On-line Supplemental Material

Brief Research Report: Interaction of Vitamin E Isoforms on Asthma and Allergic Airway Disease

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Detailed Methods

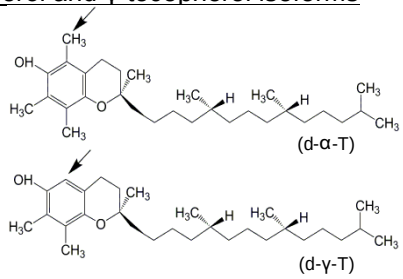
Human Study: We conducted a study designed and powered to investigate the association of dietary and plasma antioxidants on adult incident asthma, which has been previously reported.[1, 2] In brief, we used a nested case-control study design to assess the role of dietary antioxidants, including vitamin E on asthma inception. We followed women from the Shanghai Women's Asthma and Allergy Study who were between ages 40 and 70, who did not have a history of asthma at baseline. The study population from whom the nested cohort was drawn included 65,372 women with no previous diagnosis of asthma, followed over 8 years.[1,2] Incident asthmatics were ascertained prospectively by gold standard methacholine or airway reversibility testing of symptomatic women who were matched to two asymptomatic controls. The nested study was conducted including all available confirmed asthma cases (n = 150) matched to two control subjects (n = 294), who reported no new- onset symptoms of asthma. Matching variables were age, date of baseline biospecimen collection, body mass index (BMI), and self-reported smoking status. Matching on sex was not necessary as all study participants were women. Plasma concentrations of tocopherols, including the isoforms α- and γ- tocopherol were measured from samples collected prior to disease onset. We previously reported that doubling of α-tocopherol concentrations was associated with 50% decreased risk of incident asthma (adjusted Odds Ratio [OR]; 95% confidence interval [OR; 95%CI], α-tocopherol OR= 0.52; 95%CI: 0.32-0.84).[2] We did not see a statistically significant relationship with γ-tocopherol. The food isoform content of α-and γ-tocopherol are known to vary widely. In humans dietary oils influence plasma tocopherol levels. As countries assume western lifestyles, diets change, including increased consumption of soybean and corn oils high in γ-tocopherol[3, 4]. In contrast to

high levels of γ-tocopherol in soybean oil, γ-tocopherol is low in other oils, such as sunflower oil, safflower oil, and olive oil, that are used in several European and Mediterranean countries.[5, 6] The study was approved by the Vanderbilt IRB, collaborating institutions, and patients provided informed consent.

Regression modeling: To investigate the joint relationship of the two vitamin E isoforms and potential effect modification on asthma risk, we studied the interaction of α- and γ-tocopherol with a cross-product term in the multivariable conditional logistic regression model while adjusting for covariates that were selected *a priori* (work place smoking exposure, fat intake and exercise in the past 5 years).[1, 2] α- and γ-tocopherol levels were examined with non-linear term (using restricted cubic splines and three knots) to account for non-linear relationships. In addition, for graphical representation of the asthma odds ratio, we regressed asthma log odds ratio against levels of α- and γ-tocopherol. In these analyses the α-tocopherol levels were modeled with restricted cubic splines while the γ-tocopherol levels were categorized by tertiles (Figure 1). A likelihood ratio test (difference between two nested models) was used to assess the interaction between α- and γ-tocopherol on the asthma odds ratio.

Table 1

A α-tocopherol and γ-tocopherol isoforms



B γ-Tocopherol-supplemented Diet.

	g/kg diet
Casein, high nitrogen	200
L-Cystine	3
Sucrose	100
Cornstarch	397
Dyetrose	132
Corn Oil	70
Cellulose	50
Mineral Mix #210025	35
Vitamin Mix #210025	10
Choline Bitartrate	2.5
d-γ-tocopherol or d-α-tocopherol	0.25

The standard basal control diet without supplementation is 45 mg γ-tocopherol and 45 mg α-tocopherol/kg diet (Dyets, Inc., Catalog #101591). The α-tocopherol supplemented diet was 250 mg α- tocopherol and 45 mg γ-tocopherol (Dyets, Inc., Catalog #103373). The γ-tocopherol supplemented diet was 250 mg γ-tocopherol and 45 mg α-tocopherol/ kg of diet (Dyets, Inc., Catalog #103742). The diet was a modified AIN-93G Purified Rodent Diet with corn oil replacing soybean oil. Corn oil, which was commonly used in rodent diet in the past, was used in this basal diet instead of the more recent formulas with soybean oil, which contain high levels of γ-tocopherol.

Animal Study: C57BL/6 mice were from Jackson Laboratories, Bar Harbor, Maine. The studies were approved by the Northwestern University institutional review committee for animals.

Tocopherol diet (Table 1). D-α-tocopherol (>98% pure) from Sigma and d-γ-tocopherol (>98% pure) from Alpha Chemistry were sent to Dyets, Inc. (Bethlehem, PA) to make the diets with 250 mg α-tocopherol/kg diet (Dyets Catalog #103373) or 250 mg γ-tocopherol/kg diet (Dyets Catalog #103742). As in standard rodent chow, the basal diet contained 45 mg γ-tocopherol/kg diet and 45 mg α-tocopherol/ kg diet (Dyets Catalog #101591). The purity of these tocopherols that were used to make the diets was confirmed by HPLC with electrochemical detection. The lipids in the diet were extracted with an equal volume of hexane with 0.37 wt% butylated hydroxytoluene to prevent oxidation and increase recovery of tocopherol. The samples were vortexed and then centrifuged for 10 minutes

at 9,000 x g at 4°C. The hexane layer was dried under nitrogen and stored at -20°C. The samples were reconstituted in methanol, and then tocopherols were separated using a reverse phase C_{18} HPLC column (Hewlett Packard) and HPLC (Waters Co.) with 99% methanol-1% water as a mobile phase with detection with an electrochemical detector (potential 0.7V) (Waters Co.). These diets increase tissue tocopherol concentrations 2-3 fold for α-tocopherol[7] and γ-tocopherol.[8, 9]

Doses for administration to mice and humans are difficult to compare because there are differences in species metabolism. Basal α-tocopherol is necessary for mouse and human placental development.[10, 11] The standard basal rodent diet (45 mg/kg of diet) supports rodent development. A relevant dose for mouse supplementation is a physiologically reasonable, non-toxic dose that achieves fold changes in tissues similar to fold changes in humans. A 250 mg d-α-tococopherol/kg of diet dose is well within the range to elevate tissue tocopherols in adult rodents;[12-14] this dose is 30 times lower than the maternal tocopherol rodent diet dose that reduces rodent hippocampus function.[15] The 250 mg α-tocopherol/kg of diet or 250 mg γ-tocopherol/kg of diet yields a 2-3 fold increase in each of these liver tocopherols in adult mice.[7] This is similar to the fold changes achievable in the human population.[16-19]

House dust mite administration. House dust mite (HDM), *Dermatophagoides pteronyssius* extract containing DerP1 was from Greer Labs. The mice (8-10 mice/group) were provided the indicated tocopherol-supplemented diets during HDM challenge. The mice received intratracheal 10 μg HDM/50 μl saline 3 times per week for a total of 6 or 8 challenges as indicated in Figure 2. Lung tissues were collected 24

hours after the last treatment and blood eosinophils were stained and counted. Bronchoalveolar lavage (BAL) cells were counted and cytospun for differential counts.

Cytokines and Chemokines. Total RNA was isolated from 50-100 mg lung tissue using the QIAGEN RNeasy Fibrous Tissue Mini Kit. cDNA was prepared using a SuperScript II RNase H-Reverse Transcriptase kit (Invitrogen Corp.) and analyzed by PCR on an ABI 7300 Thermal Cycler (Applied Biosystems). Taqman probes and Taqman Universal Master Mix were used as directed (Applied Biosystems).

Descriptive statistics are presented as medians and interquartile ranges (IQR) or mean and standard deviations as appropriate for continuous variables and number and proportions for categorical variables. Comparisons in the animal studies were made by a one way ANOVA followed by Tukey's or Dunnett's multiple comparisons test (SigmaStat, Jandel Scientific, San Ramon, CA). Because of the small N the data were also analyzed with a Kruskal-Wallis test followed by Dunn's test. The outcomes were the same as that obtained with an ANOVA followed by Tukey's or Dunnet's.

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