Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 196–203

Journal of Nutritional Biochemistry

# Supplementation of a γ-tocopherol-rich mixture of tocopherols in healthy men protects against vascular endothelial dysfunction induced by postprandial hyperglycemia☆,☆☆

Eunice Mah[a], Sang K. Noh[b], Kevin D. Ballard[c], Hea Jin Park[a], Jeff S. Volek[c], Richard S. Bruno[a,*]

[a]Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269-4010, USA
[b]Department of Food and Nutrition, Changwon National University, Changwon 641-773, South Korea
[c]Department of Kinesiology, University of Connecticut, Storrs, CT 06269-4017, USA

Received 27 October 2011; received in revised form 25 April 2012; accepted 27 April 2012

**Abstract**

Postprandial hyperglycemia induces oxidative stress responses, impairs vascular endothelial function (VEF) and increases the risk of cardiovascular disease. We hypothesized that the antioxidant and anti-inflammatory activities of a γ-tocopherol-rich mixture of tocopherols (γ-TmT) would protect against vascular dysfunction that is otherwise caused by postprandial hyperglycemia by decreasing oxidative stress and proinflammatory responses, and improving nitric oxide (NO•) homeostasis. In a randomized, crossover study, healthy men ($n$=15; 21.8±0.8 years) completed a fasting oral glucose challenge (75 g) with or without prior supplementation of γ-TmT (5 days). Brachial artery flow-mediated dilation (FMD), plasma glucose, insulin, antioxidants, malondialdehyde (MDA), inflammatory proteins, arginine and asymmetric dimethylarginine (ADMA) were measured at regular intervals during a 3-h postprandial period. Supplementation of γ-TmT increased ($P$<.05) plasma γ-T by threefold and γ-carboxyethyl-hydroxychroman by more than ninefold without affecting α-T, glucose, arginine or ADMA. Baseline FMD, MDA, arginine and ADMA were unaffected by γ-TmT ($P$>.05). Postprandial FMD decreased 30%–44% ($P$<.05) following glucose ingestion, but was maintained with γ-TmT. Supplementation of γ-TmT also attenuated postprandial increases in MDA that occurred following glucose ingestion. Plasma arginine decreased ($P$<.05) in both trials to a similar extent regardless of γ-TmT supplementation. However, the ratio of ADMA/arginine increased time-dependently in both trials ($P$<.05), but to a lesser extent following γ-TmT supplementation ($P$<.05). Inflammatory proteins were unaffected by glucose ingestion or γ-TmT. Collectively, these findings support that short-term supplementation of γ-TmT maintains VEF during postprandial hyperglycemia possibly by attenuating lipid peroxidation and disruptions in NO• homeostasis, independent of inflammation.
Published by Elsevier Inc.

*Keywords:* Vascular endothelial function; Flow-mediated dilation; Hyperglycemia; γ-Tocopherol

## 1. Introduction

Vascular endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and is associated with traditional risk factors for cardiovascular disease (CVD) such as diabetes [1]. Chronic hyperglycemia increases the risk of CVD, and individuals with diabetes are two to four times more likely to develop CVD [2]. However, large-scale observational studies in individuals with diabetes suggest that 2-h blood glucose, but not fasting glucose concentration, predicts CVD-related mortality [3]. This relation is supported by controlled human trials indicating that acute or postprandial hyperglycemia decreases flow-mediated dilation (FMD) of the brachial artery, a noninvasive technique commonly used to assess vascular endothelial function (VEF) [4–6].

The mechanism by which postprandial hyperglycemia impairs VEF is not fully understood, but oxidative stress is implicated in its pathogenesis [5,7,8]. Lipid peroxidation is an early event contributing to atherosclerosis [9]. Degradation products of lipid hydroperoxides inhibit the activity of dimethylarginine dimethylaminohydrolase (DDAH) in endothelial cells, thereby increasing the accumulation of asymmetric dimethylarginine (ADMA) [10]. ADMA is a competitive inhibitor of endothelial nitric oxide synthase (eNOS) [11]. Greater concentrations of ADMA relative to arginine decrease the biosynthesis of nitric oxide (NO•), a key regulator of VEF [12], because it competitively inhibits arginine binding to eNOS [13]. Indeed, our studies in healthy men show that acute hyperglycemia caused by an oral glucose challenge decreases postprandial FMD responses while increasing lipid peroxidation and ADMA/arginine, an index of CVD risk [14]. This suggests that hyperglycemia-mediated impairments in VEF occur, at least in part, by increasing oxidative stress responses that dysregulate NO• homeostasis.

☆ Support provided by a grant to R.S.B. from the International Life Sciences Institute.
☆☆ Author disclosures: E. Mah, S.K. Noh, K.D. Ballard, H.J. Park, J.S. Volek and R.S. Bruno have no conflicts of interest.
* Corresponding author. Tel.: +860 486 1608; fax: +860 486 3674.
*E-mail address:* Richard.Bruno@uconn.edu (R.S. Bruno).

0955-2863/$ - see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.jnutbio.2012.04.015

The involvement of oxidative stress in disrupting vascular homeostasis suggests that antioxidants capable of suppressing lipid peroxidation and inflammation may protect against vascular endothelial dysfunction otherwise induced by acute hyperglycemia. Vitamin E consists of four tocopherols (α-, β-, γ-, δ-T) and four tocotrienols (α-, β-, γ-, δ-$T_3$) that function as a chain-breaking antioxidant to terminate lipid peroxidation [15]. α-T is the most abundant circulating form of vitamin E in humans and is the most commonly used in dietary vitamin E supplements. In contrast, dietary intakes of γ-T are greater than those of α-T, but only represent ~10% of the total circulating vitamin E because it has limited binding affinity to the hepatic α-tocopherol transfer protein [15]. γ-T is rapidly metabolized in a cytochrome-P450-dependent manner to its physiological metabolite γ-carboxyethyl-hydroxychroman (γ-CEHC) [16]. Both α-T and γ-T function as antioxidants by scavenging reactive oxygen and nitrogen species [17]. However, γ-T and γ-CEHC exert greater anti-inflammatory activity compared to α-T [18,19]. Of particular importance is that the potential beneficial effects of γ-T may be lost during periods of high α-T intakes since dietary supplementation of α-T substantially decreases circulating concentrations of γ-T [20].

The central involvement of lipid peroxidation and inflammation in CVD [9] suggests that the bioactivities of γ-T may protect against its development. However, most vitamin E intervention studies related to CVD have focused on α-T, and the findings of those studies have been equivocal in that some show beneficial effects on CVD risk, whereas other fail to support a health benefit [16]. In contrast, substantially less is known about the potential benefits of γ-T on CVD-related outcomes. Coronary heart disease patients have lower plasma γ-T compared to healthy age-matched controls [21]. In a cross-sectional study, Swedish men having concentrations of plasma γ-T that are double those of Lithuanian men have a 25% lower incidence of CVD-related mortality [22]. While these studies are promising, limited information is available from human intervention studies regarding the potential benefits of γ-T in regulating CVD risk. Thus, we hypothesized in the present study that supplementation of a γ-T-rich mixture of tocopherols (γ-TmT) would maintain VEF otherwise suppressed by postprandial hyperglycemia by decreasing oxidative stress and inflammatory responses, and improving NO• homeostasis. We examined postprandial FMD, assessed NO• homeostasis by measuring circulating arginine and ADMA levels, and evaluated markers of antioxidant status, oxidative stress and inflammation in response to an oral glucose challenge with or without prior supplementation of γ-TmT.

## 2. Methods and materials

### 2.1. Study participants and experimental design

The study protocol was approved by the Institutional Review Board at the University of Connecticut, and participants provided written consent before enrolling. An extensive description of the study protocol was reported previously where we evaluated the effects of γ-TmT supplementation and postprandial hyperglycemia on methylglyoxal accumulation [23]. In brief, healthy, nonsmoking men ($n$=16) were enrolled on the basis of age (18–35 years), stable body mass (>2 months), adiposity (8%–20%), nonuser of dietary supplements (>2 months), nonuser of medications or dietary agents that affect vasodilation, stable exercise patterns (<5 h/week aerobic activity), fasting total cholesterol (<5.18 mmol/L) and glucose (<5.55 mmol/L), and resting blood pressure (<140/90 mmHg). To control for factors that may confound FMD responses such as gender, aging and comorbidities [24], healthy young men were specifically enrolled. Waist circumference was determined at the umbilicus, body density was estimated from skinfolds at seven sites [25], and adiposity was calculated using appropriate equations for race [26,27].

In a randomized, crossover, single-blind study design, participants completed an oral glucose tolerance test following an overnight fast on two occasions separated by ≥7 days. For 5 consecutive days prior to one visit, participants ingested an encapsulated γ-TmT supplement containing 500 mg γ-T, 60 mg α-T, 170 mg δ-T and 9 mg β-T (Archer Daniels Midland; Decatur, IL, USA). Capsules were ingested with participants' evening meal in order to perform study procedures when plasma γ-T would be at its maximal concentrations [28]. Our study population was identical to that reported previously [14], with the exception that one participant was excluded in the present study. Data from this participant were omitted in the final analysis due to lack of compliance, which was determined on the basis of pill counts and by measuring plasma γ-T concentrations following γ-TmT supplementation.

### 2.2. Sample collection

Blood was collected from the antecubital vein into evacuated tubes containing EDTA, sodium heparin or lithium heparin prior to (0 min) and following the ingestion of glucose at 30, 60, 90, 120, 150 and 180 min. Plasma was separated by centrifugation (4°C, 15 min, 1500$g$) and snap-frozen in liquid nitrogen. For the analysis of vitamin C and uric acid, sodium heparinized plasma was mixed (1:1) with 10% perchloric acid (PCA) containing 1 mmol/L diethylenetriaminepentaacetic acid (DTPA) and centrifuged (5 min, 15,000$g$, 4°C), and the supernatant was snap-frozen in liquid nitrogen. All samples were stored at −80°C until analysis. FMD was also measured at the same time intervals, as described below, to assess VEF. Lastly, participants completed a food record for 3 days prior to each visit. Records were reviewed for accuracy by a Registered Dietitian, and nutrient intakes were analyzed using Food Processor SQL (ESHA Research, Salem, OR, USA).

### 2.3. Materials

High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA), as were the following chemicals: butylated hydroxytoluene, DTPA, monomethylarginine, ortho-phthalaldehyde, PCA, sodium hydroxide and trichloroacetic acid (TCA). β-Glucuronidase type HP-2S, fluorescein and thiobarbituric acid (TBA) were from Sigma (St. Louis, MO, USA). 2,2′-Azobis-2-methyl-propanimidamide (AAPH) was from Cayman Chemical (Ann Arbor, MI, USA).

### 2.4. Flow-mediated dilation

Brachial artery FMD was assessed by high-frequency ultrasonography as described [14]. In brief, a blood pressure cuff was placed on the forearm distal to the olecranon process, and the brachial artery was imaged using a cardiac ultrasound system (Acuson Corp, Elmwood Park, NJ, USA). Brachial artery diameter was collected for 30 heartbeats and then the blood pressure cuff was rapidly inflated to 200 mmHg to occlude the vessel for 5 min. Vessel diameter was assessed for 300 heartbeats (~5 min) upon cuff release. Images were electrocardiogram gated, and their analysis was performed using edge-detection software (Medical Imaging Applications, Iowa City, IA, USA). Brachial artery FMD (%) was calculated as follows: [postocclusion peak diameter (mm)−preocclusion diameter (mm)/preocclusion diameter (mm)*100. Each participant's FMD measurements were obtained by the same technician, and images were analyzed independently by two technicians in a blinded manner. Both analyses were in good agreement ($P$>.05) and were therefore averaged.

### 2.5. Clinical chemistries

Plasma triglyceride, total cholesterol and glucose were measured spectrophotometrically using clinical assays (Pointe Scientific, Canton, MI, USA) on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (Diagnostic Systems Laboratories, Webster, TX, USA).

### 2.6. Total antioxidant status

Total antioxidant status was measured using the oxygen radical absorbance capacity (ORAC) [14] and ferric-reducing ability of plasma (FRAP) [14] assays as described. Briefly, ORAC values were determined by mixing samples with fluorescein and AAPH, and the oxidative decay of fluorescein was monitored (485/520 nm, excitation/emission) on the aforementioned microplate reader. Area under the curve (AUC) for oxidative decay of fluorescein was calculated, and ORAC concentrations were determined by linear regression. FRAP was measured by mixing plasma or Trolox standard with FRAP reagent and measuring the absorbance at 593 nm on the aforementioned microplate reader after a 15-min incubation at 37°C.

### 2.7. Vitamin E and γ-CEHC

Plasma α-, β-, γ- and δ-T were measured by HPLC-Coularray (ESA Inc., Chelmsford, MA, USA) following saponification and hexane extraction as described [14]. γ-CEHC, the physiological metabolite of γ-T, was measured by HPLC-Coularray as described [14]. Briefly, a methanolic extract of plasma was evaporated under nitrogen gas, reconstituted in water and subjected to enzymatic hydrolysis with β-glucuronidase (800 U) and sulfatase (60 U). Following extraction with ethyl acetate, samples were dried under nitrogen gas, dissolved in methanol and separated isocratically (1 ml/min) on a Phenomenex Luna C18 column (250×4.6 mm i.d., 5 μm; Torrance, CA, USA) and detected at potential settings of −100, −50, 200 and 350 mV.

Table 1
Participant characteristics

| Parameter | |
|---|---|
| Age (years) | 21.8±0.8 |
| Height (m) | 1.80±0.01 |
| Weight (kg) | 90.6±6.2 |
| Adiposity (% total mass) | 16.7±1.9 |
| Waist circumference (cm) | 87.6±3.5 |
| Systolic blood pressure (mm Hg) | 117±1 |
| Diastolic blood pressure (mm Hg) | 79±1 |
| Glucose (mmol/L) | 5.31±0.10 |
| Insulin (pmol/L) | 142.5±23.6 |
| Triglyceride (mmol/L) | 3.26±1.24 |
| Cholesterol (mmol/L) | 3.7±0.2 |
| Arginine (μmol/L) | 82.9±3.5 |
| ADMA (nmol/L) | 500±11 |
| ADMA:arginine (nmol/μmol) | 6.23±0.35 |
| Baseline brachial artery diameter (mm) | 3.98±0.12 |

Data are means±S.E.M.; $n=15$.

### 2.8. Ascorbic acid and uric acid

Ascorbic acid and uric acid were analyzed from PCA-treated plasma as described [14]. Samples were separated isocratically (1 ml/min) on the aforementioned HPLC-Coularray system using a Supelcosil LC-8 column (150×4.6 mm i.d., 3 μm, Supelco; Bellefonte, PA, USA) and detected at oxidation potentials of 150, 275, 400 and 525 mV.

### 2.9. Malondialdehyde

Plasma malondialdehyde (MDA), a marker of lipid peroxidation, was measured by HPLC-fluorescence (HPLC-FL) as described [14], with minor modifications. Plasma was mixed with 40 μl 0.2% butylated hydroxytoluene and 200 μl 1 N sodium hydroxide. Following incubation for 30 min at 60°C, 2 ml 5% TCA was added, and the mixture was placed on ice for 10 min and centrifuged (1100g, 4°C, 10 min). The supernatant was mixed with 250 μl 0.6% (w/v) TBA and heated at 95°C for 30 min. After cooling, the mixture was extracted with 500 μl butanol, and the supernatant was injected on a Shimadzu LC-20XR system (Columbia, MD, USA) equipped with an RF-10AXL fluorescence detector set to 532 nm and 553 nm for excitation and emission, respectively. Isocratic HPLC separation was performed at 0.8 ml/min on a Luna C18[2] column (250×4.6 mm i.d., 5 μm; Phenomenex; Torrance, CA, USA) using 50:50 methanol and 25 mmol/L phosphate buffer (pH 6.5) as the mobile phase.

### 2.10. Arginine and ADMA

Arginine, the amino acid for eNOS-mediated NO• synthesis, and ADMA, an endogenously produced competitive inhibitor of eNOS, were measured simultaneously by HPLC-FL as described [14], with minor modification. Briefly, samples and standards were mixed with methylmonoarginine (0.6 nmol; internal standard) and subjected to solid-phase extraction on a polymeric cation-exchange column (Hypersep Retain-CX SPE column; 30 mg, 1 ml; Fisher Scientific, Pittsburgh, PA, USA) using ammonia:water:methanol (10:40:50, v/v/v). Samples were dried under nitrogen gas, and the residue was dissolved in water for derivatization using ortho-phthalaldehyde. Isocratic HPLC separation was performed on a Shim-Pack XR-ODS column (3.0 × 50 mm i.d.; Shimadzu, Columbia, MD, USA) at 1.1 ml/min on the aforementioned HPLC-FL system that was programmed to 340/455 nm (excitation/emission). After arginine was eluted at 4.7 min, the detector was programmed to a higher sensitivity to enable detection of ADMA and symmetric dimethylarginine (SDMA).

### 2.11. Inflammatory markers

Myeloperoxidase (MPO) and high-sensitivity C-reactive protein (CRP) were measured by ELISA (BioCheck, Foster City, CA, USA). Plasma interleukins (IL-6 and -10), tumor necrosis factor-α (TNF-α), soluble intracellular adhesion molecule-1 (ICAM-1), soluble vascular adhesion molecule-1 (VCAM-1) and soluble E-selectin were measured using xMAP technology on a Luminex IS200 system (Austin, TX, USA) with corresponding antibodies (Millipore, Billerica, MA, USA).

### 2.12. Statistical methods

Data (means±S.E.M.) were analyzed by SPSS Version 15.0 (SPSS Inc., Chicago, IL, USA). Two-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction was used to evaluate differences due to time, treatment and their interaction. A Student's paired $t$ test was used to evaluate baseline ($t=0$ min) and AUC data between trials. Multiple linear regression, controlling for within-subject repeated measures, was used to calculate correlation coefficients as described [29]. Prior to any analysis, normality was assessed using Shapiro-Wilk's $W$ test. Measurements that were not normally distributed (ADMA, CRP, IL-10, insulin, MPO, α-T and γ-CEHC) were normalized by log transformation using the natural base. All statistical analyses were performed simultaneously on both transformed and untransformed data. Results of untransformed values are reported because they did not qualitatively differ from those of transformed data and permit more meaningful interpretations than transformed data. An α level of $P\leq.05$ was considered statistically significant for all analyses.

## 3. Results

### 3.1. Participants and compliance

Participants had adiposity, resting blood pressure and concentrations of fasting plasma glucose, total cholesterol and triglyceride that were within normal clinical limits (Table 1). No differences in nutrient intakes were observed between trials (Table 2), indicating that participants maintained their dietary habits throughout the study. Supplementation of γ-TmT increased ($P<.05$) plasma concentrations of γ-T by about threefold and plasma γ-CEHC by more than ninefold without affecting plasma α-T (Table 3). Plasma δ-T concentrations increased from 114 to 728 nmol/L in response to supplementation, but only accounted for ~3% of the total plasma tocopherol concentration, whereas plasma β-T was below quantitative limits.

### 3.2. Plasma glucose and insulin

Fasting glucose and insulin concentrations did not differ between trials, nor were they affected by γ-TmT supplementation (Fig. 1). Regardless of supplementation, postprandial concentrations of glucose and insulin increased to a similar extent by 30 min following glucose ingestion and returned to baseline concentrations at 150 min and 180 min, respectively (Fig. 1A–B).

### 3.3. Flow-mediated dilation

Baseline ($t=0$ min) brachial artery diameter and FMD responses did not differ between trials prior to glucose ingestion ($P>.05$; Fig. 1C). As reported previously, FMD responses decreased at 30 min following the ingestion of glucose without any prior γ-TmT supplementation and were restored to baseline levels at 120 min [14]. In contrast, no time-dependent changes on FMD responses occurred during the γ-TmT trial. A significant treatment effect was also observed, indicating that FMD responses were greater in the γ-TmT trial, with post hoc differences observed at 60 min only ($P<.05$).

Table 2
Dietary intakes

| | Glucose | Glucose+γ-TmT |
|---|---|---|
| Total intake (kcal) | 2463±107 | 2267±118 |
| Calories from carbohydrate (%) | 50.8±1.3 | 50.3±2.0 |
| Calories from protein (%) | 16.6±0.8 | 17.0±1.0 |
| Calories from fat (%) | 32.8±1.3 | 33.8±1.2 |
| Total saturated fat (g) | 29.8±2.1 | 28.4±2.5 |
| Monounsaturated fat (g) | 24.5±3.9 | 24.7±1.9 |
| Polyunsaturated fat (g) | 11.2±1.6 | 10.9±1.5 |
| *trans*-Fatty acid (g) | 1.57±0.61 | 1.89±0.71 |
| Cholesterol (mg) | 270±35 | 267±34 |
| β-Carotene (μg) | 1428±484 | 839±325 |
| Vitamin C (mg) | 100±21 | 91.5±22.3 |
| α-Tocopherol (mg) | 5.50±1.10 | 5.22±1.01 |
| Selenium (μg) | 86.1±14.7 | 75.0±8.4 |
| Zinc (mg) | 9.77±1.49 | 8.71±0.82 |
| Sodium (mg) | 3585±238 | 3686±291 |

Data (means±S.E.M.; $n=15$) are obtained from 3-day dietary records completed prior to each visit. No differences in nutrient intake occurred between trials ($P>.05$).

Table 3
Plasma antioxidants and markers of oxidative stress and inflammation[a]

| | Baseline | | | AUC | | |
|---|---|---|---|---|---|---|
| | Glucose | Glucose+γ-TmT | *P* | Glucose | Glucose+γ-TmT | *P* |
| Plasma antioxidant and oxidative stress markers | | | | | | |
| ORAC (μmol/L Trolox eq.) | 3579±257 | 4022±295 | .020 | 1,001,529±40,024 | 1,076,427±24,275 | .029 |
| FRAP (μmol/L Trolox eq.) | 978±26 | 1043±30 | .022 | 176,685±4068 | 186,389±5550 | .030 |
| α-Tocopherol (μmol/L) | 15.4±1.2 | 16.6±1.6 | .207 | 2774±200 | 2944±294 | .204 |
| γ-Tocopherol (μmol/L) | 2.23±0.26 | 6.60±0.62 | .000 | 372±42 | 1147±127 | .000 |
| γ-CEHC (μmol/L) | 0.32±0.07 | 3.12±0.52 | .000 | 52.3±9.0 | 543±91 | .000 |
| Vitamin C (μmol/L) | 34.3±4.3 | 38.7±4.4 | .007 | 6079±730 | 6796±797 | .012 |
| Uric acid (μmol/L) | 354±12 | 367±15 | .160 | 64,110±2115 | 65,375±2925 | .271 |
| MDA (μmol/L) | 0.91±0.03 | 0.96±0.04 | .081 | 222±8 | 207±8 | .002 |
| Inflammatory markers | | | | | | |
| CRP (nmol/L) | 2.98±1.05 | 2.17±0.81 | .373 | 473±178 | 357±136 | .265 |
| MPO (pmol/L) | 84.0±10.4 | 76.1±7.2 | .443 | 16,107±1362 | 14,399±882 | .057 |
| IL-6 (pg/ml) | 4.78±0.77 | 4.09±0.73 | .197 | 861±148 | 683±84 | .059 |
| IL-10 (pg/ml) | 12.0±2.5 | 9.05±1.28 | .163 | 2338±457 | 1777±318 | .178 |
| TNF-α (pg/ml) | 5.40±0.50 | 5.30±0.69 | .408 | 891±81 | 902±75 | .420 |
| ICAM-1 (pg/ml) | 97.4±11.0 | 95.1±10.5 | .253 | 17,493±1980 | 17,010±1867 | .107 |
| VCAM-1 (pg/ml) | 993±33 | 1006±42 | .353 | 186005±5437 | 178233±6447 | .053 |
| E-selectin (pg/ml) | 32.8±3.6 | 32.4±3.8 | .366 | 6125±708 | 5788±751 | .078 |

Differences between trials were assessed using a Student's paired *t* test, and $P<.05$ is considered significant.
[a] Data are means±S.E.M.; $n=15$.

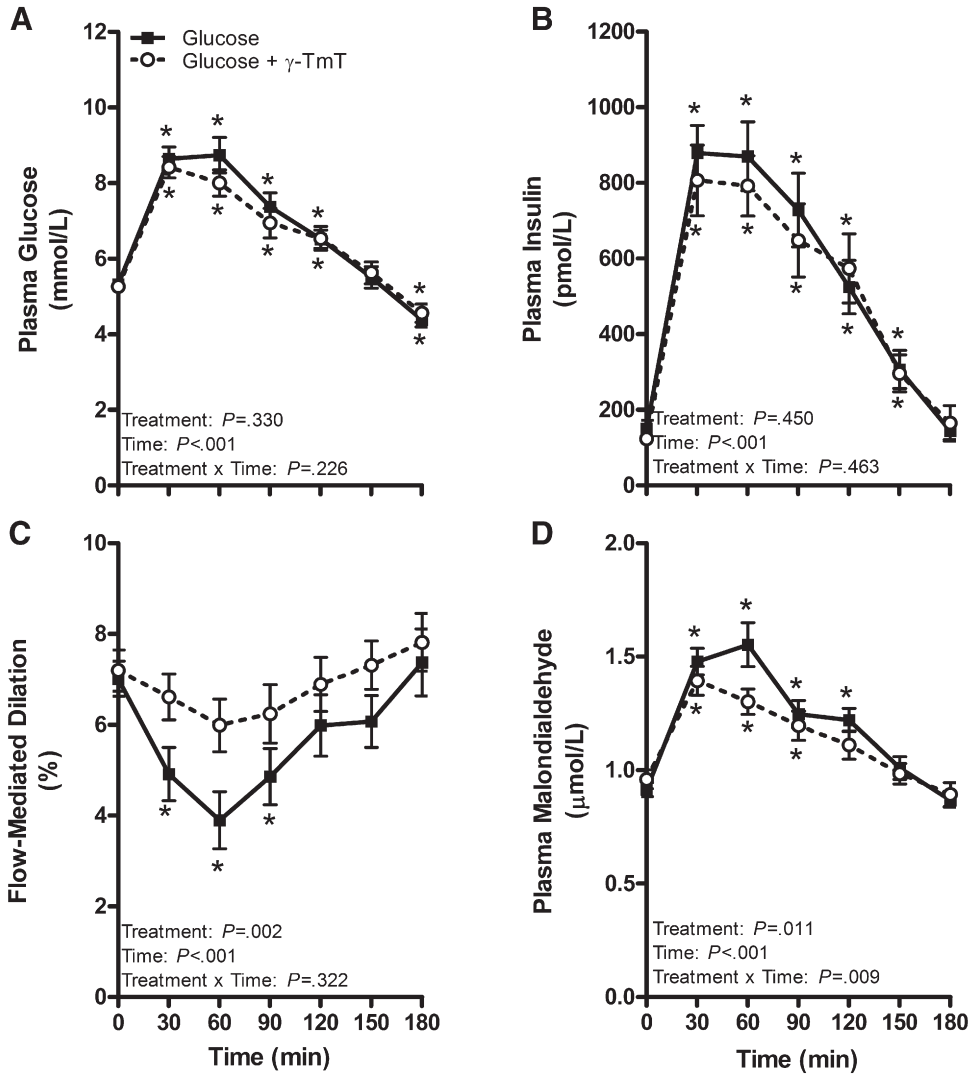


Fig. 1. Postprandial responses for (A) plasma glucose, (B) plasma insulin, (C) brachial artery FMD and (D) plasma MDA in healthy men following glucose ingestion with or without prior supplementation of a γ-TmT. Two-way repeated-measures ANOVA with Bonferroni correction was performed to evaluate time and treatment effects. Data are means±S.E.M.; $n=15$. *Significantly different from baseline, $P<.05$.

### 3.4. Markers of antioxidant status and oxidative stress

Plasma antioxidants and MDA were examined to better define whether postprandial hyperglycemia regulates VEF in an oxidative-stress-dependent manner (Table 3). Short-term γ-TmT supplementation increased baseline ($t$=0 min) concentrations of ORAC, FRAP and vitamin C. These antioxidant markers remained higher throughout the postprandial period as evidenced by greater $AUC_{0-3\ h}$ during the γ-TmT trial (Table 3) and were unaffected by glucose ingestion. There were also no significant differences between trials at baseline or throughout the postprandial period for uric acid (Table 3). In contrast, postprandial concentrations of γ-T, but not α-T or γ-CEHC, decreased significantly at 150–180 min during the γ-TmT supplementation arm of the study only.

Baseline MDA concentrations did not differ between trials (Table 3). Plasma MDA increased following glucose ingestion in both trials. However, a treatment×time interaction effect indicated that postprandial MDA increased to a lesser extent during the γ-TmT trial, with a specific treatment effect observed at 60 min only (Fig. 1D). Multiple linear regression analysis, controlling for within-subject repeated measures, indicated that MDA $AUC_{0-3\ h}$ was highly correlated with FMD $AUC_{0-3\ h}$ (mean slope=−16.4, $r$=−0.82, $P$<.05), suggesting that hyperglycemia-mediated decreases in FMD occur in an oxidative-stress-dependent manner and that γ-TmT maintains VEF, at least in part, by suppressing postprandial lipid peroxidation.

### 3.5. Markers of NO• homeostasis

Plasma arginine and ADMA were measured as an indirect index of NO• homeostasis to better define the mechanism by which γ-TmT supplementation protects against hyperglycemia-mediated decreases in FMD. Baseline concentrations of plasma arginine, ADMA and SDMA did not differ between trials (Fig. 2). Glucose ingestion, regardless of γ-TmT supplementation, decreased plasma arginine concentrations to a similar extent in both trials (Fig. 2A). γ-TmT supplementation had no effect on baseline concentrations of ADMA or SDMA (Fig. 2B and 2C). However, a small but significant decrease in plasma ADMA was observed at 150–180 min following γ-TmT supplementation only (Fig. 2B). Of particular importance was that the ratio of ADMA/arginine increased postprandially in both trials, but to a lesser extent in the γ-TmT trial at 150 min (Fig. 2D).

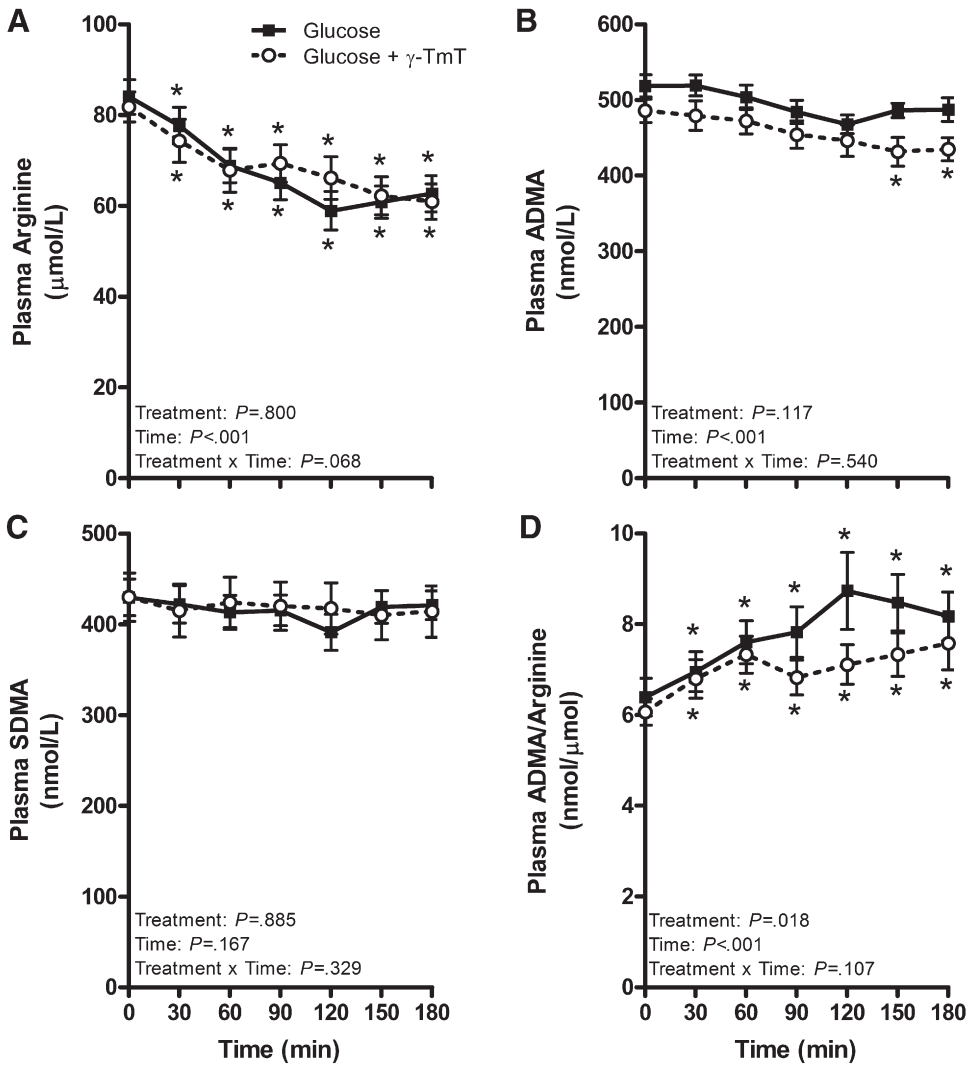


Fig. 2. Postprandial responses for (A) plasma arginine, (B) ADMA, (C) SDMA and (D) the ratio of ADMA to arginine in healthy men following glucose ingestion with or without prior supplementation of a γ-TmT. Two-way repeated-measures ANOVA with Bonferroni correction was performed to evaluate time and treatment effects. Data are means±S.E.M.; $n$=15. *Significantly different from baseline, $P$<.05.

### 3.6. Markers of inflammation

Baseline concentrations and $AUC_{0-3\ h}$ of numerous inflammatory markers did not differ between trials (Table 3). Furthermore, no time-dependent changes occurred throughout the postprandial period for any of these markers (data not shown).

## 4. Discussion

This study demonstrates that short-term γ-TmT supplementation in healthy men maintains VEF that is otherwise impaired by postprandial hyperglycemia likely by decreasing lipid peroxidation and mitigating disruptions in NO• homeostasis, without affecting inflammatory responses. γ-TmT supplementation increased plasma γ-T and γ-CEHC concentrations without affecting α-T and maintained postprandial FMD responses that were otherwise decreased following glucose ingestion. Supplementation of γ-TmT also attenuated postprandial MDA responses following glucose ingestion, supporting a mechanism whereby γ-T protects against impairments in VEF in an oxidative-stress-dependent manner. We are also providing the first evidence that γ-TmT supplementation may attenuate impairments in VEF mediated by acute hyperglycemia by decreasing ADMA/arginine, which would be expected to increase the likelihood of arginine binding to eNOS, thereby improving NO• biosynthesis and bioavailability. Collectively, the findings of this study support that improvements in γ-T status inhibit transient impairments in VEF resulting from postprandial hyperglycemia likely by decreasing lipid peroxidation and improving NO• bioavailability.

Chronic and postprandial hyperglycemia are associated with greater oxidative stress [30]. We previously showed that MDA increases rapidly during the postprandial period in response to glucose ingestion [14]. This supports that hyperglycemia impairs VEF, at least in part, by increasing lipid peroxidation. Indeed, lipid peroxidation is an early atherogenic event that contributes to the development of CVD [9]. Although lipid peroxidation was lowered in individuals with diabetes following α-T supplementation [31], no studies have investigated the independent effects of vitamin E supplementation on lipid peroxidation in association with VEF following acute hyperglycemia. Co-supplementation of vitamin C and vitamin E immediately prior to an oral glucose challenge prevented the decrease in postprandial FMD responses that otherwise occurred with hyperglycemia [6]. However, maximal plasma vitamin E occurs ~10 h following ingestion [32], whereas maximal plasma vitamin C occurs ~60 min postingestion [33]. Thus, the maintenance of VEF following glucose ingestion in combination with supplementation of vitamins C and E [6] most likely reflects improvements in vitamin C status rather than vitamin E. Thus, our study provides the first direct evidence that dietary vitamin E supplementation, provided as γ-TmT, attenuates hyperglycemia-mediated impairments in VEF in healthy young men, at least partly by reducing lipid peroxidation.

We previously showed that postprandial hyperglycemia induced by an oral glucose challenge decreases circulating arginine levels [14], possibly by inducing membrane hyperpolarization of endothelial cells, thereby resulting in greater arginine uptake [34]. However, it is unknown if this increase in intracellular arginine increases NO• production by eNOS or enhances its degradation by arginase. Arginase activity has been shown to be up-regulated in animals and humans having diabetes [35,36], but it is unknown if arginase activity is up-regulated within 3 h of an oral glucose challenge. Additionally, studies in rats showed that 3-week dietary supplementation of α-T attenuates hyperglycemia-mediated increases in hepatic arginase activity [37]. To the best of our knowledge, no studies have investigated the acute effects of γ-T on arginase activity.

The production of NO• by eNOS is dependent on not only arginine availability but also competitive inhibition by ADMA [13]. We provide novel evidence that short-term γ-TmT supplementation attenuates the ratio of ADMA/arginine that is otherwise increased by postprandial hyperglycemia. However, the mechanism by which this occurs is unclear. The formation of both ADMA and SDMA is catalyzed by protein arginine methyltransferases (PRMTs) [38]. ADMA can be degraded by DDAH, whereas SDMA is excreted directly through the urine [38]. Currently, no evidence indicates that hyperglycemia or oxidative stress regulates PRMT activity. However, studies *in vitro* demonstrate that incubation of endothelial cells with glucose or end products of lipid peroxidation increases ADMA by suppressing its DDAH-dependent degradation [39,40]. In contrast, our work indicates that postprandial concentrations of ADMA are unaffected following glucose ingestion. However, we did observe a small but significant decrease in postprandial concentrations of ADMA following γ-TmT supplementation. Indeed, treatment of endothelial cells with polyethylene-glycol-conjugated superoxide dismutase resulted in recovery of DDAH-mediated degradation of ADMA that was otherwise decreased following incubation with glucose [39]. Thus, our observation that γ-TmT supplementation decreases postprandial ADMA concentrations suggests that γ-T may improve NO• homeostasis by decreasing oxidative stress responses that otherwise inhibit DDAH activity. In support, we also observed that SDMA, an arginine metabolite that is not degraded in a DDAH-dependent manner, was unaffected by γ-TmT supplementation. Direct measurements of endothelial arginase and DDAH activity would better define the mechanism by which arginine and ADMA are regulated, but their assessment was beyond the scope of this clinical investigation. Moreover, no studies have investigated the effects of γ-T on the expression or activity of arginase or DDAH in humans. This indicates that more invasive studies are warranted to define the mechanism by which γ-T regulates ADMA and arginine metabolism in relation to NO• homeostasis and VEF.

We have also considered the possibility that the vasoprotective effects of γ-TmT are occurring indirectly. Data show that plasma vitamin C increases following γ-TmT supplementation without any changes in dietary vitamin C intake, possibly due to the sparing effect of γ-T on vitamin C oxidation [41]. Vitamin C supplementation has been shown to maintain postprandial FMD responses that were otherwise decreased by an oral glucose tolerance test in healthy individuals [6]. This supports the possibility that greater plasma vitamin C concentrations following γ-TmT supplementation may contribute to the vasoprotective effects observed in the present study. Another line of evidence suggests the possibility that the natriuretic activity of γ-CEHC observed in rodents [42] may regulate vascular function. Controlled studies in normotensive adults demonstrate that a low-sodium diet (1153 mg/day) improves FMD responses in comparison to a high-sodium diet (3450 mg/day) [43]. This suggests that greater sodium excretion by γ-CEHC [42] may improve vascular function. However, sodium excretion was unaffected in healthy adults despite an increase in plasma and urinary γ-CEHC following 28 days of γ-T supplementation [44]. The anti-inflammatory activities of γ-T [18,19,45] would also be expected to protect against vascular dysfunction. However, we show that plasma markers of inflammation at baseline and postprandially were unaffected regardless of γ-TmT supplementation. Interestingly, Ceriello et al. [4] observed that plasma ICAM-1, VCAM-1 and E-selectin concentrations increased following an oral glucose tolerance test in healthy older men (mean age=53.5 years). Similarly, studies *in vitro* demonstrate that mitogen-induced cytokine secretion from mononuclear cells isolated from elderly individuals was greater than that from younger individuals [46,47]. Thus, the lack of change in inflammatory markers in the present study likely reflects that our participants were young and healthy.

Insulin has vasoactive properties [48], but pharmacological inhibition of insulin secretion continues to suppress endothelium-

dependent vasodilation following hyperglycemia [49]. This suggests that the vascular endothelial dysfunction (VED) observed in our study is due to hyperglycemia rather than hyperinsulinemia. Harris et al. [50] demonstrated that FMD measurements repeated at 30-min intervals have no effect on brachial artery FMD, supporting that the postprandial FMD responses observed herein were not confounded by repetitive reactive hyperemia. In support, no time-dependent changes in preocclusion brachial artery diameter were observed throughout the postprandial period for either trial ($P$>.05; data not shown). Furthermore, FMD responses were measured at the same time of day during each visit because diurnal changes of FMD are known to occur [24]. Our FMD protocol is suggested to best reflect NO•-dependent vasodilation [51]. Consistent with this, our findings show that γ-TmT attenuated hyperglycemia-mediated increases in ADMA/arginine, a surrogate index of NO• bioavailability [13]. Although modest treatment differences were observed, plasma levels of ADMA/arginine may not accurately reflect intracellular levels. In support, studies in an experimental model of hyperglycemia and hyperinsulinemia showed that arginine/ADMA decreased to a larger extent in myocardial tissue compared to plasma [52]. Likewise, time-dependent responses in postprandial ADMA/arginine and FMD were not fully aligned. This does not exclude the possibility that γ-TmT protected against vascular dysfunction in a NO•-dependent manner. It does, however, emphasize the need to more directly measure changes in NO• status. The accurate assessment of NO• *in vivo* requires invasive NO• sensors [53]. Metabolites of NO• could be measured spectrophotometrically, but this approach is less specific and sensitive compared to GC-MS methods [54]. We have also considered the possibility that γ-TmT may have protected against hyperglycemia-mediated vascular dysfunction through NO•-independent mechanisms since alterations of other vasodilators and vasoconstrictors (e.g., prostacyclin, bradykinin, endothelin and angiotensin II) are known to affect FMD responses [55]. Thus, future studies should consider the use of chemical inhibitors or stimulators of NOS to better define the extent to which γ-TmT protects against vascular dysfunction through NO•-dependent and -independent mechanisms. Lastly, this study is also limited to healthy young men to control for confounding factors related to aging, gender and presence of morbidities that affect vasodilatory responses. Therefore, it is premature to extrapolate these findings to women, different age ranges, and those with underlying morbidities.

In conclusion, our findings support that γ-TmT is beneficial for maintaining VEF in healthy young men. Further study is needed to more fully define the specific mechanisms by which γ-TmT maintains VEF that is otherwise impaired by postprandial hyperglycemia. Moreover, long-term studies investigating the cardioprotective effects of γ-TmT are also needed to examine its potential benefit as a strategy to mitigate CVD risk.

## Acknowledgments

We are thankful to the participants for their cooperation and also to Stefanie Lynn, Holly Warfel and Diana Kawiecki for their expert assistance with the collection and analysis of dietary intake data. A special thanks to Manuel Matos for assisting in participant recruitment and FMD analysis. R.S.B. and J.S.V. were responsible for the study design. R.S.B. analyzed participant dietary records. R.S.B., K.D.B., S.K.N., H.J.P. and E.M. were responsible for collecting and analyzing data. E.M. and R.S.B. wrote the initial draft of the manuscript, and all authors contributed to the editing and review of this manuscript. None of the authors has a known conflict of interest.

## References

[1] Ogita H, Liao J. Endothelial function and oxidative stress. Endothelium 2004;11:123–32.
[2] Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. Heart disease and stroke statistics — 2010 update: a report from the American Heart Association. Circulation 2010;121:e46–215.
[3] DECODE Study Group; on behalf of the European Diabetes Epidemiology Group. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. Arch Intern Med 2001;161:397–405.
[4] Ceriello A, Quagliaro L, Piconi L, Assaloni R, Da Ros R, Maier A, et al. Effect of postprandial hypertriglyceridemia and hyperglycemia on circulating adhesion molecules and oxidative stress generation and the possible role of simvastatin treatment. Diabetes 2004;53:701–10.
[5] Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, et al. Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. J Am Coll Cardiol 1999;34:146–54.
[6] Title LM, Cummings PM, Giddens K, Nassar BA. Oral glucose loading acutely attenuates endothelium-dependent vasodilation in healthy adults without diabetes: an effect prevented by vitamins C and E. J Am Coll Cardiol 2000;36:2185–91.
[7] Ceriello A, Bortolotti N, Crescentini A, Motz E, Lizzio S, Russo A, et al. Antioxidant defences are reduced during the oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects. Eur J Clin Invest 1998;28:329–33.
[8] Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, et al. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment. Circulation 2002;106:1211–8.
[9] Libby P. Inflammation in atherosclerosis. Nature 2002;420:868–74.
[10] Forbes SP, Druhan LJ, Guzman JE, Parinandi N, Zhang L, Green-Church KB, et al. Mechanism of 4-HNE mediated inhibition of hDDAH-1: implications in NO regulation. Biochemistry 2008;47:1819–26.
[11] McCarty MF. Vascular endothelium is the organ chiefly responsible for the catabolism of plasma asymmetric dimethylarginine — an explanation for the elevation of plasma ADMA in disorders characterized by endothelial dysfunction. Med Hypotheses 2004;63:699–708.
[12] Green DJ, Jones H, Thijssen D, Cable NT, Atkinson G. Flow-mediated dilation and cardiovascular event prediction: does nitric oxide matter? Hypertension 2011;57:363–9.
[13] Bode-Boger SM, Scalera F, Ignarro LJ. The l-arginine paradox: importance of the L-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther 2007;114:295–306.
[14] Mah E, Noh SK, Ballard KD, Matos ME, Volek JS, Bruno RS. Postprandial hyperglycemia impairs vascular endothelial function in healthy men by inducing lipid peroxidation and increasing asymmetric dimethylarginine:arginine. J Nutr 2011;141:1961–8.
[15] Bruno RS. The role of alpha and gamma tocopherol in health. In: Wildman REC, editor. Nutraceuticals and functional foods. Boca Raton: CRC Press; 2007. p. 309–33.
[16] Jiang Q, Christen S, Shigenaga MK, Ames BN. Gamma-tocopherol, the major form of vitamin E in the us diet, deserves more attention. Am J Clin Nutr 2001;74:714–22.
[17] Goss SP, Hogg N, Kalyanaraman B. The effect of alpha-tocopherol on the nitration of gamma-tocopherol by peroxynitrite. Arch Biochem Biophys 1999;363:333–40.
[18] Jiang Q, Ames BN. Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. FASEB J 2003;17:816–22.
[19] Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proc Natl Acad Sci U S A 2000;97:11494–9.
[20] Wolf G. How an increased intake of alpha-tocopherol can suppress the bioavailability of gamma-tocopherol. Nutr Rev 2006;64:295–9.
[21] Kontush A, Spranger T, Reich A, Baum K, Beisiegel U. Lipophilic antioxidants in blood plasma as markers of atherosclerosis: the role of alpha-carotene and gamma-tocopherol. Atherosclerosis 1999;144:117–22.
[22] Kristenson M, Zieden B, Kucinskiene Z, Elinder LS, Bergdahl B, Elwing B, et al. Antioxidant state and mortality from coronary heart disease in Lithuanian and Swedish men: concomitant cross sectional study of men aged 50. BMJ 1997;314:629–33.
[23] Masterjohn C, Mah E, Guo Y, Koo SI, Bruno RS. Gamma-tocopherol abolishes postprandial increases in plasma methylglyoxal following an oral dose of glucose in healthy, college-aged men. J Nutr Biochem 2011.
[24] Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. Hypertension 2010;55:1075–85.
[25] Jackson AS, Pollock ML. Generalized equations for predicting body density of men. Br J Nutr 1978;40:497–504.
[26] Schutte JE, Townsend EJ, Hugg J, Shoup RF, Malina RM, Blomqvist CG. Density of lean body mass is greater in blacks than in whites. J Appl Physiol 1984;56:1647–9.
[27] Siri W. Body composition from fluid spaces and density: analysis of methods. In: Brozek J, Hanschel A, editors. Techniques for measuring body composition. Washington, DC: National Academy of Sciences; 1961. p. 223–45.
[28] Iuliano L, Micheletta F, Maranghi M, Frati G, Diczfalusy U, Violi F. Bioavailability of vitamin e as function of food intake in healthy subjects: effects on plasma peroxide-scavenging activity and cholesterol-oxidation products. Arterioscler Thromb Vasc Biol 2001;21:E34–7.
[29] Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: part 1 — correlation within subjects. BMJ 1995;310:446.

[30] Santini SA, Marra G, Giardina B, Cotroneo P, Mordente A, Martorana GE, et al. Defective plasma antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM. Diabetes 1997;46:1853–8.
[31] Pazdro R, Burgess JR. The role of vitamin E and oxidative stress in diabetes complications. Mech Ageing Dev 2010;131:276–86.
[32] Leonard SW, Good CK, Gugger ET, Traber MG. Vitamin E bioavailability from fortified breakfast cereal is greater than that from encapsulated supplements. Am J Clin Nutr 2004;79:86–92.
[33] Bates CJ, Jones KS, Bluck LJ. Stable isotope-labelled vitamin C as a probe for vitamin C absorption by human subjects. Br J Nutr 2004;91:699–705.
[34] Flores C, Rojas S, Aguayo C, Parodi J, Mann G, Pearson JD, et al. Rapid stimulation of L-arginine transport by D-glucose involves p42/44(MAPK) and nitric oxide in human umbilical vein endothelium. Circ Res 2003;92: 64–72.
[35] Kashyap SR, Lara A, Zhang R, Park YM, DeFronzo RA. Insulin reduces plasma arginase activity in type 2 diabetic patients. Diabetes Care 2008;31:134–9.
[36] Pieper GM, Dondlinger LA. Plasma and vascular tissue arginine are decreased in diabetes: acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. J Pharmacol Exp Ther 1997;283: 684–91.
[37] Erisir M, Naziroglu M, Tasdemir B, Simsek M. Effect of a dietary combined vitamin C and E supplementation on the liver, kidneys and brain arginase activity in non-pregnant and pregnant rats with streptozotocin-induced diabetes. Revue de Médecine Vétérinaire 2006;157:445–9.
[38] Teerlink T, Luo Z, Palm F, Wilcox CS. Cellular ADMA: regulation and action. Pharmacol Res 2009;60:448–60.
[39] Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, et al. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. Circulation 2002;106: 987–92.
[40] Pope AJ, Druhan L, Guzman JE, Forbes SP, Murugesan V, Lu D, et al. Role of DDAH-1 in lipid peroxidation product-mediated inhibition of endothelial NO generation. Am J Physiol Cell Physiol 2007;293:C1679–86.
[41] Jiang Q, Lykkesfeldt J, Shigenaga MK, Shigeno ET, Christen S, Ames BN. Gamma-tocopherol supplementation inhibits protein nitration and ascorbate oxidation in rats with inflammation. Free Radic Biol Med 2002;33:1534–42.
[42] Murray Jr ED, Wechter WJ, Kantoci D, Wang WH, Pham T, Quiggle DD, et al. Endogenous natriuretic factors 7: biospecificity of a natriuretic gamma-tocopherol metabolite LLU-alpha. J Pharmacol Exp Ther 1997;282:657–62.
[43] Dickinson KM, Keogh JB, Clifton PM. Effects of a low-salt diet on flow-mediated dilatation in humans. Am J Clin Nutr 2009;89:485–90.
[44] Yoshikawa S, Morinobu T, Hamamura K, Hirahara F, Iwamoto T, Tamai H. The effect of gamma-tocopherol administration on alpha-tocopherol levels and metabolism in humans. Eur J Clin Nutr 2005;59:900–5.
[45] Wiser J, Alexis NE, Jiang Q, Wu W, Robinette C, Roubey R, et al. In vivo gamma-tocopherol supplementation decreases systemic oxidative stress and cytokine responses of human monocytes in normal and asthmatic subjects. Free Radic Biol Med 2008;45:40–9.
[46] Bruunsgaard H, Pedersen BK. Age-related inflammatory cytokines and disease. Immunol Allergy Clin North Am 2003;23:15–39.
[47] Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, et al. Increased cytokine production in mononuclear cells of healthy elderly people. Eur J Immunol 1993;23:2375–8.
[48] Baron AD. Hemodynamic actions of insulin. Am J Physiol 1994;267:E187–202.
[49] Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, et al. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. Circulation 1998;97:1695–701.
[50] Harris RA, Padilla J, Rink LD, Wallace JP. Variability of flow-mediated dilation measurements with repetitive reactive hyperemia. Vasc Med 2006;11:1–6.
[51] Doshi SN, Naka KK, Payne N, Jones CJ, Ashton M, Lewis MJ, et al. Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. Clin Sci (Lond) 2001;101:629–35.
[52] Ellger B, Richir MC, van Leeuwen PA, Debaveye Y, Langouche L, Vanhorebeek I, et al. Glycemic control modulates arginine and asymmetrical-dimethylarginine levels during critical illness by preserving dimethylarginine-dimethylaminohydrolase activity. Endocrinology 2008;149:3148–57.
[53] Davies IR, Zhang X. Nitric oxide selective electrodes. Methods Enzymol 2008;436: 63–95.
[54] Tsikas D. Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. Free Radic Res 2005;39:797–815.
[55] Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation 2007;115:1285–95.