Title: Effect of Tocotrienols on Bone Health: A Pilot Study

Funded by American River Nutrition (ARN),
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Background

Osteoporosis is a degenerative bone condition characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures especially of the hip, spine, and wrist.[1] Women are four times more likely to develop osteoporosis than men because of a decrease in estrogen level after menopause in conjunction with generally lighter and thinner bones.[2] Over half of postmenopausal women will experience a bone fracture as the result of osteoporosis.[2] Using the WHO definition [3], the National Osteoporosis Foundation has estimated that at present, approximately 8 million women in the United States have osteoporosis, and an additional 22 million are estimated to have osteopenia placing them at increased risk for osteoporosis.[4] Therefore, improving bodily function in order to maintain bone health in the postmenopausal woman has become a major public health strategy.

Clinical application of bone densitometry such as dual-energy x-ray absorptiometry (DXA) is generally used to measure bone mineral density (BMD) at the spine, hip, and femoral neck to predict the fracture risk and to monitor the natural progression of diseases that affect BMD or to monitor the therapeutic response to osteoporosis-specific treatments 1 to 2 years after inception of therapies.[5-8] Besides BMD, serum bone turnover markers (bone formation marker and bone resorption marker) can provide additional information and indication of the effectiveness of certain therapies[9] including dietary supplements for restoring BMD. [10, 11] Bone turnover markers have shown to be promising in predicting fractures in the elderly up to 2 years before the event. [12-14] A higher level of bone resorption rate was significantly associated with faster BMD loss.[12-14] Decreased vertebral BMD and increased bone turnover have approximately equal power to predict the risk of bone loss and osteoporosis-related fracture rate in postmenopausal women.[12-14] Therefore, efforts to decrease the occurrence of dilapidation fractures should concentrate on postmenopausal women diagnosed with low BMD. Using bone turnover markers will be the first step to monitor the early response of intervention in this population.

There is an increasing body of evidence suggesting the importance of oxidative stress in bone metabolism and bone loss.[15, 16] Recent cellular [17-20] and animal studies [21-32] including the ovariectomized rat model mimicking the bone loss in postmenopausal women all indicate that tocotrienols supplement may be beneficial to bone health in postmenopausal women due to its antioxidant capability. However, no study has ever been conducted to show the antioxidant role of tocotrienols, as dietary supplement, on the structure and/or function of the body in postmenopausal women. Therefore, the purpose of this pilot study is to evaluate the effects of dietary tocotrienols, as a dietary supplement, to determine its effects on bone turnover markers and related mechanisms in postmenopausal women.

Hypotheses

1. 12 weeks of tocotrienols supplements will modulate biomarkers important for supporting bone health in postmenopausal women compared to those only receiving placebo.
2. The modulation in bone turnover biomarkers associated with bone remodeling will be correlated with the changes in oxidative stress.

Specific Aims (SA)

1. **Determine if, compared to placebo, 12 weeks of tocotrienols supplementation is safe and feasible to the study subjects.** The dose-dependent effect of dietary tocotrienols on its bioavailability will be determined by measuring serum tocotrienol levels. The activities of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) in serum will be determined for monitoring liver function (safety). In addition, quality of life (SF-36 survey) will also be assessed. All outcome measures will be determined at baseline, 6, and 12 weeks.

2. **Determine if, compared to placebo, 12 weeks of tocotrienols supplementation will modulate bone formation and resorption biomarkers in the study subjects.** Bone formation biomarker (serum bone-specific alkaline phosphatase, BALP) and bone resorption biomarker (urine N-terminal telopeptide, NTX) will be measured at baseline, 6, and 12 weeks.

3. **Determine if, compared to placebo, 12 weeks of tocotrienols supplementation will modulate oxidative stress in the study subjects.** The oxidative stress biomarkers including 8-hydroxy-2'-deoxyguanosine (8-OHdG) and urinary F2-isoprostanes (also called 8-iso-PGF2α) will be determined at baseline, 6, and 12 weeks.

4. **Determine if, compared to placebo, 12 weeks of tocotrienols supplementation will modulate serum receptor activator of nuclear factor-kappaB ligand (sRANKL), osteoprotegerin (OPG), and insulin-like growth factor-I (IGF-I), and urine calcium in the study subjects.** The concentrations sRANKL, OPG, IGF-I, and calcium will be determined at baseline, 6, and 12 weeks.

Rationale:

Studies have demonstrated that RANKL and its decoy receptor OPG constitute a complex physiological mediator system involved in the regulation of bone resorption and may be responsible for the homeostatic mechanism of normal bone remodeling (Rogers and Eastell 2005). The serum RANKL, OPG (Marques et al. 2013, Josse et al. 2012), and IGF-I (Kanellakis et al. 2012), and urine calcium (Dawson-Hughes et al. 2015) are commonly used by bone researchers to monitor the changes in bone remodeling due to treatments.

References:

- Dawson-Hughes B, Harris SS, Palermo NJ, Gilhooly CH, Shea MK, Fielding RA, Ceglia L. Potassium Bicarbonate Supplementation Lowers Bone Turnover and Calcium Excretion in Older Men and Women: A Randomized Dose-Finding Trial. J Bone Miner Res. 2015 Nov;30(11):2103-11.
- Josse AR, Atkinson SA, Tarnopolsky MA, Phillips SM. Diets higher in dairy foods and dietary protein support bone health during diet- and exercise-induced weight loss in overweight and obese premenopausal women. J Clin Endocrinol Metab. 2012 Jan;97(1):251-60.
- Kanellakis S, Moschonis G, Tenta R, Schaafsma A, van den Heuvel EG, Papaioannou N, Lyritis G, Manios Y. Changes in parameters of bone metabolism in postmenopausal women following a 12-month intervention period using dairy products enriched with calcium, vitamin D, and phylloquinone (vitamin K(1)) or menaquinone-7 (vitamin K (2)): the Postmenopausal Health Study II. Calcif Tissue Int. 2012 Apr;90(4):251-62.
- Marques EA, Mota J, Viana JL, Tuna D, Figueiredo P, Guimarães JT, Carvalho J. Response of bone mineral density, inflammatory cytokines, and biochemical bone markers to a 32-week combined loading exercise programme in older men and women. Arch Gerontol Geriatr. 2013 Sep-Oct;57(2):226-33.
- Rogers A, Eastell R. Circulating osteoprotegerin and receptor activator for nuclear factor kappaB ligand: clinical utility in metabolic bone disease assessment. J Clin Endocrinol Metab. 2005 Nov;90(11):6323-31.

5. **Determine if, compared to placebo, 12 weeks of tocotrienols supplementation will modulate oxylipid and endocannabinoid analysis of serum in the study subjects.** The profiles of oxylipids and endocannabinoid will be determined using LC-MS/MS at baseline, 6, and 12 weeks.

Rationale:

Oxylipins include a myriad of species derived from n6 polyunsaturated fatty acids, such as arachidonic acid and linoleic acid, and from n3 polyunsaturated fatty acids, such as α-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid. Bioactivity among known oxylipins varies with structure and includes pro- and anti-inflammatory action, vasodilatory/relaxant properties, and mediation of cell growth and proliferation (Tourdot 2014, Demmig-Adams 2013). On the other hand, endogenous cannabinoid-receptor ligands (endocannabinoids) and over a dozen related metabolites now comprise the "endocannabinoid metabolome". The diverse (patho)physiological roles of endocannabinoids, the predictive/diagnostic utility of systemic endocannabinoid levels, and the growing interest in endocannabinoid-related pharmacotherapeutics mandate

a valid clinical protocol for processing human blood that does not jeopardize profiling of the circulating endocannabinoid metabolome (Wood 2008).

Tocotrienol, which belongs to vitamin E group of compounds, is a lipophilic antioxidant that widely used as antioxidant supplements. Emerging evidence indicates that tocotrienols may influence cellular signaling by protecting against the propagation of lipid peroxidation in vivo (Atkinson 2008). Thus, this specific aim is to determine if tocotrienol supplementation would modulate indices of lipid peroxidation in subjects via interacting with enzymes involved in oxylipin and endocannabionoid.

References:

- Atkinson J, Epand RF, Epand RM. Tocopherols and tocotrienols in membranes: a critical review. Free Radic Biol Med. 2008 Mar 1;44(5):739-64. Review.
- Demmig-Adams B, Cohu CM, Amiard V, Zadelhoff G, Veldink GA, Muller O, Adams WW 3rd. Emerging trade-offs - impact of photoprotectants (PsbS, xanthophylls, and vitamin E) on oxylipins as regulators of development and defense. New Phytol. 2013;197(3):720-9.
- Tourdot BE, Ahmed I, Holinstat M. The emerging role of oxylipins in thrombosis and diabetes. Front Pharmacol. 2014 Jan 7;4:176.
- Wood JT, Williams JS, Pandarinathan L, Courville A, Keplinger MR, Janero DR, Vouros P, Makriyannis A, Lammi-Keefe CJ. Comprehensive profiling of the human circulating endocannabinoid metabolome: clinical sampling and sample storage parameters. Clin Chem Lab Med. 2008;46(9):1289-95.

Study Sites:

a. TTUHSC, Lubbock, TX will be the main study site including the following activities:

1. Clinical Research Institute (CRI) for study participant recruitment, initial screening, and the collection of fasting blood and urine samples, and storage and dispense of all study agents and Ca/vitamin D.

2. Lubbock Wellness Center, 4009 19th St, Suite E, Lubbock, TX, for BMD measurement by a certified radiology technician. We have used this facility to screen postmenopausal women in a recent study.

3. Biochemical analyses of blood and urine samples for pre-screening, safety monitoring purposes, and urine calcium will be performed at a certified clinical laboratory (Quest Diagnostics, Irving, TX).

4. Analyses of serum and urine biomarkers will be conducted at the PI's laboratory, Division of Comparative Pathology, TTUHSC, Lubbock.

5. Analyses of serum tocotrienols will be conducted using HPLC method in the laboratory of Dr. Shu Wang (Co-Investigator) at Texas Tech University Department of Nutritional Sciences.

6. Analyses of serum oxylipid, endocannabinoid, and other metaolomic analyses will be conducted using LC-MS/MS metabolomics measurements in the Metabolon, Inc, located in Durham, NC. Metabolon is a pioneer and leader in metabolomics (www.metabolon.com). Metabolon office scalable, customizable metabolomics research service to academic and commercial investigators. The staff at Metabolon has written and contributed to over 550 peer-reviewed journal publications in the past 15 years.

Study population and recruitment This study will be conducted in the TTUHSC OB/Gyn clinic, Lubbock, TX and the surrounding area. This study will consist of postmenopausal women (1-10 years after menopause), ambulatory, and irrespective of ethnicity/races. Direct person to person solicitation in the OB/Gyn clinic, advertisement through flyers, non-solicited e-mail system, campus announcement, local radio, newspapers,

and TV scripts will be used to recruit potential subjects. Historically, the accrual rate is about 50%, therefore, we need to recruit at least 150 subjects for BMD screening in order to have n=78 for the proposed study. We will require approximately 78 patients, with 26 assigned to each of three study groups.

Screening Assessment of potential participants will involve a brief survey on menstrual history, medication for osteoporosis treatment, age, availability for the study period, most recent BMD result if available, though a phone interview or medical record review. This pre-screening procedure will identify the potential participants who are interested in the research and are qualified for further screening. After telephone pre-screening, potential subjects will attend an informed consent session. After signing consents, participants will complete a detailed questionnaire to collect demographic, health, and dietary information. Then, the potential participants will be invited for BMD screening and follow-up fasting blood screening visits.

Inclusion criteria

1. Postmenopausal women with no menses at least 1 year.
2. Bone mass with BMD T-score < -2.5 (non-osteoporotic) at measured site (spine and/or hip).[3]
3. Normal laboratory evaluation, thyroid function: TSH > 0.3 and < 5.0 mU/L; hepatic function: bilirubin ≤ 2.0 mg/dl; SGOT (also called AST)/SGPT (also called ALT) < 3x upper limit of normal; renal function: serum creatinine ≤ 2.0 mg/dl; BUN less than 1.5 times upper limit of normal; serum calcium, and alkaline phosphatase: within normal ranges. HbA1c < 7.0%. This critera has been used in our previous GTP and Tai Chi study.
4. Serum 25-hydroxy vitamin D ≥ 20 ng/mL.
5. Age 40 and older

Exclusion criteria

1. History of, or evidence for, metabolic bone disease including recent fractures (other than low BMD).
2. Having received medication (calcitonin, raloxifene, or systemic glucocorticoids) within 3 months before the start of the study.
3. Having bisphosphonate within 12 months before the start of the study.
4. Having hormone/hormone-like replacement therapy within 3 months before the initiation of the study.
5. . History of cancer within the last 5 years.
6. History or evidence of endocrine disease or malabsorption syndrome that would be a contraindication to the investigation of tocotrienols' absorption.
7. Uncontrolled diabetes mellitus defined by an HbA1c of ≥ 7% in the last 3 months.
8. History of statin or other drug for cholesterol-control within 3 months before the start of the study.
9. Alcohol intake greater than "moderate" (one drink per day) or use of nonsteroidal anti-inflammatory drugs on a regular basis.
10. Cognitive impairment, depression or other medical/eating disorders, likely to move during the trial, lack of transportation, distance from the study site, or unavailable at sample collection times.
11. Smoking > 10 cigarettes/day.
12. Unwilling to accept randomization.
13. Subjects on anticoagulants because an interaction with tocotrienols may exist.

Study groups Qualified subjects will be randomly assigned to one of the 3 study groups (placebo, LowT3, and HighT3) (see table below).

Placebo group: Subjects will take 860 mg olive oil daily (430 mg olive oil softgel × 2 softgels/day, morning and evening) for 12 weeks. A subgroup of 15 subjects will also participate in a pharmacokinetic measurement.

LowT3 group: Subjects will take 430 mg olive oil softgel in the morning and 300 mg tocotrienols softgel in the evening daily for 12 weeks. Both the morning and evening doses will take place after a meal (breakfast and dinner). A subgroup of 15 subjects will also participate in a pharmacokinetic measurement.

HighT3 group: Subjects will take 600 mg tocotrienols (300 mg tocotrienols softgel × 2 softgels/day, morning and evening) for 12 weeks. Both the morning and evening doses will take place after a meal (breakfast and dinner). A subgroup of 15 subjects will also participate in a pharmacokinetic measurement.

	Placebo Group	300mg T3* Study Group (LowT3 group)	600mg T3 Study Group (HighT3 group)
Morning Bottle	430 mg olive Oil	430mg olive Oil	300mg T3
Evening Bottle	430 mg olive Oil	300mg T3	300mg T3

(* T3 indicates tocotrienols).

For ethical reasons, calcium (500 mg elemental Ca)/vitamin D (400 IU) supplement will be provided to all participants. Compliance of Ca/vitamin D supplement intake will be determined by pill count.

Randomization In order to minimize the consequent bias, a stratified block randomization method with varying block sizes of 3 and 6 will be employed to assign participants to one of the 3 groups: placebo, LowT3, and HighT3 groups. The strata will be constructed based on BMI (≥30 or <30 kg/m^2) and age (≥50 or <50 years old).

Blinding/unblinding All investigators, coordinators, and measurement and site personnel will be blinded to intervention allocation throughout this study. The PI will determine when unblinding of a study participant's assignment code is necessary. The blind may be removed on a participant because of an adverse event or other legitimate reason.

Study Visits and Data Collection (what's happening to the subjects)

We will send a reminder letter/text/e-mail including brief purpose of visit along with date and place to the subject about a week before each visit. Then, we will send a reminder (text, e-mail, or phone) to the subject on the day before each visit.

Visit 1: Informed consent session (informed consent form, medical history form) and Q&A at TTUHSC.

- Meet with a member of the study team to discuss the study and give informed consent.
- Sign informed consent form and HIPAA form.
- Complete medical history form about demographic info, medical history, health status, current medication (if any), menstrual history, physical activity, etc.

Visit 2: DXA bone screening at Lubbock Wellness Center, 4009 19th St, Suite E, Lubbock, TX, for BMD measurement by a certified radiology technician.

Inclusion Criteria: Bone mass with BMD T-score between 0.5 and -2.5 at the spine and/or hip.

Qualified subjects will be invited for food-fasted blood collection visit (Visit 3).

Subjects will be given the results of their bone scan at the end of the study. However, if a subject no longer qualifies to be in the study, the PI will notify her by phone and she will be mailed her bone scan results at the time of exclusion.

Visit 3: Food-fasted blood collection at Clinical Research Institute for blood chemistry screening (liver, kidney, thyroid hormone, HbA1c, and 25-OH vitamin D laboratory test). All blood samples of subjects will be sent to Quest Diagnostics Laboratory for blood chemistry profile, TSH, HbA1c, and 25-OH vitamin D (Irving, TX).

Inclusion Criteria:

- TSH > 0.3 and < 5.0 mU/L

- hepatic function: bilirubin ≤ 2.0 mg/dl; SGOT/SGPT < 3x upper limit of normal;
- renal function: serum creatinine ≤ 2.0 mg/dl; BUN less than 1.5 times upper limit of normal;
- serum calcium, and alkaline phosphatase: within normal ranges.
- HbA1c < 7.0%;
- serum 25-hydroxy vitamin D ≥ 20 ng/mL.

Qualified subjects will be invited for baseline data collection visit (Visit 4).

Subjects who are excluded because of abnormal vitamin D or TSH levels will be by the PI by phone and mailed a letter that contains these results. If a subject's PCP prescribes vitamin D supplements and thyroid medication, she will be given the opportunity to return for rescreening 2 months after taking the vitamin D supplement and thyroid hormone medication.

Visit 4 (baseline visit- no more than 3 months from visit 3) at Clinical Research Institute

- Have blood drawn and give a urine sample.
- Measure body weight and height.
- Complete a quality of life survey (SF-36).
- Complete a physical activity survey (Godin Leisure-time Exercise Questionnaire).
- Complete a diet questionnaire (FFQ).
- Assign into one of three study groups:
  - Placebo group: 430 mg olive oil softgel twice daily. On in the morning and one in the evening.
  - Low T3 group: 430 mg olive oil softgel in the morning and 300 mg tocotrienol concentrate softgel in the evening daily
  - High T3 group: 300 mg tocotrienol concentrate softgel twice daily. One in the morning and one in the evening.
- Receive a 6-week supply of the pills, along with verbal information about the pills. Also receive a 6-week supply of calcium + vitamin D tablets. Instruct to take 1 study pill twice daily for 6 weeks (one after breakfast and one after dinner) and also take 1 calcium + vitamin D tablet after a meal.
- Ask to report any problems, issues, or concerns that have occurred since your last visit.

For those in the subgroup of subjects for a pharmacokinetic measurement, when she comes for fasting blood collection, her fasting blood will be drawn first; then she will be fed a meal catered/prepared by our research team. After her meal, she will ingest a full strength of respective daily study supplements (placebo, 300 mg tocotrienols, and 600 mg tocotrienols) and she will not take any study supplement for the rest of day. Two hours after her daily study supplement is ingested, she will have a 2-teaspoon blood draw and give a urine sample.

Visit 5 (midpoint visit- 5 to 7 weeks from visit 4) at Clinical Research Institute.

- Have blood drawn and give a urine sample.
- Receive a 6-week supply of the pills, along with verbal information about the pills. Also receive a 6-week supply of calcium + vitamin D tablets. Instruct to take 1 study pill twice daily for 6 weeks (one after breakfast and one after dinner) and also take 1 calcium + vitamin D tablet after a meal.
- Bring leftover study supplement softgels and calcium + vitamin D tablets to be counted by research staff.
- Report problems, issues, or concerns, if any.

For those in the subgroup of subjects for a pharmacokinetic measurement, when she comes for fasting blood collection, her fasting blood will be drawn first; then she will be fed a meal catered/prepared by our research team. After her meal, she will ingest a full strength of respective daily study supplements (placebo, 300 mg tocotrienols, and 600 mg tocotrienols) and she will not take any study supplement for the rest of day. Two hours after her daily study supplement is ingested, she will have a 2-teaspoon blood draw and give a urine sample.

Visit 6 (end of study visit- 11 to 13 weeks from visit 5) at Clinical Research Institute

- Have blood drawn and give a urine sample.
- Measure body weight and height.
- Complete a quality of life survey.
- Complete a physical activity survey.
- Complete a diet questionnaire.
- Bring leftover study supplement softgels and calcium + vitamin D tablets to be counted by research staff.
- Report problems, issues, or concerns, if any.

For those in the subgroup of subjects for a pharmacokinetic measurement, when she comes for fasting blood collection, her fasting blood will be drawn first; then she will be fed a meal catered/prepared by our research team. After her meal, she will ingest a full strength of respective daily study supplements (placebo, 300 mg tocotrienols, and 600 mg tocotrienols) and she will not take any study supplement for the rest of day. Two hours after her daily study supplement is ingested, she will have a 2-teaspoon blood draw and give a urine sample.

Study supplements supply, masking, and administration

Supply: All study agents including 430 mg olive oil and 430 mg T3 from the same lot will be provided from American River Nutrition (ARN, 31 Campus Plaza Road, Hadley, MA).

The placebo softgel contains 430 mg of olive oil.

The tocotrienol softgel contains 430 mg of 70% tocotrienol concentrate, DeltaGold® Tocotrienol 70% **(which is equivalent to 300 mg total tocotrienols).** Annatto tocotrienol (DeltaGold ®) is developed by American River Nutrition, Inc. It has the highest concentration of delta- and gamma-tocotrienols that is commercially available and is tocopherol-free. Annatto tocotrienol is nature-sourced, patent-protected, and extracted through a solventless process (see attached documents for product formula of placebo and study supplement).

The softgel shell ingredients for both placebo and tocotrienol softgel are gelatin type B, glycerin at 99.7%, purified water, and dark natural caramel color. Also attached is the certificate of analysis for the olive oil that will be used in the placebo.

Masking: Placebo softgels will be of the same appearance (size and color) as the T3 softgels

Administration:

American River Nutrition will package the study supplements in light-protective, fully labeled, and child-resistant bottles, and dispensed double-masked. Study supplements will be distributed by nurse coordinators to participants at baseline, 6 weeks, and 12 weeks according to their identification numbers.

We will use different colored bottles to distinguish the morning bottle and evening bottle. They will have the same kind of softgels in both bottles for placebo and 600 mg T3 groups.

Educate the subjects so they will not take double pills if they forget to take either one. Just skip the pill (softgel) and continue with the same pattern for morning and evening softgels.

Subjects will take 1 softgel twice daily. At each 6-week follow-up visit, each participant will be asked to return unused softgels for a pill count. In addition, concentrations of tocotrienols composition in serum will be determined by HPLC analysis to quantify T3 compliance at various time points (baseline, 6, and 12 weeks).

Safety Monitoring:

According to GRAS reports of tocotrienols, liver toxicity in humans is minimal based on the tocotrienol doses we proposed for this study. Even though there is no current evidence showing adverse effects of tocorienols

on liver function, we will monitor the liver function by measuring SGOT (also called AST) and SGPT (also called ALT) at baseline, 6 weeks and 12 weeks as suggested by the FDA for a different study. Additionally, we will follow FDA's previous recommendation for a different study (Shen et al. 2010) as described below for the subjects' safety:

- "Withhold study agent for grade 1 (≥ 1.25-2.5, upper limit of normal, ULN) elevation in ALT or AST until recovery to normal. For grade 2 (>2.5-5.0, ULN), 3 (>5.0-10.0 ULN), and 4 (>10 ULN) elevations, discontinue drug and monitor liver function tests until resolution. A subject that has agent withheld for a grade 1 elevation may be re-continued on study after her ALT or AST value is at least ULN or less. A subject discontinued for a grade 2, 3 or 4 elevation should be monitored until ALT is at or below the ULN, but cannot to be placed back onto the study." *Source: http://livertox.nih.gov/Severity.html

*The following levels are used to assess severity, with the values expressed as multiples of the upper limit of the normal range (ULN).

FEATURE	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
ALT	<1.25	1.25-2.5	>2.5-5.0	>5.0-10	>10
AST	<1.25	1.25-2.5	>2.5-5.0	>5.0-10	>10

Measurement of serum tocotrienols: compliance assessment

We will measure the concentration of tocotrienols in serum at baseline, after 6 and 12 weeks.
Rationale: Normal serum levels of tocotrienols, nor toxic serum levels of tocotrienols, have been established. However, based on the study of Yap et al. (2011) that Human subjects taking 300 mg mixed tocotrienols had peak serum level Cmax of 5 uM with no apparent adverse effects and the half-life for blood elimination was 2.3 – 4.4 h; and according to the animal (rat) studies of Tasaki et al. (2008 and 2009), that demonstrated that at as long as 104 weeks the no-observed-adverse-effect level is 0.4% diet, or 303mg/kg/d for males rats and 472 mg/kg/d for female rats. Equal doses in human would be 300 x 70 = 21 g tocotrienols intake/day.

Therefore, with a maximum dose of 600 mg total tocotrienols in our study, we don't expect to see a toxic level in the serum tocotrienols.

Reference:
Yap SP, Yuen KH, Wong JW. Pharmacokinetics and bioavailability of alpha-, gamma- and delta-tocotrienols under different food status. J Pharm Pharmacol. 2001 Jan;53(1):67-71.

Tasaki M, Umemura T, Inoue T, Okamura T, Kuroiwa Y, Ishii Y, Maeda M, Hirose M, Nishikawa A. Induction of characteristic hepatocyte proliferative lesion with dietary exposure of Wistar Hannover rats to tocotrienol for 1 year.
Toxicology. 2008 Sep 4;250(2-3):143-50. doi: 10.1016/j.tox.2008.07.003. Epub 2008 Jul 15.

Tasaki M, Umemura T, Kijima A, Inoue T, Okamura T, Kuroiwa Y, Ishii Y, Nishikawa A. Simultaneous induction of non-neoplastic and neoplastic lesions with highly proliferative hepatocytes following dietary exposure of rats to tocotrienol for 2 years. Arch Toxicol. 2009 Nov;83(11):1021-30.

Justification for selection of supplement dosages.

Cellular [17-20] and animal studies [21-31] suggest that tocotrienols may promote bone health by maintaining BMD and reducing the risk of fracture, as shown in enhanced osteoblastic and suppressed osteoclastic activities in bone remodeling, due to it's antioxidant capacity. [32-34] However, the influence of tocotrienols on the human bone remodeling process is still unclear, because most studies used cells and animal models.[32-34]

Based on the letter from FDA (Appendix A) and report of independent and collective critical scientific evaluation by an Expert Panel (Appendix B), the study agent (T3, DeltaGold Annatto Tocotrienol) is GRAS (Generally Recognized as Safe). According to the safety evaluation and clinical trials, the ranges of T3 used in the trials are from 25 mg to 800 mg per day in humans (Appendix C-G).

A 13-week study by H. Nakamura and colleagues at the National Institute of Health Sciences (Japan) of tocotrienols' toxicity in rats found significant changes in several blood components, increases in liver weights and (in females) reductions in ovary and uterus weights, depending on the dosages. The authors estimated the no-observed-adverse-effect level (NOAEL) to be 120 mg per kg of body weight per day for males and 130 mg per kg of body weight per day for females. Since effects on the blood components were observed in all cases with non-placebos, a no-observed-effect level (NOEL) could not be determined.[35]

Based on (i) the consumption of T3 in animal studies, [27, 30, 31] (rats were given at 60 mg/kg body weight) showing osteoprotective impacts and (ii) metabolic ratio of human (70 kg) to rat (250 g) is 70:1, the expected effective dose in human for T3 consumption on potential osteoprotective effect is approximately 1000 mg T3 daily. In this study, we will test two dosages of T3 (300 and 600 mg) for 12 weeks.

We have included additional published articles to support the justification of tocotrienols doses for this study.

Justification for selection of study length and bone turnover markers. In general, it will take 1-2 years after intervention (especially dietary treatments) in order to detect the effects of supplements on BMD by DXA. Since this is a 12-week study (relatively short-term), DXA is not able to see the difference due to the supplements. On the other hand, bone turnover markers are more sensitive than DXA and can detect treatment and supplement effects in a shorter period of time (i.e., 12 weeks). Therefore, we proposed the use of bone turnover markers in this study and not DXA.

Outcome measures

Biomarkers will be assessed at baseline, 6 weeks, and 12 weeks

Primary outcome measure: urine bone resorption marker (i.e., NTX) will be measured using ELISA kits.
Secondary outcome measures: **(i)** serum bone formation marker (i.e., BALP) will be measured using ELISA kits, **(ii)** serum tocotrienols will be measured by high-pressure liquid chromatography, and **(iii)** urinary F2-isoprostanes or 8-OHdG will be measured using ELISA kits

We will monitor liver function (SGOT and SGPT) at the follow-up 6-week and 12-week visits.

In this study, we will measure urine calcium and explore a possible molecular mechanism to explore how the study interventions may have an impact in bone remodeling via assessing circulating RANKL and its decoy receptor OPG, serum IGF-I, and urine calcium at baseline, 6, and 12 weeks of intervention. The concentrations of RANKL, OPG, and IGF-I in serum will be determined using respective ELISA kits at Dr. Shen's laboratory. The urine calcium levels will be measured at Quest Diagnostic Laboratory.

In addition, we will measure creatinine concentration in the urine samples at baseline, 6, and 12 weeks of intervention. The level of urinary creatinine will be used normalize urinary NTX and thus final bone resorption marker will be presented as NTX/creatinine.

The following outcomes will be assessed at baseline and 12 weeks (end):

Quality of life will be assessed by SF-36 (version 2) survey.[36-38]

Food intake assessment will be conducted by food frequency questionnaires (FFQ) at baseline and the end of study. In the food frequency questionnaires, we will include vitamin E-related consumption items and analyze vitamin E consumption at both collection times.

Physical activity (PA) survey will be administered by Godin Leisure-time Exercise Questionnaire at baseline and the end of study.

Statistical Methods

Sample Size Calculation and Power Analysis In this study, we propose to **enroll 78 patients, 26 in each of the 3 arms.** Using Power Analysis and Sample Size software (PASS 11), a sample size of 22 in each of the placebo, LowT3 and HighT3 groups would achieve a power of 81% to detect a clinically significant difference between the group mean of serum NTX levels at the end of 12 weeks. This calculation was based on an analysis of covariance (ANCOVA), adjusted for three covariates: baseline NTX, age, and BMI.

It was assumed that final NTX levels (primary outcome) will have a mean 0.45 ng/ml in the control group and that all three groups will have a standard deviation of 0.15 ng/ml.[26] In this context, clinically significant was defined to be a relative decrease of 15% and 30% (0.38 and 0.32 ng/ml) in the LowT3 and HighT3 groups, respectively. It was assumed that the considered covariates and intervention effect will explain at least 20% of the variability with NTX since age alone exhibits a Pearson correlation coefficient of 0.45 (R^2=0.20) with NTX.[39] Taking into consideration an expected attrition rate of 15%, n=26 will be recruited for each group, a total 78 participants to start the study.

Analysis Plan In the study, the primary outcome is NTX at 12 weeks and intervention is tocotrienals involves three levels: placebo, low dose, and high dose. The outcome and other cofactors will be summarized using appropriate summary measures as per the type and distribution of the variable according to each intervention level. The baseline cofactors will be compared among the three groups using one way analysis of variance (ANOVA) or Fisher's exact test/Chi square test as appropriate.

To assess the effect of intervention on primary outcome (urine NTX) at 12 weeks, ANCOVA will be conducted after adjusting the baseline NTX and other significant cofactors if any. A *post hoc* comparison among the three intervention groups will be made using Tukey's test if there is a significant effect of intervention. The assumptions of the ANCOVA model will be assessed and appropriate transformations will be made if needed for inferential data analysis. An intention-to-treat analysis will be carried out for efficacious end point. The results will be summarized using mean differences and their 95% confidence intervals.

Similarly, the same analysis approach for NTX will be employed in serum BALP and tocotrienols, and urinary F2-isoprostanes. For secondary measures, the Pearson/Spearman rank correlation between change in NTX and change in the secondary biomarker (BALP, tocotrienols, and F2-isoprostane) will be computed and reported in tabular form. To access quality of life, the ordinal score on the SF-36 test will be used, and therefore the Kruskal-Wallis test will be employed to detect any significant differences in the distribution of this score amongst the three groups followed by post hoc multiple comparisons if needed. P-value less than 5% will be considered as significant results. All the statistical analysis will be carried out using SAS 9.3.

Potential Risks

Tocotrienols is a dietary supplement available over the counter at many stores. It is generally recognized as safe (GRAS). However, there have been reports of the following potential side effect:

- Rashes
- Trouble breathing
- Facial Swelling

- Itching
- Chest Pain
- Headaches

Additionally, the risks of a blood draw may include:

- Lightheadedness, dizziness or fainting at the sight of blood
- Pain or discomfort, bleeding, bruising at the puncture site; and
- Rarely, infection or redness at the puncture site

DXA related risk:

The technique of dual energy x-ray absorptiometry (DEXA) is a well-established method for the determination of bone mineral density. The total amount of radiation you receive during this research is about 45.7 μSv or less than the natural background exposure of 10 days.

Benefits There is no benefit to the subject.

Reimbursement for transportation

Subject will be given thirty dollars ($30) to help with transportation costs. We will mail half of this amount after Visit 4 is completed, and the other half after Visit 6 is completed.

For those in the subgroup of subjects for a pharmacokinetic measurement, the subject will be given an additional ninety dollars ($90) to compensate her time involved in this study. We will mail her 1/3 ($30) of this amount after Visit 4 is completed; 1/3 ($30) after Visit 5 is completed; and the remaining 1/3 ($30) after Visit 6 is completed.

Confidentiality Measures

We will keep subjects' involvement in this research study confidential to the extent permitted by law. In addition to the staff carrying out this study, others may learn that subjects are in the study. This might include federal regulatory agencies such as the Food and Drug Administration (FDA) and the Office for Human Research Protection (OHRP), Texas Tech University Health Sciences Center (TTUHSC) representatives, and the TTUHSC Institutional Review Board (a committee that reviews and approves research). These people may review and copy records involving this research. A copy of this document may be placed in your medical record.

The sponsor of the study, American River Nutrition Inc., can also review the study records, but the sponsor is not allowed to remove or copy information that identifies subjects by name.

Study results that are used in publications or presentations will not use subject's name.

Key Personnel

Dr. **Chwan-Li (Leslie) Shen,** PhD (Principal Investigator) Associate Professor of Pathology, School of Medicine, TTUHSC, Lubbock, TX. Dr. Shen (principal investigator) has more than 20 years of research experience in bioactive components and bone using cellular, animal, and human models. Dr. Shen has a broad expertise in nutrition, exercise, and bone health. Her research includes investigation of effects of dietary supplements and exercise on bone heath by translating the findings from animal models to human population. She is the principal investigator of two NIH/NCCAM funded projects on bone health including animal study (nutrition) and human clinical trial (nutrition and exercise). Currently, she is the sponsor for FDA IND (number: 77,470) to study the effects of green tea polyphenols on bone health. Dr. Shen is a fellow of United States Bone and Joint Decade (USBJD) to receive training to conduct successful clinical studies on musculoskeletal diseases. She is also a Certified Clinical Research Professional (C.C.R.P.) with Society of Clinical Research

Associates (SoCRA). She has translational/clinical research experience in nutrition and exercise intervention on chronic diseases, including osteoporosis, osteoarthritis, and diabetes.

Dr. **Ima N. Soelaiman**, PhD (co-investigator), Professor of Pharmacology, Medical Center, Universiti Kebangsaan Malaysia. Dr. Soelaiman has 18 years of experience in studies on tocotrienols and bone metabolism in experimental osteoporosis and related human studies. She will participate in experimental design, study quality control, and data interpretation.

Dr. **Chun Guo**, (co-investigator) The First Affiliated Hospital, Hunan University of Chinese Medicine, Hunan China. Dr. Guo will perform laboratory analysis of collected samples.

Dr. **Carol K. Felton,** MD (collaborator), Associate Professor of Obstetrics and Gynecology, School of Medicine, TTUHSC, Lubbock, TX. Dr.Felton is a Certified Menopause Practitioner with expertise in the area of menopause, osteoporosis, and related health concerns. Her responsibility is to serve as a study physician and to assist in patient recruitment, screening, safety monitoring, and medical compliance.

Dr. **Shengping Yang**, PhD (collaborator), Assistant Professor of Pathology, School of Medicinie, Texas Tech University Health Sciences Center, Lubbock, TX. Dr. Yang's experience in biostatistics will assist in experimental design, data analysis, results interpretation, and preparation of reports.

Dr. **Barbara C. Pence**, PhD, CCRP, Professor of Pathology, School of Medicine TTUHSC, Lubbock, TX. Dr. Pence will serve as a consultant for this study to provide consultation for IRB and other regulatory issues.

Dr. **Shu Wang**, PhD, Associate Professor of Nutritional Sciences, Texas Tech University. Dr. Wang will perform laboratory analysis of serum tocotrienols using HPLC method.

Clinical Research Institute (CRI). The goal of the CRI is to increase sponsored (both industry and federal) investigator-initiated clinical trials conducted at TTUHSC. CRI staff is available as a resource to TTUHSC faculty beginning with pre-study activities and contract negotiation through study completion and close out. The CRI typically executes more than 20-30 NIH-funded and industry-sponsored clinical studies per year and oversees many more. In these completed and on-going clinical trials, the CRI has demonstrated its ability to provide high quality clinical research service in (a) protocol/project development, (b) project management including IRB preparation and submission, entire IRB support, recruitment, consenting, clinical research coordination, account management, dietary supplement dispensing and management, etc., and (c) data management and biostatistics consultation.

In the proposed study, dedicated nurse research coordinators from CRI will be responsible for medical history pre-screening, consenting, drawing blood, collecting urine samples, dispensing study agents (placebo/tocotrienols) and Calcium/Vitamin D supplement, IRB support, etc.

Timeline The proposed study period is two years. In Year 1, the first three months will be occupied with IRB approvals, the next 4 months will be used for recruitment, screening, randomization, and baseline assessment and followed by a 12-week intervention period. In Year 2, all sample analyses will be conducted and data analyses will be completed by the end of the project.

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