolism. If they change any medications/supplements after the baseline visit that will affect lipid metabolism, their study participation will end.
3. Taking anticoagulants that may interact with TT.
4. Serious chronic disease (eg, unstable cardiovascular disease, uncontrolled diabetes and hypertension, and active cancer).

Sample size

Sample size is determined to provide adequate power for the study. Data from previous studies suggest that the TT intervention would yield moderate to large changes (median f=0.31) in total fat mass and body weight. The power analysis for the present study is considered to observe similar changes in other previous studies[20 37–39] and assumed 0.20 correlations among repeated measures. The analysis results revealed that the sample size of n=46 will produce 80% power while controlling type I error under 5%. Conservatively assuming a high attrition rate of 20%, therefore, we plan to recruit 60 participants (30 in each

group) at baseline—that is, anticipated final n≥46 (power ≥80%) at the end of the study with up to 20% attrition. Missing data, either attrition or non-response, will be fully recovered via multiple imputation as described later, which will remove or minimise (if present) confounding effects of missingness on our statistical power.

Randomisation and allocation concealment

Participants who meet the inclusion criteria will be randomly assigned 1:1 to placebo and TT groups using a stratified randomisation with BMI (≥35 or <35 kg/m^2), age (≥60 or <60 years), use of hormone replace therapy (yes or no) and use of cholesterol-lowering drug (yes or no). All participants, investigators, coordinators and assessors will be blinded to the treatments. Deidentified data with codes will be used to facilitate blinded randomisation and data analysis. The blinding on a participant will be removed in the case of an adverse event or other legitimate reason.

Intervention

Purchasing and masking of study agents

Placebo and TT supplements of the same lot, respectively, will be provided by American River Nutrition (Hadley, MA; Investigational New Drug (IND) No 120761 by Food and Drug Administration (FDA)). Each placebo capsule of 430 mg olive oil contains no TT ingredient at detectable levels. Each TT capsule contains 430 mg DeltaGold Tocotrienol 70% (90% δ-TT and 10% γ-TT) with a 70% purity, representing 300 mg TT. In the present study, we will include the placebo group to mask the control group in order to keep participants and evaluators blinded to the treatment assignment. Placebo softgels will be made of the same size and colour as the active TT softgels for identical appearance and taste. We will compare the results of blood and urinary outcome measures in the placebo group with those in the TT group.

Treatment arms

The study agent (annatto-extracted TT, DeltaGold Tocotrienol 70%) has Generally Recognized as Safe status. A TT has an unsaturated isoprenoid side chain, which is different from tocopherols. Both TTs and tocopherols exist in four different forms in nature: α, β, γ and δ isomers. Our study agent, the DeltaGold Tocotrienol 70%, consists of 90% δ-TT and 10% γ-TT with 70% purity.

Based on (1) TT fed at 400 mg/kg diet to high-fat-diet-induced obese mice showed reduced total fat mass relative to the no-TT group[19] and (2) the use of body surface area for dose translation from mouse to human,[40] the estimated effective dose of TT in humans for antiobesity is approximately 300 mg daily. Therefore, we will use a dosage of TT (300 mg TT daily) for 24 weeks in this study. The current recommended daily allowance value of vitamin E, 15 mg/day, for this study population is largely focused on α-tocopherol. No specific recommendation exists for TT. However, no adverse effects were observed with daily intake of 3.2 g or less TT.[41] Qualified participants will be randomly assigned into one of the two study groups (placebo and TT). Participants in the placebo group will receive 430 mg of olive oil daily for 24 weeks and participants in the TT group will receive a 430 mg DeltaGold Tocotrienol 70% softgel for 24 weeks.

Dietary intake, physical activity and concomitant medication assessment

We will take the same strategy that we used in our previous TT for bone health study[42] into this obesity study to monitor participants' dietary intake using a food frequency questionnaire, physical activity using a log and over-the-counter medications/dietary supplement at the baseline and 24 weeks' visit.

Blinding and unblinding

Study participants and investigators including biostatistician, coordinators and measurement and site personnel will be blinded to intervention allocation throughout this study. The investigators will be supplied with a blind code-breaker envelope for each subject. The blind code will not be broken except in a medical emergency or a potential study-related adverse event determined by the principal investigator.

Sample collection

Fasting blood will be drawn from a superficial arm vein at Clinical Research Institute, Texas Tech University Health Sciences Center, Lubbock, and blood will be allowed to clot in a vacutainer at room temperature. After centrifugation of blood samples, serum samples will be aliquoted and stored in −80°C freezers for later analysis. Urine and stool specimen will also be collected and stored in −80°C freezers for later analysis. Under local anaesthesia, the fat biopsy specimen will be obtained from visceral fat with Bard Magnum instrument and needle (CR Bard, Covington, GA).

Evaluation of adherence and compliance

We will count the softgels consumed and measure serum TT concentration at baseline and 24 weeks for adherence and compliance of participants. We will not use the results of the percentage of softgels consumed or serum concentration of TT and tocopherol as our exclusion criteria.

Evaluation of adverse events

We will measure the AST and ALT at 0, 12 and 24 weeks to monitor the liver function of participants. In addition to abnormal liver function, all self-reported adverse events and medical emergencies will be recorded throughout the study period. In our previous study,[43] we reported TT supplementation at both 300 and 600 mg TT daily for 12 weeks did not affect liver or kidney function parameters and no adverse event due to treatments was reported by study participants.

Outcome measures

In the present study, the primary outcome is total/regional fat mass and visceral adipose tissue (VAT). The

secondary outcomes include lipid profile (total cholesterol, high-density lipoprotein and triglycerides) in serum, mRNA expression of fatty acid synthase (FAS) and carnitine palmitoyltransferase 1A (CPT-1) in fat tissue, OxL and endocannabinoids (eCB) in plasma and adipose tissue, abundance and composition of intestinal microbiome in faeces, hs-CRP in serum and leptin in serum. Every participant will be evaluated at 0 (prior to starting intervention) and 24 weeks of intervention, except for serum lipid profile and hs-CRP at 0, 12 and 24 weeks.

Fat mass

Rationale

δ-TT supplementation has been shown to improve glucose tolerance, insulin sensitivity and lipid profile, and to reduce total fat mass, abdominal circumference and visceral adiposity index in obese rats.[39] Such an improvement in glucose utilisation and insulin sensitivity contributes to the reduced proinflammatory microenvironment and subsequent reduction in total fat mass and VAT.[19] Our animal study further confirmed δ-TT's antiobesity potential in obese mice.[19] These animal studies indicate that δ-TT plays an important role in reducing VAT in obese population.

Methods

Total and regional fat mass, relative body fat and VAT will be quantified via dual-energy X-ray absorptiometry (DXA; GE Lunar Prodigy with enCore software V.16.2).[44 45] Body regions for analysis include the trunk, legs and arms. VAT will be estimated using the built-in functionality of the enCore software. In the same DXA report, we will also collect data of bone mineral mass/density and lean mass. Additional total fat and VAT estimates will be obtained via bioimpedance analysis (SC-331S Body Composition Analyzer, Tanita Corporation of America, Arlington Heights, IL, USA).

Serum lipid profile, adiponectin, leptin, and hs-CRP, and urine 8-OHdG

Rationale

In our previous animal study, we have shown that TT supplementation to diet reduced serum triglycerides and proinflammatory adipokines in obese mice.[19] In a randomised, double-blinded, placebo-controlled trial, we reported that 12-week TT supplementation (90% δ-TT+10% γ-TT) at 300 mg/day lowered urine 8-OHdG, an oxidative stress biomarker in osteopenic PMW.[46]

Methods

We will ship blood samples to Covenant Medical Laboratory, Lubbock, TX, for lipid profile (total cholesterol, triglycerides, low-density lipoprotein and high-density lipoprotein) and hs-CRP analysis. The level of serum adiponectin and leptin, and urine 8-OHdG (oxidative stress biomarker) will be measured at Dr Shen's lab using ELISA.

Lipid metabolism-related gene expression in adipose tissue

Rationale

Obesity is characterised by altered mitochondrial activity and lipid metabolism, promoting accumulation of triglycerides in tissues, mainly white adipose tissue.[47] Defective mitochondrial biogenesis and fatty acid oxidative metabolic pathways in subcutaneous adipose tissue during acquired obesity lead to the metabolic disturbance in obesity.[48] Based on our animal findings,[19] we will focus on gene expression associated with lipid β-oxidation and fatty acid synthesis of subcutaneous adipose tissue sampled before and after TT regime.

Methods

Total RNA will be isolated from frozen adipose samples using Qiagen RNeasy Lipid Kit (Qiagen, Valencia, CA) and reversely transcribed into cDNA using iScript Reverse Transcription Supermix. Conventional quantitative real-time PCR will be performed using SYBR Green with respective primers for FAS and CPT-1 (Human Probe-Library Gene).[49] The relative gene expression will be assessed using the ΔΔCT method[50] and glyceraldehyde 3-phosphate dehydrogenase will be used as internal control for normalisation.[49] In addition, total RNA will be extracted from deidentified serum and adipose tissue for later stranded mRNA sequencing.

Concentrations of OxL and eCB in plasma and adipose tissue

Rationale

Ageing and decline of oestrogen are factors that contribute to weight gain in PMW, and approaches, such as anti-inflammatory potential in TT, to reduce inflammation, may likely combat obesity. OxLs and eCBs are classes of bioactive fatty acid metabolites with many structural members that influence insulin signalling, adipose function and inflammation through autocrine, paracrine and endocrine mechanisms.[4 5] In addition, physical activity is reported to increase specific levels of eCB[51] leading to responses of well-being. In our recent animal study, male mice fed with TT-supplemented diet experienced reduced thromboxane B2 (TXB2) and 13-hydroxyoctadecadienoic acid (13-HODE), and increased 12-HEPE (antiplatelet) and some essential amino acids (ie, lysine and methionine) (Shen *et al*, unpublished findings). The former exerting complex functions like 13-HODE but lowering TXB2, a product of TX2, could have implications on the function of TXA2 (target of aspirin). Thus, higher TXB2 means increased catabolism of TXA2 and less thrombosis may result in reducing the risk for atherosclerosis. Our animal study suggests that TT may influence macronutrient metabolism in obese PMW (Shen *et al*, unpublished findings). Identification of novel metabolites and metabolic pathways, especially antioxidant and oxidative stress metabolites, of TT, in serum and adipose tissue of subjects will help understand the composition and roles of these bioactive compounds in management of obesity. However, there has been no study to evaluate the effect of TT on any metabolomics profiling in obese population.

Herein, we will use the same targeted metabolomics to measure OxL and eCB.

Methods

Targeted metabolomics analyses of OxL and eCBs. The levels of OxL and eCB in plasma and adipose tissue will be quantitatively analysed using ultra-performance liquid chromatography-tandem mass spectrometers based on our previously published work.[4] Briefly, 60 mg Oasis-HLB solid phase extraction (SPE) columns (Waters, Milford, MA) are placed on a vacuum manifold, cleaned and conditioned, then spiked with deuterated OxLs and eCBs internal standards. The serum samples are thawed on ice and a 200 µL volume transferred to the SPE, updiluted to 3 mL with 5% MeOH/0.1% acetic acid, and gravity extracted. Columns are then washed with 3 mL of the 5% MeOH solution. OxLs and eCBs are eluted from the SPE with 0.2 mL MeOH followed by 1.5 mL ethyl acetate, by gravity. Solvent is removed by vacuum and samples are reconstituted in 50 µL MeOH containing the internal standard 1-cyclohexyl-3-dodecyl-urea (Sigma, Aldrich, St Louis, MO), then filtered at 0.1 µm. Analytes are chromatographically separated on a 2.1×150 mm, 1.7 µm Acquity BEH C18 (Waters) column using 0.1% acetic acid and acetonitrile gradient. The OxL and eCB profiles are acquired using electrospray ionisation and tandem mass spectrometry by back-to-back (+)-mode/(−)-mode injections on a Sciex API 4000-QTRAP (Pleasanton, CA). A complete list of measured analytes with retention times, acquisition parameters and detection limits is shown for the OxLs and eCBs in a previously published article, respectively.

Abundance and the composition of intestinal bacteria in faeces

Rationale

There has been a growing body of evidence to implicate altered intestinal microbiota in the development of obesity. The commensal microflora of the human gastrointestinal tract actively participate in the processes of energy harvest and expenditure. Biochemical crosstalk between gut bacteria and the host has a critical impact on the metabolic status (OxLs and eCBs) of the human body. In our previous animal study, we reported that relative to the high-fat diet (HFD) control group, the HFD+TT at 800 mg/kg diet group increased abundance of *Akkermansia g* (of the phylum Verrucomicrobia) and S24-7 family (class Bacteroidetes), and decreased that of Clostridiales order, and *Oscillospira* genus (of the phylum Firmicutes). Our animal data were in line with δ-TT's anti-inflammatory properties to improve intestinal dysbiosis of obese mice with insulin resistance (Shen *et al*, unpublished findings). Therefore, in this pilot study, we would like to explore how dietary TT supplement affects the abundance and composition of gut microbiome in PMW.

Methods

16S metagenome analysis of faecal samples. Bacterial DNA will be isolated from faeces using a MoBio PowerFecal DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The microbial diversity of faecal samples will be evaluated using metagenomic 16S rDNA gene sequencing technique. Variability in the bacterial V3–V4 region will be used to identify bacterial abundances in both treated and untreated subjects. Quantitative insights into microbial ecology (QIIME V.1.8.0) will be used for data analysis according to previous published method.[52] In brief, 1 µL of DNA sample (20 ng/µL) will be used for a 25 µL PCR reaction. The V3–V4 regions of the 16S rRNA gene region will be amplified using gene-specific primer pairs. PCR amplicons from all samples will be normalised using SequalPrep Plate Normalization Kit (Thermo Fisher Scientific, MA). Equal volumes of samples will be pooled and quantified using Qubit 2.0 (Thermo Fisher Scientific) and quality will be checked using TapeStation (Agilent Technologies, CA). Libraries will be sequenced on MiSeq (Illumina, CA) using a 600-cycle v3 sequencing kit. After sequencing, reads will be filtered based on quality score and aligned on 16S database using our custom pipeline involving PEAR, and QIIME software's. Final operational taxonomic units (OTU) will be taxonomically classified using BLASTn against the GreenGenes database, and compiled into each taxonomic level into both 'counts' and 'percentage' files.

Statistical analysis

Sample demographics and all outcome measures will be summarised by descriptive statistics and bivariate tests within the whole sample and between treatment groups (placebo vs TT) and demographic subgroups (eg, age, BMI). The correlations among outcomes and potential covariates (eg, demographic variables, dietary and lifestyle changes, concomitant medications) will be examined in a preliminary analysis. In order to examine the effects of the TT intervention, hierarchical linear modelling will be conducted to examine overall treatment group difference across time (ie, group effect), linear or non-linear change over time (ie, time effect) and group difference in this change (ie, group-by-time interaction). Models will be adjusted for identified covariates, thereby providing unbiased effect estimates. If present, missing data will be handled by Monte Carlo Markov chain (MCMC) multiple imputation.[53] A large number (eg, 200) of imputed data sets will be generated and then analysis results from each imputed data set will be combined together to make valid statistical inference. Gut microbiome data will be analysed designed to pinpoint similarities and differences of bacterial communities between the treated and untreated subjects.

Data for OxL and eCB will be expressed by descriptive statistics. If necessary, OxL and eCB data will be transformed to normality by an iterative process using imDEV V.1.4.2[54] and missing data will be handled by MCMC multiple imputation. Statistical significance of the means will be evaluated using two-tailed t-test at 0.05 alpha level. To account for multiple testing, the false discovery rate will be computed and reported for each comparison

using the q-value method.[55] A summary of the numbers of biochemicals (or called metabolites) that achieved statistical significance will be reported. OxL and eCB data will be combined with measurements for partial least squares discriminate analysis. To improve clustering performance, the reduced data matrix variables (complete data set) will be subjected to orthogonal signal correction using the procedures of Wehrens[56] and partial least squares discriminate analysis of variable importance plot filtered data set using the orthogonal score PLSR (partial least squares regression) component of the R 'pls' package.[57] Iterative rotations for uniform projection of discriminate variables onto a single latent variable will be done.

Data of microbiota OTU reads will be imported into R V.3.4.3, and all statistical analyses will be performed using the vegan and phyloseq packages unless specifically noted. OTU richness will be determined by Chao1 and the OTU evenness will be determined by several diversity indices (ie, Shannon, Simpson, Inverse Simpson and Fisher). Group differences in α-diversity (richness and diversity) will be evaluated by analysis of variance. Between-specimen diversity (β-diversity) will be assessed by the Bray-Curtis method and then visualised using non-metric multidimensional scaling. Group differences in β-diversity will be measured using permutational multivariate analysis of variance with 500 permutations. Group differences among taxa and genus-level OTUs will be assessed by pairwise comparisons on read counts using negative binomial Wald tests from the DESeq2 package. All tests will be corrected for multiple comparisons using the false discovery rate correction by Benjamini and Hochberg. Associations among selected variables will be assessed with Spearman correlations. The statistical significance is as determined at $p \leq 0.05$.

Data management and data collection

Researchers will keep information obtained in this study confidential except as required by law. All participant questionnaires, records and data will be coded and properly stored in locked cabinets. Only study investigators will have access to those files linking a person's study number to his/her name. Those forms will include Informed Consent Form, HIPAA Authorization Form, all recruitment questionnaires and all other related materials.

The principle investigator will be responsible for oversight of data management of the trial. A certified monitor from Clinical Research Institute, independent of research team and sponsor, will assist the principle investigator in monitoring data collection before the data sets are delivered to study biostatistician. Questionnaire data will be entered on various forms and then entered into the Excel database. Laboratory data will also be verified and entered into an Excel database. Data of eligibility, medical records, attrition rate and compliance rate will all be entered into an Excel database. Data queries including missing values will be referred to the principle investigator. The principle investigator will have access to the final trial data set and disclosure of contractual agreements. The principle investigator will also incorporate any correction or addition into the data sets. Finally, a clean data set without any identification will be generated and delivered to the biostatistician for statistical analysis.

Ethics

The study protocol and template consent forms have been reviewed and approved by the Bioethics Committee of the Texas Tech University Health Sciences Center. An informed consent form will be signed by a participant before enrolling in the study. Any modifications to the protocol, which may affect the conduct of the study, potential benefits to the study participants or their safety, will be reported to the ethics committee for all necessary amendments. All study-related information will be stored securely at the study site in local cabinets, in an area with limited access (databases will be secured with a password-protected access system).

Patient and public involvement

The patients and the public will not be involved in the design, assessment or conduction of this trial. Recruitment information is available on local newspaper, senior newsletter and clinics. This information will allow PMW to contact research coordinator if they are willing to participant. PMW with adverse outcomes will be followed up throughout their intervention and will be referred to experts. Feedback will be sought from all participants at the end of the study to assess burden of intervention and to help develop future trials.

Dissemination

The findings of this study will be submitted to a peer-referred journal in the areas of obesity or nutrition/functional food. Abstracts will be submitted to relevant national and international conferences.

The effectiveness of annatto-extracted TTs for obesity-associated parameters in postmenopausal obese women is still unknown. Our study, carried out at a single research centre with experience in conducting independent, investigator-initiated FDA-IND clinical trial, is intended to address a gap in the field and will test the safety and efficacy of annatto-extracted TT supplementation for antiobesity capacities in postmenopausal obese women.

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REFERENCES

1 New who report: deaths from noncommunicable diseases on the rise, with developing world hit hardest. *Cent Eur J Public Health* 2011;19:114–20.
2 Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. *Annu Rev Immunol* 2011;29:415–45.
3 Powell WS, Rokach J. Biosynthesis, biological effects, and receptors of hydroxyeicosatetraenoic acids (HETEs) and oxoeicosatetraenoic acids (oxo-ETEs) derived from arachidonic acid. *Biochim Biophys Acta* 2015;1851:340–55.
4 Watkins BA, Kim J, Kenny A, *et al*. Circulating levels of endocannabinoids and oxylipins altered by dietary lipids in older women are likely associated with previously identified gene targets. *Biochim Biophys Acta* 2016;1861:1693–704.
5 Kim J, Carlson ME, Kuchel GA, *et al*. Dietary DHA reduces downstream endocannabinoid and inflammatory gene expression and epididymal fat mass while improving aspects of glucose use in muscle in C57BL/6J mice. *Int J Obes* 2016;40:129–37.
6 Heemskerk MM, Giera M, Bouazzaoui FE, *et al*. Increased PUFA content and 5-lipoxygenase pathway expression are associated with subcutaneous adipose tissue inflammation in obese women with type 2 diabetes. *Nutrients* 2015;7:7676–90.
7 Möller K, Ostermann AI, Rund K, *et al*. Influence of weight reduction on blood levels of C-reactive protein, tumor necrosis factor-α, interleukin-6, and oxylipins in obese subjects. *Prostaglandins Leukot Essent Fatty Acids* 2016;106:39–49.
8 Horrillo R, González-Périz A, Martínez-Clemente M, *et al*. 5-Lipoxygenase activating protein signals adipose tissue inflammation and lipid dysfunction in experimental obesity. *J Immunol* 2010;184:3978–87.
9 Poeckel D, Funk CD. The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease. *Cardiovasc Res* 2010;86:243–53.
10 Chakrabarti SK, Cole BK, Wen Y, *et al*. 12/15-Lipoxygenase products induce inflammation and impair insulin signaling in 3T3-L1 adipocytes. *Obesity* 2009;17:1657–63.
11 Dobrian AD, Lieb DC, Cole BK, *et al*. Functional and pathological roles of the 12- and 15-lipoxygenases. *Prog Lipid Res* 2011;50:115–31.
12 Nunemaker CS, Chen M, Pei H, *et al*. 12-Lipoxygenase-knockout mice are resistant to inflammatory effects of obesity induced by Western diet. *Am J Physiol Endocrinol Metab* 2008;295:E1065–75.
13 Sears DD, Miles PD, Chapman J, *et al*. 12/15-Lipoxygenase is required for the early onset of high fat diet-induced adipose tissue inflammation and insulin resistance in mice. *PLoS One* 2009;4:e7250.
14 Börgeson E, Godson C. Resolution of inflammation: therapeutic potential of pro-resolving lipids in type 2 diabetes mellitus and associated renal complications. *Front Immunol* 2012;3:318.
15 Newman JW, Pedersen TL, Brandenburg VR, *et al*. Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects. *PLoS One* 2014;9:e111471.
16 Suzuki YJ, Tsuchiya M, Wassall SR, *et al*. Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: implication to the molecular mechanism of their antioxidant potency. *Biochemistry* 1993;32:10692–9.
17 Meydani M, Vitamin E. Vitamin E.. *Lancet* 1995;345:170–5.
18 Burdeos GC, Nakagawa K, Kimura F, *et al*. Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats. *Lipids* 2012;47:471–81.
19 Allen L, Ramalingam L, Menikdiwela K, *et al*. Effects of delta-tocotrienol on obesity-related adipocyte hypertrophy, inflammation and hepatic steatosis in high-fat-fed mice. *J Nutr Biochem* 2017;48:128–37.
20 Wong W-Y, Poudyal H, Ward LC, *et al*. Tocotrienols reverse cardiovascular, metabolic and liver changes in high carbohydrate, high fat diet-fed rats. *Nutrients* 2012;4:1527–41.
21 Chou T-W, Ma C-Y, Cheng H-H, *et al*. A rice bran oil diet improves lipid abnormalities and suppress hyperinsulinemic responses in rats with streptozotocin/nicotinamide-induced type 2 diabetes. *J Clin Biochem Nutr* 2009;45:29–36.
22 Das S, Mukherjee S, Lekli I, *et al*. Tocotrienols confer resistance to ischemia in hypercholesterolemic hearts: insight with genomics. *Mol Cell Biochem* 2012;360:35–45.
23 Qureshi AA, Qureshi N, Hasler-Rapacz JO, *et al*. Dietary tocotrienols reduce concentrations of plasma cholesterol, apolipoprotein B, thromboxane B2, and platelet factor 4 in pigs with inherited hyperlipidemias. *Am J Clin Nutr* 1991;53:1042S–6.
24 Watkins T, Lenz P, Gapor A, *et al*. Gamma-Tocotrienol as a hypocholesterolemic and antioxidant agent in rats fed atherogenic diets. *Lipids* 1993;28:1113–8.
25 Wong RSY, Radhakrishnan AK, Ibrahim TAT, *et al*. δ- and γ-tocotrienols induce classical ultrastructural apoptotic changes in human T lymphoblastic leukemic cells. *Microsc Microanal* 2012;18:462–9.
26 Budin SB, Othman F, Louis SR, *et al*. The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats. *Clinics* 2009;64:235-44.
27 Heng EC, Karsani SA, Abdul Rahman M, *et al*. Supplementation with tocotrienol-rich fraction alters the plasma levels of apolipoprotein A-I precursor, apolipoprotein E precursor, and C-reactive protein precursor from young and old individuals. *Eur J Nutr* 2013;52:1811–20.
28 Qureshi AA, Bradlow BA, Brace L, *et al*. Response of hypercholesterolemic subjects to administration of tocotrienols. *Lipids* 1995;30:1171–7.
29 Qureshi AA, Khan DA, Mahjabeen W, *et al*. Qureshi, dose-dependent modulation of lipid parameters, cytokines and RNA by ¦Ä-tocotrienol in hypercholesterolemic subjects restricted to AHA Step-1 diet, 2015 2015.
30 Qureshi AA, Khan DA, Mahjabeen W, *et al*. Impact of δ-tocotrienol on inflammatory biomarkers and oxidative stress in hypercholesterolemic subjects, Journal of clinical and experimental cardiology 2015;6.
31 Zaiden N, Yap WN, Ong S, *et al*. Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion. *J Atheroscler Thromb* 2010;17:1019–32.
32 Haghighat N, Vafa M, Eghtesadi S, *et al*. The effects of tocotrienols added to canola oil on microalbuminuria, inflammation, and nitrosative stress in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *Int J Prev Med* 2014;5:617–23.
33 Irandoost P, Ebrahimi-Mameghani M, Pirouzpanah S. Does grape seed oil improve inflammation and insulin resistance in overweight or obese women? *Int J Food Sci Nutr* 2013;64:706–10.
34 Aggarwal BB, Sundaram C, Prasad S, *et al*. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem Pharmacol* 2010;80:1613–31.
35 Shen C-L, Yang S, Tomison MD, *et al*. Tocotrienol supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: a 12-week randomized double-blinded placebo-controlled trial. *Osteoporosis International* 2018;29:881–91.
36 Craig CL, Marshall AL, Sjöström M, *et al*. International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc* 2003;35:1381–95.
37 Choquette S, Riesco Éléonor, Cormier Éric, *et al*. Effects of soya isoflavones and exercise on body composition and clinical risk factors of cardiovascular diseases in overweight postmenopausal

women: a 6-month double-blind controlled trial. *Br J Nutr* 2011;105:1199–209.
38 Pervez MA, Khan DA, Ijaz A, *et al*. Effects of delta-tocotrienol supplementation on liver enzymes, inflammation, oxidative stress and hepatic steatosis in patients with nonalcoholic fatty liver disease. *Turk J Gastroenterol* 2018;29:170–6.
39 Wong W-Y, Ward LC, Fong CW, *et al*. Anti-Inflammatory γ- and δ-tocotrienols improve cardiovascular, liver and metabolic function in diet-induced obese rats. *Eur J Nutr* 2017;56:133–50.
40 Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *Faseb J* 2008;22:659–61.
41 Mahipal A, Klapman J, Vignesh S, *et al*. Pharmacokinetics and safety of vitamin E δ-tocotrienol after single and multiple doses in healthy subjects with measurement of vitamin E metabolites. *Cancer Chemother Pharmacol* 2016;78:157–65.
42 Shen C-L, Mo H, Yang S, *et al*. Safety and efficacy of tocotrienol supplementation for bone health in postmenopausal women: protocol for a dose-response double-blinded placebo-controlled randomised trial. *BMJ Open* 2016;6:e012572.
43 Shen C-L, Wang S, Yang S, *et al*. A 12-week evaluation of annatto tocotrienol supplementation for postmenopausal women: safety, quality of life, body composition, physical activity, and nutrient intake. *BMC Complement Altern Med* 2018;18:198.
44 Tinsley GM. Proportional bias between dual-energy X-ray absorptiometry and bioelectrical impedance analysis varies based on sex in active adults consuming high- and low-carbohydrate diets. *Nutr Res* 2017;42:85–100.
45 Tinsley GM. Reliability and agreement between DXA-derived body volumes and their usage in 4-compartment body composition models produced from DXA and BIA values. *J Sports Sci* 2018;36:1235–40.
46 Shen C-L, Yang S, Tomison MD, *et al*. Tocotrienol supplementation suppressed bone resorption and oxidative stress in postmenopausal osteopenic women: a 12-week randomized double-blinded placebo-controlled trial. *Osteoporos Int* 2018;29:881–91.
47 Christodoulides C, Lagathu C, Sethi JK, *et al*. And Wnt signalling. *Trends Endocrinol Metab* 2009;20:16–24.
48 Heinonen S, Buzkova J, Muniandy M, *et al*. Impaired mitochondrial biogenesis in adipose tissue in acquired obesity. *Diabetes* 2015;64:3135–45.
49 Walhin J-P, Richardson JD, Betts JA, *et al*. Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. *J Physiol* 2013;591:6231–43.
50 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8.
51 Watkins BA. Endocannabinoids, exercise, pain, and a path to health with aging. *Mol Aspects Med* 2018;64:68–78.
52 Al-Obaide MAI, Singh R, Datta P, *et al*. Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. *J Clin Med* 2017;6. doi:10.3390/jcm6090086. [Epub ahead of print: 19 Sep 2017].
53 Enders CK. *Applied missing data analysis*. New York: Guilford Publications, 2010.
54 Capitanio D, Vasso M, De Palma S, *et al*. Specific protein changes contribute to the differential muscle mass loss during ageing. *Proteomics* 2016;16:645–56.
55 Adams GR, Hather BM, Baldwin KM, *et al*. Skeletal muscle myosin heavy chain composition and resistance training. *J Appl Physiol* 1993;74:911–5.
56 Wehrens R. Chemometrics with R multivariate data analysis in the natural sciences and life sciences 2011.
57 Mevik B-H, Wehrens R. The PLS package: principal component and partial least squares regression in R 2007;18:23.